

1    **Rescue of Tomato spotted wilt tospovirus entirely from cDNA clones,**  
2    **establishment of the first reverse genetics system for a segmented**  
3    **(-)RNA plant virus**

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18

19    **Running title**

20    Establishment of a reverse genetics system for TSWV

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22

23 **Abstract**

24 The group of negative strand RNA viruses (NSVs) includes not only dangerous  
25 pathogens of medical importance but also serious plant pathogens of agronomical  
26 importance. Tomato spotted wilt tospovirus (TSWV) is one of those plant NSVs that  
27 cause severe diseases on agronomic crops and pose major threats to global food  
28 security. Its negative-strand segmented RNA genome has, however, always posed a  
29 major obstacle to molecular genetic manipulation. In this study, we report the  
30 complete recovery of infectious TSWV entirely from cDNA clones, the first reverse  
31 genetics (RG) system for a segmented plant NSV. First, a replication and transcription  
32 competent mini-genome replication system was established based on 35S-driven  
33 constructs of the S<sub>(-)</sub>-genomic (g) or S<sub>(+)</sub>-antigenomic (ag) RNA template, flanked by a  
34 5' Hammerhead and 3' Ribozyme sequence of Hepatitis Delta virus, a nucleocapsid  
35 (N) protein gene and codon-optimized viral RNA dependent RNA polymerase (RdRp)  
36 gene. Next, a movement competent mini-genome replication system was developed  
37 based on M<sub>(-)</sub>-gRNA, which was able to complement cell-to-cell and systemic  
38 movement of reconstituted ribonucleoprotein complexes (RNPs) of S RNA replicon.  
39 After further optimization, infectious TSWV and derivatives carrying eGFP reporters  
40 were successfully rescued *in planta* via simultaneous expression of full-length cDNA  
41 constructs coding for S<sub>(+)</sub>-agRNA, M<sub>(-)</sub>-gRNA and L<sub>(+)</sub>-agRNA. Viral rescue occurred  
42 in the additional presence of various viral suppressors of RNAi, but TSWV NSs  
43 interfered with the rescue of genomic RNA. The establishment of a RG system for  
44 TSWV now allows detailed molecular genetic analysis of all aspects of tospovirus life

45 cycle and their pathogenicity.

46 **Key words:** Reverse genetics system, Tomato spotted wilt tospovirus, negative-strand

47 RNA virus, tripartite RNA genome, mini-replicon, genome-length infectious cDNA

48 clones

49

50 **Significance**

51 For many different animal-infecting segmented negative-strand viruses (NSVs), a

52 reverse genetics system has been established that allows the generation of mutant

53 viruses to study disease pathology and the role of *cis*- and *trans*-acting elements in the

54 virus life cycle. In contrast to the relative ease to establish RG systems for

55 animal-infecting NSVs, establishment of such system for the plant-infecting NSVs

56 with a segmented RNA genome so far has not been successful. Here we report the

57 first reverse genetics system for a segmented plant NSV, the Tomato spotted wilt

58 tospovirus, a virus with a tripartite RNA genome. The establishment of this RG

59 system now provides us with a new and powerful platform to study their disease

60 pathology during a natural infection.

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67 **Introduction**

68 Negative-stranded RNA viruses (NSVs) present of a large group of viruses that  
69 include well known members of medical importance such as Ebola (EBOV), Rabies  
70 (RV), Influenza A (FLUAV) and Rift valley fever virus (RVFV) (1, 2). Infections with  
71 these viruses may cause considerable morbidity and mortality in humans and form an  
72 important burden on national health care budgets. The group also contains plant  
73 viruses of agronomical importance such as Tomato spotted wilt virus (TSWV) and  
74 Rice stripe virus (RSV) that cause severe diseases on agronomic crops and pose major  
75 threats to global food security (3-10).

76 Tospoviruses belong to the NSV with a segmented (tripartite) RNA genome and  
77 rank among the most devastating plant viruses worldwide (11, 12). They are classified  
78 in the family of *Tospoviridae* within the order *Bunyavirales* (13). TSWV is the type  
79 member of the only genus *Orthotospovirus*, in the family of *Tospoviridae* (11, 13).  
80 TSWV has very broad host range infecting more than one thousand plant species over  
81 80 families (14) and is transmitted by thrips in a persistent, propagative manner (6, 9,  
82 15, 16). Crops losses due to this virus have been estimated more than one billion  
83 dollars annually (7, 14).

84 TSWV consists of spherical, enveloped virus particles (80-120 nm) that contain a  
85 tripartite genome consisting of a large- (L), medium- (M), and small-sized (S) RNA  
86 segment (11). The L segment is of entire negative polarity, whereas the S and M  
87 segments are ambisense. The L segment encodes the viral RNA-dependent RNA  
88 polymerase (RdRp, ~330 kDa) that is required for viral RNA replication and mRNA

89 transcription (17, 18). The viral (v) strand of the M segment encodes the precursor to  
90 the glycoproteins (Gn and Gc, with n and c referring to the amino- and  
91 carboxy-terminal end of the precursor, respectively) in the negative sense and a  
92 nonstructural protein (NSm) in the positive sense. The glycoproteins are required for  
93 particle maturation and are presented as spikes on the surface of the virus envelope  
94 membrane (19, 20). They also play a major role as determinants for thrips vector  
95 transmission (21). The NSm plays pivotal roles in cell-to-cell and long distance  
96 movement of TSWV (22-26). The vRNA of the S segment codes for the nucleocapsid  
97 protein (N) in the negative sense and a nonstructural protein (NSs) in the positive  
98 sense. The N protein participates in the formation of ribonucleoprotein complexes  
99 (RNPs) (27-29) and is required for viral intracellular movement (30, 31). The NSs  
100 protein functions as a RNA silencing suppressor to defend against the plant innate  
101 immunity system (32-34), and triggers a defence response and concomitant  
102 programmed cell-death mediated by the dominant resistance gene *Tsw* from *Capsicum*  
103 *chinense* (35-37).

104 As a virus documented for almost a century (7, 38), TSWV has served as an  
105 important model for studying the molecular biology of tospovirus and other plant  
106 NSVs with segmented genomes (6-8, 11). However, its negative-strand tripartite RNA  
107 genome has posed a major obstacle to genetic manipulation of the virus. To initiate an  
108 infection cycle with this virus requires at least RNPs, the minimal infectious units that  
109 consist of viral RNA encapsidated by the N protein and associated with a few copies  
110 of the viral RNA-dependent RNA polymerase (6, 11). TSWV RNPs can be

111 mechanically transferred from infected to healthy plants, however, transmission by  
112 thrips requires RNPs to be enveloped and spiked with the glycoproteins (21).

113 The first animal-infecting and related counterpart of TSWV with a segmented  
114 RNA genome to be rescued entirely from cDNA was from Bunyamwera virus in 1996  
115 (39). Following this study, soon other segmented NSV were rescued from plasmid  
116 DNA. The influenza virus, containing a genome of eight RNA segments, was  
117 recovered in 1999 (40), while the first Arenavirus, with a bipartite RNA genome, was  
118 recovered in 2006 (41). Just recently, the first nonsegmented plant NSVs from the  
119 *Mononegavirales* have been rescued, *i.e.* the Sonchus yellow net nucleorhabdovirus  
120 (SYNV) (42, 43). A recent study also reported on the establishment of a TSWV S  
121 RNA-based mini-replicon in yeast (44), but in contrast to replication, no  
122 transcriptional activity was observed.

123 In contrast to the relative ease to establish RG systems for animal-infecting NSVs,  
124 reconstitution of infectious RNPs *in planta* for the plant-infecting viruses with a  
125 segmented RNA genome seems particularly difficult. Not only have all DNA  
126 constructs to be delivered into one and the same plant cell but for TSWV the RdRp is  
127 exceptionally large (~330 kDa) compared to the RdRp of most other related  
128 bunyaviruses (~240-260 kDa) and to typical open reading frames (ORFs) from the  
129 plant genome. Expression of such a large protein gene may not only be very  
130 inefficient but mRNA transcripts resulting from RNA polymerase II transcription of  
131 35S promoter-constructs in the nucleus may also face splicing of cryptic splicing sites.  
132 Moreover, achieving proper ratios of all three genome segments in plant cells is not

133 easily and consistently achieved by agrobacterium-mediated delivery of several  
134 constructs, which will affect the outcome of each individual experiment. All these  
135 obstacles may hamper the construction of a reverse genetics system for TSWV in  
136 plants.

137 In this study, we report the complete recovery of infectious TSWV entirely from  
138 cDNA clones in plants, the first reverse genetics system for a segmented plant NSV.  
139 The establishment of this system presents the start of a new research era for TSWV  
140 and provides us an entirely new and powerful platform to study the basic principles of  
141 the tospovirus life cycle and viral pathogenicity.

142

## 143 **Results**

### 144 **Development of a TSWV S<sub>(-)</sub>-genomic RNA mini-replicon system in *Nicotiana* 145 *benthamiana***

146 Prior to the rescuing of TSWV entirely from cDNA clones, a mini-replicon system  
147 based on the S RNA-template was established. To this end, a DNA copy of the TSWV  
148 S<sub>(-)</sub>-genomic RNA (gRNA) was cloned and flanked with a self-cleaving hammerhead  
149 (HH) ribozyme at the 5'-terminus and a hepatitis delta virus (HDV) ribozyme at the  
150 3'-terminus. For visual monitoring, quantification purposes, and discrimination  
151 between primary and secondary genome transcription, the NSs and N genes were  
152 replaced with mCherry and eGFP, respectively (Fig. 1A). The resulting S<sub>(-)</sub>  
153 mini-replicon reporter was cloned in a binary vector pCB301 downstream a double  
154 35S promoter (2×35S) and denoted 35S:SR<sub>(-)</sub>mCherry&eGFP (Fig. 1A), or a T7 promoter

155 and denoted T7: SR<sub>(-)</sub>mCherry&eGFP (Fig. S1A). The RdRp and N ORFs were amplified  
156 from cDNA of TSWV infected tissue and cloned into pCambia 2300 binary vector  
157 downstream a double 35S promoter. Binary vector constructs of the RdRp, N and four  
158 viral RNA silencing suppressor genes (VSRs: NSs from TSWV, P19 from Tomato  
159 bushy stunt tombusvirus (TBSV), HcPro from Tobacco etch potyvirus (TEV) and  $\gamma$ b  
160 from Barley yellow mosaic hordeivirus (BYMV)) were agroinfiltrated in *N.*  
161 *benthamiana* either with 35S:SR<sub>(-)</sub>mCherry&eGFP or with T7:SR<sub>(-)</sub>mCherry&eGFP and a  
162 35S-driven T7 RNA polymerase gene construct and next monitored for eGFP  
163 fluorescence. However, during repeated experiments no eGFP fluorescence was  
164 observed from 35S:SR<sub>(-)</sub>mCherry&eGFP nor T7:SR<sub>(-)</sub>mCherry&eGFP (Fig. S1B and C).

165 The possibility of failures to establish a mini-replicon system for TSWV could  
166 be due to low (unstable) expression of the TSWV RdRp protein, therefore, the codon  
167 usage of the RdRp gene was optimized for *N. benthamiana* and potential intron  
168 splicing sites were removed. The optimized RdRp gene (RdRp<sub>opt</sub>) was cloned in a  
169 binary, 35S-driven expression vector and next, again agroinfiltrated in *N.*  
170 *benthamiana* leaves together with binary expression constructs of the N gene, the four  
171 VSR gene constructs and the 35S:SR<sub>(-)</sub>mCherry&eGFP mini-replicon reporter. At 5 days  
172 post infiltration (dpi), expression of the reporter genes was analyzed by monitoring  
173 for mCherry and eGFP fluorescence in the *N. benthamiana* leaves (Fig. 1A and B).  
174 Whereas no fluorescence was observed in the controls, *i.e.* leaves agroinfiltrated with  
175 35S:SR<sub>(-)</sub>mCherry&eGFP alone or co-expressing 35S:SR<sub>(-)</sub>mCherry&eGFP with RdRp<sub>opt</sub> or N  
176 only, eGFP and mCherry fluorescence was consistently observed in leaves

177 agroinfiltrated with 35S:SR<sub>(-)</sub>mCherry&eGFP and both N and RdRp<sub>opt</sub> (Fig. 1C). This was  
178 confirmed by Western immunoblot analysis (Fig. 1D).

179 Northern blot analysis showed that both the genomic RNA and anti-genomic  
180 RNA of the SR<sub>(-)</sub>mCherry&eGFP mini-replicon were detected in the leaves that  
181 co-expressed both N and RdRp (here after RdRp represent optimized RdRp) at 5 dpi,  
182 but not in the leaves co-expressing RdRp or N only (Fig. 1E). In addition, using an  
183 anti-sense eGFP probe genome length S RNA subgenomic-sized RNA, likely  
184 presenting eGFP transcripts, were detected (Fig. 1E, upper panel). A time course  
185 analysis showed that eGFP and mCherry fluorescence in *N. benthamiana* leaves was  
186 visible from 3 dpi onwards and gradually increased to 12 dpi (Fig. S2A). This was  
187 also confirmed by immunoblot analysis (Fig. S2B).

188 Altogether the results indicated that in *N. benthamiana* the 35S replicon  
189 transcript SR<sub>(-)</sub>mCherry&eGFP was properly processed by the HH and RZ, and next used  
190 by the RdRp as template for primary transcription of mRNA<sup>eGFP</sup>, as well replicated  
191 into SR<sub>(-)</sub>mCherry&eGFP for secondary transcription of mRNA<sup>mCherry</sup>. Furthermore, the  
192 codon-optimized RdRp clearly exhibited full functionality in supporting viral genome  
193 transcription and replication, while the wild type RdRp for unknown reasons didn't.  
194 When the T7:SR<sub>(-)</sub>mCherry&eGFP mini-replicon reporter was co-expressed with T7 RNA  
195 polymerase, RdRp, N and four VSRs in *N. benthamiana* leaves, somewhat  
196 unexpected, no mCherry or eGFP fluorescence was detected (Fig. S1C and D).

197

198 **The optimization of the concentration of N and RdRp, and VSRs on TSWV**

199 **SR<sub>(-)</sub>mCherry&eGFP mini-replicon**

200 Having established a TSWV S<sub>(-)</sub>-gRNA based mini-replicon system in *N.*  
201 *benthamiana*, attempts were made to further optimize the system. To this end, binary  
202 constructs of the TSWV S<sub>(-)</sub> mini-replicon were agroinfiltrated into *N. benthamiana* in  
203 the additional presence of varying amounts of N and RdRp gene expression constructs.  
204 During these experiments the amounts of *Agrobacterium* carrying the N expression  
205 construct were increased (from OD<sub>600</sub> 0.2 to 0.8) while the *Agrobacterium* harboring  
206 the RdRp expression construct was kept fixed at OD<sub>600</sub> 0.2, and vice versa. The results  
207 showed highest eGFP reporter gene expression from the 35S:SR<sub>(-)</sub>mCherry&eGFP  
208 mini-replicon when *Agrobacterium* suspensions bearing N and RdRp expression  
209 constructs were both infiltrated at OD<sub>600</sub> 0.2. When *Agrobacterium* harboring either N  
210 or RdRp was infiltrated onto *N. benthamiana* leaves at OD<sub>600</sub>>0.4 the expression of  
211 eGFP from the S<sub>(-)</sub>-mini-replicon greatly decreased (Fig. 2 A, B and C ). Furthermore,  
212 at high concentrations (OD<sub>600</sub> > 0.6) of *Agrobacterium*, the visible cell death was  
213 triggered in the infiltrated leaves (data not shown).

214 Next, in a similar approach and using the optimized setting, the effects of VSRs  
215 on the replication and transcription of the SR<sub>(-)</sub>mCherry&eGFP mini-replicon were  
216 investigated. Without the addition of VSRs, mCherry and eGFP fluorescence was only  
217 observed in a small number of cells, but these numbers increased in the addition of  
218 TSWV NSs and/or the three VSRs P19, HcPro and  $\gamma$ b. The largest number of cells  
219 showing eGFP expression from the S<sub>(-)</sub> mini-replicon, as monitored by fluorescence,  
220 were obtained when all four VSRs were added (Fig. 2D and E). These observations

221 were further confirmed by immunoblot assays (Fig. 2E).

222 **The role of *cis*-acting sequences in transcription and replication of the TSWV**

223 **SR<sub>(-)</sub>mCherry&eGFP replicon**

224 Using the optimized SR<sub>(-)</sub>mCherry&eGFP mini-replicon system, the role of the 5'-  
225 untranslated region (5'-UTR), 3'-UTR and the non-coding A/U-rich intergenic region  
226 (IGR) between NSs and N genes (45, 46) in replication-transcription was examined.

227 To this end, SR<sub>(-)</sub>mCherry&eGFP derivatives were made from which the 5'-UTR, IGR or  
228 3'-UTR, respectively, were deleted and next tested on transcription-replication using

229 the mini-replicon assay (Fig. S3A). No eGFP and mCherry fluorescence was observed  
230 when the 5'-UTR or 3'-UTR of SR<sub>(-)</sub>mCherry&eGFP was removed. However, eGFP  
231 reporter expression could still be detected when the IGR of SR<sub>(-)</sub>mCherry&eGFP was  
232 deleted (Fig. S3B). Immunoblot analysis confirmed the expression of eGFP from  
233 SR<sub>(-)</sub>mCherry&eGFPΔIGR (ΔIGR), and lack of expression from SR<sub>(-)</sub>mCherry&eGFPΔ5'UTR  
234 (Δ5'UTR) and SR<sub>(-)</sub>mCherry&eGFPΔ3'UTR (Δ3'UTR) (Fig. S3C). To analyze whether the

235 lack of eGFP expression was a matter of translation or transcription-replication,  
236 samples from infiltrated *N. benthamiana* were collected and analyzed by Northern

237 blot. The results showed that for SR<sub>(-)</sub>mCherry&eGFPΔ5'UTR (Δ5'UTR) and  
238 SR<sub>(-)</sub>mCherry&eGFPΔ3'UTR (Δ3'UTR), weak signals of genomic RNA could be detected but

239 they are not similar to the signal strength obtained for the genome length and mRNA  
240 molecules as seen with the SR<sub>(-)</sub>mCherry&eGFP replicon, while anti-genomic RNAs could

241 not be detected (Fig. S3D). For SR<sub>(-)</sub>mCherry&eGFPΔIGR (ΔIGR) both RNA strands were  
242 detected (Fig. S3D), suggesting that IGR is not essential for viral RNA synthesis,

243 while no signal is obtained for the mRNA length molecules as seen with the  
244 SR<sub>(-)</sub>mCherry&eGFP replicon. Taken together, these findings suggest that the 5'-UTR and  
245 3'-UTR play an essential role in viral transcription and replication of TSWV RNA  
246 segments.

247

#### 248 **Development of a TSWV S<sub>(+)</sub>-agRNA based mini-replicon system**

249 For many reverse genetics systems of NSV, mini-replicons have initially been  
250 established based on gRNA (vRNA). Here, we managed to develop a first system for  
251 TSWV based on antigenomic (ag)RNA (vcRNA). In order to analyze whether a  
252 system could be developed based on agRNA, a S<sub>(+)</sub>-agRNA mini-replicon was  
253 constructed similarly to the one based on S<sub>(-)</sub>-gRNA but in which the N gene was  
254 maintained and only the NSs gene was replaced by eGFP, denoted SR<sub>(+)</sub>eGFP (Fig. 3A).

255 In analogy to the replicon assays with SR<sub>(-)</sub>mCherry&eGFP, *N. benthamiana* leaves were  
256 agro-infiltrated with binary expression constructs of SR<sub>(+)</sub>eGFP, four VSRs and either N  
257 or RdRp separately or together, respectively, and monitored for eGFP fluorescence.

258 Whereas eGFP fluorescence, resulting from primary transcription of the replicon  
259 transcript by viral RdRp, was not detected when SR<sub>(+)</sub>eGFP was expressed alone or in  
260 the additional presence of N, eGFP fluorescence was observed when SR<sub>(+)</sub>eGFP was  
261 co-expressed with both RdRp and N, but also when SR<sub>(+)</sub>eGFP was co-expressed with  
262 RdRp alone (Fig. 3B). This strongly indicated that a certain (residual) amount of  
263 SR<sub>(+)</sub>eGFP transcripts, resulting from 35S transcription, did not become fully processed  
264 by the HH and RZ and remained functional in translation, thereby giving rise to N

265 protein. This was confirmed by Western immunoblot analysis (Fig. 3C). Northern blot  
266 analysis furthermore showed that samples from the replicon assays performed in the  
267 presence of RdRp and N or RdRp alone, besides eGFP mRNA transcripts, also  
268 contained agRNA and gRNA of SR<sub>(+)</sub>eGFP, indicating the occurrence of replication  
269 (Fig. 3D). Altogether, these results demonstrate that the N protein can also be  
270 expressed from the SR<sub>(+)</sub>eGFP replicon to support its transcription and replication. This  
271 provides an attractive alternative to the S<sub>(-)</sub>-gRNA based mini-replicon as additional  
272 binary expression constructs for N do not have to be supplied anymore.

273

274 **Development of a M<sub>(-)</sub>-gRNA based mini-replicon for cell-to-cell movement of**  
275 **TSWV in *N. benthamiana***

276 As a first step towards development of a reverse genetics system to rescue TSWV  
277 virus entirely from cDNA, a movement competent mini-replicon was also established.  
278 To this end, a TSWV M<sub>(-)</sub>-gRNA based mini-replicon was constructed, similar as the  
279 ones made for S<sub>(-)</sub> and S<sub>(+)</sub>. Within this construct the NSm cell-to-cell movement  
280 protein gene was maintained but the GP ORF was exchanged for eGFP, resulting in a  
281 mini-replicon designated as MR<sub>(-)</sub>eGFP (Fig. 4A). After *Agrobacterium*-mediated  
282 delivery into *N. benthamiana*, no eGFP fluorescence was observed in leaves  
283 containing the MR<sub>(-)</sub>eGFP replicon with RdRp or N. However, in the presence of both  
284 RdRp and N, eGFP fluorescence was observed in many cells that connected each  
285 other (Fig. 4B and C). In comparison to the eGFP fluorescence always expressed in a  
286 single plant cells from SR<sub>(-)</sub>mCherry&eGFP or SR<sub>(+)</sub>eGFP reporter, the results suggested that

287 the MR<sub>(-)</sub>eGFP mini-replicon has moved from cell-to-cell in *N. benthamiana* leaves.  
288 Northern blot analysis confirmed the synthesis of gRNA, agRNA and  
289 (subgenomic-length) eGFP mRNA transcripts of the MR<sub>(-)</sub>eGFP replicon in the  
290 presence of both RdRp and N, but not with RdRp or N only (Fig. 4D).

291 To further substantiate the findings on possible cell-to-cell movement of the  
292 MR<sub>(-)</sub>eGFP mini-replicon, a stop codon was introduced immediately downstream the  
293 start codon of NSm and the construct designated MR<sub>(-)</sub>eGFP&NSmMut (Fig. 4A). When  
294 the MR<sub>(-)</sub>eGFP&NSmMut replicon was delivered and co-expressed with RdRp and N in *N.*  
295 *benthamiana* leaves, eGFP fluorescence was only detected in a single cells (Fig. 4B).  
296 As expected, Western immunoblot analysis confirmed the production of eGFP protein  
297 in leaves containing the MR<sub>(-)</sub>eGFP&NSmMut replicon, and in significantly lower amounts  
298 compared to the MR<sub>(-)</sub>eGFP replicon (Fig. 4C).

299

300 **Establishment of the systemic infection of M<sub>(-)</sub>- and S<sub>(+)</sub>-mini-replicon reporters**  
301 **by co-expression of full-length antigenomic L<sub>(+)</sub> in *N. benthamiana***

302 With the establishment of S (g/ag)RNA-based mini-replicon systems, and a  
303 movement-competent M gRNA-based mini-replicon, we set out to construct full  
304 length genomic cDNA clones of L<sub>(-)</sub>, M<sub>(-)</sub> and S<sub>(-)</sub>, flanked by HH and HDV at 5'- and  
305 3'-terminus, as a first step towards the rescue of TSWV entirely from cDNA clones.

306 At the same time, similar constructs were made for the anti-genomic L<sub>(+)</sub>, M<sub>(+)</sub> and  
307 S<sub>(+)</sub>. However, attempts to recover infectious TSWV from *N. benthamiana* after  
308 agrobacterium-mediated delivery of all binary expression constructs of L<sub>(-)</sub>, M<sub>(-)</sub>, S<sub>(-)</sub>

309 together with N, RdRp and four VSRs, but also with the anti-genomic L<sub>(+)</sub>, M<sub>(+)</sub> and  
310 S<sub>(+)</sub> constructs, all failed ([Table 1](#)).

311 Since MR<sub>(-)eGFP</sub> was earlier shown to be movement competent, it was next  
312 investigated whether the M<sub>(-)</sub>- and S<sub>(-)</sub>-minigenomes moved into the same plant cell in  
313 the presence of both RdRp and N. Upon co-expression of MR<sub>(-)mCherry</sub>, SR<sub>(-)eGFP</sub>, RdRp  
314 and N in *N. benthamiana* leaves, expression of both mCherry and eGFP from the  
315 MR<sub>(-)mCherry</sub> and SR<sub>(-)eGFP</sub> mini-replicons, respectively, could be discerned. However,  
316 the foci of mCherry fluorescence were separate from those showing eGFP  
317 fluorescence ([Fig. S4](#)). Previously, ectopic expression of Tobacco crinkle virus (TCV)  
318 RdRp was reported to cause superinfection exclusion, and prevented the entry of  
319 progeny virus into the original cell expressing the RdRp ([47](#)). Ectopic expression of  
320 TSWV RdRp and N would possibly cause superinfection exclusions and block  
321 intercellular movement of SR<sub>(-)eGFP</sub> into cells containing MR<sub>(-)mCherry</sub>. To avoid that, a  
322 new strategy was employed in which RdRp and N were expressed from viral agRNAs.  
323 To this end, a construct was made of the full-length L agRNA containing the  
324 optimized RdRp and flanked by the HH and HDV ribozymes, denoted L<sub>(+)opt</sub> ([Fig. 5A](#)).  
325 To test the expression and functionality of RdRp from this construct, L<sub>(+)opt</sub> was  
326 co-expressed with the SR<sub>(-)mCherry&eGFP</sub> mini-replicon, N and VSRs in *N. benthamiana*  
327 leaves. The results showed clear eGFP and mCherry fluorescence and indicated that  
328 L<sub>(+)opt</sub> was able to support SR<sub>(-)mCherry&eGFP</sub> transcription and replication ([Fig. S5A](#)).  
329 Furthermore, L<sub>(+)opt</sub> was also able to support the replication and transcription of the  
330 SR<sub>(+)eGFP</sub> mini-replicon, without the additional ectopic expression of N ([Fig. S5B](#)),

331 and of the movement competent  $MR_{(-)}eGFP$  (Fig. S5C).

332 In a next experiment  $L_{(+)}opt$  was co-expressed with  $MR_{(-)}mCherry$ ,  $SR_{(+)}eGFP$  and four  
333 VSRs in *N. benthamiana* and plants analyzed for a systemic infection (Fig. 5A). At 6  
334 dpi, mCherry and eGFP fluorescence were detected in the locally agroinfiltrated *N.*  
335 *benthamiana* leaves and in which some foci were found to express mCherry and eGFP  
336 together (Fig. S6A). At 15 dpi, necrotic symptoms became visual in systemic leaves of  
337 *N. benthamiana* (Fig. 5B, a and c). Using a handheld UV lamp, a clear eGFP  
338 fluorescence was also observed in those leaves (Fig. 5B, b and d). The eGFP signal  
339 was detected in 24 out of 30 agro-infiltrated *N. benthamiana* plants (Table 1). Both  
340 eGFP and mCherry fluorescence were detected in veins/stems and systemic leaves  
341 under a fluorescence microscope (Fig. 5B, e). The systemic infection of *N.*  
342 *benthamiana* with  $SR_{(+)}eGFP$ ,  $MR_{(-)}mCherry$  and  $L_{(+)}opt$  was further confirmed by RT-PCR  
343 analysis (Fig. S6B).

344

### 345 Recovery of infectious TSWV from the full-length cDNA clones

346 Based on the establishment of a systemic infection after *Agrobacterium*-mediated  
347 delivery of replicons  $SR_{(+)}eGFP$ ,  $MR_{(-)}mCherry$  and  $L_{(+)}opt$ , we next generated a full-length  
348 construct for S agRNA without any reporter gene, designated as  $S_{(+)}$  and co-expressed  
349 it with replicon constructs  $L_{(+)}opt$ ,  $M_{(-)}$  and four VSRs in *N. benthamiana* leaves.  
350 However, and surprisingly, no infectious TSWV was recovered from systemic leaves  
351 of *N. benthamiana* that were infiltrated with these constructs (Table 1). To find out  
352 whether this was due to failure of  $S_{(+)}$ , we next examined whether  $S_{(+)}$  was able to

353 establish a systemic infection in combination with the functional  $MR_{(-)}eGFP$  and  $L_{(+)}opt$   
354 constructs. When  $L_{(+)}opt$ ,  $MR_{(-)}eGFP$ ,  $S_{(+)}$  and three VSRs (P19-HcPro- $\gamma b$ ) were  
355 co-expressed in *N. benthamiana* leaves, eGFP fluorescence was visible at 18 dpi in  
356 systemic leaves of *N. benthamiana*, although not as efficient as in the case with the  
357  $SR_{(+)}eGFP$  replicon, since only 7 out of 60 plants showed systemic infection (Fig. 5D  
358 and Table 1). RT-PCR analysis confirmed the systemic infection with  $S_{(+)}$ ,  $MR_{(-)}eGFP$   
359 and  $L_{(+)}opt$  in those *N. benthamiana* (Fig. S6C). When  $L_{(+)}opt$ ,  $MR_{(-)}eGFP$  and  $S_{(+)}$  were  
360 co-expressed with four VSRs (P19-HcPro- $\gamma b$  and NSs) in *N. benthamiana* leaves,  
361 intriguingly, no eGFP fluorescence was observed in the systemic leaves (Table 1)  
362 suggesting that ectopic expression of NSs interfered with the rescue of virus from full  
363 length  $S_{(+)}$ ,  $MR_{(-)}eGFP$  and  $L_{(+)}opt$ .

364 Next, we tested the rescuing of virus from full length  $M_{(-)}$ ,  $SR_{(+)}eGFP$  and  $L_{(+)}opt$ . To  
365 this end, the constructs of  $L_{(+)}opt$ ,  $M_{(-)}$  and  $SR_{(+)}eGFP$  were delivered and co-expressed  
366 in *N. benthamiana* in the presence of either four (P19-HcPro- $\gamma b$ +NSs) or three  
367 (P19-HcPro- $\gamma b$ ) VSRs. The results showed no eGFP fluorescence in systemic leaves  
368 of *N. benthamiana* at 15-50 dpi, indicating that  $M_{(-)}$  was not able to complement and  
369 rescue the  $S_{(+)}$ -mini-replicons into systemic leaves (Table 1). Northern blot analysis  
370 showed that neither gRNAs nor agRNAs were detected for  $M_{(-)}$  while, in contrast,  
371 gRNAs and agRNAs were detected for  $S_{(+)}$  (Fig. S7A and B). Earlier, the  $MR_{(-)}eGFP$   
372 mini-replicon was shown to replicate and transcribe (Fig. 4D). The only difference  
373 between  $M_{(-)}$  and  $MR_{(-)}eGFP$  mini-replicon was the GP gene, which was exchanged for  
374 eGFP in the second construct. Considering that primary  $M_{(-)}$  transcripts were

375 produced in the nucleus by the 35S promoter, and putative splice sites were also  
376 predicted in the GP sequence (*SI Appendix, Table S2*), it was likely that primary M<sub>(-)</sub>  
377 transcripts were prone to splicing before sufficient replication of the mini-replicon  
378 and transcriptional-translational expression of the cell-to-cell movement protein gene  
379 could take place. For this reason, codon optimization was performed on the GP gene  
380 sequence in M<sub>(-)</sub>, leading to a new construct designated as M<sub>(-)</sub>opt (*Fig. 5A*). Upon  
381 co-expression of L<sub>(+)</sub>opt, M<sub>(-)</sub>opt and the SR<sub>(+)</sub>eGFP mini-replicon in *N. benthamiana*  
382 eGFP fluorescence was observed in systemic leaves (*Fig. 5C*). Fluorescence was  
383 observed in 27 out of 30 agroinfiltrated plants, and demonstrated that M<sub>(-)</sub>opt  
384 produced a functional and stable M genomic RNA, able to replicate and support  
385 systemic movement of S and L RNP molecules by its encoded NSm protein (*Table 1*).  
386 RT-PCR analysis further confirmed a systemic infection of *N. benthamiana* with  
387 SR<sub>(+)</sub>eGFP, M<sub>(-)</sub>opt and L<sub>(+)</sub>opt (*Fig. S6D*).

388 In a final experiment, aiming to rescue “wild type” TSWV entirely from cDNA  
389 clones, the binary constructs of L<sub>(+)</sub>opt, M<sub>(-)</sub>opt and S<sub>(+)</sub> were agroinfiltrated into *N.*  
390 *benthamiana* leaves together with three VSRs (P19-HcPro- $\gamma$ b). At 19 dpi, typical leaf  
391 curling was observed in systemic leaves from *N. benthamiana* plants (*Fig. 6A* and  
392 *Table 1*). Upon disease progression, plants started to exhibit a stunted phenotype  
393 between 19-30 dpi. When the experiment was repeated with a large batch of plants, a  
394 systemic infection was observed in 6 out of 60 plants (*Table 1*). Northern blot  
395 analyses on samples collected from systemically infected leaves showed the presence  
396 of gRNA and agRNA of S, M and L RNA segments (*Fig. 6B*), which was also

397 confirmed by RT-PCR (Fig. S8). Moreover, sequence analysis of the amplicons  
398 derived from the L and M RNA confirmed the presence of codon optimized RdRp and  
399 GP gene sequences (Fig. 6C). Immunoblot analysis on systemically infected leaf  
400 samples showed the presence of N, NSs, NSm, Gn and Gc proteins *N. benthamiana*  
401 (Fig. 6D), altogether indicating a successful systemic infection with rescued TSWV  
402 (rTSWV).

403 To demonstrate genuine virus particle rescue of rTSWV, samples were collected  
404 from newly infected systemic leaf tissues and subjected to transmission electron  
405 microscopy. As shown in Fig. 6E, typical enveloped and spherical virus particles were  
406 observed in rTSWV-infected tissue, altogether indicating that infectious TSWV  
407 (rTSWV) was successfully rescued from full-length cDNA clones of L<sub>(+)</sub>opt, M<sub>(-)</sub>opt and  
408 S<sub>(+)</sub>.

409

## 410 **Discussion**

411 The establishment of a reverse genetics system for a segmented NSV basically  
412 requires two steps. The first one involves the *in vivo* reconstitution of transcriptionally  
413 active RNPs, often managed by development of a mini-genome replication system.  
414 The second step involves virus rescue entirely from full-length "infectious" cDNA  
415 clones, based on tools developed and optimized with the mini-genome replication  
416 system. In this study, we first successfully reconstituted infectious RNPs based on  
417 TSWV S<sub>(-)</sub>-gRNA and S<sub>(+)</sub>-agRNA after having optimized the sequence of RdRp.  
418 Next, a movement competent mini-genome replication system was developed based

419 on M<sub>(-)</sub>-gRNA, which was also able to complement and systemically rescue  
420 reconstituted S RNPs. In a third step, full length constructs were made for  
421 S<sub>(+)</sub>eGFP-agRNA, M<sub>(-)</sub>mCherry-gRNA and L<sub>(+)</sub>-agRNA, to directly accommodate for  
422 translation of (small amounts of) all three genomic (35S) transcripts into N, NSm and  
423 RdRp proteins, respectively, and leave out the additional need of ectopically  
424 expressed N and RdRp. *Agrobacterium*-mediated delivery of these constructs lead to a  
425 successful systemic infection of *N. benthamiana* with rTSWV carrying eGFP  
426 reporters. In a last step, the GP gene sequence of M<sub>(-)</sub> was optimized, that allowed the  
427 final rescuing of infectious rTSWV particles entirely from full-length cDNA clones in  
428 *N. benthamiana*.

429 The genomic RNAs of segmented NSVs possess neither a 5' cap-structure nor  
430 3'-poly(A) tail (2, 48). Instead, their termini contain highly conserved sequences that  
431 show inverted sequence complementary and fold into a panhandle structure with a  
432 major role in RNA transcription and replication. Any additional nucleotide residues at  
433 those termini in the past have been shown to disrupt/affect transcription-replication of  
434 animal-infecting segmented NSVs (49). For this reason, the choice of plant promoter  
435 to generate the first primary full-length genomic RNA templates (mimicking authentic  
436 genomic RNA molecules) for initiating viral replication is one of the major and  
437 critical factors for the construction of a reverse genetics system for TSWV. For  
438 animal-infecting segmented NSVs, researchers in the past have been using various  
439 systems. One of the first strategies employed bacteriophage T7 promoter constructs  
440 co-expressed with a T7 RNA polymerase and later followed by the use RNA

441 polymerase I (Pol I) promoter constructs to generate the initial viral genome length  
442 RNA transcripts in mammalian cells (39, 40, 50-52). Unfortunately, attempts to  
443 establish the TSWV mini-replicon system based on the T7 promoter and T7 RNA  
444 polymerase strategy was unsuccessful (Fig. S1A, C and D). The activity of the Pol I  
445 promoter was shown to be species-dependent (53). Although a Pol I promoter has  
446 been reported from *Arabidopsis* (54, 55), while the transcription initiation +1 site is  
447 still not known. For *N. benthamiana* no Pol I promoter has been characterized yet.  
448 The 35S promoter, an RNA Pol II promoter, is well characterized and hence remains  
449 the only choice to establish a reverse genetics system for TSWV in plants. This is be  
450 in contrast to reverse genetics of segmented NSVs in animal cells, where all viruses  
451 have been reconstituted after T7/Pol I driven production of primary viral RNA  
452 templates for replication. The Pol II promoter has been used to produce the initial  
453 viral RNA transcripts of an animal-infecting nonsegmented NSV (56). Recently, the  
454 35S/Pol II promoter was also successfully employed to produce primary viral RNA  
455 template of the first non-segmented plant NSV reconstituted, the SYNV rhabdovirus  
456 (42, 43). Here, we successfully deployed the 35S/Pol II promoter and two ribozymes  
457 at 5' and 3' ends of viral RNA sequences, to generate full length viral RNA transcripts  
458 that are recognized as initial/"authentic" RNA templates for TSWV replication and  
459 transcription by viral N and RdRp.

460 Besides the right promoter, the RdRp protein may present another bottleneck for  
461 the establishment of a reverse genetics system. Tospoviruses code for a single,  
462 unprocessed ~330 kDa RdRp from the 8.9 kb-sized L RNA (17, 18). The RdRp gene

463 sequence of TSWV was predicted to contain numerous intron splicing sites (*SI*  
464 *Appendix, Table S1*). Since the first animal segmented negative strand RNA virus was  
465 rescued in 1996 (39), numerous groups worldwide have attempted to construct a  
466 reverse genetics system for a tospovirus in plants. Here, it is shown that codon  
467 optimization and removal of potential intron splicing sites have been crucial for the  
468 expression of a functional RdRp of tospovirus from 35S-driven constructs *in planta*  
469 (*Fig. 1B*). While codon optimization may have contributed to increased protein  
470 expression levels, removal of predicted potential intron splicing sites from the RdRp  
471 genemay have helped to further stabilize and increase expression levels. After all,  
472 TSWV is known to replicate in the cytoplasm (2, 48), and its RdRp gene may not  
473 have been evolved to escape from the nuclear (pre-mRNA) splicing machinery.  
474 However, after nuclear transcription of the RdRp gene by the 35S promoter, any  
475 intron splicing site in the wild type RdRp transcript could thus be spliced and result in  
476 a truncated, non-functional RdRp.

477 Not only for RdRp, but also an optimized GP gene sequence turned out to be  
478 crucial to rescue a full length M RNA-based transcriptionally active RNP. Whereas  
479 the M<sub>(-)</sub>mCherry mini-replicon was able to establish a systemic infection in *N.*  
480 *benthamiana* when co-expressed with S<sub>(+)</sub>eGFP mini-replicon and L<sub>(+)</sub>opt, the wild type  
481 full length M segment did not. Like in the case with the RdRp gene sequence, the GP  
482 gene sequence of TSWV was also predicted to contain numerous intron splicing sites  
483 (*SI Appendix, Table S2*). The absence of antigenomic and genomic RNA strands from  
484 the wild type full length M replicon on Northern blots (*Fig. S7B*) indicated the

485 possibility that primary transcripts could have been prone to splicing in the GP  
486 sequence. This would not only lead to a loss of genome length RNA molecules, but  
487 also inhibit the production of NSm protein (either from direct translation of the  
488 primary M transcript, or after secondary transcription of NSm mRNA), needed for  
489 cell-to-cell and systemic movement of viral RNPs.

490 Not only the wild type sequence of L and M RNA segments may be spliced in the  
491 nucleus, also the S RNA segment generated by the 35S promoter could be prone to  
492 splicing. This is supported by the experiments in which *N. benthamiana* where  
493 infiltrated with the S<sub>(+)</sub>eGFP mini-replicon, M<sub>(-)</sub>opt and L<sub>(+)</sub>opt and resulted in 80% virus  
494 recovery (Table 1), but when only the S<sub>(+)</sub>eGFP mini-replicon was exchanged for the  
495 full length S<sub>(+)</sub> virus recovery dropped to 11.37 %. The very same reason may explain  
496 the low infection rate (10 %) when full length S<sub>(+)</sub>, M<sub>(-)</sub>opt and L<sub>(+)</sub>opt are co-expressed  
497 (Table 1). Although this could be due to the splicing of S RNA, the (residual) levels of  
498 full length S produced apparently have been sufficient to initiate viral replication.

499 Similar to other bunyaviruses (39, 52, 57), both the RdRp and N proteins are  
500 required for reconstitution of infectious RNPs complexes for TSWV (Fig 1C).  
501 However, high expression of either N or RdRp results in cell death and cause negative  
502 effects on the replication and transcription of TSWV. Moreover, ectopic expression of  
503 N and RdRp by the strong 35S promoter also seems to cause superinfection exclusion,  
504 as earlier observed and reported with various viruses infecting humans, animals, and  
505 plants (47, 58-60). During superinfection exclusion a preexisting infection of virus  
506 prevents a secondary infection with the same or a highly similar virus. It is an active

507 virus-controlled process that may be determined by a specific viral protein. For  
508 example, for potyvirus the coat protein and NIa protease have been identified to  
509 control superinfection exclusion (60). For TCV, the p28, involved in replication  
510 protein, was shown to confer superinfection exclusion as *a priori* expression of p28  
511 blocked (re-)infection with TCV (47). Ectopic expression of N and RdRp may also  
512 have blocked progeny L-, M- and S- RNAs from moving into neighboring plant cells.  
513 However, the presence of L-, M- and S- RNA segments in the same cells is a  
514 pre-requisite for the reconstitution of infectious TSWV and to systemic spread in *N.*  
515 *benthamiana*. Fortunately, direct expression of N from S<sub>(+)</sub> and RdRp from L<sub>(+)</sub><sup>opt</sup> have  
516 helped to overcome superinfection exclusion and to recover infectious TSWV.  
517 Whether this is due to the fact that N and RdRp are directly expressed from primary  
518 viral genome transcripts and simultaneously associate to progeny L-, M- and S- RNA  
519 segments in the same plant cells into infectious RNPs and/or involves a more  
520 fine-tuned protein expression relative to RNA segment replication remains unclear.  
521 Ectopic expression of NSs also inhibited the rescue of full length S<sub>(+)</sub> segment from  
522 cDNA. Since TSWV NSs significantly enhanced the replication of S and M  
523 mini-replicons lacking the NSs ORF, this indicated that the inhibition could relate to  
524 (simultaneous / *a priori*) ectopic expression of NSs gene sequences with overlap to  
525 the full length S<sub>(+)</sub> replicon. This could be tested by ectopic expression of an  
526 untranslatable NSs<sup>ΔATG</sup> construct.

527 In summary, a series of issues has hampered the construction of a successful  
528 TSWV reverse genetics system: the choice of promoter and construct design to

529 generate primary viral RNA transcripts in plants that mimick authentic viral RNA  
530 molecules, the expression of a very large viral RdRp, negative effects of ectopic  
531 expression of RdRp, N and NSs, and the absence of viral RNA synthesis of the wild  
532 type M<sub>(-)</sub> segment. In this study, we have been able to solve all these issues and  
533 successfully managed to establish a reverse genetics system for the tripartite RNA  
534 genome of TSWV. Using the S RNA mini-replicon system containing eGFP and  
535 mCherry reporter genes, the role of *cis*-and *trans*-acting elements for viral replication  
536 and transcription can be studied. Using the M-RNA mini-replicon system cell-to-cell  
537 movement of TSWV RNPs *in planta* can be studied. To track the virus during  
538 systemic infection of plants rTSWV can be generated containing fluorescent reporter  
539 genes at the genetic loci of either GP or NSs. The establishment of this RG system  
540 now provides us with a new and powerful platform to generate mutant viruses and  
541 study their disease pathology in a natural setting, including basic principles of all  
542 tospovirus life cycle and viral pathogenicity. As a personal communication, Jeanmarie  
543 Verchot's group has also recovered the Rose rosette emaravirus entirely from cDNA  
544 clones, a plant NSV with 7 RNA segments. The establishment of these RG systems  
545 presents the start of a new research era for the segmented plant NSVs.

546

## 547 **Materials and Methods**

548 Details of the methodology used are provided in *SI Appendix, Materials and Methods*,  
549 and include plasmid construction, plant material and growth conditions,  
550 agro-infiltration, immunoblot analysis, Northern blot analysis, RT-PCR, GFP imaging,

551 fluorescence microscopy and Electron microscopy. Primers used in this study are  
552 listed in *SI Appendix, Table S3*.

553

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560

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714 **Table 1.** Systemic infection rate of recombinant TSWV rescued in *N. benthamiana* in  
715 the presence of viral suppressors of RNA silencing (VSRs).

Antigenome and genome derivatives	VSRs	Systemic infection (No. of infected/inoculated plants)
S <sub>(-)</sub> +M <sub>(-)</sub> +L <sub>(-)</sub>	N+RdRp+NSs+P19-HcPro- $\gamma$ b	0 % (0/30)
S <sub>(+)</sub> +M <sub>(+)</sub> +L <sub>(+)</sub>	N+RdRp+NSs+P19-HcPro- $\gamma$ b	0 % (0/30)
SM <sub>(+)</sub> eGFP+MR <sub>(-)</sub> mCherry+L <sub>(+)</sub> opt	NSs+P19-HcPro- $\gamma$ b	80 % (24/30)
S <sub>(+)</sub> +MR <sub>(-)</sub> eGFP+L <sub>(+)</sub> opt	P19-HcPro- $\gamma$ b	11.37 % (7/60)
S <sub>(+)</sub> +MR <sub>(-)</sub> eGFP+L <sub>(+)</sub> opt	NSs+P19-HcPro- $\gamma$ b	0 % (0/60)
SR <sub>(+)</sub> eGFP+M <sub>(-)</sub> +L <sub>(+)</sub> opt	P19-HcPro- $\gamma$ b	0 % (0/60)
SR <sub>(+)</sub> eGFP+M <sub>(-)</sub> +L <sub>(+)</sub> opt	NSs+P19-HcPro- $\gamma$ b	0 % (0/60)
SR <sub>(+)</sub> eGFP+M <sub>(-)</sub> opt+L <sub>(+)</sub> opt	NSs+P19-HcPro- $\gamma$ b	90 % (27/30)
S <sub>(+)</sub> +M <sub>(-)</sub> opt+L <sub>(+)</sub> opt	P19-HcPro- $\gamma$ b	10 % (6/60)

716

717 Mixture of *Agrobacterium* cultures harboring the plasmids encoding each of the S, M, L and  
718 derivatives (final concentration OD<sub>600</sub>=0.2), RdRp (OD<sub>600</sub>=0.2), N (OD<sub>600</sub>=0.2) and VSRs  
719 (OD<sub>600</sub>=0.05) were infiltrated into *N. benthamiana* leaves. Systemic infection was scored  
720 15-30 dpi.

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728 **Figure Legends:**

729 **Fig. 1** Construction of a TSWV S<sub>(-)</sub> RNA-based mini-replicon system in *N.*  
730 *benthamiana*. (A) Schematic representation of binary constructs to express TSWV S<sub>(-)</sub>  
731 mini-replicon, TSWV N, RdRp and four RNA silencing suppressors (VSRs: NSs, P19,  
732 HcPro and  $\gamma$ b) proteins by agroinfiltration into *N. benthamiana*. The S<sub>(-)</sub>-gRNA of  
733 TSWV is shown on the top. SR<sub>(-)</sub>mCherry&eGFP: the NSs and N of S<sub>(-)</sub>-gRNA were  
734 replaced by mCherry and eGFP, respectively. (-) refers to the negative  
735 (genomic)-strand of S RNA; 2 $\times$ 35S: a double 35S promoter; HH: hammerhead  
736 ribozyme; RZ: Hepatitis Delta virus (HDV) ribozyme; NOS: nopaline synthase  
737 terminator. (B) Foci of eGFP and mCherry fluorescence in *N. benthamiana* leaves  
738 co-expressing SR<sub>(-)</sub>mCherry&eGFP, RdRp, N and four VSRs at 5 days post infiltration (dpi)  
739 under a fluorescence microscope. The bar represents 400  $\mu$ m. (C) Analysis of RdRp  
740 and N requirement for SR<sub>(-)</sub>mCherry&eGFP mini-genome replication in *N. benthamiana*  
741 leaves. SR<sub>(-)</sub>mCherry&eGFP was coexpressed with pCB301 empty vector (Vec), N, RdRp  
742 or both in *N. benthamiana* leaves by agroinfiltration. Agro-infiltrated leaves were  
743 examined and photographed at 5 dpi under a fluorescence microscope. Signal shown  
744 reflects a merge of mCherry and eGFP fluorescence from both reporter genes. Bar  
745 represents 400  $\mu$ m. (D) Immunoblot analysis on the expression of N and eGFP  
746 proteins in the leaves shown in panel (C) using specific antibodies against N and GFP,  
747 respectively. Ponceau S staining of rubisco large subunit is shown for protein loading  
748 control. (E) Northern blot analysis of S<sub>(-)</sub>-mini-replicon replication and transcription  
749 in the presence of N, RdRp or both in *N. benthamiana*. The S RNA genomic,

750 anti-genomic and subgenomic transcripts (eGFP mRNA) were detected by  
751 DIG-labeled sense eGFP or anti-sense eGFP probes. The red and blue arrows indicate  
752 the anti-genomic and genomic RNAs of SR<sub>(-)m</sub>Cherry&eGFP, respectively. The green  
753 arrow indicates the eGFP mRNA transcript. Ethidium bromide staining of ribosomal  
754 RNA (rRNA) was used as RNA loading control.

755

756 **Fig. 2** Optimization of the SR<sub>(-)m</sub>Cherry&eGFP mini-replicon system. (A) Optimizing the  
757 concentration of N and RdRp proteins for replication and transcription of  
758 SR<sub>(-)m</sub>Cherry&eGFP in *N. benthamiana* leaves. Increasing amounts of *Agrobacterium*,  
759 from OD<sub>600</sub>= 0.2 to 0.8 and containing the binary expression constructs for N (upper  
760 panels) or RdRp (bottom panels), were mixed with fixed amounts of *Agrobacterium*  
761 containing the RdRp or N construct (OD<sub>600</sub> 0.2), respectively, and their effects on  
762 eGFP reporter expression were visualized under a fluorescence microscope at 5 dpi.  
763 Bars represent 400  $\mu$ m. (B) and (C) Western immunoblot detection of the N and eGFP  
764 proteins expressed in the leaves shown in panel (A) using specific antibodies against  
765 N and GFP, respectively. (D) Optimization of RNA silencing suppressors (VSRs) on  
766 SR<sub>(-)m</sub>Cherry&eGFP mini-reporter replication and transcriptions as measured by eGFP and  
767 mCherry expression. The SR<sub>(-)m</sub>Cherry&eGFP, N and RdRp proteins were co-expressed  
768 with pCB301 empty vector (Vec), NSs, P19-HcPro- $\gamma$ b or all four VSRs in *N.*  
769 *benthamiana* leaves. Foci expressing eGFP and mCherry in agroinfiltrated leaves  
770 were visualized under a fluorescence microscope at 5 dpi. Bars represent 400  $\mu$ m. (E)  
771 Western immunoblot detection of N and eGFP protein synthesis in the leaves shown

772 in panel (D) using N and GFP-specific antibodies, respectively. Ponceau S staining  
773 was used as protein loading control.

774

775 **Fig. 3** Development of the S<sub>(+)</sub>-agRNA mini-replicon system in *N. benthamiana*. (A)  
776 Schematic representation of the TSWV SR<sub>(+)</sub>eGFP mini-replicon. The NSs gene of  
777 TSWV S agRNA was replaced by eGFP. Anti-genomic RNA strands of the SR<sub>(+)</sub>eGFP  
778 mini-replicon are transcribed from a double 35S promoter (2×35S), and flanked by a  
779 HH ribozyme and HDV ribozyme (RZ) sequence. (+) refers to the positive  
780 (antigenomic)-strand of S RNA. (B) Foci of eGFP fluorescence in *N. benthamiana*  
781 leaves co-expressing TSWV SR<sub>(+)</sub>eGFP with pCB301 empty vector (Vec), N, RdRp or  
782 N+RdRp by agroinfiltration. The agroinfiltrated leaves were photographed at 3 dpi  
783 under a fluorescence microscope. Bars represent 400 μm. (C) Western immunoblot  
784 detection of N and eGFP protein synthesis in the leaves shown in panel (B) using N-  
785 and GFP-specific antibodies, respectively. Ponceau S staining was used as a protein  
786 loading control. (D) Northern blot analysis of the replication and transcription of  
787 SR<sub>(+)</sub>eGFP mini-replicon in *N. benthamiana* co-expressed with empty vector (Vec), N,  
788 RdRp or both. The anti-genomic RNAs (red arrow), genomic RNAs (blue arrow) and  
789 eGFP mRNA transcripts (green arrow) were detected with DIG-labeled sense and  
790 anti-sense eGFP probes, respectively. Ethidium bromide staining was used as RNA  
791 loading control.

792

793 **Fig. 4** Establishment of a TSWV M<sub>(-)</sub>-gRNA based mini-replicon system with

794 cell-to-cell movement competency in *N. benthamiana*. (A) Schematic representation  
795 of the TSWV MR<sub>(-)</sub>eGFP mini-replicon and its mutant derivative MR<sub>(-)</sub>eGFP&NSmMut. The  
796 GP gene of TSWV M<sub>(-)</sub>gRNA was substituted by eGFP. The genomic RNA of the  
797 mini-replicon is transcribed from a double 35S promoter (2×35S) and flanked by a  
798 Hammerhead (HH) ribozyme and HDV ribozyme (RZ). For MR<sub>(-)</sub>eGFP&NSmMut, a stop  
799 codon was introduced immediately after the start codon of the NSm gene in the  
800 MR<sub>(-)</sub>eGFP mini-replicon. (B) Foci of eGFP fluorescence expressed from the MR<sub>(-)</sub>eGFP  
801 or MR<sub>(-)</sub>eGFP&NSmMut mini-replicon in *N. benthamiana* leaves co-expressed with the  
802 empty vector (Vec), N, RdRp or N+RdRp by agroinfiltration. Agroinfiltrated leaves  
803 were photographed at 4 dpi under a fluorescence microscope. Bars represent 400 μm.  
804 (C) Western immunoblot detection of N and eGFP protein synthesis in the leaves  
805 shown in panel (B) with specific antibodies against N and GFP, respectively. Ponceau  
806 S staining was used as protein loading control. (D) Northern blot analysis of the  
807 replication and transcription of the MR<sub>(-)</sub>eGFP mini-replicon co-expressed with Vec, N,  
808 RdRp or N+RdRp in *N. benthamiana* leaves. The anti-genomic RNAs (red arrow),  
809 genomic RNAs (blue arrow) and eGFP mRNA transcripts (green arrow) were detected  
810 with DIG labeled sense and anti-sense eGFP probes, respectively. Ethidium bromide  
811 staining was used as RNA loading control.

812

813 **Fig. 5** Establishment of a systemic infection in *N. benthamiana* with replicons S<sub>(+)</sub> and  
814 M<sub>(-)</sub> co-expressed with full length antigenomic L<sub>(+)</sub>opt. (A) Schematic representation of  
815 constructs expressing TSWV full length antigenomic L<sub>(+)</sub>opt with optimized RdRp, full

816 length genomic  $M_{(-)opt}$  with optimized GP,  $MR_{(-)eGFP}$ ,  $MR_{(-)mCherry}$ , full length  
817 antigenomic  $S_{(+)}$  and  $SR_{(+)eGFP}$ . Primary viral RNA transcripts are transcribed from a  
818 double 35S promoter ( $2 \times 35S$ ) and flanked by a HH and HDV ribozyme (RZ). (B)  
819 eGFP and mCherry fluorescence in *N. benthamiana* resulting from systemic infection  
820 of agroinfiltrated  $SR_{(+)eGFP}$ ,  $MR_{(-)mCherry}$  and  $L_{(+)opt}$  constructs. The systemic infected  
821 plants (*a* and *b*) and leaves (*c* and *d*) were photographed at 21 dpi under white light  
822 and (hand-held) ultraviolet (UV) light. Foci of eGFP and mCherry fluorescence in  
823 leaves shown in panel *d* as visualized under a fluorescence microscope. Bar represents  
824 400  $\mu$ m. (C) eGFP fluorescence in *N. benthamiana* resulting from systemic infection  
825 of agroinfiltrated  $SR_{(+)eGFP}$ ,  $M_{(-)opt}$  and  $L_{(+)opt}$  constructs. Infected plants (*a* and *b*) and  
826 leaves (*c* and *d*) were photographed at 18 dpi under white light and (hand-held) UV  
827 light, respectively. (D) eGFP fluorescence in *N. benthamiana* resulting from systemic  
828 infection of agroinfiltrated  $S_{(+)}$ ,  $MR_{(-)eGFP}$  and  $L_{(+)opt}$  constructs. Infected plants (*a* and  
829 *b*) and leaves (*c* and *d*) were photographed at 50 dpi under white light and (hand-held)  
830 UV light, respectively.

831

832 **Fig. 6** Rescue of infectious TSWV from full-length cDNA clones in *N. benthamiana*.  
833 (A) Systemic infection of *N. benthamiana* plants with rescued TSWV (rTSWV)  
834 resulting from agoinfiltration of  $S_{(+)}$ ,  $M_{(-)opt}$  and  $L_{(+)opt}$  and three VSRs (P19, HcPro  
835 and  $\gamma b$ ). The plant agroinfiltrated with pCB301 empty vector was used as a mock  
836 control. Images were taken at 19 dpi. Boxed areas of the plants that show stunting,  
837 mosaic and leaf curling are shown enlarged in the right panels. (B) Sequence

838 confirmation of codon optimized sequences of the GP gene (from the M<sub>(-)</sub>opt RNA  
839 segment) and the RdRp gene (from the L<sub>(+)</sub>opt RNA segment) on RT-PCR fragments  
840 obtained from systemic leaves of *N. benthamiana* infected with rTSWV. The  
841 optimized sequence of GP from rTSWV is underlined by a blue dashed line, and wild  
842 type GP sequence underlined in blue. The 3'-untranslated region (UTR) sequence of  
843 the M genomic RNA is marked with a yellow line. The optimized sequence of RdRp  
844 from rTSWV is underlined by a red dashed line, and wild type RdRp sequence  
845 underlined in red. The 3'-UTR sequence of the L genomic RNA is marked with a  
846 purple line. The stars indicate the codon optimization sites of GP and RdRp gene  
847 sequences. (C) Northern blot detection of viral RNA from the S, M and L RNA  
848 segment, respectively, in systemic leaves of *N. benthamiana* infected with rTSWV.  
849 The anti-genomic RNAs (red arrow) and genomic RNAs (blue arrow) were detected  
850 with DIG labeled sense and anti-sense NSs-, NSm- and L-5'UTR probes, respectively.  
851 Lane 1 and 2 refer to two independent replicates. Ethidium bromide staining was used  
852 as RNA loading control. (D) Western immunoblot detection of the N, NSm, NSs, Gc,  
853 and Gn proteins from leaves systemically infected with rTSWV, using specific  
854 antibodies against N, NSm, NSs, Gc, and Gn, respectively. Leaves infected with  
855 wild-type TSWV were used as a positive control. Ponceau S staining was used as  
856 protein loading control. (E) Electron micrographs of thin sections of *N. benthamiana*  
857 plants infected with rTSWV. Boxed regions in the left panels and showing the  
858 presence of virions, are shown enlarged in the right panels. Spherical enveloped virus  
859 particles are indicated (white arrow head). Bars represent 0.2  $\mu$ m.

860 **Supplemental Figure Legends**

861 **Fig. S1** Functional analysis of wild type RdRp and the use of T7 promoter in a  
862 mini-genome replication assay. (A) Schematic diagram of TSWV 35S:SR<sub>(-)</sub>mCherry&eGFP  
863 and T7:SR<sub>(-)</sub>mCherry&eGFP mini-replicon reporters. (B) The wild type RdRp (RdRp<sub>wt</sub>) or  
864 the empty vector (Vec) was co-expressed with 35S:SR<sub>(-)</sub>mCherry&eGFP, N, VSRs (VSRs:  
865 NSs, P19, HcPro and  $\gamma$ b) in *N. benthamiana* leaves. The expression of eGFP was  
866 examined by a fluorescence microscope. (C) Constructs coding for  
867 T7:SR<sub>(-)</sub>mCherry&eGFP, T7 RNA polymerase (pol), N and VSRs were co-expressed with  
868 RdRp<sub>wt</sub> or RdRp<sub>opt</sub> in *N. benthamiana* leaves. Replication of T7:SR<sub>(-)</sub>mCherry&eGFP was  
869 examined by monitoring for eGFP fluorescence with a fluorescence microscope.  
870 Empty vector (Vec) pCB301 was used as a negative control. Bars represent 200  $\mu$ m.  
871 (D) Western immunoblot detection of T7 RNA pol using a T7 RNA pol specific  
872 antibody. Ponceau S staining was used as protein loading control. Lane 1: sample  
873 from leaves co-expressing T7:SR<sub>(-)</sub>mCherry&eGFP, T7 RNA Pol, N, VSRs and RdRp<sub>wt</sub>;  
874 lane 2: sample from leaves co-expressing T7:SR<sub>(-)</sub>mCherry&eGFP, T7 RNA Pol, N, VSRs  
875 and RdRp<sub>opt</sub>.

876

877 **Fig. S2** Time course analysis on gene expression from the SR<sub>(-)</sub>mCherry&eGFP  
878 mini-replicon in *N. benthamiana* leaves. (A) Foci of eGFP and mCherry fluorescence  
879 expressed from SR<sub>(-)</sub>mCherry&eGFP in *N. benthamiana* leaves co-expressing N, RdRp and  
880 the VSRs at 3, 6, 9 and 12 dpi, respectively. Fluorescence of eGFP and mCherry were  
881 photographed under a fluorescence microscope using GFP and RFP filters,

882 respectively. Bars represent 400  $\mu$ m. (B) Western immunoblot detection of the N,  
883 eGFP and mCherry proteins in leaves shown in panel A, using specific antibodies  
884 against N, GFP and mCherry, respectively. The empty vector (Vec) was used as a  
885 negative control. Ponceau S staining was used as protein loading control.

886

887 **Fig. S3** The role of 5'-UTR, 3'-UTR and IGR on viral RNA synthesis from the  
888 SR<sub>(-)</sub>mCherry&eGFP mini-replicon. (A) Schematic representation of TSWV  
889 SR<sub>(-)</sub>mCherry&eGFP and derivatives with deletions of the 5'UTR, IGR or 3'UTR. (B) eGFP  
890 and mCherry fluorescence expressed from TSWV SR<sub>(-)</sub>mCherry&eGFP and mutant  
891 derivatives in *N. benthamiana*. The SR<sub>(-)</sub>mCherry&eGFP or its mutants were coexpressed  
892 with N, RdRp and the four VSRs in *N. benthamiana* leaves. The agroinfiltrated leaves  
893 were examined and photographed at 5 dpi under a fluorescence microscope using  
894 GFP and RFP filters, respectively. Bars represent 200  $\mu$ m. (C) Western immunoblot  
895 detection of the N and eGFP proteins expressed from the SR<sub>(-)</sub>mCherry&eGFP  
896 mini-replicon and mutant derivatives, using specific antibodies against N and GFP,  
897 respectively. Ponceau S staining was used as protein loading control. (D) Northern  
898 blot analysis of viral RNA synthesis from SR<sub>(-)</sub>mCherry&eGFP and mutant derivatives. The  
899 anti-genomic RNAs (red arrow), genomic RNAs (blue arrow) and eGFP mRNA  
900 transcripts (green arrow) were detected with DIG-labeled sense eGFP or anti-sense  
901 eGFP probes. Ethidium bromide staining was used as RNA loading control.

902

903 **Fig. S4** Cell-to-cell movement analysis of SR<sub>(-)</sub>GFP and MR<sub>(-)</sub>mCherry in *N. benthamiana*

904 co-expressing RdRp, N and four VSRs in *N. benthamiana*. Agroinfiltrated leaves were  
905 examined and photographed at 5 dpi under a fluorescence microscope. Bars represent  
906 400  $\mu$ m.

907

908 **Fig. S5** Functional analysis of RdRp expressed from TSWV L<sub>(+)</sub>opt, NSm from  
909 MR<sub>(-)</sub>eGFP and N from SR<sub>(+)</sub>eGFP using the mini-genome replication system in *N.*  
910 *benthamiana*. (A) Functional analysis of RdRp expressed from TSWV L<sub>(+)</sub>opt using the  
911 S RNA mini-replicon system in *N. benthamiana*. The L<sub>(+)</sub>opt, RdRp, or pCB301 empty  
912 vector (Vec) was co-expressed with N, SR<sub>(-)</sub>mCherry&eGFP and the four VSRs in *N.*  
913 *benthamiana* leaves. (B) Functional analysis of N expressed from SR<sub>(+)</sub>eGFP in *N.*  
914 *benthamiana*. SR<sub>(+)</sub>eGFP was co-expressed with the empty vector (Vec), N, RdRp or  
915 N+L<sub>(+)</sub>opt in *N. benthamiana* leaves in the presence of four VSRs. (C) Functional  
916 analysis of NSm expressed from MR<sub>(-)</sub>eGFP in *N. benthamiana*. MR<sub>(-)</sub>eGFP was  
917 co-expressed with the empty vector (Vec), N, RdRp or N+L<sub>(+)</sub>opt in *N. benthamiana*  
918 leaves in the presence of four VSRs. Foci showing mCherry and eGFP fluorescence in  
919 agroinfiltrated *N. benthamiana* leaves were examined at 3 dpi by a fluorescence  
920 microscope. Bars represent 400  $\mu$ m.

921

922 **Fig. S6** Analysis of *N. benthamiana* leaves agroinfiltrated with constructs of SR<sub>(+)</sub>eGFP,  
923 MR<sub>(-)</sub>mCherry and L<sub>(+)</sub>opt, or SR<sub>(+)</sub>eGFP, MR<sub>(-)</sub>opt and L<sub>(+)</sub>opt or S<sub>(+)</sub>, MR<sub>(-)</sub>eGFP and L<sub>(+)</sub>opt. (A)  
924 Local infection analysis of cell-to-cell movement of SR<sub>(+)</sub>eGFP and MR<sub>(-)</sub>mCherry  
925 co-expressing with L<sub>(+)</sub>opt and four VSRs in *N. benthamiana* by agroinfiltration. The

926 agro-infiltrated leaves were examined and photographed at 5 dpi under a fluorescence  
927 microscope. Bars represent 400  $\mu$ m. (B) RT-PCR analysis on systemically infected  
928 leaves from *N. benthamiana* plants agroinfiltrated with SR<sub>(+)</sub>eGFP, MR<sub>(-)</sub>mCherry and  
929 L<sub>(+)</sub>opt. (C) RT-PCR analysis on systemically infected leaves from *N. benthamiana*  
930 plants agroinfiltrated with S<sub>(+)</sub>, MR<sub>(-)</sub>eGFP and L<sub>(+)</sub>opt (D) RT-PCR analysis on  
931 systemically infected leaves from *N. benthamiana* plants agroinfiltrated with SR<sub>(+)</sub>eGFP,  
932 M<sub>(-)</sub>opt and L<sub>(+)</sub>opt. All agroinfiltrations were performed in the additional presence four  
933 VSRs constructs. RT PCR was performed on total RNA purified from systemic leaves  
934 for detection of S, M or L segments using segment-specific primers. Amplicons were  
935 resolved by electrophoresis in a 1 % agarose gel. Lanes 1-2 represent two biological  
936 replicates of systemic infected leaf samples; As positive controls (CK<sup>+</sup>) for proper  
937 fragment size, PCR was performed on plasmids carrying S, M , L<sub>(+)</sub>opt or derivatives.  
938 As negative control (CK<sup>-</sup>), RT-PCR was performed in the absence of nucleic acids.  
939 DNA size markers are shown on the left hand side.

940

941 **Fig. S7** Northern blot detection of viral RNA synthesis produced from full length S<sub>(+)</sub>  
942 and wild type M<sub>(-)</sub> replicons. (A and B) The full length S<sub>(+)</sub> or wild type M<sub>(-)</sub> was  
943 co-expressed with the empty vector (Vec), N, RdRp<sub>opt</sub> or N+RdRp<sub>opt</sub> in *N.*  
944 *benthamiana* leaves in the presence of four VSRs. The genomic RNAs (blue arrow),  
945 anti-genomic RNAs (red arrow) of S (A) and M (B) were detected with DIG-labeled  
946 sense or anti-sense NSs and NSm probes, respectively. Ethidium bromide staining  
947 was used as RNA loading control.

948

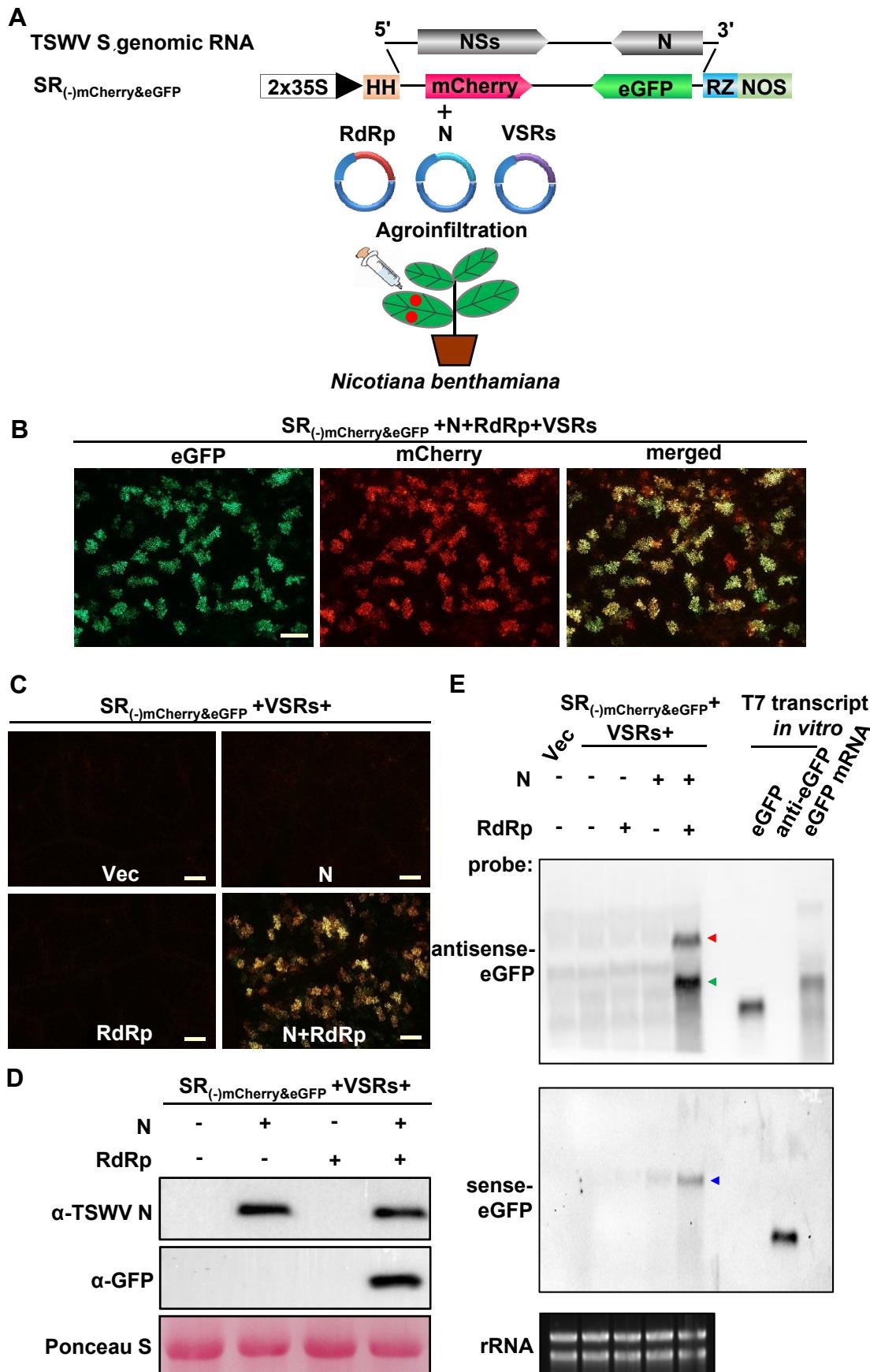
949 **Fig. S8** RT-PCR detection of S<sub>(+)</sub>, M<sub>(-)opt</sub> and L<sub>(+)opt</sub> genomic RNA in systemic leaves  
950 of *N. benthamiana* infected by rTSWV. The S<sub>(+)</sub>, M<sub>(-)opt</sub> and L<sub>(+)opt</sub> and the four VSRs  
951 were co-expressed in *N. benthamiana* leaves by agroinfiltration. Total RNA was  
952 purified from systemic leaves of agroinfiltrated plants and the presence of S<sub>(+)</sub>, M<sub>(-)opt</sub>  
953 and L<sub>(+)opt</sub> were detected by RT-PCR using segment-specific primers. RT-PCR  
954 products were resolved by electrophoresis in a 1% agarose gel. Lanes 1-2, two  
955 biological replicates of systemic infected leaf samples; RT-PCR on plasmids carrying  
956 S, M and L as DNA template were used as positive controls (CK<sup>+</sup>). RT-PCR without  
957 adding the DNA template was used as negative controls (CK<sup>-</sup>). DNA size markers are  
958 shown on the left hand side.

959

960 **Fig. S9** Optimized RdRp gene sequence used in the study.

961

962 **Fig. S10** Optimized GP gene sequence used in the study.



**Fig. 1**

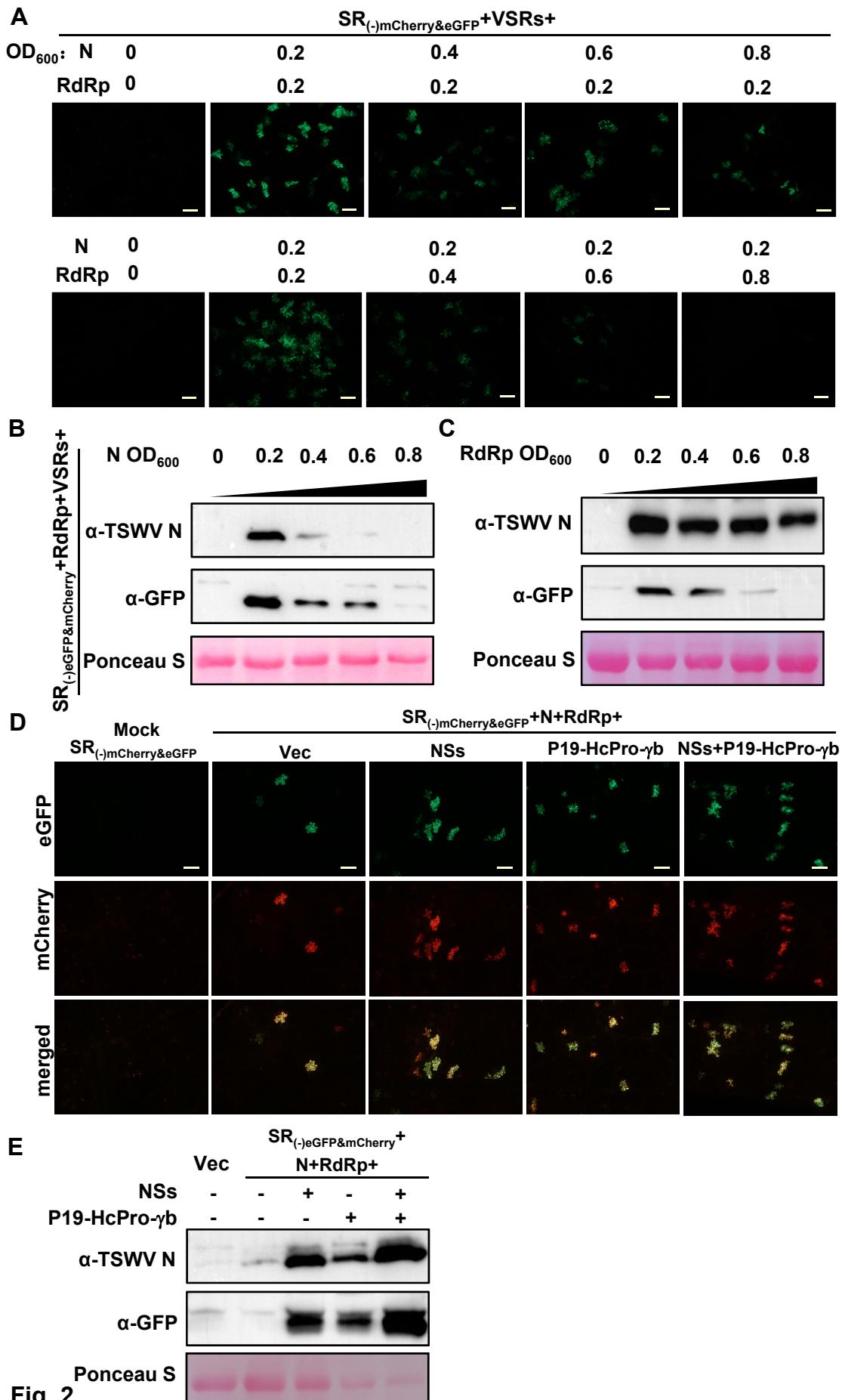
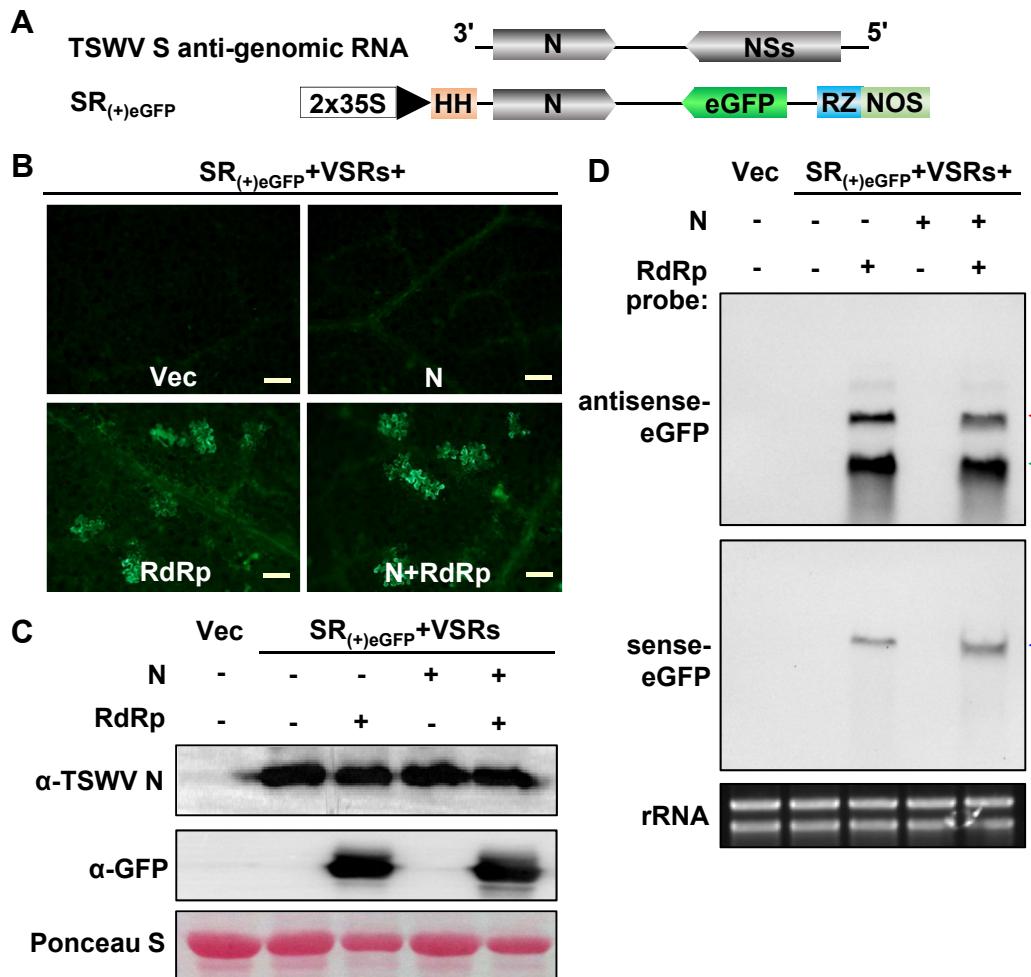
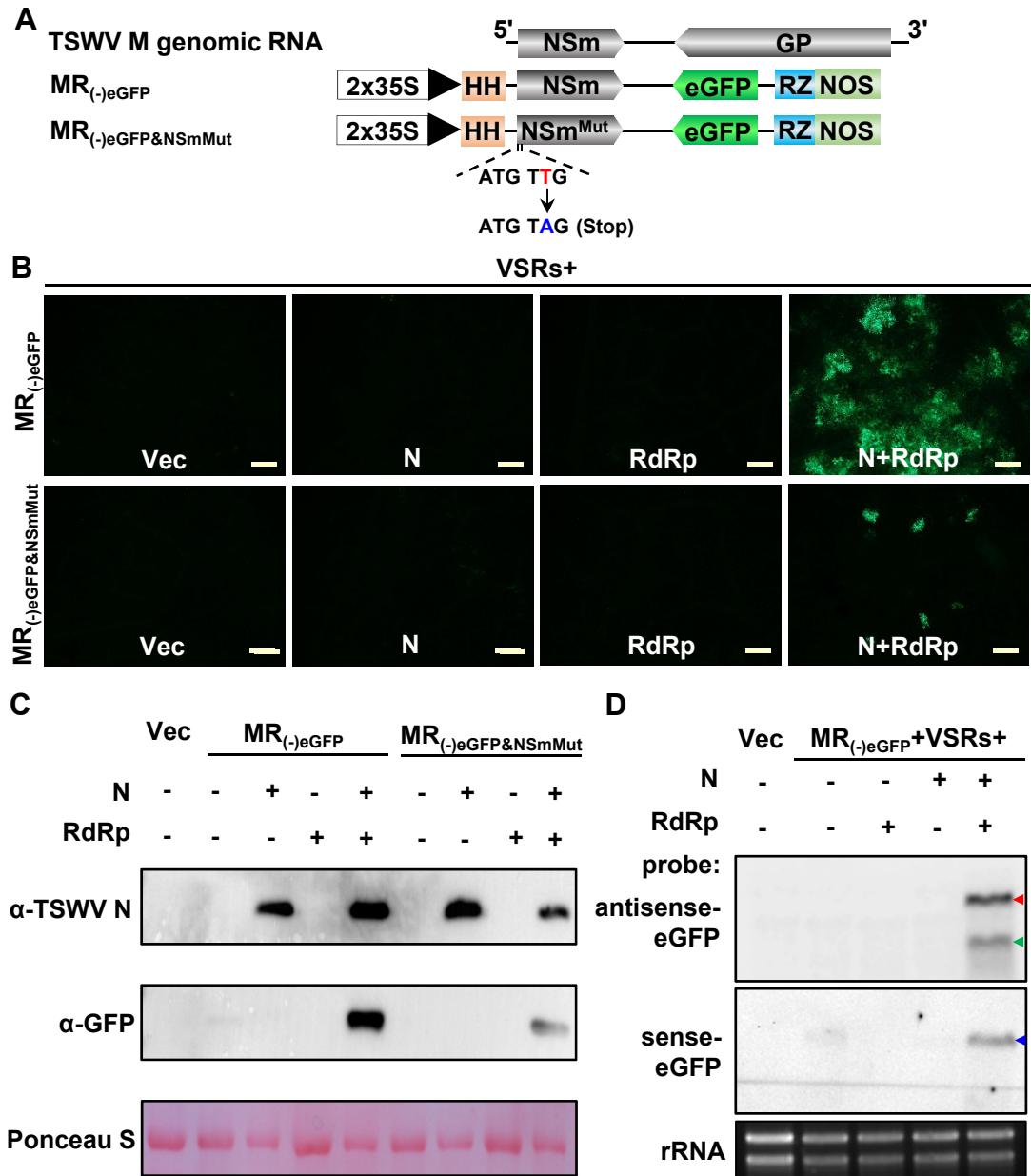


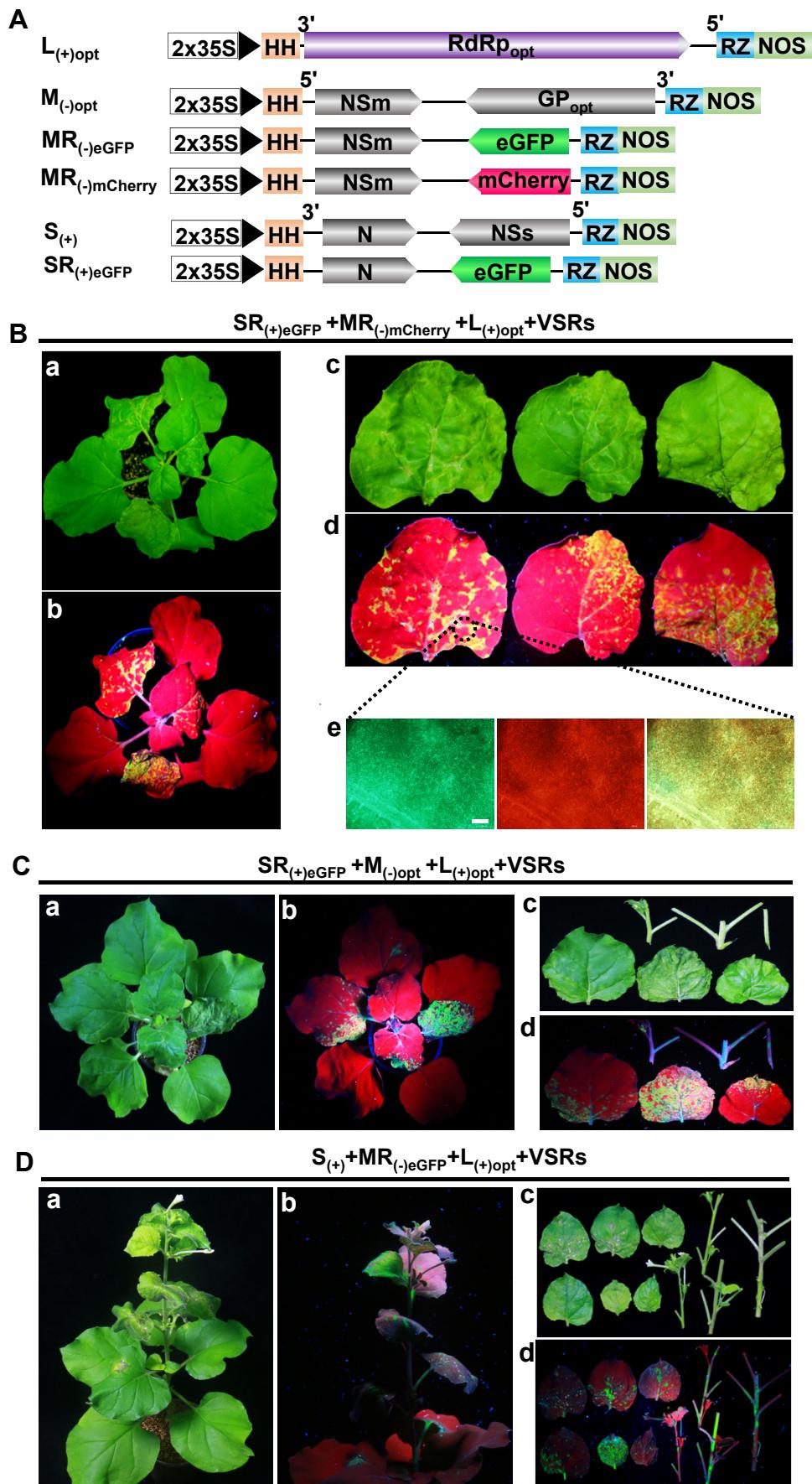
Fig. 2



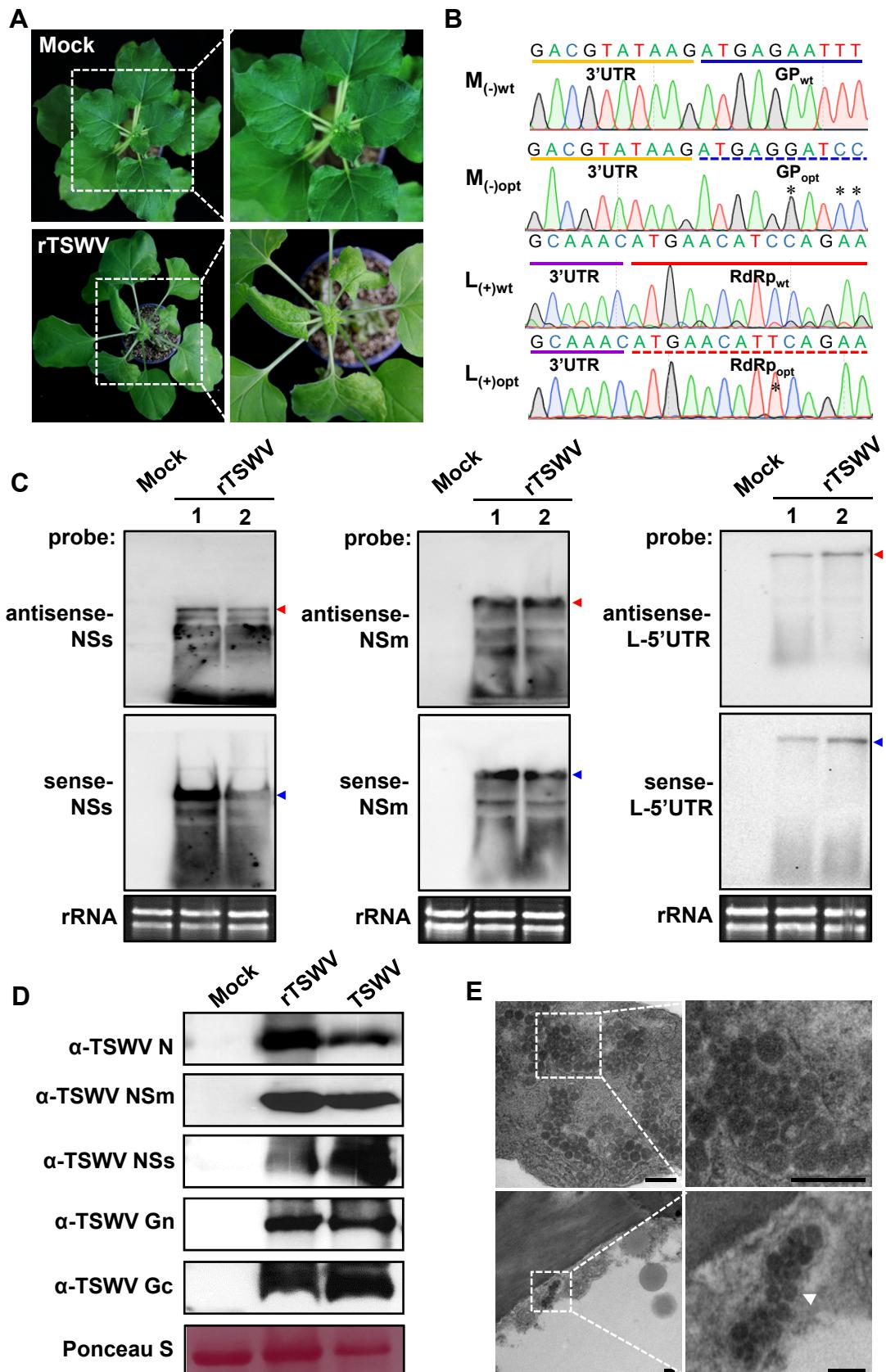
**Fig. 3**



**Fig. 4**



**Fig. 5**



**Fig. 6**

1    **Supplementary Materials and Methods**

2    **Plasmid construction**

3    **Construction of RdRp, RdRp<sub>opt</sub>, N, NSs and VSRs.** The cDNA of the RdRp, N, and NSs  
4    genes was amplified from the total RNA of TSWV-lettuce isolate infected tissues and  
5    inserted into a binary vector pCambia2300 or pCXSN to generate p2300-RdRp<sub>wt</sub>, p2300-  
6    N, and pCXSN-NSs downstream a double 35S promoter (2×35S). The P19-HcPro- $\gamma$ b  
7    carrying three VSRs simultaneously in the pCB301 vector was kindly provided by Dr.  
8    Xianbing Wang in College of Biological Sciences of China Agricultural University. The  
9    codon usage and intron-splicing sites optimized sequence of RdRp (*SI Appendix, Fig. S9*)  
10   was *de novo* synthesized by GenScript Biotech Corp (Nanjing, China) and was inserted  
11   into a binary vector pCambia2300 to generate p2300S-RdRp<sub>opt</sub> downstream a 2×35S  
12   promoter.

13   **Construction of full length TSWV genomic S<sub>(-)</sub>, M<sub>(-)</sub>, L<sub>(-)</sub>, and anti-genomic S<sub>(+)</sub>, M<sub>(+)</sub> and**  
14   **L<sub>(+)</sub> cDNA clones.** To generate constructs to express full length TSWV genomic RNA and  
15   antigenomic RNA S, M and L segments, total RNA extracted from TSWV-lettuce infected  
16   leaves of *N. benthamiana* plants was reverse transcribed into cDNA, followed by PCR  
17   amplification with specific primers (*SI Appendix, Table S3*) using Phanta Super-Fidelity  
18   DNA Polymerase (Vazyme Biotech, Nanjing, China). The PCR products were fused with  
19   a self-cleaving hammerhead (HH) ribozyme (1) and inserted into a binary expression  
20   vector pCB301-2×35S-RZ-NOS linearized by two restriction endonucleases *Stu* I and *Sma*  
21   I (2). The pCB301-2×35S-HH-S<sub>(-)</sub>-RZ-NOS [S<sub>(-)</sub>], pCB301-2×35S-HH-M<sub>(-)</sub>-RZ-NOS  
22   [M<sub>(-)</sub>], pCB301-2×35S-HH-L<sub>(-)</sub>-RZ-NOS [L<sub>(-)</sub>], pCB301- pCB301-2×35S-HH-S<sub>(+)</sub>-RZ-  
23   NOS [S<sub>(+)</sub>], 2×35S-HH-M<sub>(+)</sub>-RZ-NOS [M<sub>(+)</sub>] and pCB301-2×35S-HH-L<sub>(+)</sub>-RZ-NOS [L<sub>(+)</sub>]

24 cDNA clones were generated. The full length TSWV genomic S<sub>(-)</sub>, M<sub>(-)</sub> and L<sub>(-)</sub>, and anti-  
25 genomic S<sub>(+)</sub>, M<sub>(+)</sub> and L<sub>(+)</sub> were expressed downstream a double 35S promoter (2×35S)  
26 and franked with a self-cleaving hammerhead (HH) ribozyme at 5'-terminus and a Hepatitis  
27 delta virus (HDV) ribozyme at 3'-terminus.

28 ***Construction of TSWV SR<sub>(-)eGFP</sub>, SR<sub>(-)mCherry&eGFP</sub> and SR<sub>(+)eGFP</sub> minireplicons.*** To  
29 generate SR<sub>(-)eGFP</sub>-genomic RNA minireplicon, the eGFP ORF was amplified and used to  
30 replace the N gene in the pCB301-2×35S-HH-S<sub>(-)</sub>-RZ-NOS by the *in vitro* recombination  
31 using the In-Fusion Cloning mixture (Clontech, Japan). The construct pCB301-2×35S-HH-  
32 SR<sub>(-)eGFP</sub>-RZ-NOS [SR<sub>(-)eGFP</sub>] was generated.

33 To generate SR<sub>(-)mCherry&eGFP</sub> in which the NSs and N genes in S gRNA were replaced  
34 with mCherry and eGFP, respectively, the mCherry ORF was amplified and used to  
35 exchange the NSs gene in the pCB301-2×35S-HH-SR<sub>(-)eGFP</sub>-RZ-NOS by recombination  
36 using In-Fusion Cloning mixture (Clontech). The construct pCB301-2×35S-HH-  
37 SR<sub>(-)mCherry&eGFP</sub>-RZ-NOS [35S:SR<sub>(-)mCherry&eGFP</sub>] was generated. The T7:SR<sub>(-)mCherry&eGFP</sub>  
38 minireplicon (pCB301-T7-HH-SR<sub>(-)mCherry&eGFP</sub>-RZ-NOS) controlled by T7 promoter was  
39 constructed by the same strategy as 35S:SR<sub>(-)mCherry&eGFP</sub>.

40 To generate antigenomic S<sub>(+)eGFP</sub>-minireplicon, the eGFP ORF was amplified and used  
41 to replace the NSs gene in the pCB301-2×35S-HH-S<sub>(-)</sub>-RZ-NOS by recombination using  
42 In-Fusion Cloning mixture (Clontech). The construct pCB301-2×35S-HH-SR<sub>(+)eGFP</sub>-RZ-  
43 NOS [SR<sub>(+)eGFP</sub>] was generated. The primers used above are listed in *SI Appendix, Table*  
44 [S3](#).

45 ***Construction of SR<sub>(-)mCherry&eGFPΔ5'UTR</sub>, SR<sub>(-)mCherry&eGFPΔIGR</sub> and SR<sub>(-)mCherry&eGFPΔ3'UTR</sub>***  
46 ***mutants.*** To generate SR<sub>(-)mCherry&eGFPΔ5'UTR</sub>, the DNA copy of SR<sub>(-)mCherry&eGFP</sub> without 5'-

47 UTR (88 nt) was amplified from pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS and used  
48 to recombine with backbone vector of pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS by  
49 recombination using In-Fusion Cloning mixture (Clontech). The construct pCB301-  
50 2×35S-HH-SR<sub>(-)</sub>mCherry&eGFPΔ5'UTR-RZ-NOS [SR<sub>(-)</sub>mCherry&eGFPΔ5'UTR] was generated.

51 To generate SR<sub>(-)</sub>mCherry&eGFPΔIGR, the DNA copy of SR<sub>(-)</sub>mCherry&eGFP without IGR (550  
52 nt) was amplified from pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS and used to  
53 recombine with the vector backbone from pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS  
54 by recombination using In-Fusion Cloning mixture (Clontech). The construct pCB301-  
55 2×35S-HH-SR<sub>(-)</sub>mCherry&eGFPΔIGR-RZ-NOS [SR<sub>(-)</sub>mCherry&eGFPΔIGR] was generated.

56 To generate SR<sub>(-)</sub>mCherry&eGFPΔ3'UTR, the DNA copy of SR<sub>(-)</sub>mCherry&eGFP without 3'-UTR  
57 (151 nt) was amplified from pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS and used to  
58 recombine with the vector backbone from pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFP-RZ-NOS  
59 using the primer pair FMF48/P3382 by recombination using In-Fusion Cloning mixture  
60 (Clontech). The construct pCB301-2×35S-HH-SR<sub>(-)</sub>mCherry&eGFPΔ3'UTR-RZ-NOS  
61 [SR<sub>(-)</sub>mCherry&eGFPΔ3'UTR] was generated. The primers used above are listed in *SI Appendix*,  
62 [Table S3](#).

63 **Construction of TSWV MR<sub>(-)</sub>eGFP, MR<sub>(-)</sub>mCherry and MR<sub>(-)</sub>eGFPNSmMut minireplicons.** To  
64 generate MR<sub>(-)</sub>eGFP and MR<sub>(-)</sub>mCherry minireplicons, the eGFP and mCherry ORFs were  
65 amplified and used to replace the GP gene in pCB3012×35S-HH-M<sub>(-)</sub>-RZ-NOS,  
66 respectively, by recombination using In-Fusion Cloning mixture (Clontech). The  
67 constructs pCB301-2×35S-HH-MR<sub>(-)</sub>eGFP-RZ-NOS [MR<sub>(-)</sub>eGFP] and pCB301-2×35S-HH-  
68 MR<sub>(-)</sub>mCherry-RZ-NOS [MR<sub>(-)</sub>mCherry] was generated.

69 To generate MR<sub>(-)</sub>eGFPNSmMut in which a stop codon was introduced immediately after

70 the start codon of NSm, the NSm<sup>Mut</sup> was amplified and used to replace the wild type NSm  
71 sequence in pCB301-2×35S-HH-MR<sub>(-)</sub>eGFP-RZ-NOS by recombination using In-Fusion  
72 Cloning mixture (Clontech). The construct pCB301-2×35S-HH-MR<sub>(-)</sub>eGFPNSmMut-RZ-NOS  
73 [MR<sub>(-)</sub>eGFPNSmMut] was generated. All primers used above are listed in *SI Appendix*, Table  
74 S3.

75 **Construction of full length L<sub>(+)</sub>opt and M<sub>(-)</sub>opt cDNA clones.** To generate full length L<sub>(+)</sub>opt  
76 cDNA clone, the sequence codon and intron-splicing sites optimized RdRp was amplified  
77 and used to replace the wild type RdRp sequence in pCB301-2×35S-HH-L<sub>(+)</sub>-RZ-NOS by  
78 recombination using the In-Fusion Cloning mixture (Clontech). The pCB301-2×35S-HH-  
79 L<sub>(-)</sub>opt-RZ-NOS [L<sub>(-)</sub>opt] was generated.

80 To generate full length M<sub>(-)</sub>opt cDNA clone, the codon and intron-splicing sites  
81 optimized GP gene was de novo synthesized by GenScript Biotech Corp (Nanjing, China)  
82 (*SI Appendix*, Fig. S10) and used to replace the wild type GP sequence in pCB301-2×35S-  
83 HH-M<sub>(-)</sub>-RZ-NOS by the *in vitro* recombination using In-Fusion Cloning mixture  
84 (Clontech). The pCB301-2×35S-HH-M<sub>(-)</sub>opt-RZ-NOS [M<sub>(-)</sub>opt] was generated. The primers  
85 used above are listed in *SI Appendix*, Table S3.

## 86 **Plant material and virus source**

87 Six to eight weeks of *Nicotiana benthamiana* was used in all agroinfiltration assay. *N.*  
88 *benthamiana* plants were grown in a growth chamber setting at 25 °C, a 16 h light and 8 h  
89 dark photoperiod (3). The TSWV isolate from asparagus lettuce (TSWV-LE) was used in  
90 this study (GenBank accession number: KU976396 for S, JN664253 for M and KU976394  
91 for L) (4). The TSWV-LE isolate was maintained on *N. benthamiana*. For long-term  
92 storage, the infected new leaves of *N. benthamiana* were kept in an 80 °C refrigerator.

## 93 **Agrobacterium infiltration**

94 Recombinant plasmids were electroporated into *Agrobacterium tumefaciens* strain  
95 GV3101 and agroinfiltrations were performed essentially as described (5, 6). *A.*  
96 *tumefaciens* cells were resuspended by agroinfiltration buffer [10 mM MgCl<sub>2</sub>, 10 mM  
97 MES (pH 5.6) and 100 µM acetosyringone] adjusted to an optical density OD<sub>600</sub> of 1.0 and  
98 incubated for 2 to 3 h in dark at room temperature. Equal volumes of *Agrobacterium*  
99 cultures (final concentration OD<sub>600</sub>=0.2) harboring the p2300-N, p2300-RdRp, pCB301-  
100 derived reporter or full-length infectious clone vector(s), were mixed with one volume of  
101 bacterial mixture (final concentration OD<sub>600</sub>=0.05) containing the NSs and P19-HcPro-γb.  
102 The *Agrobacterium* cultures were infiltrated into fully expanded leaves of 6-7 leaf stage *N.*  
103 *benthamiana* plants using 1 mL needleless syringes.

104 **Immunoblot analysis**

105 Total protein was extracted from 1 g *Agrobacterium*-infiltrated leaf patches, healthy or  
106 TSWV-infected *N. benthamiana* systemic leaves in a 1 mL extraction buffer [10 % (v/v)  
107 glycerol, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10 mM dithiothreitol, 2 %  
108 (w/v) polyvinylpolypyrrolidone, 0.5 % (v/v) Triton X-100 and 1× protease inhibitors  
109 cocktail] (7). Protein samples were separated by SDS-PAGE gels, transferred to PVDF  
110 membranes (GE Healthcare, UK), blocked with 5 % skim milk solution and incubated with  
111 a polyclonal antiserum specific to the TSWV N, NSm, NSs, Gn, Gc, GFP, mCherry or T7  
112 RNA pol at room temperature for 1 h or overnight at 4 °C and washed three times. After  
113 incubation in a secondary antibody containing HRP-conjugated goat anti-rabbit (1:10000)  
114 for 1 h, the blots were detected using the ECL Substrate Kit (Thermo Scientific, Hudson,  
115 NH, USA). To evaluate protein loading, the blots were stained with Ponceau S.

116 **Northern blot analysis**

117 For Northern blot analysis of TSWV gRNAs, agRNAs or viral mRNA transcripts, total

118 RNAs were extracted from *Agrobacterium*-infiltrated leaf patches, healthy or TSWV-  
119 infected systemic leaves using an RNApreg Pure Plant Kit (Tiangen Biotech, Beijing,  
120 China), respectively. DIG-labeled specific probes for sense or antisense GFP, NSs, NSm,  
121 L-5'UTR was synthesized by DIG High Prime RNA labeling kit (Roche, Basel,  
122 Switzerland). The total RNAs were separated on 1 % formaldehyde agarose gels and  
123 transferred to Hybond-N+ membranes (GE Healthcare, UK) (8). The membrane blots were  
124 hybridized with a DIG-labeled specific probe and detected using a DIG-High Prime  
125 Detection Starter Kit II (Roche), following the manufacturer's protocol.

126 **RT-PCR and sequencing analysis**

127 To detect the virus in systemic leaves of *N. benthamiana* infected with  
128 SR<sub>(+)</sub>eGFP+MR<sub>(-)</sub>mCherry+L<sub>(+)</sub>opt, S<sub>(+)</sub>+MR<sub>(-)</sub>eGFP+L<sub>(+)</sub>opt, SR<sub>(+)</sub>eGFP+M<sub>(-)</sub>opt+L<sub>(+)</sub>opt or rTSWV  
129 recovered from the full-length cDNA clones, total RNAs were extracted from systemic  
130 symptoms plant leaves. First-strand cDNAs were synthesized using M-MLV Reverse  
131 Transcriptase (Promega, USA). RT-PCRs were performed to detect the SR<sub>(+)</sub>eGFP,  
132 MR<sub>(-)</sub>mCherry, MR<sub>(-)</sub>eGFP, S<sub>(+)</sub>, M<sub>(-)</sub>opt and L<sub>(+)</sub>opt minigenome and genomic RNA using their  
133 specific-primers. The PCR products were inserted into a pMD19-T vector (Takara, Dalian,  
134 China) and sequenced by Sanger dideoxy-mediated chain-termination DNA sequencing  
135 method at Sangon Biotech (Shanghai, China). The primers used in this study are listed in  
136 *SI Appendix, Table S3*.

137 **Fluorescence microscopy**

138 The agro-infiltrated *N. benthamiana* leaves were examined for fluorescence expression  
139 using an OLYMPUS IX71-F22FL/DIC Inverted Fluorescence Microscope (OLYMPUS,  
140 Tokyo, Japan) with a green or red barrier filter. The leaf sample was fixed in water on a

141 microslider under a coverslip to detect the eGFP and mCherry fluorescence, respectively.  
142 Fluorescence images were processed using ImagePro (OLYMPUS, Tokyo, Japan) and  
143 Adobe (San Jose, CA, USA) Photoshop programs.

144 **Electron microscopy**

145 Small tissues (1 mm × 4 mm) were excised from leaves of the *N. benthamiana* with infected  
146 rTSWV rescued from the full-length infectious clones. The sample tissues were fixed in  
147 2.5 % glutaraldehyde and 1 % osmium tetroxide dissolving into 100 mM phosphate buffer  
148 (pH 7.0) as described by Li *et al* (5, 9) and then embedded in Epon 812 resin as instructed  
149 by the manufacture (SPI-EM, Division of Structure Probe, Inc., West Chester, USA).  
150 Ultrathin sections (70 nm) were mounted on formvar-coate grids and then stained with  
151 uranyl acetate for 10 min followed by lead citrate for 10 min. The stained sections were  
152 examined under a transmission electron microscope (TEM; H-7650, Hitachi, Japan).

153 **Imaging GFP in infected plant by hand-held UV lamp**

154 GFP fluorescence in leaves was monitored with a hand-held 100 W, long-wave UV lamp  
155 (UV Products, Upland, CA, USA) and the leaves were photographed using a Canon EOS  
156 70D digital camera (Canon, Japan) with a 58 mm UV filter.

157

158 **References**

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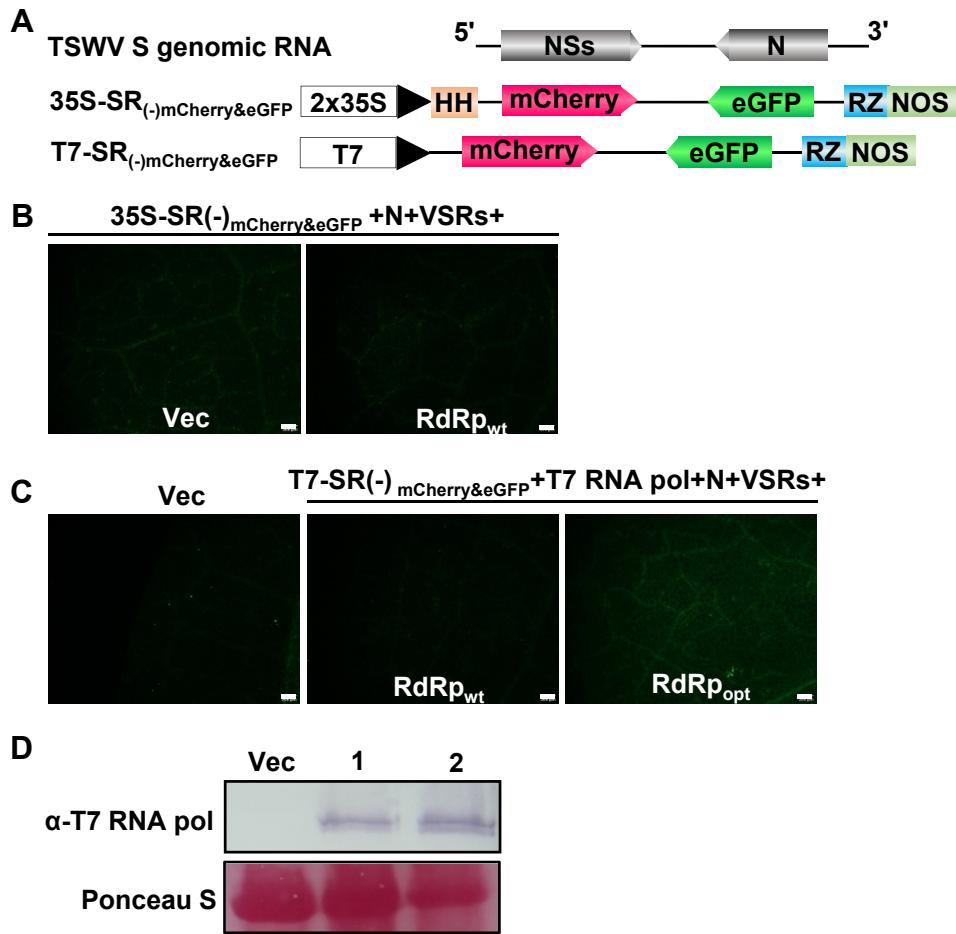
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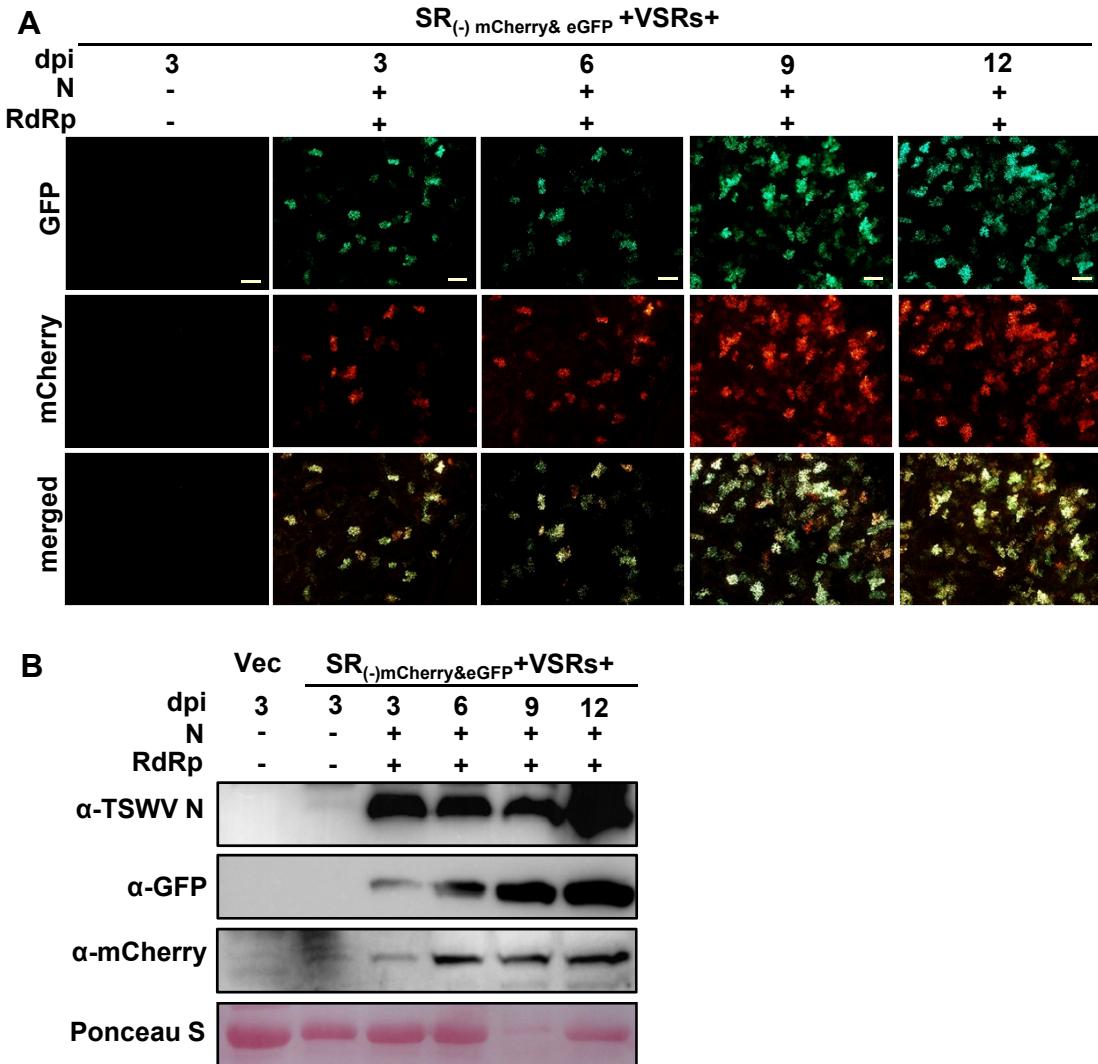
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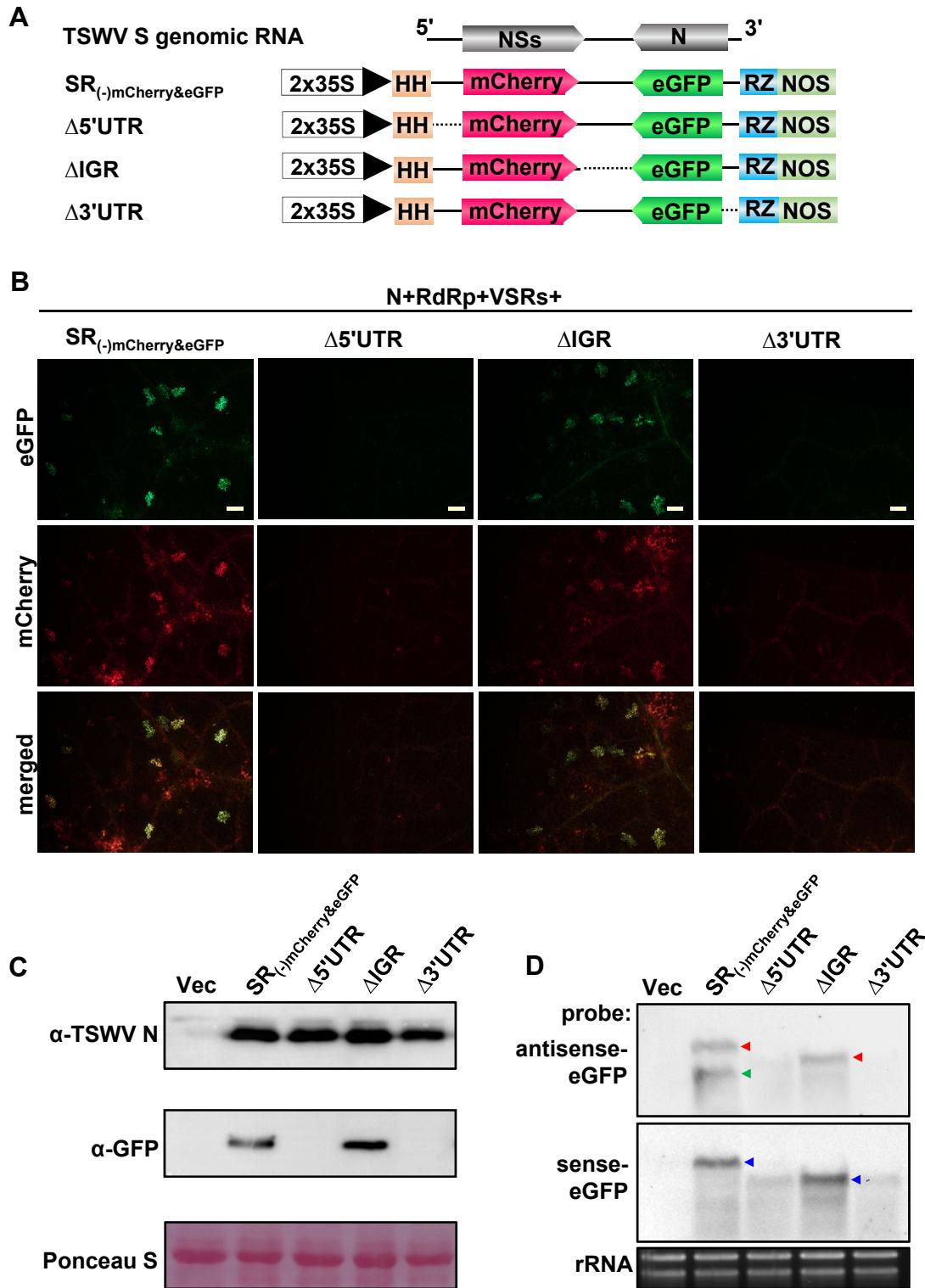
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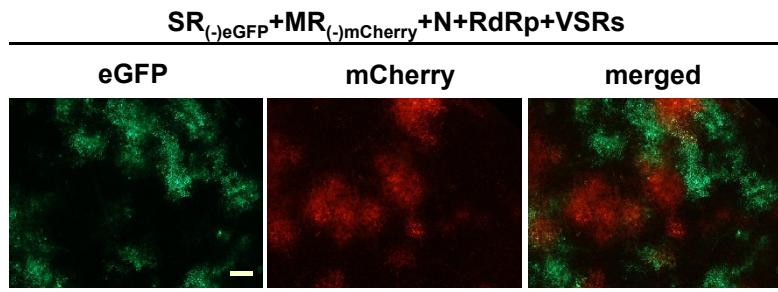
**Fig. S1**



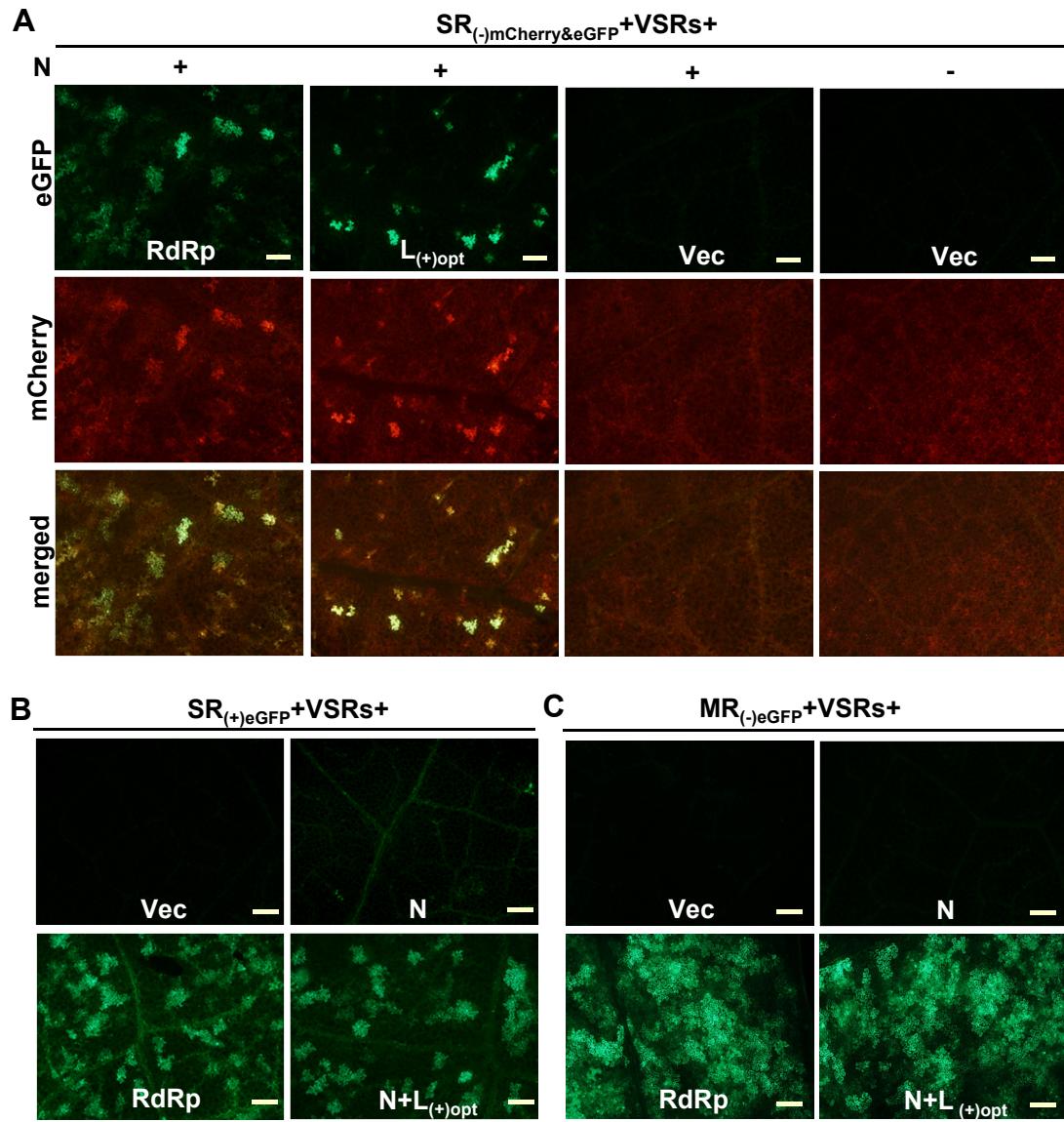
**Fig. S2**



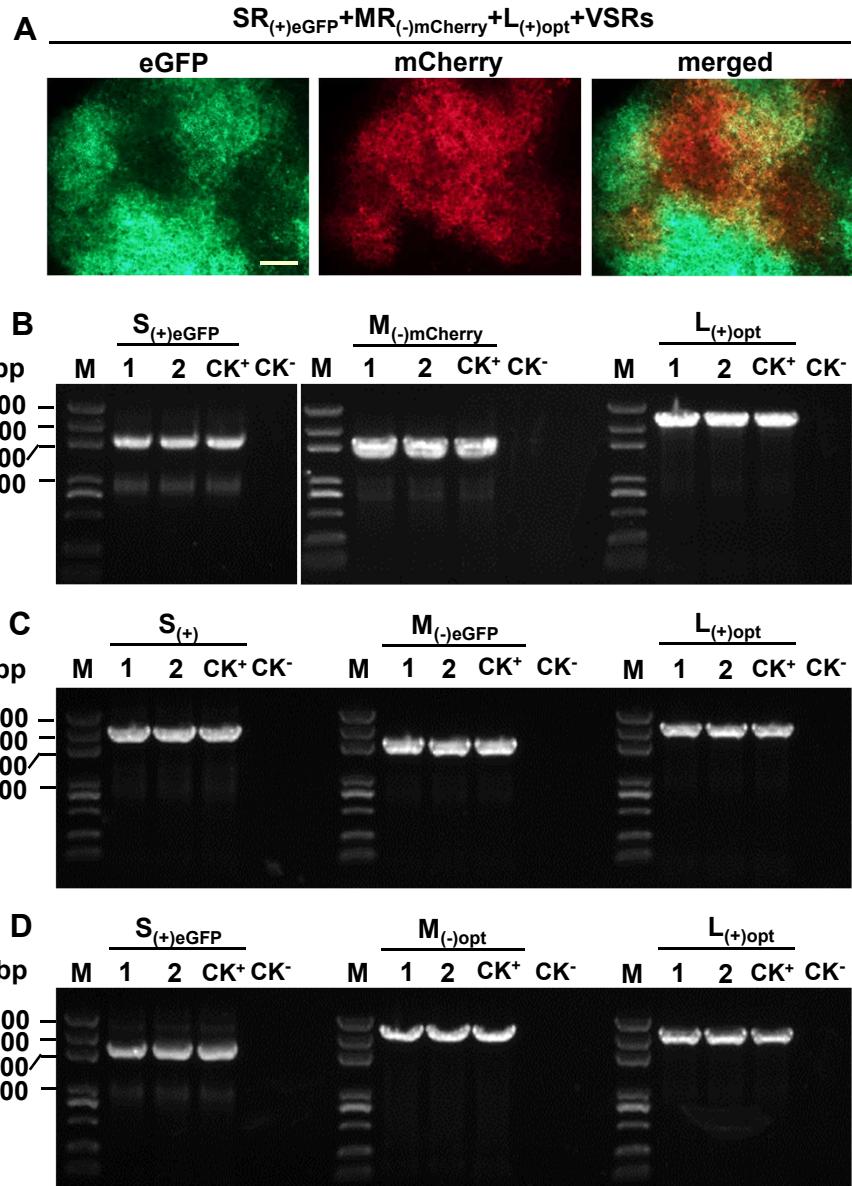
**Fig. S3**



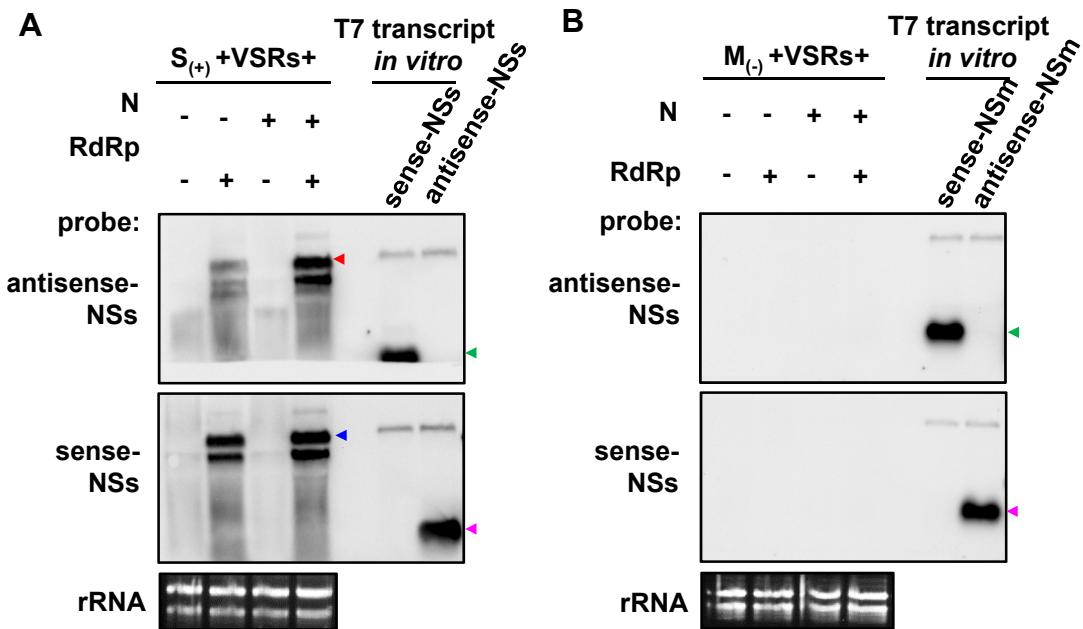
**Fig. S4**



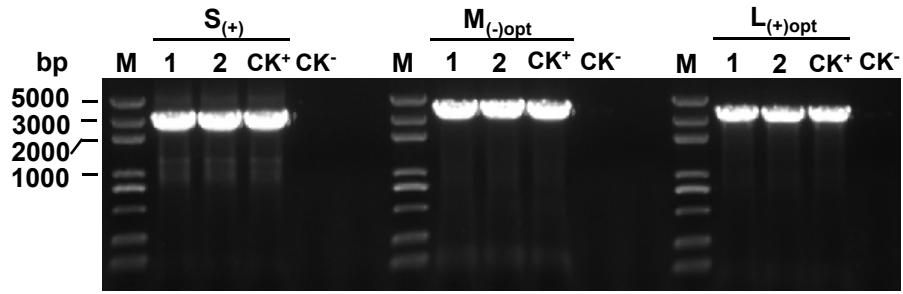
**Fig. S5**



**Fig. S6**



**Fig. S7**



**Fig. S8**

**Fig. S9. Optimized RdRp gene sequence used in the study.**

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CCTGCAAGAAAATCAACAAACTGTGCAACGATCTGCCATCTACCAACTACAGCGAGGACATGATGCAAGTTCTCCAAGGGT  
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CCAACGCAAAGCTGTTGGATCACGAGAACGATATCATCGTGGCATACCGACCTGAACATCAAGTGCCTATT  
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ATCATTGAGTGCCTGAGAACGCAAAGATTCAAGGACATCGAAAAGTGAAGGGCAACAAACCTGGTATTATCAAGGGCT  
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TGCTGACCAAGGGACTTACCAACACTTACCCGGTGTATGAACTGGCTGCAGGGTAACCTGAACTACCTCTTTCT  
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GACAACCTCGAGAACGAGTACTCTCCACTGTCAGCGACATGTATAAGACCGCTGACAGCGAGACTGAGACAATCA  
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TTTCGGCAGGGGATCAGGTGAATCACGATCTGGATCTGAGAACACCTATGGAAAAGAGCTACGACTTACGACTCAAGACCT  
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CCTTAAGATGGACAGGTCCGATGAAGAGTTCGAGGGCTCGCTTCAATTGAGCTGCTTAGGTTGGACGAGGAAGAGATG  
TACGAGGGGCTGATCAAAGAAATGAAGATCAAGCGGAAGAAGAACGGCTCTCTGTTCCCTGCTTAAGGTGTACGCCACT  
CGAGTTGATCAAGTTCTGATCGCGGTATTAAGGGCACCGCTTCGATATTGAGACTCTGCTCCGGAACTCATTCAAG  
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AGTACAAGAACGTCAACTGCCCTCTGAACGAGATGCCGATTCTCTTGAGGGTTACCTGAAGCTCACCAAGTCCAAGAG  
CAAAGAACACTCTTGAGCGCAGGGTGAAGAACGGCTTATTCAAGTTGAGGGACGAGCAGTCCAGGACCAAAAGCTC  
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CTACAGCGATATCAACGATTACATCATGCAGACCCGGAAATCATCCTCTCTAACGATCTCCGAGTTGGATGAGGTGG  
AAACTGACGAGGACAACCTCCTGCTCTTACCTTAGGGCGAAGAGGATGCTTCGATGAGGACGATTCTGATGAGGA  
AGAGGACACCGATTAG

**Fig. S10. Optimized GP gene sequence used in the study.**

ATGAGGATCCTGAAGCTCTTGAGCTGGTGAAGGTGAGCCTGTTACCATGCTCTGCTCTGTGCTCTGGCCTT  
CCTTATTTCAAGGGCTACCGACGCTAAGGTCGAGATCATTAGAGCGATCATCCTGAGGTGACGACTCTGCTGAGA  
ATGAAGTTCTCCTACCGCTGCTAGCATTAGCGGAAGGCTATTCTTGAGACTCTGACCTCTGATGCTCGAGTCTCAA  
CCTGGAACCCAGGCAGATTCTGAGGAAGAGTCTACCATTCTATCTTGGCAGCACTACCCAGAAGATTATCTCCG  
TAGCGACCTGCCTAACAACTGCCTGAACGCTTCATCTGAAGTGCGAGATCAAGGGCATCAGCACCTACACGTTACT  
ACCAGGTCGAGAACAAACGGCGTGAATCTACTCTGCGTGCAGATTCTGCTGAGGGCCTGAGAAGTGCAGAACACTCT  
TAACCTGCCGAAGCGGTTCTAAGGTGCCAGTGATTCTATACCAAGCTGGACAACAAGCGGCACTTCTGTGGCA  
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CTCTCTCAGACCGTTAAGCTGTCTGGCATTGCAAGATACCAAGAGCAACTTCGCTAACCGTACCGTGAGCATTAC  
CTCTCCAGAGAAGATCATGGCTACCTGATCAAGAAGCCTGGCGAAACGCTGAGGACAAGGTGATCTCATTCTCCGGCT  
CTGCTCCATTACCTCACCGAAGAGATGCTGGATGGTGAGCACAACCTCTGCGGTGATAAGTCTGCTAAGATCCCT  
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CTTGTGGTACGATCTGATCGGCTGATTACTTACCAATCCTGCTGCTGATTAACCGCTGTGGAAGTACTTCCGTTCAA  
GTGCAGCAACTGCGGCAACCTGTGCATTACTCACGAGTGCACCAAGATCTGCATCTGCAACAAAGAGCAAGGCCAGC  
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ATCCTTAGCCAGATGCCATGCTATGGCTCAGACTACCCAGTGCCTAGCGGTTGCTTTATGTTCTGGTGGCCCTGTG  
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CCATCTCGGCACCAACATCGTATTGAGGGCTCTAACGACTGCATCGAGAACCGAATTGTTGGCTCACCGAGCATC  
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CTACACCGGTTCTTACGGTCTACCTCTGAGGACTGTACGAGGCTAAGAGGCTTAGGAACGGCATCATCGACTCT  
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CTGCCAAGGCAGTCTGATTTGACTCTGAGGTTGATGGCAAGTACCGGTATATGGTTGAGCAGAGCCTTCTGGAGG  
TGGTGGAACTGTGTTCATGCTAACGATAAGACCTCTGAGAAGGCCAAGAACGTTGCTCATCTACATCAAGAGCGTGGC  
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TGTGCTGCTACGGCAAGAACGACTACCATCAAGACCTGCGGTTACGACACTTACAGGTTCAAGGTTCAAGGCTGGTCTGAGCA  
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TCGTGGTGGATCTGCCAGCGATCTTCAAGGTTGACCTAACGCGCCGTATCACTTCACTAGGCTTAACGCAAC  
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ATCGTTGAGGAACCTGAGAACATCATCGATCAGAACGACGAGTACGCTCACGAGGAACAGCAGTACAACAGCGATTCATC  
TGCTTGGGGATTCTGGGATTACATCAAGTCCCCGTTCAACTTCATTGCCAGCTACTCGGCAGCTTCTCGATACAATCAG  
GGTGATCCTGCTTATGCCCTCATCTTCTGGTTACTTCTGCAGCATTGACCACCATCTGCAAGGGTTACGTGAA  
GAACAAGTCCTACAAGAGCCGGTCCAAGATCGAGGATGATGACGACTCAGAGATTAAGGCCCCGATGCTGATGAAGGAC  
ACTATGACTAGAAGGGGGCCCTCCGATGGATTCTCATCTTGTAG

**Table S1. The predicted intron splicing sites of wild-type RdRp gene.**

Position (bp)	Putative splice site	Sequence	Score*	Intron GC*	Activations**		
					Alt./Cryptic	Constitutive	Confidence**
69	Alt. isoform/cryptic donor	TGAGGATTGCgtggcagca	4.835	0.529	0.954	0.035	0.963
175	Alt. isoform/cryptic donor	GAGACTATGCgtgagctgt	4.841	0.514	0.933	0.051	0.946
205	Constitutive donor	ACTGCTGATGgtgaggct	12.212	0.500	0.370	0.546	0.322
223	Alt. isoform/cryptic donor	CTGAACAAAGgtatggctac	10.886	0.500	0.802	0.142	0.823
261	Alt. isoform/cryptic donor	GTCTGAGATGgtgtcttgt	5.341	0.486	0.882	0.086	0.903
280	Alt. isoform/cryptic acceptor	gttcgagcagAAGTACCTTG	5.730	0.514	0.943	0.055	0.942
281	Alt. isoform/cryptic donor	TCGAGCAGAAgtacctgag	5.847	0.500	0.816	0.135	0.834
336	Constitutive acceptor	ttatttcttagGCACCTGAGG	4.932	0.486	0.212	0.770	0.725
579	Alt. isoform/cryptic acceptor	tggatgccagGACTGAGACT	4.087	0.500	0.696	0.295	0.576
602	Alt. isoform/cryptic donor	CCATGGAAAAGtactacaag	6.059	0.457	0.939	0.044	0.953
642	Alt. isoform/cryptic donor	GGACATCAAAGgtgaacggca	7.873	0.514	0.821	0.135	0.835
681	Alt. isoform/cryptic donor	TCCTGTGTTGgtgacatcg	6.292	0.529	0.839	0.123	0.853
738	Alt. isoform/cryptic acceptor	tgacctcttagCAGAGTGCTC	3.060	0.500	0.836	0.157	0.812
741	Alt. isoform/cryptic acceptor	cctcttagcAGTGCTCGAG	3.393	0.514	0.762	0.227	0.703
831	Alt. isoform/cryptic donor	CATTCTCACgtgggtcaga	5.665	0.500	0.943	0.042	0.955
835	Alt. isoform/cryptic donor	TCTCACGTGGgtcagatcg	6.026	0.500	0.936	0.048	0.949
843	Alt. isoform/cryptic donor	GGGTCAGATCgtgggtacta	5.056	0.514	0.903	0.074	0.918
864	Alt. isoform/cryptic donor	TCCTACTGTGgtgaggaact	11.320	0.500	0.901	0.072	0.920
906	Alt. isoform/cryptic donor	CAAGTCCGAGgttagggta	10.127	0.514	0.709	0.226	0.682
913	Alt. isoform/cryptic donor	GAGGTTAGGGgtatccctgg	4.945	0.514	0.953	0.032	0.967
971	Alt. isoform/cryptic donor	GGACCTCCAAGtacaaagag	5.665	0.514	0.934	0.049	0.948
1185	Alt. isoform/cryptic donor	CGAGCCTAAAGgtggacactgg	4.643	0.514	0.716	0.215	0.700
1207	Alt. isoform/cryptic acceptor	tgatcttaagGATCACCTGA	2.743	0.500	0.596	0.388	0.349
1249	Alt. isoform/cryptic donor	AGCCTGTACGgtaaagcacct	11.990	0.457	0.579	0.329	0.431
1361	Alt. isoform/cryptic donor	ACGAAGAGAAAGtacccgaac	5.393	0.529	0.904	0.069	0.923
1388	Alt. isoform/cryptic donor	CCAACGACCTGtcagagaca	5.572	0.500	0.910	0.068	0.925
1421	Alt. isoform/cryptic donor	CTATGACCTGgtcacccgagc	5.097	0.471	0.952	0.036	0.962
1492	Alt. isoform/cryptic donor	AACAAGTTCCgtgagagctt	6.085	0.500	0.565	0.376	0.334
1515	Alt. isoform/cryptic acceptor	agtccagcagCAGGGTGATC	2.527	0.486	0.940	0.057	0.939
1542	Alt. isoform/cryptic acceptor	gccttacagCTGCATTAAC	3.273	0.486	0.526	0.454	0.136

1587	Alt. isoform/cryptic donor	CACTAACCTTgtgaggctt	7.336	0.514	0.944	0.040	0.958
1672	Constitutive donor	ATCAACACCGgttagcattaa	5.999	0.500	0.395	0.516	0.233
1740	Constitutive acceptor	gccttactagGAACAAGAAC	7.612	0.529	0.146	0.841	0.827
1801	Alt. isoform/cryptic acceptor	ctactcaagGGTCTTGCCG	3.996	0.471	0.717	0.270	0.624
1889	Alt. isoform/cryptic donor	AGGGCCTCGAgtacgatact	5.278	0.500	0.956	0.032	0.966
2006	Alt. isoform/cryptic donor	CACTTTCTTgtgcgagcga	6.544	0.529	0.958	0.031	0.968
2125	Alt. isoform/cryptic acceptor	gtacatccagGATTCTGTGC	6.304	0.514	0.555	0.432	0.222
2205	Alt. isoform/cryptic acceptor	accactacagCGAGGACATG	4.878	0.457	0.847	0.147	0.827
2230	Alt. isoform/cryptic acceptor	gttctcaagGGTTTGATGG	2.346	0.471	0.692	0.290	0.580
2321	Constitutive acceptor	gtttcaagGCGACGGTAT	6.287	0.457	0.385	0.587	0.344
2326	Alt. isoform/cryptic donor	AAAGGCGACGgtatgaacac	7.959	0.486	0.760	0.181	0.761
2487	Constitutive donor	GCTTAACCAAGtgaggctt	14.109	0.500	0.246	0.676	0.637
2512	Alt. isoform/cryptic acceptor	actgttcaagACCCCTTCTA	4.626	0.514	0.933	0.062	0.933
2523	Alt. isoform/cryptic donor	CCCTTCTAAAGgtgccagtt	10.939	0.471	0.614	0.306	0.501
2545	Alt. isoform/cryptic acceptor	cttgcgccagTTCAGCAAAA	6.490	0.529	0.692	0.300	0.566
2550	Alt. isoform/cryptic acceptor	ccagttcagCAAAAAGGCC	4.578	0.500	0.800	0.194	0.758
2601	Alt. isoform/cryptic donor	CATTGAGAAAGgtcaacgtgt	6.294	0.500	0.944	0.041	0.956
2664	Constitutive acceptor	tgttgcgcagCGTGATGATT	6.507	0.486	0.462	0.524	0.117
2720	Alt. isoform/cryptic donor	ACTTCATGAGgtacgctgg	9.451	0.543	0.706	0.226	0.680
2765	Alt. isoform/cryptic donor	ACATCAAAGAgtacatccgg	4.583	0.500	0.944	0.041	0.956
2942	Constitutive donor	TGAACATCAAgtgccctatt	6.540	0.500	0.357	0.573	0.378
3217	Alt. isoform/cryptic donor	TAATACCTGGgtaacatcga	6.561	0.500	0.885	0.083	0.906
3292	Alt. isoform/cryptic acceptor	gtgtacaagATCTCTACCC	3.251	0.514	0.960	0.038	0.961
3307	Alt. isoform/cryptic acceptor	taccctgaagTCCAGTAAGA	3.209	0.500	0.930	0.067	0.928
3310	Alt. isoform/cryptic donor	CTGAAGTCCAGtaagaagt	9.173	0.471	0.712	0.218	0.694
3370	Constitutive acceptor	gtgcctgcagAACGCAAAGA	3.539	0.500	0.477	0.500	0.047
3557	Alt. isoform/cryptic donor	CCGTGGGAAAGtctgtgact	6.027	0.500	0.919	0.059	0.935
3642	Alt. isoform/cryptic donor	TCGGAACAAACgtgaccgtgc	5.533	0.500	0.919	0.061	0.934
3666	unclassified donor	GTCTAAGAAAGtgagcgagg	13.918	0.500	0.418	0.490	0.000
3745	Alt. isoform/cryptic donor	ATGAACCTTgttagggta	11.753	0.500	0.609	0.303	0.502
3751	Alt. isoform/cryptic donor	CTTGGTAAGGgtactgagg	6.065	0.500	0.952	0.033	0.966
3784	Constitutive acceptor	tttctgcagATGCTTGAGT	7.888	0.514	0.201	0.782	0.742
3816	Alt. isoform/cryptic donor	GGCCAAGAAATgtgaccgtt	6.207	0.543	0.936	0.047	0.950

3840	Alt. isoform/cryptic donor	GGATTCCTCgtgaggctgt	5.915	0.514	0.899	0.079	0.912
3880	Alt. isoform/cryptic donor	AAGACCGACCgtgagatcta	5.588	0.457	0.542	0.380	0.299
3906	Alt. isoform/cryptic donor	GAGCATGAAAGgtgaagatga	8.763	0.500	0.560	0.358	0.361
4023	Alt. isoform/cryptic acceptor	ctacccttagCCTGGACACC	6.193	0.514	0.626	0.365	0.416
4136	Alt. isoform/cryptic donor	TGACCTACAAAGtacgtctg	9.678	0.500	0.596	0.323	0.459
4211	Alt. isoform/cryptic donor	GCATTCTGATgtacgtgaag	7.123	0.514	0.847	0.112	0.868
4230	Alt. isoform/cryptic donor	GCTGAAGAAAGtgtgcaccc	6.203	0.529	0.934	0.048	0.949
4266	Alt. isoform/cryptic acceptor	tgaaccttagGAAGGCTCAG	7.185	0.457	0.849	0.138	0.837
4306	Alt. isoform/cryptic donor	ACCGCTATTGgtctgtcgtac	5.056	0.529	0.865	0.097	0.888
4344	Alt. isoform/cryptic donor	CACTTACCCGgtgtctatga	6.061	0.514	0.900	0.077	0.914
4366	Alt. isoform/cryptic donor	TGGCTGCAGGgttaacctgaa	6.041	0.500	0.750	0.183	0.755
4414	Alt. isoform/cryptic acceptor	cgctatgaagGCTTACCAACA	3.152	0.514	0.945	0.052	0.945
4459	Alt. isoform/cryptic acceptor	cgacttcagACCCGGTGGAA	3.313	0.500	0.787	0.200	0.746
4510	Constitutive donor	ATCGCTTCAGgtgagggtga	11.745	0.500	0.262	0.665	0.607
4511	Constitutive acceptor	atcgcttcagGTGAGGTTGA	6.293	0.529	0.441	0.541	0.185
4701	Constitutive acceptor	ttaactgcagGCATCTTGCT	8.159	0.514	0.411	0.576	0.288
4773	Alt. isoform/cryptic donor	GTCTATCCACgtgaccatgt	6.070	0.529	0.520	0.403	0.224
4809	Alt. isoform/cryptic donor	CCCTAATGAGgtgatccctt	6.780	0.529	0.856	0.106	0.877
4873	Alt. isoform/cryptic donor	ATGCTGCCTGgtgagggtgaa	9.597	0.529	0.762	0.188	0.753
4878	Alt. isoform/cryptic donor	GCCTGGTGAGgtgaacgaca	5.312	0.500	0.940	0.044	0.953
5001	Alt. isoform/cryptic acceptor	tggccccagCAGCAACGAC	4.531	0.529	0.765	0.227	0.703
5004	Alt. isoform/cryptic acceptor	gccccageagCAACGACCAG	3.016	0.529	0.837	0.157	0.813
5067	Alt. isoform/cryptic donor	CCTGGAAGAGgtgaaggact	8.199	0.514	0.764	0.180	0.764
5104	Alt. isoform/cryptic acceptor	ttacctgcagATGAGGTTCA	11.438	0.514	0.591	0.398	0.325
5140	Alt. isoform/cryptic donor	TACGAGAAGGgtactctgga	5.097	0.471	0.916	0.059	0.936
5263	Alt. isoform/cryptic acceptor	gctgaccctagATCATCAAGC	2.350	0.557	0.593	0.388	0.345
5416	Alt. isoform/cryptic donor	ATCCTCGAGGgtgtgatgact	5.258	0.471	0.829	0.126	0.848
5542	Alt. isoform/cryptic acceptor	gctgtctcagCTGTCATGT	8.742	0.500	0.683	0.304	0.555
5549	Alt. isoform/cryptic donor	AGCTGTTCATgtacacctct	5.135	0.514	0.868	0.098	0.887
5569	Alt. isoform/cryptic acceptor	tccgtctaagAGGAACCAGC	3.653	0.514	0.927	0.070	0.924
5616	Alt. isoform/cryptic acceptor	ctttgatagGGTGTGAGA	3.796	0.486	0.802	0.185	0.769
5616	Alt. isoform/cryptic donor	TCTTGATAGGgtgtgagat	4.789	0.457	0.911	0.062	0.931
5668	Alt. isoform/cryptic acceptor	taccgttaagATGACCTATG	4.339	0.500	0.796	0.196	0.754

5730	Alt. isoform/cryptic acceptor	atctcgacagCTACTGCAGC	3.975	0.414	0.845	0.148	0.825
5829	Alt. isoform/cryptic acceptor	gcgagcttagGAAGCGGGAC	4.419	0.486	0.590	0.389	0.340
5863	Alt. isoform/cryptic acceptor	ttggttccagACTGAGAAAGT	7.317	0.543	0.600	0.390	0.350
5921	Alt. isoform/cryptic donor	TGCATGCTGTgtacgggtct	4.883	0.500	0.957	0.031	0.968
6139	Alt. isoform/cryptic acceptor	cgctgttcagAACAGGCTCC	2.442	0.529	0.816	0.176	0.784
6199	Alt. isoform/cryptic acceptor	ctactccaagTTCAATCTTG	4.578	0.529	0.930	0.066	0.929
6213	Alt. isoform/cryptic acceptor	atcttggcagGGGCTTCATC	4.671	0.500	0.678	0.310	0.543
6258	Alt. isoform/cryptic acceptor	ccatctacagCAAAGAGGAA	4.973	0.500	0.822	0.171	0.793
6337	Alt. isoform/cryptic acceptor	ctctgcacagCAGGATATGA	3.577	0.529	0.610	0.372	0.390
6340	Alt. isoform/cryptic acceptor	tgcacageagGATATGAACC	5.307	0.543	0.575	0.395	0.313
6420	Alt. isoform/cryptic acceptor	ccatcaccagAGAGGACATC	2.479	0.514	0.962	0.036	0.963
6445	Constitutive acceptor	catttcacagAACGTGTGCC	9.539	0.500	0.209	0.784	0.733
6624	Constitutive acceptor	gtcttcatagGACCCTGGAT	10.048	0.500	0.176	0.817	0.784
6654	Alt. isoform/cryptic acceptor	gttacatcagGCGGTCCGAT	3.376	0.500	0.765	0.221	0.712
6663	Alt. isoform/cryptic donor	GCGGTCCGATgtgagatacg	6.227	0.486	0.936	0.047	0.950
6722	Alt. isoform/cryptic donor	GCGGAACCATgtacaagatt	5.221	0.514	0.960	0.028	0.971
6763	Alt. isoform/cryptic acceptor	ctacgtcagTTGATCGCAT	5.764	0.529	0.844	0.148	0.824
6801	Constitutive acceptor	tgtctcttagGACCCCTTTC	5.629	0.514	0.255	0.731	0.652
6850	Alt. isoform/cryptic donor	GACACCTACCgtgagtccat	8.138	0.443	0.627	0.307	0.511
6894	Alt. isoform/cryptic donor	GTTCGACAAAGgtcaacatta	6.897	0.471	0.855	0.108	0.874
6895	Alt. isoform/cryptic acceptor	gttcgacaagGTCAACATTA	2.380	0.486	0.870	0.126	0.855
6943	Alt. isoform/cryptic donor	CTCGAGCCAGgtgatgttg	8.185	0.486	0.833	0.125	0.850
6944	Alt. isoform/cryptic acceptor	ctcgagccagGTGATGCTTG	5.597	0.457	0.761	0.232	0.696
6960	Constitutive acceptor	cttgcattagGATGACCACC	2.622	0.471	0.452	0.510	0.115
6990	unclassified donor	GATCGTCAAAGgttaacgcca	6.349	0.471	0.500	0.414	0.000
7038	Alt. isoform/cryptic donor	TAAGCTGGTGgtgaagatta	5.500	0.471	0.942	0.041	0.957
7071	Alt. isoform/cryptic donor	GAACCTCCGACgtgtggaca	4.732	0.543	0.918	0.059	0.936
7124	Alt. isoform/cryptic acceptor	cttcctgaagTTGGTGAGTG	3.877	0.543	0.743	0.247	0.667
7126	Alt. isoform/cryptic donor	CCTGAAGTTGgtgagtgtt	12.153	0.500	0.727	0.210	0.711
7242	Alt. isoform/cryptic acceptor	gcaacacctagCCAGCAGATC	3.540	0.500	0.601	0.379	0.370
7355	Alt. isoform/cryptic donor	GCGTGGAAAGAtacgactcc	4.790	0.514	0.943	0.040	0.957
7464	Alt. isoform/cryptic acceptor	cactgtcagCGAGATCGTG	6.043	0.529	0.637	0.349	0.453
7535	Alt. isoform/cryptic donor	TGATGCTGAAgtactctcg	4.763	0.529	0.907	0.069	0.924

7585	Alt. isoform/cryptic donor	AGAGCTACTGgtatgcacgc	9.638	0.500	0.752	0.188	0.750
7648	Alt. isoform/cryptic donor	AACCTGCTGGgtatgattca	7.568	0.500	0.698	0.229	0.672
7722	Alt. isoform/cryptic acceptor	ccagccttagGAATGTGCTG	3.055	0.500	0.807	0.185	0.771
7758	Alt. isoform/cryptic acceptor	cttcggcagGC GGATCAGG	6.039	0.514	0.607	0.379	0.375
7792	Alt. isoform/cryptic acceptor	ggatctgcagAACAAACCTTA	3.876	0.514	0.696	0.289	0.586
7922	Alt. isoform/cryptic donor	AGATGGACAGgtccgatgaa	6.684	0.514	0.941	0.042	0.955
7968	Constitutive acceptor	atgtgettagGTTGGACGAG	2.272	0.529	0.343	0.637	0.461
8169	Alt. isoform/cryptic acceptor	ctacggacagGCTTGGTAGG	8.327	0.529	0.960	0.038	0.961
8173	Alt. isoform/cryptic donor	GACAGGCTTGGtaggctc	8.257	0.486	0.774	0.169	0.782
8205	Alt. isoform/cryptic donor	TGCTCTTAAGgtgtacgcca	6.332	0.514	0.932	0.049	0.948
8206	Alt. isoform/cryptic acceptor	tgctctaagGTGTACGCCA	5.395	0.514	0.524	0.455	0.133
8331	Alt. isoform/cryptic acceptor	tgagcggcagGGTGAAGAAG	5.008	0.514	0.856	0.139	0.838
8353	Alt. isoform/cryptic acceptor	tcttattcagTTGAGGGACG	2.472	0.500	0.856	0.133	0.845
8418	Alt. isoform/cryptic acceptor	acttccttagCAGGCACCCT	2.679	0.500	0.944	0.052	0.945
8421	Alt. isoform/cryptic acceptor	tccttagcagGCACCCCTTT	6.406	0.514	0.782	0.208	0.734
8459	Alt. isoform/cryptic donor	TGTACGGCCGgtacacctac	4.997	0.471	0.903	0.074	0.918
8472	Alt. isoform/cryptic acceptor	acacctacagCGATATCAAC	3.154	0.543	0.614	0.363	0.409
8497	Alt. isoform/cryptic acceptor	catcatgcagACCCGGGAAA	2.271	0.471	0.944	0.053	0.944
8521	Constitutive acceptor	cctctctaagATCTCCGAGT	5.017	0.500	0.424	0.562	0.245
8538	Alt. isoform/cryptic donor	GTTGGATGAGgtggaaa	4.678	0.529	0.945	0.039	0.958
8583	Constitutive acceptor	cttaccttagGGCGAAGAG	8.039	0.486	0.196	0.792	0.753

The putative intron splicing sites of wild-type RdRp gene sequence was predicted by Alternative Splice Site

Predictor (ASSP) (<http://wangcomputing.com/assp/>).

\* Scores of the preprocessing models reflecting splice site strength, i.e. a PSSM for putative acceptor sites, and an

MDD model for putative donor sites. Intron GC values correspond to 70 nt of the neighboring intron.

\*\* Activations are output values of the backpropagation networks used for classification. High values for one class

with low values of the other class imply a good classification. Confidence is a simple measure expressing the

differences between output activations. Confidence ranges between zero (undecided) to one (perfect classification).



**Table S2. The predicted intron splicing sites of wild-type GP gene.**

Position (bp)	Putative splice site	Sequence	Score*	Intron GC*	Activations**		
					Alt./Cryptic	Constitutive	Confidence**
36	unclassified donor	GGTGGTGAAAGgtgaggctgt	13.078	0.514	0.408	0.496	0.000
93	Constitutive acceptor	ttattttcagGGCTACCGAC	11.782	0.500	0.182	0.810	0.775
183	Alt. isoform/cryptic acceptor	ccgctgttagCATTAGCGG	3.056	0.500	0.929	0.067	0.928
259	Alt. isoform/cryptic donor	AGGCAGATTGtgaggaaaga	4.761	0.500	0.860	0.108	0.875
297	Alt. isoform/cryptic acceptor	tgcgtggcagCACTACCCAG	5.498	0.514	0.952	0.046	0.952
318	Alt. isoform/cryptic donor	GATTATCTCCgttagcgacc	4.792	0.529	0.928	0.056	0.940
324	Alt. isoform/cryptic acceptor	tctecgttagCGACCTGCCT	2.244	0.471	0.679	0.308	0.547
364	Alt. isoform/cryptic acceptor	atctctgaagTGCGAGATCA	3.458	0.500	0.845	0.145	0.828
405	Alt. isoform/cryptic donor	TTACTACCAAGgtcgagaaca	5.199	0.529	0.954	0.034	0.965
406	Alt. isoform/cryptic acceptor	ttactaccagGTCGAGAACAA	5.238	0.500	0.592	0.383	0.354
437	Alt. isoform/cryptic donor	ACTCTTGCGTgtcagattct	4.710	0.529	0.923	0.057	0.938
443	Alt. isoform/cryptic acceptor	tgcgtgtcagATTCTGCTGA	5.120	0.514	0.630	0.357	0.433
504	Alt. isoform/cryptic donor	GTTCTCTAAAGgtgccagtga	9.430	0.500	0.831	0.124	0.851
529	Alt. isoform/cryptic acceptor	tatcacaagCTGGACAAACA	2.328	0.500	0.853	0.139	0.837
552	Alt. isoform/cryptic donor	GCACTTCTCTgtggccacca	6.302	0.514	0.893	0.079	0.912
592	Constitutive acceptor	tctgaccaggACAACTTACCC	3.778	0.543	0.341	0.630	0.460
649	Alt. isoform/cryptic acceptor	gtctttcagACCGTTAACG	6.880	0.529	0.693	0.295	0.574
708	Alt. isoform/cryptic donor	CCCGTATACCgtgaggcatta	8.383	0.514	0.655	0.278	0.576
728	Constitutive acceptor	accttccaggAGAAGATCAT	6.756	0.514	0.388	0.598	0.352
780	Alt. isoform/cryptic donor	TGAGCACAAAGgtgatctat	9.170	0.514	0.526	0.387	0.264
824	Alt. isoform/cryptic acceptor	tteaccggaggAGATGCTGGA	3.783	0.529	0.805	0.185	0.770
835	Alt. isoform/cryptic donor	ATGCTGGATGgtgaggcaca	9.886	0.514	0.525	0.389	0.260
894	Alt. isoform/cryptic donor	CAACAAGCGTgtgaggact	8.781	0.500	0.825	0.132	0.840
914	Alt. isoform/cryptic donor	GCATCATCAAgtacacaaag	6.287	0.514	0.880	0.089	0.899
969	Constitutive acceptor	cttggatttagGCTGATCCTG	5.033	0.486	0.190	0.798	0.761
1008	Constitutive acceptor	tccctttagATGGCTGGTG	7.158	0.500	0.176	0.808	0.782
1046	Alt. isoform/cryptic donor	TTTTCTTGTGgtacatctg	8.598	0.486	0.789	0.158	0.800
1106	Alt. isoform/cryptic donor	GCCTGTGGAAgtacttccg	5.205	0.500	0.934	0.049	0.948
1123	Alt. isoform/cryptic acceptor	tccgttcaaggTGCAGCAACT	4.432	0.471	0.799	0.192	0.760
1128	Alt. isoform/cryptic acceptor	tcaagtggcagCAACTGCGGC	3.238	0.486	0.858	0.136	0.842

1328	Alt. isoform/cryptic donor	GCCTGCTTAAGttcgtagacc	5.154	0.514	0.929	0.051	0.945
1356	Alt. isoform/cryptic donor	GATCGGTCTGgtgatcccta	4.853	0.486	0.642	0.284	0.558
1368	Alt. isoform/cryptic acceptor	tgcacccatggCCAGATGCCT	2.891	0.500	0.795	0.198	0.751
1372	Alt. isoform/cryptic acceptor	ccttagccagATGCCTATGT	3.041	0.529	0.730	0.259	0.646
1443	Alt. isoform/cryptic donor	CCCTGTGCTGgtgaccctta	6.777	0.471	0.866	0.100	0.884
1463	Alt. isoform/cryptic donor	AGTTTGAAAAAGtgccttag	5.015	0.543	0.964	0.026	0.973
1631	Alt. isoform/cryptic donor	TGGGCTGCGAGtacctggac	4.582	0.500	0.967	0.024	0.975
1783	Alt. isoform/cryptic donor	AAGATCAGCGgtatgategc	7.260	0.529	0.671	0.262	0.610
1795	Alt. isoform/cryptic donor	ATGATCGCTGgtgacagect	7.023	0.500	0.803	0.149	0.814
1883	Alt. isoform/cryptic donor	TGGATGGCAAAGtaccgtat	5.025	0.500	0.926	0.053	0.943
1889	Alt. isoform/cryptic donor	GCAAGTACCGgtatatggtt	5.618	0.486	0.814	0.135	0.834
1992	Alt. isoform/cryptic donor	CATCAAGAGCgtggcatcc	5.600	0.514	0.825	0.138	0.833
2010	Alt. isoform/cryptic donor	CCACTACGAGGgtgtcagaga	7.706	0.500	0.765	0.178	0.767
2044	Alt. isoform/cryptic acceptor	tcctattcagTCTACCCACA	4.199	0.514	0.801	0.186	0.768
2077	Constitutive donor	ACCTGCACCGgttaattgcga	7.342	0.500	0.414	0.502	0.176
2097	Alt. isoform/cryptic acceptor	atacctgcagAAAGAACCAAG	4.833	0.529	0.869	0.122	0.860
2125	Constitutive acceptor	aggcttcagGACTTCTGCA	3.662	0.500	0.451	0.529	0.148
2191	Alt. isoform/cryptic donor	ATTAACGAGGgtgacttg	4.511	0.529	0.955	0.033	0.966
2253	Alt. isoform/cryptic acceptor	ggatctacagCGTGCTGAAG	3.191	0.514	0.910	0.086	0.906
2280	Alt. isoform/cryptic donor	CGTTGCCGACgtgtcact	6.880	0.514	0.904	0.073	0.919
2331	Alt. isoform/cryptic donor	TACCGAAGAGgttccatacg	4.991	0.500	0.959	0.029	0.970
2359	Constitutive acceptor	tctgttccagGCTGATATT	5.600	0.514	0.373	0.611	0.389
2401	Alt. isoform/cryptic donor	ATCACCATTGgtgagettat	11.534	0.529	0.522	0.395	0.242
2443	Alt. isoform/cryptic donor	ATCTACTCCGgtaacattgc	9.301	0.529	0.591	0.337	0.430
2469	Alt. isoform/cryptic donor	GAACGGACCCGgtgaagatgt	5.862	0.500	0.788	0.167	0.788
2494	Alt. isoform/cryptic acceptor	tcatcctcagCTTACTCATG	6.466	0.514	0.705	0.287	0.594
2618	Alt. isoform/cryptic donor	ACACTTACAGgttcaggct	7.293	0.500	0.904	0.068	0.925
2619	Alt. isoform/cryptic acceptor	acacttacagGTTCAAGGTCT	5.949	0.500	0.773	0.215	0.722
2624	Alt. isoform/cryptic donor	ACAGGTTCAAGgtctggctt	8.772	0.486	0.925	0.054	0.942
2625	Alt. isoform/cryptic acceptor	acaggttcagGTCTGGTCTT	3.359	0.514	0.684	0.296	0.567
2641	Alt. isoform/cryptic acceptor	tcttgacccatcgCTTCAAGGAC	4.449	0.529	0.945	0.051	0.946
2661	Alt. isoform/cryptic acceptor	tcccccacatcgCTTCAAGGAC	3.473	0.514	0.769	0.222	0.711
2668	Alt. isoform/cryptic acceptor	cagttcaagGACTTCAGCT	3.339	0.514	0.785	0.206	0.737

2676	Alt. isoform/cryptic acceptor	aggacttcagCTCATTCTTC	2.357	0.500	0.795	0.197	0.753
2751	Alt. isoform/cryptic donor	TCTTTCAAGgttgcaecta	5.168	0.529	0.935	0.047	0.950
2752	Alt. isoform/cryptic acceptor	tctttcaagGTTGCACCTA	3.558	0.500	0.699	0.281	0.598
2787	Constitutive acceptor	cttctactagGCTTAACACTGC	5.710	0.500	0.455	0.528	0.138
2815	Alt. isoform/cryptic donor	CTTCTTGTTGtcagggcct	9.649	0.529	0.757	0.184	0.757
2821	Alt. isoform/cryptic acceptor	tttgttgcagGGCCTTTCTT	6.984	0.529	0.502	0.483	0.039
2865	Alt. isoform/cryptic acceptor	tgacccctcagCACCGCCATC	4.796	0.529	0.876	0.117	0.867
2908	Alt. isoform/cryptic acceptor	tacctaccagCTGGCTGTGA	6.831	0.500	0.696	0.293	0.579
2933	Alt. isoform/cryptic donor	GCAGCAACAAgtacaacatc	6.089	0.500	0.851	0.108	0.873
2958	Alt. isoform/cryptic acceptor	tgttctgcagCGCAAACCCG	4.988	0.500	0.635	0.350	0.448
3021	Alt. isoform/cryptic donor	TTCTGTTGAGgtgtcgta	5.200	0.514	0.963	0.028	0.971
3146	Alt. isoform/cryptic donor	ATTACATCAAgtccccgttc	5.088	0.500	0.945	0.041	0.956
3171	Alt. isoform/cryptic acceptor	tcattgccagCTACTTCGGC	3.870	0.471	0.816	0.173	0.788
3183	Alt. isoform/cryptic acceptor	acttcggcagCTTCTTCGAT	3.003	0.486	0.868	0.128	0.853
3201	Alt. isoform/cryptic acceptor	atacaatcgGGTGATCCTG	2.559	0.471	0.787	0.200	0.745
3201	Alt. isoform/cryptic donor	TACAATCAGGgtgtatctgc	5.357	0.500	0.829	0.130	0.843
3249	Alt. isoform/cryptic acceptor	acttctgcagCATCCTGACC	7.746	0.471	0.516	0.472	0.086

The putative intron splicing sites of wild type GP gene sequence was predicted by ASSP).

\* Scores of the preprocessing models reflecting splice site strength, i.e. a PSSM for putative acceptor sites, and an

MDD model for putative donor sites. Intron GC values correspond to 70 nt of the neighboring intron.

\*\* Activations are output values of the backpropagation networks used for classification. High values for one class

with low values of the other class imply a good classification. Confidence is a simple measure expressing the

differences between output activations. Confidence ranges between zero (undecided) to one (perfect classification).

**Table S3. List of primers used in the study.**

Construct	Abbreviation	Primer sequence (5' to 3')	Purpose
p2300-N	N	<b>F:</b> GGGGTACCATGTCTAAGGTTAAGCTC A <b>R:</b> ACGTCGACTTAAGCAAGTTCTGCAA GTTTG	To amplify TSWV N and cloned into p2300S
p2300-RdRp <sup>wt</sup>	RdRp <sup>wt</sup>	<b>F:</b> CGGGATCCATGAACATCCAGAAAATA C <b>R:</b> GACGTCGACTTAATCCGTGTCTCTT CTTC	To amplify TSWV wildtype RdRp and cloned into p2300S
p2300-RdRp <sup>opt</sup>	RdRp <sup>opt</sup>	<b>F:</b> CTCGGTACCATGAACATTCAAGAGAT CCAAAAGC <b>R:</b> GACTCTAGACTAAATCGGTGTCTCTT CCTC	To amplify TSWV optimized RdRp and cloned into p2300S
pCXSN-NSs	NSs	<b>F:</b> CTCGGTACCATGTCTCAAGTGTAA TGAG <b>R:</b> GACTCTAGATTATTTGATCCTGAAG ATATG	To amplify TSWV NSs and cloned into pCXSN
pCB301-HH-S <sub>(-)</sub> -RZ-NOS	S <sub>(-)</sub>	<b>F:</b> CGAAAACCCGGTATCCGGGTTCAAG AGCAATTGTGTCAATTATTAA <b>R:</b> GGTGGAGATGCCATGCCGACCCAGA GCAATTGTGTCAATTATTAA <b>F:</b> GTTTGAATAAAATTGACACAATTGCT CTGGGTCGGCATGGCATCTCC <b>R:</b> GAATAAAATTATGACACAATTGCTCT GAACCCGGGATACCGGGTTTCG	To amplify the TSWV genomic RNA sequence for construction of S <sub>(-)</sub>
pCB301-HH-S <sub>(+)</sub> -RZ-NOS	S <sub>(+)</sub>	<b>F:</b> CGAAAACCCGGTATCCGGGTTCAAG AGCAATTGTGTCAATTATTAA <b>R:</b> GGTGGAGATGCCATGCCGACCCAGA GCAATTGTGTCAATTATTAA <b>F:</b> GAATAAAATTATGACACAATTGCT GGGTGGCATGGCATCTC <b>R:</b> GTTTGAATAAAATTGACACAATTGCT CTGAACCCGGGATACCGGGTTTCG	To amplify the pCB301 backbone for construction of S <sub>(+)</sub>
pCB301-HH-S <sub>(-)</sub> eGFP-RZ-NOS	SR <sub>(-)</sub> eGFP	<b>F:</b> GCTTTTTATAATTAACTTACA GCTTTACTTGACAGCTCGTCATGCC GAGA <b>R:</b> GTCAAAGCATATAACAACCTCTACG ATCATCATGGTGAGCAAGGGCGAGGAG CTGTTC	To amplify the eGFP for construction of SR <sub>(-)</sub> eGFP
		<b>F:</b> GAACAGCTCCTGCCCTGCTCACC	To amplify the pCB301 backbone for

		ATGATGATCGTAGAAGTTGTTATATGCT	construction of SR <sub>(-)eGFP</sub>
		TTGAC	
		<b>R:</b> TCTCGGCATGGACGAGCTGTACAAG	
		TAAAAGCAGTTGAAGTTAAATTATAAA	
		AAAGC	
		<b>F:</b> CACAGTACCAATAACCATAATGGTGA	
		GCAAGGGCGAGGAGGATAAC	To amplify the mCherry for
		<b>R:</b> GAAAAGCTGGACACGGCAAGATTA	construction of SR <sub>(-)mCherry&amp;eGFP</sub>
		AGATCTGTACAGCTCGTCCATGCCGC	
pCB301-HH-S <sub>(-)mCherry&amp;eGFP</sub> -RZ-NOS	SR <sub>(-)mCherry&amp;eGFP</sub>	<b>F:</b> GCGGCATGGACGAGCTGTACAGATC	
		TTAACCTTGCCTGTCAGCTTTTC	To amplify the pCB301 backbone for
		<b>R:</b> GTTATCCTCCTGCCCTGCTCACCA	construction of SR <sub>(-)mCherry&amp;eGFP</sub>
		TTATGGTTATTGGTACTGTG	
		<b>F:</b> GAAATTAAATACGACTCACTATAGGAG	
		AGCAATTGTGCAATTATTATTCAAAC	To amplify the eGFP and mCherry
		<b>R:</b> GGTGGAGATGCCATGCCGACCCAGA	expression cassette for construction of
		GCAATTGTGTCTATAATTATTATTCTTA	T7:SR <sub>(-)mCherry&amp;eGFP</sub>
pCB301-T7-S <sub>(-)mCherry&amp;eGFP</sub> -RZ-NOS	T7:SR <sub>(-)mCherry&amp;eGFP</sub>	<b>F:</b> GTTTGAATAAAATTGACACAATTGCT	
		CTCCTATAGTGAGTCGTATTAATTTC	To amplify the pCB301 backbone for
		<b>R:</b> GAATAAAAATTGACACAATTGCTCT	construction of T7:SR <sub>(-)mCherry&amp;eGFP</sub>
		GGGTGGCATGGCATCTC	
		<b>F:</b> GGAAAAGCTGGACACGGCAAGATTA	
		CTTGACAGCTCGTCCATGCCGAG	To amplify the eGFP for construction of
		<b>R:</b> GAACACAGTACCAATAACCATAATG	SR <sub>(+)eGFP</sub>
		GTGAGCAAGGGCGAGGAGCTGTT	
pCB301-HH-S <sub>(+)eGFP</sub> -RZ-NOS	SR <sub>(+)eGFP</sub>	<b>F:</b> GAACAGCTCCTGCCCTGCTCACCA	
		TTATGGTTATTGGTACTGTGTT	To amplify the pCB301 backbone for
		<b>R:</b> CTCGGCATGGACGAGCTGTACAAGT	construction of SR <sub>(+)eGFP</sub>
		AATCTGCCGTGTCCAGCTTTCC	
		<b>F:</b> CGAAAACCCGGTATCCGGGTTCAT	
		GGTGAGCAAGGGCGAGGAGGATAAC	To amplify the Δ5'UTR expression
		<b>R:</b> GGTGGAGATGCCATGCCGACCCAGA	cassette for construction of
		GCAATTGTGTCAATTATTCAAAC	SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup>
pCB301-HH-S <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup> -RZ-	SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup>	<b>F:</b> GTTTGAATAAAATTGACACAATTGCT	
NOS		CTGGGTGGCATGGCATCTCACC	To amplify the pCB301 backbone for
		<b>R:</b> GTTATCCTCCTGCCCTGCTCACCA	construction of SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ5'UTR</sup>
		TGAACCCGGGATACCGGGTTTCG	
		<b>F:</b> CGAAAACCCGGTATCCGGGTTCAT	
		AGCAATTGTGTCTATAATTATTTC	To amplify the Δ1GR expression
		<b>R:</b> GCATGGACGAGCTGTACAAGTAATT	cassette for construction of
		AAGATCTGTACAGCTCGTC	SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ1GR</sup>
pCB301-HH-S <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ1GR</sup> -RZ-	SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ1GR</sup>	<b>F:</b> GACGAGCTGTACAGATCTTAATTAC	
NOS		TTGTACAGCTCGTCCATGCCGAGA	To amplify the pCB301 backbone for
			construction of SR <sub>(-)mCherry&amp;eGFP</sub> <sup>Δ1GR</sup>

		<b>R:</b> GAATAAAATTATGACACAATTGCTCT GAACCCGGATACCGGGTTTCG	
		<b>F:</b> CGAAAACCCGGTATCCCGGGTCAG AGCAATTGTGTCATAATTTTATTC <b>R:</b> GGTGGAGATGCCATGCCGACCCATG GTGAGCAAGGGCGAGGAGCTGTTC	To amplify the Δ3'UTR expression cassette for construction of SR <sub>(-)</sub> mCherry&eGFP <sup>Δ3'UTR</sup>
pCB301-HH-S <sub>(-)</sub> mCherry&eGFP <sup>Δ5'UTR</sup> -RZ-NOS	SR <sub>(-)</sub> mCherry&eGFP <sup>Δ3'UTR</sup>	<b>F:</b> GAACAGCTCCTGCCCTGTCACC ATGGGTCGGCATGGCATCTCCACC <b>R:</b> GAATAAAATTATGACACAATTGCTCT GAACCCGGATACCGGGTTTCG	To amplify the pCB301 backbone for construction of SR <sub>(-)</sub> mCherry&eGFP <sup>Δ3'UTR</sup>
		<b>F:</b> CGAAAACCCGGTATCCCGGGTCAG AGCAATCAGTCATCAGAAATATACC <b>R:</b> GGTGGAGATGCCATGCCGACCCAGA GCAATCAGTGAAACAAAAAC	To amplify the TSWV genomic M- RNA sequence for construction of M <sub>(-)</sub>
pCB301-HH-M <sub>(-)</sub> -RZ-NOS	M <sub>(-)</sub>	<b>F:</b> GTTTTGTGCACTGATTGCTCTGG GTCGGCATGGCATCTCCACC <b>R:</b> GGTATATTCTGATGCACTGATTGCT CTGAACCCGGATACCGGGTTTCG	To amplify the pCB301 backbone for construction of M <sub>(-)</sub>
		<b>F:</b> CGAAAACCCGGTATCCCGGGTCAG AGCAATCAGTGAAACAAAAACTC <b>R:</b> GGTGGAGATGCCATGCCGACCCAGA GCAATCAGTGCCTCAGAAATATAC	To amplify the TSWV antigenomic M+ RNA sequence for construction of M <sub>(+)</sub>
pCB301-HH-M <sub>(+)</sub> -RZ-NOS	M <sub>(+)</sub>	<b>F:</b> GTATATTCTGACGCACTGATTGCTC TGGGTGGCATGGCATCTCCACC <b>R:</b> GAGTTTTGTTGCACTGATTGCTCT GAACCCGGATACCGGGTTTCG	To amplify the pCB301 backbone for construction of M <sub>(+)</sub>
		<b>F:</b> GAATCAAATTAGCCTGTGACAAGC AGACTTACTTGTACAGCTCGTCATGC <b>R:</b> CCATTATAATCTGAGCAGACGTATA AGATGGTGAGCAAGGGCGAGGAGCTG	To amplify the eGFP for construction of MR <sub>(-)</sub> eGFP
pCB301-HH-M <sub>(-)</sub> eGFP-RZ-NOS	MR <sub>(-)</sub> eGFP	<b>F:</b> CAGCTCTGCCCTGTCACCACATCT TATACGTCTGCTCAGATTATAATGG <b>R:</b> GCATGGACGAGCTGTACAGCTCGTCATGC CTGCTTGTACAGGCTAAATTGATTC	To amplify the pCB301 backbone for construction of MR <sub>(-)</sub> eGFP
		<b>F:</b> GAATCAAATTAGCCTGTGACAAGC AGACTTAAGATCTGTACAGCTCGTCAT GC <b>R:</b> CCATTATAATCTGAGCAGACGTATA AGATGGTGAGCAAGGGCGAGGAGGAT AAC	To amplify the mCherry for construction of MR <sub>(-)</sub> mCherry
pCB301-HH-M <sub>(-)</sub> mCherry-RZ-NOS	MR <sub>(-)</sub> mCherry	<b>F:</b> GTTATCCTCTGCCCTGTCACCA TCTTATACGTCTGCTCAGATTATAATGG <b>R:</b> GCATGGACGAGCTGTACAGATCTTA	To amplify the pCB301 backbone for construction of MR <sub>(-)</sub> mCherry

		AGTCTGCTTGTACAGGCTAAATTGAT
TC		
pCB301-HH-M <sub>(-)</sub> eGFP&NSmMut-RZ-NOS		<b>F:</b> CTCTACCTTAGGCTGTTGAACTCAA AATGTAGACTCTTCGGTAATAAGG <b>R:</b> GCATGGACGAGCTGTACAAGTAAGT CTGCTTGTACAGGCTAAATTGATTC
MR <sub>(-)</sub> eGFP&NSmMut		<b>F:</b> GAATCAAATTAGCTGTGACAAGC AGACTTACTTGTACAGCTCGTCATGC <b>R:</b> CCTTATTACCGAAAAGAGTCTACAT TTTGAGTTAACAGCTAACGGTAGAG
pCB301-HH-L <sub>(-)</sub> -RZ-NOS		<b>F:</b> CGAAAACCCGGTATCCCGGGTCAG AGCAATCAGGTACAACAAAC <b>R:</b> GGTGGAGATGCCATGCCGACCCAGA GCAATCAGGTAAACAAACGAT
L <sub>(-)</sub>		<b>F:</b> ATCGTTGTTACCTGATTGCTCTGGGT CGGCATGGCATCTCCACC <b>R:</b> GTTTAGTTGTACCTGATTGCTCTGA ACCCGGGATACGGGTTTCG
pCB301-HH-L <sub>(+)</sub> -RZ-NOS		<b>F:</b> CGAAAACCCGGTATCCCGGGTCAG AGCAATCAGGTAAACAAACGAT <b>R:</b> GTGGAGATGCCATGCCGACCCAGAG CAATCAGGTAAACAAAC
L <sub>(+)</sub>		<b>F:</b> GTTTAGTTGTACCTGATTGCTCTGG GTCGGCATGGCATCTCCAC <b>R:</b> ATCGTTGTTACCTGATTGCTCTGAAC CCGGGATACGGGTTTCG
pCB301-HH-L <sub>(+)</sub> opt-RZ-NOS		<b>F:</b> ATCAGGTAAACAACGATTAAAGCAA ACATGAACATTCAAGAAGATCCAAAAGC TG <b>R:</b> CATGCATTGTTAGGCATTACTTTAA TCTAATCGGTGTCCTCTCCTCATCAG
L <sub>(+)</sub> opt		<b>F:</b> CTGATGAGGAAGAGGACACCGATT AGATTAAGTAATGCCAACAAATGCA TG <b>R:</b> CAGCTTTGGATCTCTGAATGTTCA TGTTGCTAAAATCGTGTACCTGAT
pCB301-HH-M <sub>(-)</sub> opt-RZ-NOS		<b>F:</b> ACCATTATAATCTGAGCAGACGTAT AAGATGAGGATCCTGAAGCTTCTG <b>R:</b> GAATCAAATTAGCCTGTGACAAGC AGACCTAAACAAGATGAGAGAAATC
M <sub>(-)</sub> opt		<b>F:</b> GATTCTCTCATCTGTTAGGTCTG CTTGTACAGGCTAAATTGATTC <b>R:</b> CAAGAAGCTTCAGGATCCTCATCTT

ATACGTCTGCTCAGATTATAATGGT

		<b>F:</b> GTTAATACTAACGGAGTGAAC	To amplify the sense-NSs for
pGEM-NSs	-	<b>R:</b> GATTGAAATTGGCTGAAACAGTA	construction of pGEM-NSs to generate the DIG-tabled probes of S vRNA in Northern blotting
	C		
		<b>F:</b> GATTGAAATTGGCTGAAACAGTA	To amplify the antisense-NSs for
pGEM-anti-NSs	-	<b>R:</b> GTTAATACTAACGGAGTGAAC	construction of pGEM-anti-NSs to generate the DIG-tabled probes of S cRNA in Northern blotting
pGEM-NSm	-	<b>F:</b> GCTTGACTAAAGCTATGGATAC	To amplify the sense-NSm for
		<b>R:</b> TCTTGTATTCTGGCTGCACATC	construction of pGEM-NSm to generate the DIG-tabled probes of M vRNA in Northern blotting
pGEM-anti-NSm	-	<b>F:</b> TCTTGTATTCTGGCTGCACATC	To amplify the antisense-NSm for
		<b>R:</b> GCTTGACTAAAGCTATGGATAC	construction of pGEM-anti-NSm to generate the DIG-tabled probes of M cRNA in Northern blotting
pGEM-L 3'UTR	-	<b>F:</b> AGAGCAATCAGGTACAACAAAC	To amplify the L 3'UTR for
		<b>R:</b> AAGTAATGCCTAACAAATGCATGA	construction of pGEM- L 3'UTR to generate the DIG-tabled probes of L vRNA in Northern blotting
pGEM-anti-L 3'UTR	-	<b>F:</b> AAGTAATGCCTAACAAATGCATGA	To amplify the antisense-L 3'UTR for
		<b>R:</b> AGAGCAATCAGGTACAACAAAC	construction of pGEM-anti-L 3'UTR to generate the DIG-tabled probes of L cRNA in Northern blotting
pGEM-eGFP	-	<b>F:</b> ATGGTGAGCAAGGGCGAGGAGCTGTT	To amplify the sense-eGFP for
		<b>R:</b> ATGGTGAGCAAGGGCGAGGAGCTGTT	construction of pGEM-eGFP to generate the DIG-tabled probes of antisense-eGFP RNA in Northern blotting
pGEM-anti-eGFP	-	<b>F:</b> ATGGTGAGCAAGGGCGAGGAGCTGTT	To amplify the antisense-eGFP for
		<b>R:</b> ATGGTGAGCAAGGGCGAGGAGCTGTT	construction of pGEM-anti-eGFP to generate the DIG-tabled probes of sense-eGFP RNA in Northern blotting
		<b>F:</b> GGTGGAGATGCCATGCCGACCCAGA	
		GCAATTGTGTCAATTATTATTCTTA	To amplify the of S <sub>(+)</sub> eGFP minigenome
		<b>R:</b> GGTGGAGATGCCATGCCGACCCAGA	by RT-PCR
		GCAATTGTGTCAATTATTCAAAC	
		<b>F:</b> GTTCATTCATTTGGAGAGGAGAC	
		ATCAGTGAAACAAAAAC	To amplify the of M <sub>(-)</sub> mCherry and M <sub>(-)</sub> eGFP
		<b>R:</b> GGTGGAGATGCCATGCCGACCCAGA	minigenome by RT-PCR
		GCAATCAGTGCCTGAGAAATATAC	
		<b>F:</b> GAATCAAATTAGCCTGTGACAAGC	To amplify the of M <sub>(-)</sub> opt genome by RT-

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AGACCTAACAGATGAGAGAAATC PCR

**R:** GGTGGAGATGCCATGCCGACCCAGA

GCAATCAGTGCAACAAAAAC

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**F:** GATCAAGGATGTTAATTTCAGCATGC

TTATCCGATCCTCGAC

To amplify the of L<sub>(+)</sub>opt antigenome by

**R:** GAATCAAATTAGCCTGTGACAAGC RT-PCR

AGACCTAACAGATGAGAGAAATC

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