

1 **Development of a mini-replicon-based reverse-genetics system for** 2 **rice stripe tenuivirus**

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20 **Running Title**

21 **A mini-replicon-based reverse-genetics system for RSV**

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23

24 **ABSTRACT**

25 Negative-stranded RNA (NSR) viruses include both animal- and plant-infecting
 26 viruses that often cause serious diseases in human and livestock, and in agronomic
 27 crops. Rice stripe tenuivirus (RSV), a plant NSR virus with four
 28 negative-stranded/ambisense RNA segments, is one of the most destructive rice
 29 pathogens in many Asian countries. Due to the lack of a reliable reverse-genetics
 30 technology, molecular studies of RSV gene functions and its interaction with host
 31 plants are severely hampered. To overcome this obstacle, we developed a
 32 mini-replicon-based reverse-genetics system for RSV gene functional analysis in
 33 *Nicotiana benthamiana*. We first developed a mini-replicon system expressing RSV
 34 genomic RNA3 eGFP reporter (MR3_{(-)eGFP}), a nucleocapsid (NP), and a codon usage
 35 optimized RNA-dependent RNA polymerase (RdRp_{opt}), respectively. Using this
 36 mini-replicon system we determined that RSV NP and RdRp_{opt} are indispensable for
 37 the eGFP expression from MR3_{(-)eGFP}. The expression of eGFP from MR3_{(-)eGFP} can be
 38 significantly enhanced in the presence of NSs and P19-HcPro-γb. In addition, NSvc4,
 39 the movement protein of RSV, facilitated eGFP trafficking between cells. We also
 40 developed an antigenomic RNA3-based replicon in *N. benthamiana*. However, we
 41 found that the RSV NS3 coding sequence acts as a *cis*-element to regulate viral RNA
 42 expression. Finally, we made mini-replicons representing all four RSV genomic
 43 RNAs. This is the first mini-replicon-based reverse-genetics system for
 44 monocot-infecting tenuivirus. We believe that this mini-replicon system described
 45 here will allow the studies of RSV replication, transcription, cell-to-cell movement

46 and host machinery underpinning RSV infection in plants.

47 **KEY WORDS:** *Rice stripe tenuivirus*, reverse-genetics system, mini-replicon,
48 negative-sense/ambisense RNA virus

49

50 **IMPORTANCE**

51 Plant-infecting segmented negative-stranded RNA (NSR) viruses are grouped into 3
52 genera: *Orthospovirus*, *Tenuivirus* and *Emaravirus*. The reverse-genetics systems
53 have been established for members in the genera *Orthospovirus* and *Emaravirus*,
54 respectively. However, there is still no reverse-genetics system available for
55 *Tenuivirus*. Rice stripe virus (RSV) is a monocot-infecting tenuivirus with four
56 negative-stranded/ambisense RNA segments. It is one of the most destructive rice
57 pathogens and causes significant damages to rice industry in Asian countries. Due to
58 the lack of a reliable reverse-genetics system, molecular characterizations of RSV
59 gene functions and the host machinery underpinning RSV infection in plants are
60 extremely difficult. To overcome this obstacle, we developed a mini-replicon-based
61 reverse-genetics system for RSV in *Nicotiana benthamiana*. This is the first
62 mini-replicon-based reverse-genetics system for tenuivirus. We consider that this
63 system will provide researchers a new working platform to elucidate the molecular
64 mechanisms dictating segmented tenuivirus infections in plant.

65

66 Introduction

67 Negative-sense RNA (NSR) viruses include well-known members of medical
68 importance such as Ebola virus (EBOV), vesicular stomatitis virus (VSV), influenza
69 A virus (FLUAV) and Rift Valley fever virus (RVFV) (1, 2) and include serious plant
70 pathogens of agronomical importance such as Tomato spotted wilt virus (TSWV),
71 Rice stripe virus (RSV), and Rose rosette virus (RRV) (3-5). There are 3 genera of
72 segmented NSR viruses infecting plants: *Orthospovirus*, *Tenuivirus* and *Emaravirus*.
73 TSWV and RSV are the representative viruses for *Orthospovirus* and *Tenuivirus*,
74 respectively (6, 7). RRV and *European mountain ash ringspot-associated virus*
75 (EMARV) are important members in the genus *Emaravirus* (8, 9).

76 Tenuiviruses are classified in the genus *Tenuivirus*, family *Phenuiviridae* within
77 the order *Bunyavirales*. Most viruses in the family *Phenuiviridae* infect animals. RSV
78 is one of most devastating causal agents of rice and often causes severe damages to
79 rice production in China and many other Asian countries (6, 10, 11). RSV is
80 transmitted by small brown planthopper (*Laodelphax striatellus*) in a persistent and
81 circulative-propagative manner (6, 12-14). RSV genome consists of four RNA
82 segments and encodes seven proteins through an antisense or an ambisense coding
83 strategy (15-18). RSV RNA1 is of negative polarity and encodes the RNA-dependent
84 RNA polymerase (RdRp) (17). RSV RNA2 encodes the NS2 protein in the viral (v)
85 strand and the NSvc2 protein from the viral complementary (vc) strand (19). The NS2
86 protein is a weak viral suppressor of RNA silencing (VSR) and is required for RSV
87 systemic infection in plants. The NSvc2 protein is a putative glycoprotein that targets

88 endoplasmic reticulum (ER) and Golgi apparatus, and function as a helper factor to
 89 conquer insect midgut barriers after being cleaved into two mature proteins (i.e.,
 90 NSvc2-N, the amino-terminal half, and NSvc2-C, the carboxyl-terminal half) (12, 20).
 91 RSV RNA3 encodes the NS3 protein in the v strand, and is known as a VSR that
 92 binds single- and double-stranded RNAs to suppress RNA silencing (21, 22), and the
 93 NP protein in the vc strand that interacts with viral genomic RNAs to form viral
 94 ribonucleoprotein complexes (RNPs) (23). RSV RNA4 encodes the SP protein in the
 95 v strand, a nonstructural and disease-specific protein that interacts with a host
 96 oxygen-evolving complex protein to interfere host photosynthesis, and the NSvc4
 97 protein in the vc strand, a protein involved in RSV cell-to-cell and long-distance
 98 movement in plant (4, 21). The four RSV genomic RNAs all contain highly conserved
 99 5'- and 3'-untranslated regions (UTR), important for the initiation of viral RNA
 100 transcriptions. RSV RNA2, 3 and 4 all have a noncoding intergenic region (IGR) with
 101 multiple AU-rich regions that form secondary hairpin-like structures to act as
 102 transcription termination signals (24, 25).

103 Viral reverse-genetics systems are important tools for the studies of viral gene
 104 functions, disease inductions and host factors involved in virus infection in plants
 105 (26-32). Although reverse-genetics systems have been firstly reported for animal
 106 segmented negative-stranded RNA viruses over 20 years ago (26, 33-43), to establish
 107 similar systems for plant segmented negative-stranded/ambisense RNA viruses turned
 108 out to be very challenging. However, just recently the reverse-genetics systems have
 109 been established for a few of non-segmented and segmented plant NSR viruses. The

110 first reverse-genetics system of the non-segmented plant NSR viruses was established
111 for *Sonchus yellow net virus* (SYNV, a nucleorhabdovirus) and followed with
112 BYSMV (a cytorhabdovirus) (27, 44-47). Recently we established the first
113 reverse-genetics system of segmented plant NSR viruses for TSWV (48). Soon after,
114 the reverse-genetics system for RRV was also established, allowing for studies of
115 emaravirus gene function and disease pathology in whole plants (9).

116 For 3 genera of segmented NSR viruses infecting plants, reverse-genetics model
117 has been established for 2 of the genera, *Orthotospovirus* and *Emaravirus* (9, 48, 49).
118 However, there is still no reverse-genetics system available for the genus *Tenuivirus*.
119 The recent progresses on TSWV and RRV reverse-genetics system encouraged us to
120 establish a reverse-genetics system for RSV. In this study, we developed a
121 mini-replicon-based reverse-genetics system for RSV gene function analyses in
122 *Nicotiana benthamiana*. This represents the first mini-replicon-based reverse-genetics
123 system for monocot-infecting tenuivirus. The developed mini-replicon
124 reverse-genetics system will provide researchers a novel platform for studies of RSV
125 replication, transcription, movement and host factors involved in the interactions
126 between the virus and the host plant. This system also provides useful basis for the
127 development of infectious RSV clones for the assays in plants, including rice.

128

129

130 **Results**

131 **Development of a genomic RNA3 mini-replicon-based reverse-genetics system** 132 **for RSV**

133 To establish a mini-replicon-based reverse-genetics system to investigate RSV
134 infection in plant, we RT-PCR-amplified the full-length RSV RNA3 sequence and
135 inserted it between an HH sequence and an RZ sequence in the
136 pCB301-2×35S-RZ-NOS vector to produce RNA3₍₋₎. We then replaced the NP gene in
137 RNA3₍₋₎ with an *eGFP* gene to produce MR3₍₋₎eGFP mini-replicon reporter (Fig. 1A).
138 The expressions of RNA3₍₋₎ and MR3₍₋₎eGFP from these two vectors are driven by a
139 doubled *Cauliflower mosaic virus* (CaMV) 35S promoter (2×35S) (Fig. 1A).

140 We constructed p2300-RdRp_{wt}, pBIN-NS3, p2300-NP, and pCXSNSvc4 to
141 express the wild-type RSV RdRp (RdRp_{wt}), NS3, NP, and NSvc4, respectively, in
142 cells through agro-infiltration. After infiltration of a mixed *Agrobacterium* culture
143 carrying the plasmids of MR3₍₋₎eGFP, RdRp_{wt}, NP and four VSRs (NSs and
144 P19-HcPro-γb) into *N. benthamiana* leaves (Fig 1B), no eGFP fluorescence was
145 observed in the infiltrated leaves by 5 days post agro-infiltration (dpi) (Fig. 1C). As
146 reported for TSWV RdRp (48), the wild-type RSV RdRp was also predicted to have
147 numerous putative intron splicing sites. This prediction prompted us to optimize its
148 codon usage and to remove the predicted intron splicing sites. The optimized *RdRp*
149 ORF was then inserted into the p2300 vector to produce p2300-RdRp_{opt} (RdRp_{opt}).
150 After infiltrating *N. benthamiana* leaves with a mixed *Agrobacterium* culture carrying
151 plasmids of MR3₍₋₎eGFP, RdRp_{opt}, NP and four VSRs, we observed the eGFP

152 fluorescence using a microscope at 5 dpi (Fig. 1B), suggesting that the sequence
153 optimized RdRp_{opt} is now functional in *N. benthamiana* cells.

154

155 **RSV NP and RdRp_{opt} are required for MR3_{(-)eGFP} expression**

156 To investigate the roles of NP and RdRp_{opt} in RSV replication in plant, we
157 co-expressed MR3_{(-)eGFP} and VSRs with empty vector (Vec), NP, RdRp_{opt}, or both NP
158 and RdRp_{opt}, respectively, in *N. benthamiana* leaves through agro-infiltration. The
159 eGFP fluorescence was again detected in the leaves co-expressing MR3_{(-)eGFP} with NP
160 and RdRp_{opt}. In contrast, the control leaves expressing MR3_{(-)eGFP} alone, or
161 co-expressing MR3_{(-)eGFP} with NP or RdRp_{opt}, did not show any eGFP fluorescence
162 (Fig. 2A), indicating that the presence of both NP and RdRp_{opt} is necessary for
163 MR3_{(-)eGFP} expression from the mini-replicon.

164 To confirm this observation, we analyzed the accumulation levels of eGFP
165 protein, *eGFP* mRNA, and MR3_{(-)eGFP} genomic RNA (gRNA) and anti-genomic RNA
166 (agRNA) in the infiltrated *N. benthamiana* leaf tissues. Western blot showed high
167 levels of eGFP in the leaves co-expressing MR3_{(-)eGFP}, NP, RdRp_{opt} and four VSRs
168 (NSs+P19-HcPro-γb). In contrast, eGFP protein was not detected in the control leaves
169 co-expressing MR3_{(-)eGFP} with Vec, NP or RdRp_{opt} only by 5 dpi (Fig. 2B). Northern
170 blot results showed that the gRNA and agRNA were detected in the leaf tissues
171 co-expressing MR3_{(-)eGFP}, NP, RdRp_{opt} and VSRs by 5 dpi (Fig. 2C). In contrast, no
172 amplified gRNA and agRNA were detected in the control leaves co-expressing
173 MR3_{(-)eGFP} with Vec, NP or RdRp only, respectively (Fig. 2C). Only primary

transcripts of agRNA from MR3_{(-)eGFP} was detected in these leaves. These results indicate that the presence of RSV NP and RdRp_{opt} are required for the replication of both gRNA and agRNA from the MR3_{(-)eGFP} mini-replicon. Intrinsically, the *eGFP* mRNA was not detected in the leaf tissues co-expressing MR3_{(-)eGFP}, NP, RdRp_{opt} and VSRs.

179

180 **Effect of viral suppressors of RNA silencing on MR3_{(-)eGFP} expression**

Viral suppressors of RNA silencing (VSRs) inhibit host RNAi machinery and enhance non-viral gene expressions in plants (44, 46). To investigate the roles of different VSRs on MR3_{(-)eGFP} expression in plant, we infiltrated *N. benthamiana* leaves with mixed agrobacterium cultures carrying MR3_{(-)eGFP}, RdRp_{opt}, NP and VSRs including NS3, NSs, and P19-HcPro- γ b, respectively. By 5 dpi, the leaves co-expressing MR3_{(-)eGFP}, RdRp_{opt} and NP with Vec showed a few cells with eGFP fluorescence. Many cells with eGFP fluorescence were observed in the leaves co-expressing MR3_{(-)eGFP}, RdRp_{opt} and NP with three VSRs (P19-HcPro- γ b) or four VSRs (NSs and P19-HcPro- γ b) (Fig. 3A and 3B). Although RSV NS3 is a VSR (21, 22), co-expression of MR3_{(-)eGFP}, RdRp_{opt}, NP with NS3 or both NS3 and NSs resulted in almost no cell with eGFP fluorescence. Moreover, co-expression of MR3_{(-)eGFP}, RdRp_{opt}, NP with both NS3 and P19-HcPro- γ b resulted in some cells with eGFP fluorescence. These findings indicate that RSV NS3 can suppress eGFP expression from the MR3_{(-)eGFP} mini-replicon, and are supported by the Western blot result (Fig. 3B). Based on these results, we decided to use VSRs including NSs, P19, HcPro and

196 γ b but not NS3 in the following experiments.

197

198 **Dosage effects of NP and RdRp_{opt} on MR3_{(-)eGFP} expression**

199 To optimize the expression of MR3_{(-)eGFP} mini-replicon in plant cells, we mixed the
200 Agrobacterium culture carrying MR3_{(-)eGFP} and four VSRs (NSs+P19-HcPro- γ b) with
201 the Agrobacterium cultures carrying NP (OD₆₀₀ 0, 0.05, 0.1, 0.2 or 0.4) or RdRp_{opt}
202 (OD₆₀₀ 0, 0.05, 0.1, 0.2 or 0.4), and then infiltrated them individually into *N.*
203 *benthamiana* leaves. When the concentration of Agrobacterium culture carrying RdRp
204 was fixed at OD₆₀₀ 0.05 and the concentration of Agrobacterium culture carrying NP
205 was increased from OD₆₀₀ 0.05 to OD₆₀₀ 0.4, the results showed that the strongest
206 eGFP fluorescence was observed in the leaves co-expressing MR3_{(-)eGFP} with NP at
207 OD₆₀₀ 0.2 and RdRp_{opt} at OD₆₀₀ 0.05 (Fig. 4A). When the concentration of
208 Agrobacterium culture carrying NP was maintained at OD₆₀₀ 0.2, while the
209 concentration of Agrobacterium culture carrying RdRp_{opt} was increased from OD₆₀₀
210 0.05 to OD₆₀₀ 0.4, the number of cells with eGFP fluorescence decreased as the
211 concentration of RdRp_{opt} increased (Fig. 4B). These results were supported by the
212 results from Western blot assays (Fig. 4C and 4D).

213

214 **RSV NSvc4 supports MR3_{(-)eGFP} cell-to-cell movement**

215 NSvc4 is the movement protein of RSV (18, 50). To investigate whether NSvc4 can
216 also influence MR3_{(-)eGFP} expression, we infiltrated *N. benthamiana* leaves with the
217 mixed Agrobacterium cultures carrying MR3_{(-)eGFP} (OD₆₀₀ 0.2), NP (OD₆₀₀ 0.2),

RdRp_{opt} (OD₆₀₀ 0.05), NSs (OD₆₀₀ 0.05), P19-HcPro-γb (OD₆₀₀ 0.05) and NSvc4 (OD₆₀₀ 0.025, 0.05, 0.1 or 0.15). In this experiment, the MR3_{(-)eGFP} started to move out the original cell in the addition of NSvc4 at OD₆₀₀ 0.025, compared with that in the leaves co-expressing MR3_{(-)eGFP}, NP and RdRp_{opt} without NSvc4 (Fig. 5A and Table 1). When the concentration of Agrobacterium culture carrying NSvc4 was further increased, stronger cell-to-cell movement of MR3_{(-)eGFP} was observed (Fig. 5A and Table 1). Results of the Western blot assays agreed with the microscopic observations (Fig. 5B).

Development of an RSV antigenomic RNA3-based replicon system

To develop an RSV antigenomic (ag) RNA-based mini-replicon, we replaced the NS3 gene in the RNA3₍₊₎-agRNA vector (RNA3₍₊₎) with an *eGFP* gene to produce MR3_{(+)eGFP} (Fig. 6A). We then transiently co-expressed MR3_{(+)eGFP}, NP, RdRp_{opt}, and four VSRs (NSs+P19-HcPro-γb) in *N. benthamiana* leaves through agro-infiltration. By 5 dpi, no eGFP fluorescence was observed in the infiltrated leaves. In contrast, strong eGFP fluorescence was observed in the leaves co-expressing MR3_{(-)eGFP}, NP, RdRp_{opt} and four VSRs (Fig. 6B), indicating that the MR3_{(+)eGFP} min-replicon is incapable of expressing eGFP in plant cells.

Next, we replaced the NS3 gene in MR3_{(-)eGFP} with a *mCherry* gene (MR3_{(-)mCherry&eGFP}) (Fig. 6C), and co-expressed MR3_{(-)mCherry&eGFP} or MR3_{(-)eGFP} with NP, RdRp_{opt} and four VSRs in *N. benthamiana* leaves through agro-infiltration. By 5 dpi, in contrast to the leaves co-expressing MR3_{(-)eGFP}, NP, RdRp_{opt} and VSRs, no

eGFP fluorescence was observed for MR3_{(-)mCherry&eGFP} (Fig. 6D), indicating that without the NS3 gene, this MR3_{(-)mCherry&eGFP} min-replicon is unable to express eGFP in *N. benthamiana* leaf cells. To confirm this result, we generated RNA3₍₊₎ to express full-length RSV agRNA3 (Fig. 6A), and transiently co-expressed RNA3₍₊₎, VSRs, NSvc4 with one or two of the three plasmids (i.e., Vec, NP and RdRp_{opt}), respectively in *N. benthamiana* leaves via agro-infiltration. The infiltrated leaves were harvested at 5 dpi and analyzed for the accumulations of RNA3₍₊₎-derived gRNA3 and agRNA3 through Northern blot assays using DIG labelled antisense- and sense-probes, respectively. The result showed that high levels of gRNA3 and agRNA3 were detected in the leaves co-expressing RNA3₍₊₎, NP, RdRp_{opt}, VSRs and NSvc4 (Fig. 6E). In contrast, No amplified gRNA3 and agRNA3 were detected in the leaves co-expressing RNA3₍₊₎, four VSRs, NSvc4 with Vec, NP or RdRp_{opt} only) (Fig. 6E). Only primary transcripts of agRNA3 from RNA3₍₊₎ was detected in these leaves. Based on these results, we conclude that the RNA3₍₊₎ replicon is functional in *N. benthamiana* in the presence of NP, RdRp_{opt}, NSs, P19-HcPro-γb and NSvc4.

256 **The NS3 gene is required for eGFP expression from MR3_{(-)eGFP}**

To investigate the function of NS3 in eGFP expression from the MR3_{(-)eGFP} mini-replicon, we introduced a stop codon (TAA) at the downstream of the start codon of NS3 ORF (MR3_{(-)eGFP&NS3stop}) (Fig. 7A), and co-expressed it with NP, RdRp_{opt} and VSRs in *N. benthamiana* leaves through agroinfiltration. By 5 dpi, strong eGFP fluorescence was observed in the infiltrated leaves, while the leaves

co-expressing MR3_{(-)eGFPΔNS3}, NP, RdRp_{opt} and VSRs did not (Fig. 7B). Western blot results showed that the eGFP were expressed in the leaves co-expressing MR3_{(-)eGFP&NS3stop}, NP, RdRp_{opt} and VSRs, while no eGFP was accumulated in the leaves co-expressing MR3_{(-)eGFPΔNS3}, NP, RdRp_{opt} and VSRs (Fig. 7C). This finding indicates that the NS3 is dispensable for eGFP expression from the MR3_{(-)eGFP} mini-replicon. Deletion of NS3 gene sequence from MR3_{(-)eGFP} (MR3_{(-)eGFPΔNS3}) abolished the expression of eGFP.

To further confirm the role of NS3 ORF in MR3_{(-)eGFP} expression, we divided NS3 ORF into four segments and generated four truncated MR3_{(-)eGFP} mutant constructs: MR3_{(-)eGFPMut1}, MR3_{(-)eGFPMut2}, MR3_{(-)eGFPMut3}, and MR3_{(-)eGFPMut4} (Fig. 7D). Each mutant was co-expressed with NP, RdRp_{opt}, NSvc4 and VSRs, respectively, in *N. benthamiana* leaves. By 5 dpi, eGFP fluorescence was observed in the leaves co-expressing MR3_{(-)eGFPMut2}, MR3_{(-)eGFPMut3}, or MR3_{(-)eGFPMut4} with NP, RdRp_{opt}, NSvc4 and VSRs. However, the leaves co-expressing MR3_{(-)eGFPMut1}, NP, RdRp_{opt}, NSvc4 and VSRs did not show eGFP fluorescence (Fig. 7E). Western blot results agreed with the microscopic observations and showed that eGFP was not expressed from MR3_{(-)eGFPMut1} in cells (Fig. 7F), indicating that deletion of the first 159 aa residues of NS3 significantly affect MR3_{(-)eGFP} mini-replicon to express the eGFP. It is noteworthy that the Northern blot result showed that the expression of gRNA and agRNA from MR3_{(-)eGFPMut1} was not affected (Fig. 7G). We also noticed that the mobility of gRNA and agRNA from MR3_{(-)eGFPMut2} and MR3_{(-)eGFPMut4} was altered compared with that from MR3_{(-)eGFPMut1}, MR3_{(-)eGFPMut3} or MR3_{(-)eGFP}. Therefore, we

284 conclude that the NS3 ORF sequence is not necessary for viral replication but is
285 required for the expression of eGFP from the RNA3₍₋₎-derived mini-replicons in cells.

286 RSV RNA2, 3 and 4 IGRs can form secondary hairpin-like structures which is
287 postulated to act as transcription termination signals (24, 25). To further investigate
288 the possible role of RSV NS3 in viral transcription regulation, we predicted the RNA
289 secondary structure and examined the possible RNA-RNA interaction between NS3
290 coding sequence, IGR and 3'-UTR of RSV RNA3. The 3'-UTR, NS3, and IGR of
291 RSV RNA3 have 65, 636 and 742 nucleotides (nt), respectively. The secondary
292 structure analysis showed that the 1-295 nt sequence of IGR formed a very long
293 hairpin structure (Fig. 8). Strikingly, the 1-40 nt coding region sequence of NS3
294 base-paired with the 575-596 nt sequence of IGR and the 1-65 nt sequence of 3'-UTR
295 and formed a long hairpin structure (Fig. 8). The 619-636 nt coding region sequence
296 of NS3 base-paired with the 396-403 nt and the 537-543 nt sequences of IGR and
297 formed a small hairpin-like structure. The 41-203 nt coding region sequence of NS3
298 itself formed four long and short hairpin structures. The 205-618 nt coding region
299 sequence of NS3 formed sector structure containing at least 9 hairpins (Fig. 8). The
300 secondary structure analysis suggested that the coding sequence of NS3 likely
301 interacts with IGR and 3'-UTR of RNA3 in forming hairpin-like structure.

302

303 **Development of RSV RNA1, RNA2 and RNA4 mini-replicons**

304 RSV genome consists of four RNA segments encoding seven proteins using an
305 antisense or an ambisense coding strategy (15-18). Because the MR3₍₋₎eGFP

mini-replicon is functional in *N. benthamiana* leaf cells (Fig. 1 and Fig. 2), we decided to develop mini-replicons for other three RSV genomic RNAs. We first produced MR1_{(-)eGFP} and then replaced the *RdRp* ORF with the *eGFP* gene to produce MR1_{(-)eGFP} (Fig. 9A). For RSV genomic RNA2 and 4, we first cloned the full-length RNA2 or 4 segments individually into the vector, and then replaced the *NSvc2* ORF with the *eGFP* gene to produce MR2_{(-)eGFP} or replaced the *NSvc4* ORF with the *eGFP* gene to produce MR4_{(-)eGFP} (Fig. 9A). *N. benthamiana* leaves were then infiltrated with the mixed *Agrobacterium* cultures carrying various combinations of plasmids (Fig. 9B). By 5 dpi, strong eGFP fluorescence was observed in the leaves co-expressing MR1_{(-)eGFP}, MR2_{(-)eGFP}, or MR4_{(-)eGFP} with NP, RdRp_{opt} and four VSRs (Fig. 9B). As expected, the leaves infiltrated with the mixed *Agrobacterium* cultures lacking NP, RdRp_{opt} or NP+RdRp_{opt} did not show eGFP fluorescence.

Collectively, the mini-replicon-based reverse-genetics system, representing all four RSV genomic RNAs, has been created.

320

321 Discussion

There are 3 genera of segmented NSR viruses infecting plants: *Orthospovirus*, *Tenuivirus* and *Emaravirus*. The reverse-genetics systems have been established for TSWV and RRV in the genera *Orthospovirus* and *Emaravirus* (9, 48, 49), respectively. Here, we established a mini-replicon-based reverse-genetics system for RSV, the representative virus for the genus *Tenuivirus*. RSV is an important rice virus and poses significant threat to rice productions in China and many other Asian countries (6, 10, 11). During the past 20 years, the lack of a reliable reverse-genetics

329 system significantly hampered the studies of RSV gene functions and disease
330 induction in plant. To overcome this obstacle, we launched a multiyear research that
331 finally yielded a functional mini-replicon-based reversed genetics system for RSV
332 studies. We first developed a mini-replicon system to express RSV MR3_{(-)eGFP}, NP,
333 and a codon usage optimized RdRp (RdRp_{opt}), respectively. Using this mini-replicon
334 systems we determined that RSV NP and RdRp_{opt} are indispensable for the eGFP
335 expression from MR3_{(-)eGFP}. The expression of eGFP from MR3_{(-)eGFP} was
336 significantly enhanced in the presence of NSs and P19-HcPro- γ b. In addition, NSvc4,
337 the movement protein of RSV, facilitated eGFP trafficking between cells. Interestingly,
338 co-expression of RSV NS3 inhibited eGFP expression from MR3_{(-)eGFP}. We also found
339 that the RSV NS3 gene sequence is not necessary for viral replication, but regulates
340 viral RNA expression. The secondary structure analysis showed that the coding
341 sequence of NS3 base-paired with the sequence of IGR and 3'-UTR of RNA3 to form
342 a long hairpin structure. The phenomenon of coding sequence as a *cis*-element in
343 regulating viral RNA expression has not been reported previously for the
344 negative-stranded/ambisense RNA viruses. Finally, based on the system of RNA3₍₋₎,
345 we have also produced mini-replicons representing all RSV RNA genomic segments,
346 allowing RSV functional studies in plant.

347 The choice of promoter for RNA transcriptions is critical for the development of
348 reverse-genetics systems for plant NSR viruses. The genomic and antigenomic RNAs
349 generated from the negative-stranded RNA virus clones were not infectious because
350 their infectious ribonucleoprotein complexes (RNPs) contain not only viral gRNA,

351 but also NP and RdRp proteins (36, 51). Genomic RNAs of the same segmented NSV
352 contain highly conserved 5' and 3' terminal untranslated sequences that have eight
353 complementary nucleotides, capable of forming panhandle-like structures. These
354 structures are known to play critical roles in viral gRNA and agRNA replications (51).
355 Moreover, the NSR viruses RNAs do not possess 5' cap-structures and 3' poly(A) tails.
356 The classical bacteriophage T7 promoter can produce accurate viral RNA 5' end
357 sequences without a cap. We initially produced an RSV mini-replicon systems using
358 the T7 promoter. This mini-replicon system, however, did not express the *eGFP* gene
359 from an RNA3-based mini-replicon in plant cells. In our recent report, we also
360 reported that the T7 promoter-based system was unable to generate infectious TSWV
361 RNA transcripts in plant cells (48). It is possible that the synthesis of viral genomic
362 RNA transcripts through the T7 promoter and T7 RNA polymerase is incomplete or is
363 inefficient in *planta*.

364 In a recent report, we described an expressing vector with a double CaMV 35S
365 promoter (an RNA Pol II promoter), a hammerhead (HH) ribozyme, and an HDV
366 ribozyme to produce infectious TSWV viral RNAs in plant cells (48). The
367 mini-replicon system produced in this study also has an HH ribozyme and an HDV
368 ribozyme before and after the viral sequence to ensure the correct ends. The results
369 shown in Fig. 1 and Fig. 2 suggest that the vector transcribed RSV genomic RNA did
370 bind to viral NP and RdRp to form functional RNPs, which is needed for the synthesis
371 of functional viral gRNA and agRNA. This finding supports earlier reports that the
372 Pol II promoter can not only replicate non-segmented plant NSR viruses (44, 46), but

373 also segmented plant NSR viruses (9, 48).

374 RSV RdRp is one of the major components needed for the initiation of viral
375 genomic RNA replication (17). RSV RdRp is a 337 kDa protein. When this protein
376 was co-expressed with the mini-replicon in plant cells, no eGFP fluorescence was
377 observed in the infiltrated leaves (Fig. 1A-C). Our computer prediction suggested that
378 the RSV *RdRp* sequence contained many putative cryptic intron splicing sites.
379 Because the segmented plant NSR viruses replicate in cytoplasm, their *RdRp* gene
380 sequences should not been evolved to remove those cryptic intron splicing sites that
381 could be spliced in cell nucleus. We speculated that after the wild-type RSV *RdRp*
382 sequences were expressed, through the 2×35S promoter, in nucleus, they were quickly
383 spliced, resulting in non-functional *RdRp* fragments. After the putative intron splicing
384 sites were removed and the codon usage was optimized, the expressed RdRp_{opt} is
385 capable of supporting *eGFP* expression from the mini-replicon (Fig. 1D). In this study,
386 we also determined that the less concentrated *Agrobacterium* culture carrying RdRp_{opt}
387 (OD₆₀₀ 0.05) caused higher eGFP expression in cells. In contrast, increase of
388 *Agrobacterium* culture carrying RdRp_{opt} from OD₆₀₀ 0.1 to OD₆₀₀ 0.4 decreased eGFP
389 expression from the mini-replicon (Fig. 4B and 4C), suggesting that an optimum
390 concentration of RdRp_{opt} is required during RSV infection in plant.

391 Analyses of the five different VSRs have indicated that in the presence of NSs or
392 P19-HcPro-γb, the eGFP expression from the mini-replicon was significantly
393 enhanced (Fig. 3B and 3C). These VSRs are known to function at different steps in
394 host RNA interference (RNAi) pathway during virus infection in plant (44, 52).

Consequently, we conclude that these steps in the RNAi pathway can all affect eGFP expression from the mini-replicon. It is noteworthy that the presence of RSV NS3 alone or NS3 plus one or three of the four VSRs (i.e., NSs or P19-HcPro- γ b) suppressed eGFP expression from the RNA3 mini-replicon (3B and 3C) suggesting that RSV NS3 is a negative regulator of RSV RNA3 mini-replicon expression. Similar phenomenon was also reported for TSWV NSs during virus rescue assays using cDNA clones (48). We hypothesize that this negative regulation is caused by the co-suppression of NS3 gene expression. In this study, addition of NSvc4 significantly increased the number of cells with eGFP fluorescence (Fig. 5A and 5B), further conforming its role in cell-to-cell trafficking.

When the NS3 gene was replaced with a *mCherry* gene (MR3_{(-)mCherry&eGFP}) or with an *eGFP* gene in MR3_{(+)eGFP}, no mCherry or eGFP fluorescence was observed in the leaf tissues co-expressing NP, RdRp_{opt} and VSRs (Fig. 6C and 6D). Deletion of the NS3 gene sequence abolished the function of MR3_{(-)eGFP}. However, gRNA and agRNA were detected in the leaves co-expressing RNA3₍₊₎, NP, RdRp_{opt}, NSvc4 and VSRs (Fig. 6A and 6E). The MR3_{(-)eGFP&NS3stop} mini-replicon contains a translation stop codon immediately after the start codon of NS3 and is still functional in *N. benthamiana* leaves (Fig. 7A, 7B and 7C). This finding suggests that the NS3 coding sequence is required for the eGFP expression of the mini-replicon, probably required for viral transcription. Through nucleotide deletion assays, we determined that the region encompassing nt 1-477 in the NS3 ORF is not sufficient to express eGFP from the MR3_{(-)eGFP} mini-replicon (Fig. 7D, and 7E-G), even though the mutant

mini-replicon (MR3_{(-)eGFPMut1}) is capable of expressing RNA in cells, based on the Northern blot results. Because the positions of the gRNA and agRNA bands from MR3_{(-)eGFPMut2} and MR3_{(-)eGFPMut4} were altered, we speculate that the NS3 ORF sequence may act as a *cis*-regulatory element during RSV RNA3 viral transcription. Importantly, the secondary structure analysis suggested that the coding sequence of NS3 interacts with IGR and 3'-UTR to form a hairpin-like structure and NS3 itself also forms sophisticated hairpin-like structure (Fig 8). Hairpin structure of TSWV and RSV IGR have been shown to regulate viral transcription termination (53). This is consistent with our findings that NS3 coding sequence involved in regulation of viral RNA transcription. In our earlier studies, deletion of NSs coding sequence from the TSWV S-based mini-replicon system had no clear effect on RNA synthesis (48). Although the genome structure of RSV RNA3 is similar to that of TSWV S RNA, this is the first evidence showing that the NS3 coding region can act as *cis*-element to regulate the synthesis of viral RNA transcripts of plant segmented NSR viruses.

Based the established mini-replicon system for MR3_{(-)eGFP}, we have also produced mini-replicons to express MR1_{(-)eGFP}, MR2_{(-)eGFP}, and MR4_{(-)eGFP} as described in (Fig. 9A). In the presence of NP, RdRp_{opt} and VSRs, eGFP was expressed from MR1_{(-)eGFP}, MR2_{(-)eGFP}, and MR4_{(-)eGFP} mini-replicons, respectively (Fig. 9B). We also constructed full-length infectious cDNA clones representing RSV genomic RNA1, RNA2 and RNA4. Infiltration of *N. benthamiana* leaves with the mixed Agrobacterium culture carrying RNA1₍₋₎, RNA2₍₋₎, RNA3₍₋₎, RNA4₍₋₎ and NP+RdRp_{opt}+VSRs did not yield a systemic infection. Because *N. benthamiana* is an

439 experimental host of RSV and the accumulations of RSV RNAs and proteins are
440 lower than that in the rice plants, it is possible that the low level of RSV RNAs and
441 proteins in the infiltrated *N. benthamiana* leaves fails to rescue of RSV systemic
442 infection. It is also possible that the difficulty of delivering four RSV RNA segments
443 into the same cells prevents the systemic infection. We have also infiltrated rice callus
444 tissues with the mixed *Agrobacterium* culture carrying MR1_{(-)eGFP}, MR2_{(-)eGFP},
445 MR3_{(-)eGFP}, MR4_{(-)eGFP} and NP+RdRp_{opt}+VSRs, none of them work in rice callus
446 tissues.

447 In conclusion, there are 3 genera of segmented NSR viruses infecting plants and
448 the replicon-based reverse-genetics system has been established for 2 genera. We now
449 established a mini-replicon-based reverse-genetics system for a tenuivirus in *N.*
450 *benthamiana*. This is the first mini-replicon-based reverse-genetics system for the
451 segmented monocot-infecting tenuivirus, and will provide a useful platform for
452 studies of RSV gene functions during viral replication, cell-to-cell movement and
453 interactions between RSV and host factors in plants. Knowledge learned from this
454 study also benefit the future constructions of full-length multi-segmented infectious
455 clones for tenuivirus in plants.

456

457 **Materials and Methods**

458

459 **Plant growth and virus source.**

460 *Nicotiana benthamiana* plants were grown inside a growth chamber maintained at
461 25°C and a 16/8 h light and dark photoperiod, and used for assays at about 7-leaf

stage. RSV was originally isolated from an RSV-infected rice plant as reported (12). Optimization of RSV *RdRp* ORF codon usage and deletion of putative intron splicing sites were performed based on the predictions using the GeneArt™ Project Manager online software (<https://www.thermofisher.com/order/geneartgenes/projectmanager>).

Plasmid construction

Constructions of RSV RdRp, RdRp_{opt}, NP, NSvc4, NS3 and VSRs mini-replicons.

Complementary DNAs (cDNAs) of *RSV*, *NP*, *RdRp*, *RdRp_{opt}*, *NS3* and *NSvc4* gene were individually amplified from a total RNA sample isolated from an RSV-infected rice plant through reverse transcription polymerase chain reaction (RT-PCR) using gene specific primers. The resulting RT-PCR products were inserted individually into the expression vector pCambia2300 (refers to as p2300 thereafter), pBINplus, or pCXSN to generate p2300-RdRp_{wt}, pBIN-NS3, p2300-NP and pCXSN-NSvc4, respectively. The pCXSV-NSs vector was constructed by inserting a NSs fragment amplified from a cDNA from a TSWV-infected *N. benthamiana* plant into the pCXSN vector. Plasmid pCB301-P19-HcPro-γb (P19-HcPro-γb) that can simultaneously expresses the *Tomato bushy stunt virus* P19 protein, the *Tobacco etch virus* HcPro protein, and the *Barley stripe mosaic virus* γb protein is from a previously published source (46). To construct p2300-RdRp_{opt}, we first optimized RdRp_{wt} codon usage and deleted the putative intron splicing sites in it at the GenScript Biotech Corp (Nanjing, China) followed by inserting the synthesized sequence into the p2300 vector to produce p2300-RdRp_{opt} (RdRp_{opt}).

Constructions of MR3_{(-)eGFP}, MR3_{(-)mCherry&eGFP and MR3_{(+)eGFP} mini-replicons.} To

485 generate the MR3_{(-)eGFP} and the MR3_{(+)eGFP} mini-replicons, we first prepared cDNAs
486 from a total RNA sample isolated from an RSV-infected rice plant using the Reverse
487 Transcription Kit as instructed (Promega, Madison, WI, USA). We then amplify the
488 full-length RSV RNA3₍₋₎ and RNA3₍₊₎ sequences from this cDNA through PCR using
489 RSV RNA3 specific primers (Table S1) and a Phanta Super-Fidelity DNA Polymerase
490 (Vazyme Biotech, Nanjing, China). Both PCR products contained a hammerhead (HH)
491 ribozyme (54) before the 5' end, and then cloned individually behind the 2×35S
492 promoter in the pCB301-2×35S-RZ-NOS vector to generate
493 pCB301-2×35S-HH-RNA3₍₋₎-RZ-NOS (refers to as RNA3₍₋₎) or
494 pCB301-2×35S-HH-RNA3₍₊₎-RZ-NOS (RNA3₍₊₎). The presence of an HH and a
495 Hepatitis delta virus ribozyme (RZ) in these two vectors allow the productions of
496 RNA3₍₋₎ and RNA3₍₊₎ with near perfect ends. To produce a MR3_{(-)eGFP} and a
497 MR3_{(+)eGFP} mini-replicon, we first amplified the *eGFP* gene from SR_{(+)eGFP} (48) using
498 primer FMF37 and FMF38, and then used it to replace the *NP* gene in the RNA3₍₋₎
499 and RNA3₍₊₎ through *in vitro* homologous recombination using a ClonExpress II One
500 Step Cloning Kit (Vazyme Biotech, Nanjing, China). The resulting plasmids were
501 referred to as MR3_{(-)eGFP} and MR3_{(+)eGFP}, respectively. To produce an
502 MR3_{(-)mCherry&eGFP} min-replicon with both *mCherry* and *eGFP* gene, we
503 PCR-amplified the *mCherry* gene from the TSWV SR_{(-)mCherry&eGFP} mini-replicon as
504 reported previously (48) using primer FMF267 and FMF269, and used it to replace
505 the *NS3* gene in MR3_{(-)eGFP} through homologous recombination as describe above. All
506 primers used in this study are listed in Table S1.

507 **Constructions of mutant MR3_{(-)eGFP} mini-replicons.** To produce these mutant
508 mini-replicons, we first introduced a stop codon (TAA) after the original start codon
509 of the NS3 gene through PCR using MR3_{(-)eGFP} as the template, and primer FMF528
510 and FMF36. This NS3stop fragment was then used to replace the NS3 gene in
511 MR3_{(-)eGFP} through homologous recombination using prime FMF40 and FMF529. The
512 resulting plasmid is named as MR3_{(-)eGFP&NS3stop}. We then deleted a 636 nucleotides
513 (nt) fragment from the NS3 gene in MR3_{(-)eGFP} through PCR using primer LLY40 and
514 FMF42, and then inserted it into the pCB301 vector through homologous
515 recombination using prime FMF37 and LLY105 to produce MR3_{(-)eGFPΔNS3}.

516 To further investigate the role of RSV NS3 gene in viral RNA replication and
517 transcription from RNA3₍₋₎, we divided NS3 ORF into four fragments and amplified
518 them individually through PCR using specific primers. These four fragments are:
519 nucleotide position 1-159, 160-318, 319-477, and 478-636, respectively. These
520 fragments were then used to generate MR3_{(-)eGFPMut1}, MR3_{(-)eGFPMut2}, MR3_{(-)eGFPMut3},
521 and MR3_{(-)eGFPMut4}, respectively. Primers used in this study are listed in Table S1. The
522 plasmids were transformed individually into *Agrobacterium tumefaciens* strain
523 GV3101 through electroporation, and the transformants are maintained at -80°C until
524 use.

525

526 **Agrobacterium infiltration**

527 *A. tumefaciens* culture were prepared as described previously (44, 45). Briefly,
528 agrobacterium cultures carrying specific plasmids were grown individually in a

529 culture medium and then diluted to $OD_{600} = 1.0$, or as indicated in the text, in an
530 infiltration buffer (10 mM MES and 10 mM $MgCl_2$, pH 5.6, supplemented with 100
531 μM acetosyringone). After 2-3 h incubation in the dark and at room temperature (RT),
532 *Agrobacterium* cultures harboring p2300-NP ($OD_{600} = 0.2$), p2300-RdRp ($OD_{600} =$
533 0.05), p2300-NSs ($OD_{600} = 0.1$), pCB301-P19-HcPro- γb ($OD_{600} = 0.1$) or one of the
534 vectors ($OD_{600} = 0.2$ each) with an *eGFP* and/or an *mCherry* report gene were mixed
535 in equal volumes, or as indicated in the text. The mixed cultures were then infiltrated
536 individually into leaves of 6-7 leaf-stage-old *N. benthamiana* plants using 1 mL
537 needleless syringes. The infiltrated plants were grown inside a growth chamber with the
538 same growth conditions as described above.

539

540 **Western blot assay**

541 The agro-infiltrated *N. benthamiana* leaves were harvested at 5 days post
542 agro-infiltration (dpi) and homogenized (1 mg/sample) individually in 1 mL
543 extraction buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2%
544 polyvinylpolypyrrolidone, 10 mM dithiothreitol, 10% glycerol, 0.5% Triton X-100,
545 and 1 $\times$ protease inhibitor cocktail reagent). The crude extract from each sample was
546 mixed with a 5 $\times$ loading buffer at a 1:4 ratio (v/v). All the samples were boiled
547 for 10 min and then incubated on ice for 5 min followed by electrophoresis in 12%
548 SDS-PAGE gels. After the protein bands were transferred onto PVDF membranes
549 (GE Healthcare, UK), the blots were probed with an RSV NP specific (1:5000 diluted)
550 or an eGFP specific (1:3000 diluted) antibody followed by a horseradish peroxidase

(HRP)-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:10000 diluted). The detection signal was visualized using the ECL Substrate Kit as instructed (Thermo Fisher Scientific, Rockford, USA). The ponceau S-stained Rubisco large subunit gels were used to show sample loadings.

555

556 **Northern blot assay**

557 To detect the expressions of RSV gRNAs, agRNAs, and *eGFP* mRNA, we isolated
558 total RNA from the agro-infiltrated *N. benthamiana* leaf tissues using the RNAprep
559 Pure Plant Kit (Tiangen Biotech, Beijing, China). The isolated total RNA samples
560 were separated in 1% formaldehyde agarose gels through electrophoresis, and then
561 transferred onto Hybond-N⁺ membranes (GE Healthcare, UK) (55). DIG-labeled
562 RNA probes specific for the sense or antisense *eGFP* mRNA were *in vitro* synthesized
563 using the DIG High Prime RNA labeling kit (Roche, Basel, Switzerland). The blotted
564 membranes were probed with DIG-labeled RNA probes specific for the sense or
565 antisense *eGFP* mRNA. The detection signal was visualized using a DIG-High Prime
566 Detection Starter Kit II as instructed (Roche).

567

568 **Fluorescence microscopy**

569 The agro-infiltrated *N. benthamiana* leaf tissues were harvested at 5 dpi, and
570 examined under an inverted fluorescence microscope (OLYMPUS IX71-F22FL/DIC,
571 Tokyo, Japan) equipped with a green barrier filter. The captured images were
572 processed using the ImagePro system (OLYMPUS, Tokyo, Japan) and then the Adobe

573 Photoshop CS4 (San Jose, CA, USA).

574

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581

582 **Author contributions**

583 M.F., L.L. and X.T. conceived and designed the experiments and M.Y., Y.Z., J.W. and
584 Y.X. provided input. M.F., L.L., R.C., Y.Y., Y.D., M.C., and G.R. performed the
585 experiments. M.F., X.D., X.Z. and X.T. wrote the manuscript.

586

587 **Competing interests:**

588 The authors declare that no competing interests exist.

589

590 **Data availability**

591 All data produced in this study are presented in this manuscript or as the supporting
592 files.

593

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Table 1. RSV NSvc4 enhances the cell-to-cell movement of MR3_{(-)eGFP} mini-replicon in the presence of NP, RdRp_{opt} and four VSRs.

NSvc4 ^a OD ₆₀₀	Total eGFP fluorescent cells	No. of clusters with 1 cell (% of total)	No. of clusters with 2 cells (% of total)	No. of clusters with 3≥cells (% of total)
0	18	18 (100%) ^b	0 (100)	0 (100)
0.025	54	15 (27.8%)	6 (22.2%)	5 (50%)
0.05	76	12 (15.8%)	7 (18.4%)	10 (65.8%)
0.1	95	8 (8.4%)	11 (23.2%)	13 (68.4%)
0.15	140	17 (12.1%)	9 (12.9%)	18 (75%)

^a Agrobacterium cultures harboring the plasmid encoding NSvc4 (final concentration, OD₆₀₀ = 0, 0.025, 0.05, 0.1 and 0.15) were used to infiltrate *N. benthamiana* leaves.

^b eGFP fluorescent foci of RSV MR3_{(-)eGFP} at 5 days post-infiltration.

764

765 **Figure legends**

766 **Fig. 1** Construction of an RSV RNA3₍₋₎-based mini-replicon. (A) Schematics
767 representing the RdRp_{wt}, RdRp_{opt}, NP, RSV RNA3₍₋₎ and MR3₍₋₎eGFP mini-replicon.
768 For MR3₍₋₎eGFP, we replaced the NP gene in the gRNA3 with an eGFP gene. The 5'
769 untranslated, the 3' untranslated, and the intergenic region in RNA3₍₋₎ were indicated
770 with a thin black line. 2×35S, doubled 35S promoter; HH, hammerhead ribozyme; RZ,
771 hepatitis delta virus (HDV) ribozyme; 35S Ter, 35S terminator; NOS, nopaline
772 synthase terminator. Minus sign (-) and 5' to 3' designation represent the viral
773 (genomic)-strand of RNA3. (B) Illustration of agro-infiltration using a mixed
774 Agrobacterium culture carrying various min-replicons into *N. benthamiana* leaves.
775 VSRs, NSs plus P19-HcPro-γb. (C) A *N. benthamiana* leaf infiltrated with a mixed
776 Agrobacterium culture carrying MR3₍₋₎eGFP, RdRp_{wt}, NP and VSRs (NSs and
777 P19-HcPro-γb). (D) A *N. benthamiana* leaf infiltrated with a mixed Agrobacterium
778 culture carrying MR3₍₋₎eGFP, RdRp_{opt}, NP and VSRs (NSs and P19-HcPro-γb) and
779 was showing eGFP fluorescence at 5 dpi under an inverted fluorescence microscope.
780 Bar represents 200 μm.

781

782 **Fig. 2** RSV NP and RdRp_{opt} are required for MR3₍₋₎eGFP expression in *N. benthamiana*
783 leaves. (A) *N. benthamiana* leaves were infiltrated with mixed *Agrobacterium* cultures
784 carrying MR3₍₋₎eGFP+NSs+P19-HcPro-γb+Vec (empty vector),
785 MR3₍₋₎eGFP+NSs+P19-HcPro-γb+NP, MR3₍₋₎eGFP+NSs+P19-HcPro-γb+RdRp_{opt}, or

MR3_{(-)eGFP}+NSs+P19-HcPro-γb+RdRp_{opt}. The infiltrated leaves were examined and photographed under an inverted fluorescence microscope at 5 dpi. Bars = 200 μm. (B) Western blot analyses using the samples described in (A) and an NP or an eGFP specific antibody. The ponceau S-stained Rubisco large subunit gel was used to show sample loadings. (C) Northern blot analyses using the samples described in (A) and a DIG-labeled sense- or an antisense-eGFP probe. The red and blue arrows indicate the antigenomic and genomic RNA3 expressed the infiltrated leaves. The ethidium bromide stained ribosomal RNA gel was used to show sample loadings.

Fig. 3 Effects of VSRs on pMR3_{(-)eGFP} expression. (A) *N. benthamiana* leaves were infiltrated with mixed *Agrobacterium* cultures as indicated in the figure. The infiltrated *N. benthamiana* leaves were harvested at 5 dpi, and examined and photographed under an inverted fluorescence microscope. Bars = 200 μm. (B) Western blot analyses using the samples described in (A), and an NP specific and an eGFP specific antibodies, respectively. Proteins in the leaves shown in panel (A) using NP and GFP-specific antibodies, respectively. The ponceau S-stained Rubisco large subunit gel was used to show sample loadings.

Fig. 4 Concentrations of NP and RdRp_{opt} needed for the maximum expression MR3_{(-)eGFP}. (A and B) *N. benthamiana* leaves were infiltrated with various mixed *Agrobacterium* cultures as indicated in the figure. The concentration of *Agrobacterium* cultures carrying NP or RdRp_{opt} ranged from OD₆₀₀ = 0 to 0.4. The

808 infiltrated leaves were harvested at 5 dpi, and examined and photographed under an
809 inverted fluorescence microscope. Bars = 200 μ m. (C and D) Western blot analyses of
810 NP and eGFP expressions in the infiltrated leaves described in (A and B) using an NP
811 specific and an eGFP specific antibodies, respectively. The ponceau S-stained Rubisco
812 large subunit gel was used to show sample loadings.

813

814 **Fig. 5** Effect of RSV NSvc4 on eGFP expression from MR3_{(-)eGFP} in cells. (A) *N.*
815 *benthamiana* leaves were infiltrated with various mixed Agrobacterium cultures as
816 described in the figure. The concentration of Agrobacterium culture carrying NSvc4
817 ranged from OD₆₀₀ = 0 to 0.15. The infiltrated leaves were harvested at 5 dpi, and
818 examined and photographed under an inverted fluorescence microscope. Bars = 200
819 μ m. (B) Western blot analyses using the infiltrated leaf samples described in (A), and
820 an NP specific and an eGFP specific antibodies, respectively. The ponceau S-stained
821 Rubisco large subunit gel was used to show sample loadings.

822

823 **Fig. 6** Construction and test of RNA3₍₊₎-based mini-replicon in *N. benthamiana*
824 leaves. (A) Schematics representing RNA3₍₊₎ and MR3_{(+)eGFP} mini-replicons. The
825 MR3_{(+)eGFP} mini-replicon was made by replacing the NS3 gene in RNA3₍₊₎ with an
826 *eGFP* gene. Plus sign (+) and 3' to 5' designation represent the viral complementary
827 (antigenomic)-strand of RNA3. (B) *N. benthamiana* leaves were infiltrated with
828 various mixed Agrobacterium cultures as described in the figure. The infiltrated
829 leaves were observed at 5 dpi, and examined and photographed under an inverted

fluorescence microscope. Bars = 200 μ m. (C) Schematics representing MR3_{(-)eGFP} and MR3_{(-)mCherry&eGFP} mini-replicons. The MR3_{(-)mCherry&eGFP} mini-replicon was constructed by replacing the *NS3* gene with an *mCherry* gene. Minus sign (-) and 5' to 3' designation represent the viral (genomic)-strand of RNA3. (D) The infiltrated *N. benthamiana* leaves were harvested at 5 dpi, and examined and photographed under an inverted fluorescence microscope. Bars = 200 μ m. (E) Northern blot analyses of RNA3 expressions from RNA3₍₊₎ in the infiltrated leaves as described in (C). After electrophoresis, the blots were probed with a DIG-labeled antisense and a sense RNA3 probes, respectively. The red and blue arrow heads indicate the RNA3 bands probed with DIG-labeled probes. The ethidium bromide stained ribosomal RNA (rRNA) gel was used to show sample loadings.

841

Fig. 7 Effect of NS3 on *eGFP* gene expression from the mini-replicons. (A and D) Schematics representing MR3_{(-)eGFP} and its mutants. For MR3_{(-)eGFP Δ NS3}, the *NS3* gene was deleted from MR3_{(-)eGFP}. For MR3_{(-)eGFP&NS3stop}, a stop codon was introduced at the downstream of the start codon of the *NS3* gene in pMR3_{(-)eGFP}. For MR3_{(-)eGFPMut1} to 4 mutant mini-replicons, a quarter of the *NS3* gene in MR3_{(-)eGFP} was deleted as shown in the figure. Minus sign (-) and 5' to 3' designation represent the viral (genomic)-strand of RNA3. (B and E) *N. benthamiana* leaves were infiltrated with various mixed *Agrobacterium* cultures as described in the figure. The infiltrated leaves were harvested at 5 dpi, and examined and photographed under an inverted fluorescence microscope. Bars = 200 μ m. (C and F) Western blot analyses using the

852 leaf samples described in (B and E), and an NP specific and an eGFP specific
853 antibodies, respectively. The ponceau S-stained Rubisco large subunit gels were used
854 to show sample loadings. (G) Northern blot analyses of antigenomic and genomic
855 RNA expressions from MR3_{(-)eGFP} using a DIG-labeled antisense- and a sense-eGFP
856 probe, respectively. The ethidium bromide-stained ribosomal RNA gel was used to
857 show sample loadings.

858

859 **Fig. 8** RNA secondary structures of the 3'-UTR, NS3 ORF and IGR region of RSV
860 RNA3 segment. Secondary structure was predicted using the RNA fold web server
861 (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>) based on
862 thermodynamic prediction of minimal free energy (MFE). Red, black and blue arrows
863 indicated the nucleotide positions in 3'-UTR, NS3 and IGR regions, respectively.
864 Dotted boxes indicated that Hairpin 1 and 2 were formed by IGR, NS3 and 3'-UTR of
865 RSV RNA3, respectively.

866

867 **Fig. 9** Establishment of complete mini-replicon systems representing RSV RNA1,
868 RNA2, RNA3, and RNA4 genomic RNA segments in *N. benthamiana* leaves. (A)
869 Schematics representing MR1_{(-)eGFP}, MR2_{(-)eGFP}, and MR4_{(-)eGFP} mini-replicons. The
870 RSV *RdRp* gene in MR1_{(-)eGFP}, the *NSvc2* gene in MR2_{(-)eGFP}, and the *NSvc4* gene in
871 MR4_{(-)eGFP} were replaced with an *eGFP* gene to produce MR1_{(-)eGFP}, MR2_{(-)eGFP}, and
872 MR4_{(-)eGFP}, respectively. Minus sign (-) and 5' to 3' designation represent the viral
873 (genomic)-strand of RNA1, 2 and 4. (B) *N. benthamiana* leaves were infiltrated with

874 various mixed *Agrobacterium* cultures as described in the figures. The infiltrated leaf
875 tissues were harvested at 5 dpi, and examined and photographed under an inverted
876 fluorescence microscope. Bars = 200 μ m.

877

878 **Table S1.** List of primers used in the study.

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

881

882 **Table S3** Optimized RdRp gene sequence made in this study.

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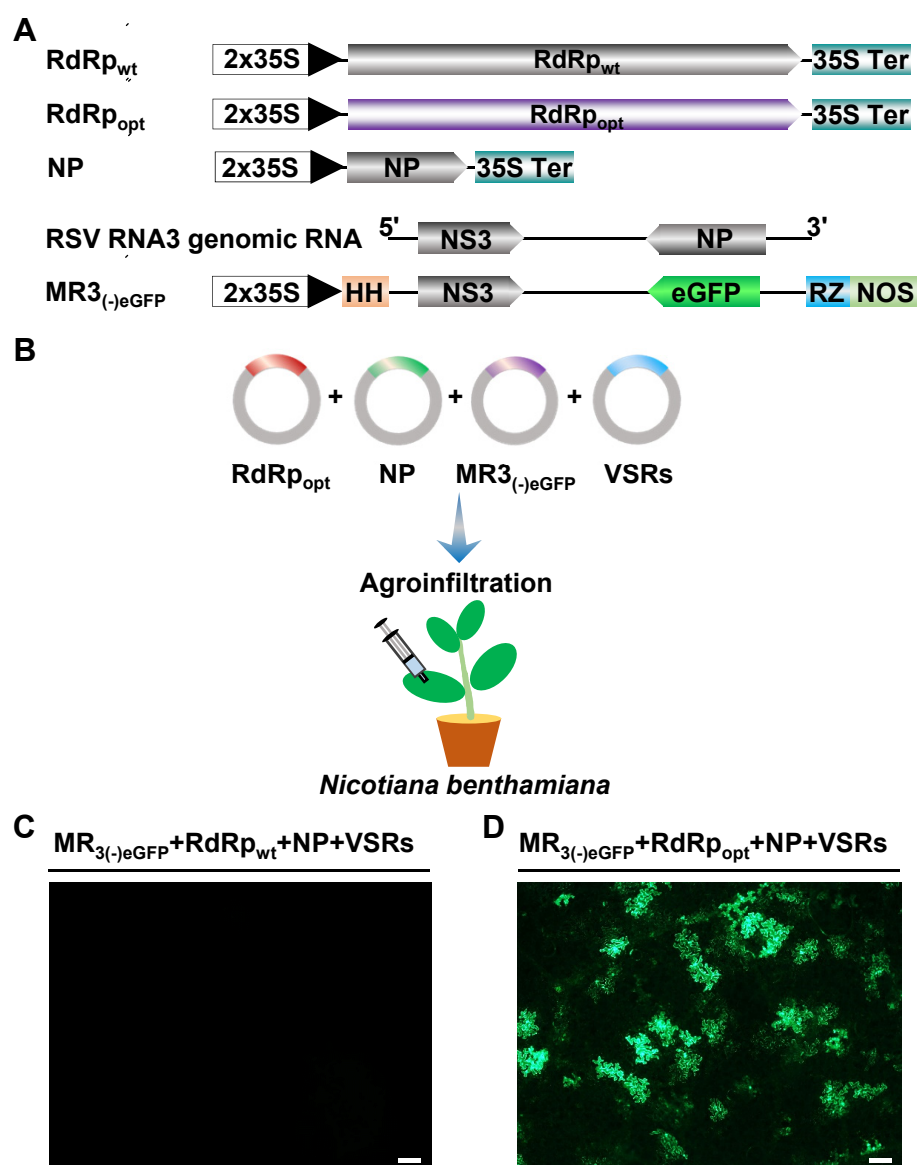


Fig. 1

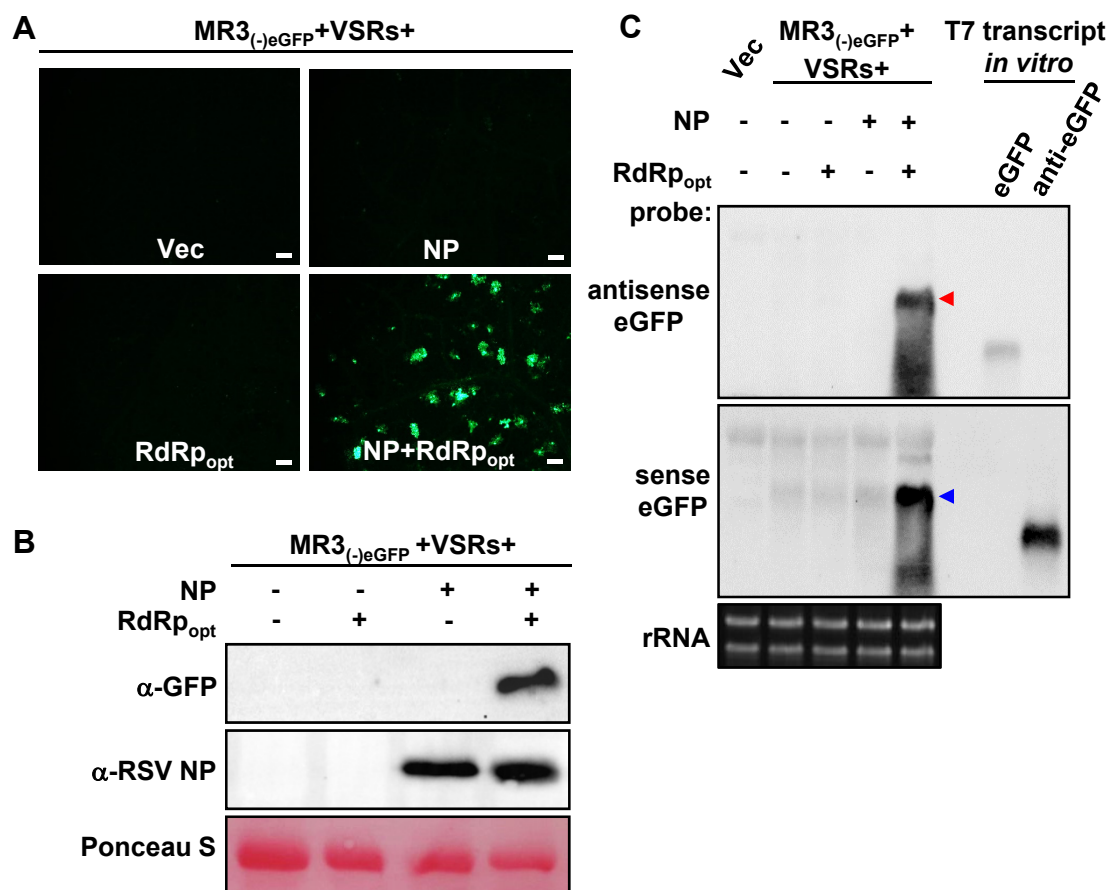


Fig. 2

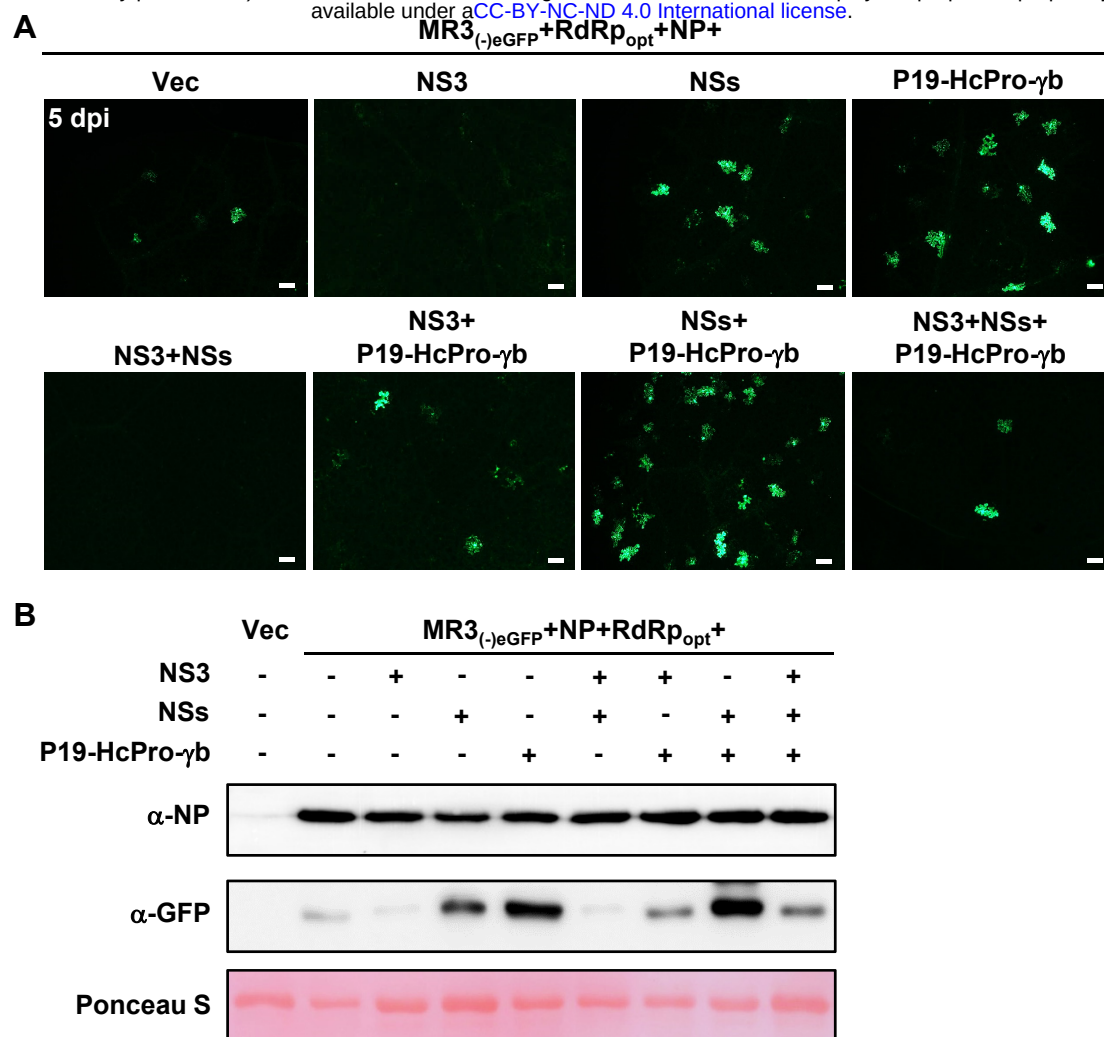


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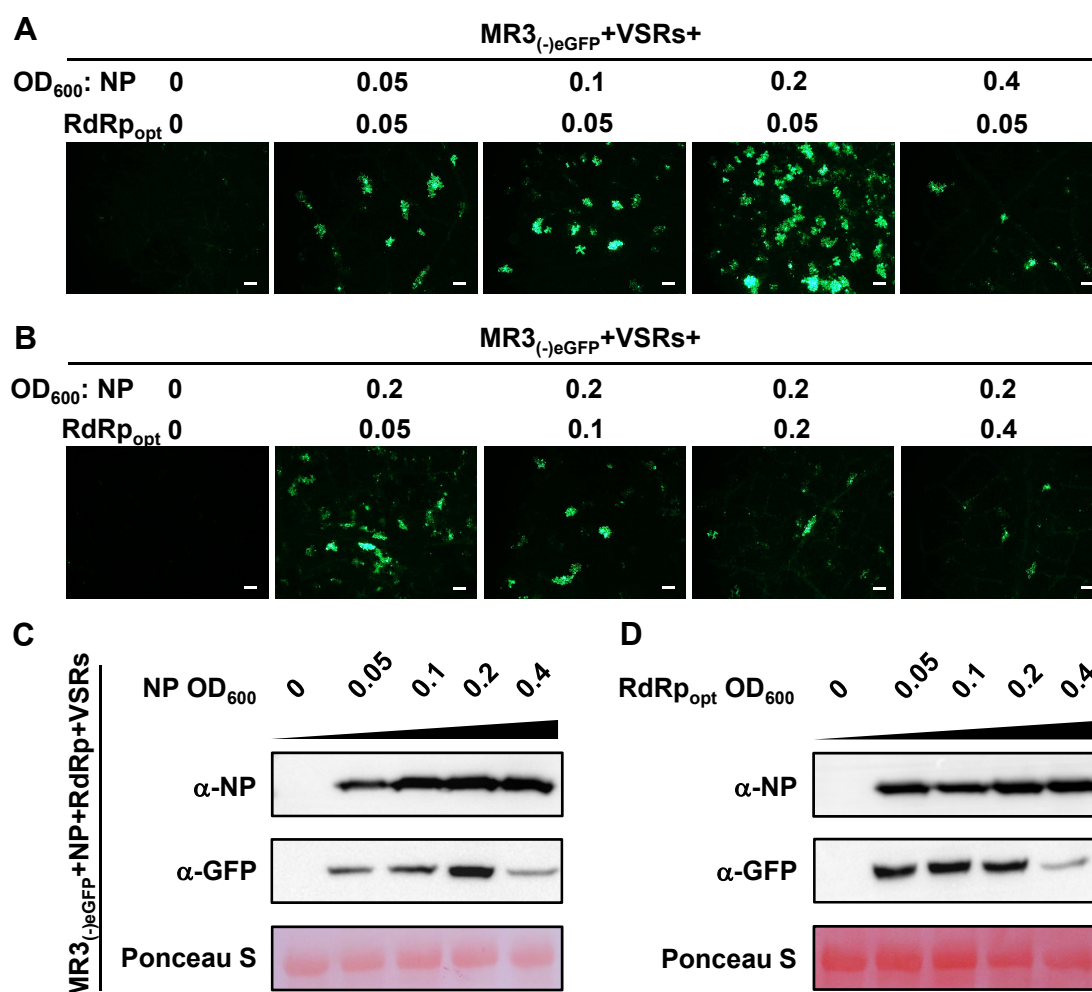


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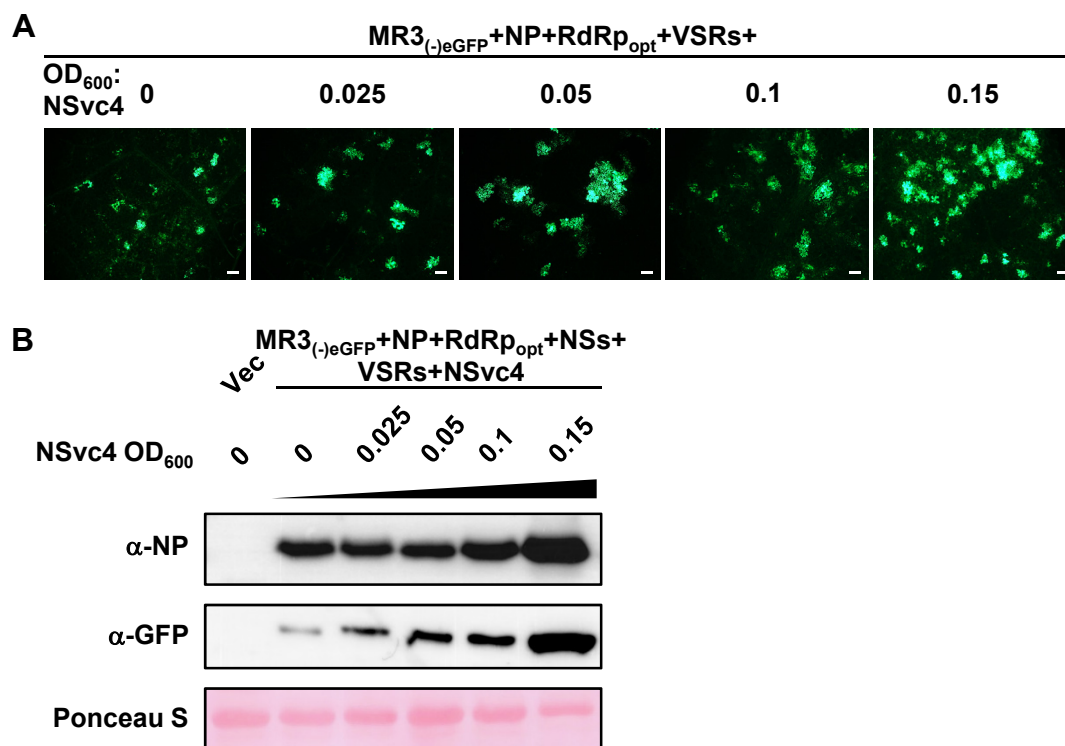


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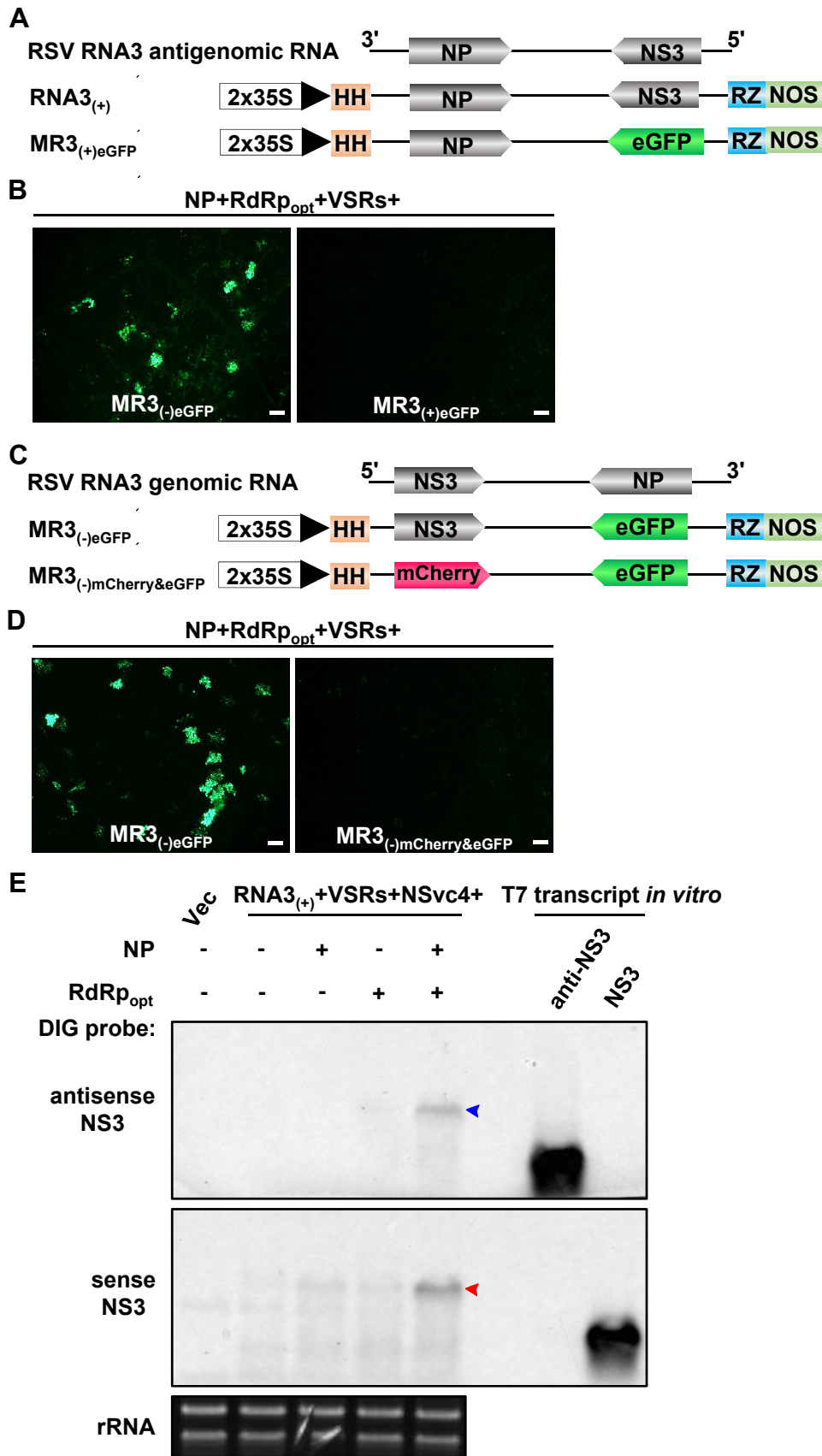


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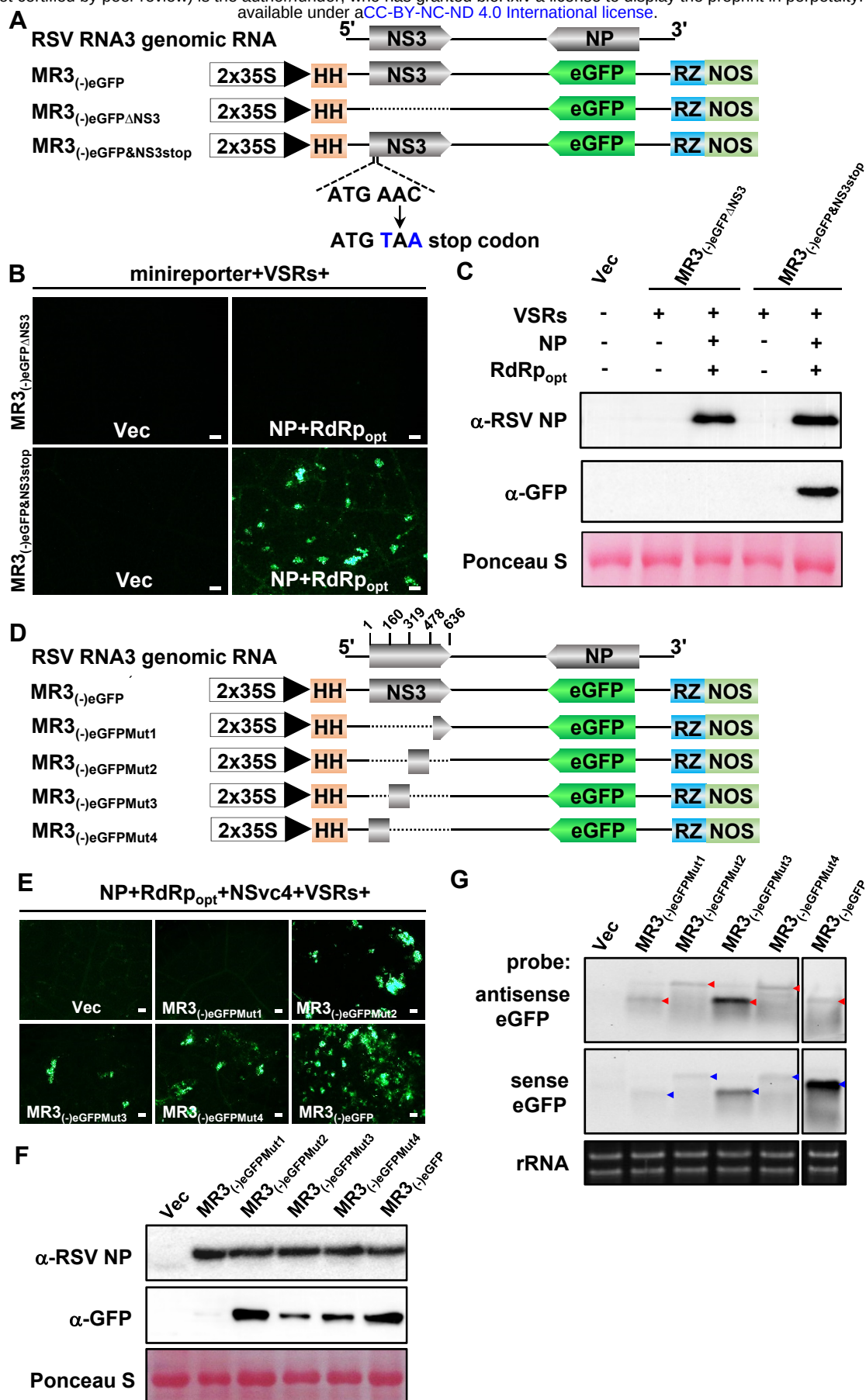


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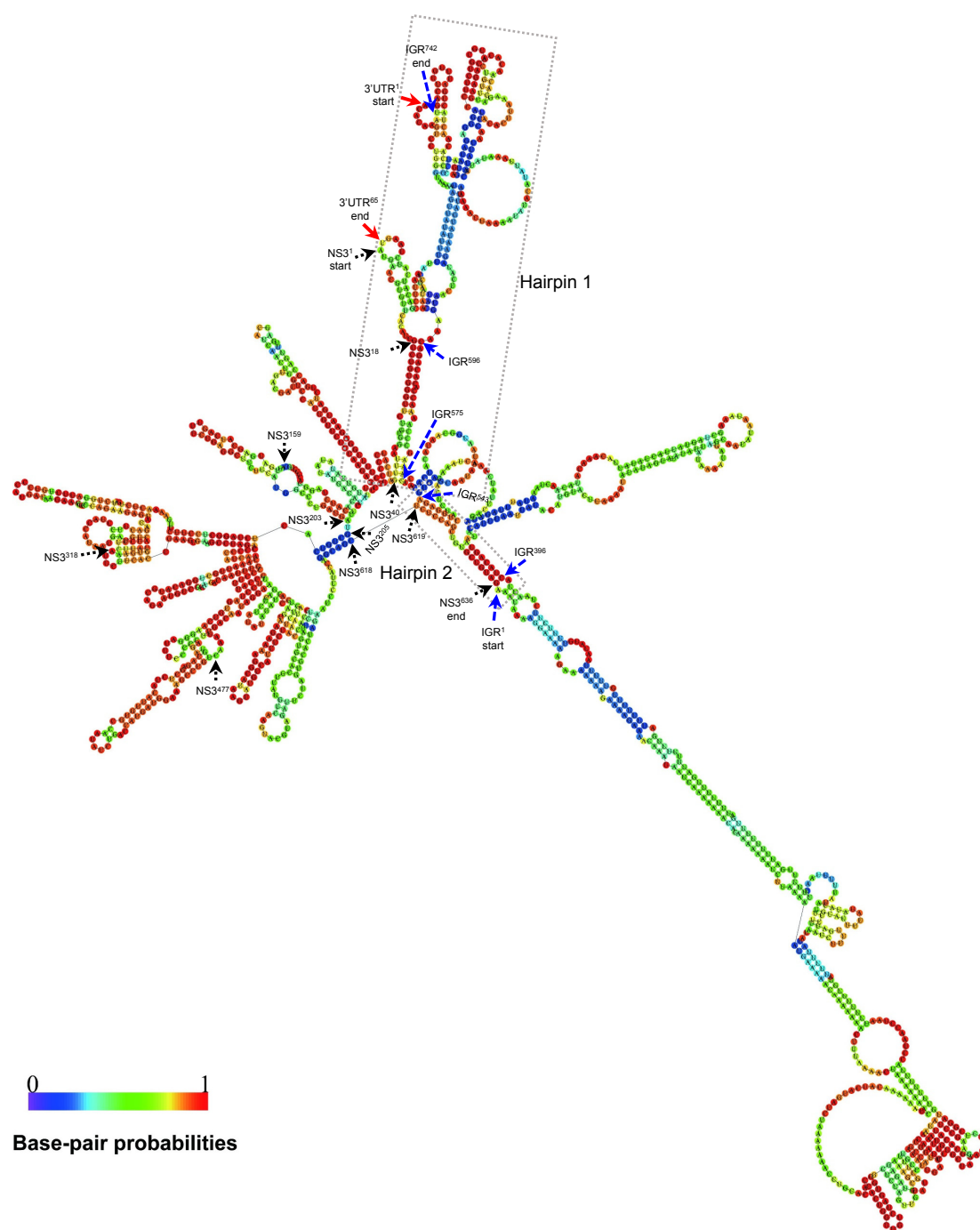


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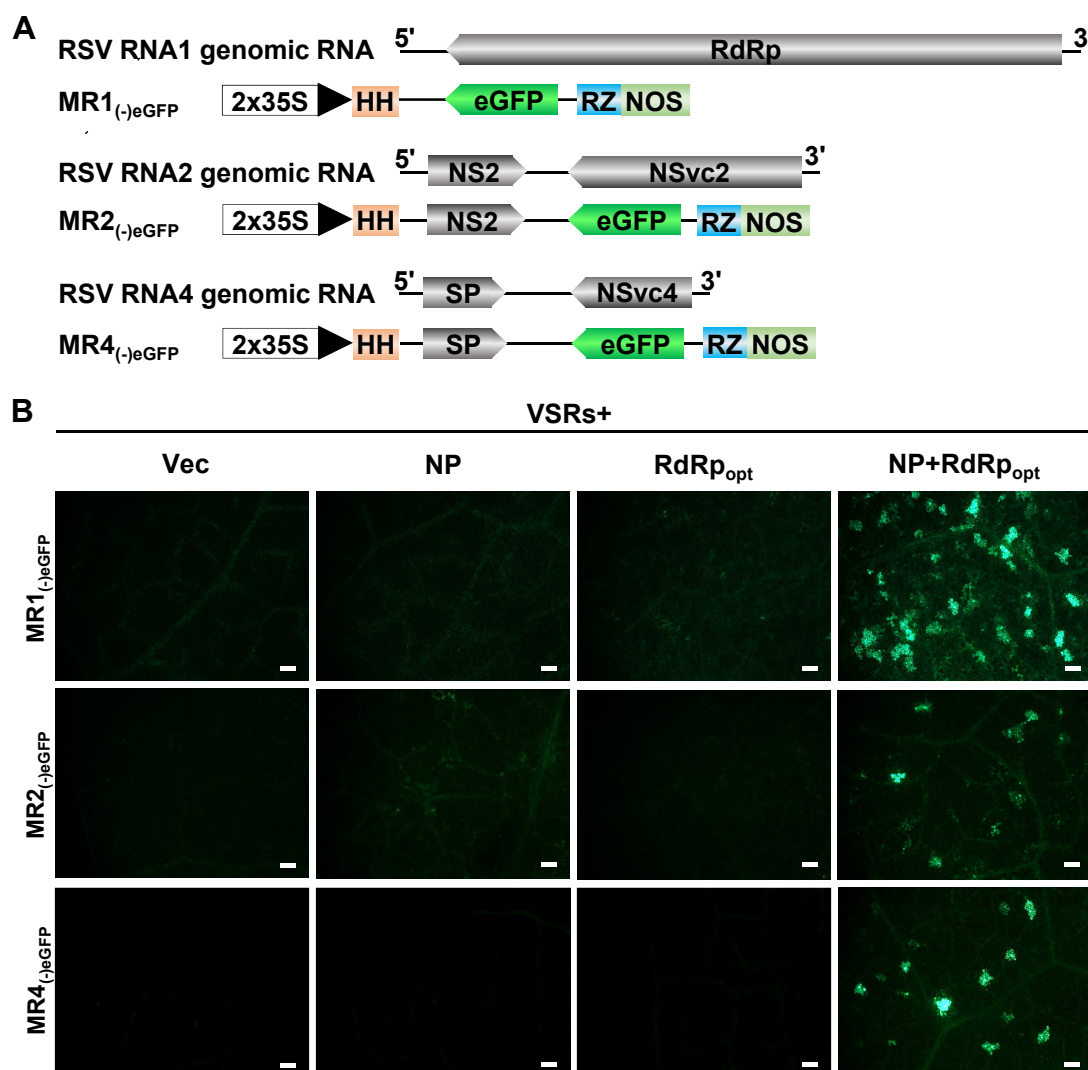


Fig. 9