Functional analysis of a weak viral RNA silencing suppressor using two GFP variants as silencing inducers

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24 **Abstract**

25 RNA silencing in plants can be triggered by the introduction of an exogenous gene. Green
26 fluorescent protein (GFP) has been widely used as a visual reporter to study RNA silencing and
27 viral-mediated suppression of RNA silencing in the model plant *Nicotiana benthamiana*. In
28 transgenic *N. benthamiana* plants expressing an endoplasmic reticulum targeted GFP variant (16c)
29 known as mGFP5, RNA silencing can be induced by ectopic over-expression of mGFP5. However,
30 other GFP variants can also be used to induce GFP silencing in these plants. We compared the
31 efficiency to induce local and systemic silencing of two commonly used GFP variants: enhanced
32 GFP (eGFP) and mGFP5. Using lettuce necrotic yellows virus (LNYV) P protein to suppress GFP
33 silencing, we demonstrate that *eGFP* gene, which is 76% identical at the nucleotide level to the
34 endogenously expressed mGFP5 in 16c plants, triggers silencing more slowly and concurrently
35 prolongs detectable silencing suppressor activity of the weak LNYV P suppressor, compared to the
36 homologous *mGFP5* gene. The use of eGFP as RNA silencing inducer in wild type or 16c plants
37 appears to be a useful tool in identifying and analysing weak viral RNA silencing suppressor
38 proteins whose activity might otherwise have been masked when challenged by a stronger RNA
39 silencing response. We also show that reducing the dosage of strong dsRNA silencing inducers in
40 conjunction with their homologous GFP targets facilitates the discovery and analysis of “weaker”
41 RNA silencing suppressor activities.

42 **Keywords**

43 Cytorhabdovirus, phosphoprotein, GFP silencing, RNA silencing suppression, *N. benthamiana* 16c,
44 green fluorescent protein.

45 **1. Introduction**

46
47 Plant RNA silencing pathways are involved in modulating host gene expression, defence
48 against invading viruses and transposons, and silencing of transgenes (Burgyan and Havelda, 2011;

49 Voinnet, 2001). Briefly, long double-stranded RNAs (dsRNAs), which act as the trigger for RNA
50 silencing, are cleaved by Dicer-like (DCL) RNases into small-interfering RNAs (siRNAs). These
51 siRNAs are then incorporated into an RNA-induced silencing complex (RISC) and guide Argonaute
52 (AGO) proteins to degrade complementary mRNAs or suppress their translation (Aukerman and
53 Sakai, 2003; Li and Ding, 2006; Vaucheret, 2006). In plants, RNA silencing can also be amplified
54 by the activity of endogenous RNA-dependent RNA polymerases (RDRs) (Dalmay et al., 2000;
55 Wang et al., 2010). This generates additional long dsRNAs that are cleaved by DCLs to form
56 secondary siRNAs, which leads to transitivity of siRNA biogenesis along the mRNA target beyond
57 the initial inducer sequence (Himber et al., 2003; Sijen et al., 2001; Vaistij et al., 2002), and may
58 potentially lead to systemic silencing of the target mRNA.

59 Viral RNA silencing suppressors (RSS) have been collectively demonstrated to interfere
60 with almost every step of the RNA silencing pathway. However, it is thought that the majority of
61 viral RSS function by binding to and sequestering siRNAs or microRNAs (miRNAs), preventing
62 their incorporation into RISC. Examples of this category of RSSs include the tombusvirus P19
63 (Silhavy et al., 2002), cucumovirus 2b (Chen et al., 2008; Hamera et al., 2012) and potyvirus HC-
64 Pro proteins (Mallory et al., 2001). Recently, we demonstrated that lettuce necrotic yellows virus
65 (LNYV) phosphoprotein (P) is a weak local RSS in plants that does not affect the accumulation of
66 siRNAs suggesting a target downstream of siRNA biogenesis. In addition, LNYV P was shown to
67 delay but not prevent systemic silencing of ER-targeted green fluorescent protein (mGFP5-ER) in
68 transgenic *Nicotiana benthamiana* 16c plants (Mann et al., 2015). LNYV is the type species of the
69 genus *Cytorhabdovirus*, family *Rhabdoviridae*. LNYV contains a negative-sense, single-stranded
70 RNA genome that consists of 12,807 nucleotides (nt) and encodes six proteins: nucleoprotein (N),
71 phosphoprotein (P), movement protein (4b or P3) (Mann et al., 2016a), matrix protein (M),
72 glycoprotein (G) and polymerase protein (L) flanked by complementary 3' leader (l) and 5' trailer
73 (t) sequences in the order 3'l-N-P-4b-M-G-L-t 5' (Dietzgen et al., 2007; Jackson et al., 2005; Mann
74 and Dietzgen, 2014).

75 GFP is widely used as reporter in transient expression assays for RNA silencing and its
76 suppression in *N. benthamiana* using agrobacterium-mediated delivery (Johansen and Carrington,
77 2001). Various versions of the *GFP* coding sequence have been used in reporter genes, including
78 the *eGFP* (GenBank accession no. U43284) and *mGFP5* (GenBank accession no. U87973) genes
79 that share 76% nucleotide sequence identity. Enhanced GFP (eGFP) has been primarily used for
80 local RSS assays in wild-type plants (Duan et al., 2012), while mGFP5 is generally used for local
81 and systemic RSS assays in 16c plants (Deng et al., 2014; Lacombe et al., 2010; Lu et al., 2004;
82 Ruiz et al., 1998). However, eGFP has been shown to have sufficient nucleotide sequence identity
83 to also trigger silencing of mGFP5 in 16c plants (Ding et al., 2004; Guo and Ding, 2002). No
84 reported studies have used eGFP to test viral RSS activity in 16c plants (Ding et al., 2004), likely
85 owing to the slower rate of silencing induction. Here, we compare differences in RNA silencing
86 induction efficiency between eGFP and mGFP5 variants, and demonstrate that weaker RSS such as
87 LNYV P, can be more clearly identified by using a less potent silencing inducer. Indeed, our data
88 suggests that slower inducers of reporter gene silencing are more useful in identifying the full
89 spectrum of viral suppressors of RNA silencing.

90 **2. Materials and methods**

91 **2.1 Gateway cloning**

92 Gateway entry clones in pDONR221 for LNYV N, P, 4b, M and G open reading frames
93 (ORFs) (Martin et al., 2012) (Thermo Fisher Scientific) were cloned into the plant expression vector
94 pSITE-Flag-C1 (kindly provided by Michael Goodin, University of Kentucky, USA) to yield N-
95 terminal flag-tagged proteins following instructions described in the Gateway LR Clonase II
96 Enzyme Mix kit (Thermo Fisher Scientific). Positive RSS controls tomato bushy stunt virus
97 (TBSV) P19 and cucumber mosaic virus (CMV)-207 2b (Australian isolate 207) ORFs were cloned
98 as previously described (Mann et al., 2015).

2.2 Agroinfiltration of *Nicotiana benthamiana*

N. benthamiana plants were grown in glasshouse conditions at 25°C with 16 hour photoperiod until they reached 4 – 5 weeks. *Agrobacterium tumefaciens* GV3101 cultures harbouring LNYV Gateway expression constructs or GFP variants were diluted and mixed in equal volumes to a final OD₆₀₀ of 0.4 – 0.5 prior to agroinfiltration into the underside of wild type *N. benthamiana* or GFP-expressing 16c *N. benthamiana* leaves as previously described (Mann et al., 2015). Post-agroinfiltration plants were moved into plant growth chambers (Conviron) and grown at 25°C with 16 hour photoperiod. Agrobacteria harbouring plasmids encoding enhanced GFP (eGFP, GenBank accession no. U43284; pUQC-214), hairpin or dsRNA GF (GF; pUQC-251), eGFP plus GF (eGFP/GF; pUQC-218) and ER-targeted mGFP (mGFP5, GenBank accession no. U87973; pBIN-mGFP5) were used as inducers and reporters of local and systemic RNA silencing (Fig. 1A).

2.3 RNA analysis

Total RNA was separated into high and low molecular weight RNA from 2 g of plant tissue using TriReagent (Sigma) following the protocol described previously (Mitter and Dietzgen, 2012). RNA blot detection of GFP mRNA was carried out using 8 µg of total plant RNA, following the recommended materials and protocols suggested by the manufacturer of the NorthernMax Kit (Ambion). In addition, GFP siRNA detection was carried out using ~8 µg of low molecular weight plant RNA following the protocol described previously (Mitter and Dietzgen, 2012), with the exception that smaller polyacrylamide gels were used and electrophoresed in the XCell SureLock™ Mini Cell (Thermo Fisher Scientific). Also, small RNAs were transferred using the BioRad Transblot SD transfer system and were detected using the BrightStar BioDetect detection kit (Thermo Fisher Scientific). Lastly, non-isotopic full-length GFP DNA biotin probes were generated to detect GFP mRNA and GFP siRNAs (Thermo Fisher Scientific). All protein and RNA analyses were independently repeated at least two times.

123 **2.4 GFP fluorescence**

124 GFP fluorescence in *N. benthamiana* leaves was observed using a long wavelength Black
125 Ray model B 100AP UV lamp (UVP, California, USA) at 365 nm and images were captured using
126 a Canon EOS 450D camera equipped with a yellow filter.

127 **3. Results**

128 **3.1 LNYV P protein suppresses local GFP silencing more efficiently when induced with eGFP** 129 **than with mGFP5 in *N. benthamiana* 16c plants**

130 The introduction of exogenous genes into plants by agroinfiltration triggers RNA silencing.
131 GFP is both an inducer and target of RNA silencing in *N. benthamiana*, and has been widely used
132 as a reporter to study RNA silencing (Johansen and Carrington, 2001). This approach has provided
133 researchers with a tool to study viral RSS proteins, and has led to a better understanding of the RNA
134 silencing pathway. Using well-established silencing reporter assays, a recent report has
135 demonstrated that LNYV P protein is a weak RSS compared with the potent TBSV P19 protein
136 (Mann et al., 2015). The efficiency of viral suppression of silencing appears to be a relative measure
137 that is dependent on the amount of inducer or speed at which RNA silencing is established. We
138 propose that the strong gene transcription provided by current 35S promoter-based silencing assays
139 will trigger a potent RNA silencing response, which may make the detection of weaker suppressor
140 activity more difficult. To investigate this hypothesis, we compared the efficiency of local RNA
141 silencing induced by eGFP in both non-transgenic *N. benthamiana* and mGFP5-expressing
142 transgenic *N. benthamiana* 16c plants. The eGFP silencing inducer is 76% identical at the
143 nucleotide level to the transgenically expressed silencing target mGFP5 in 16c plants (Fig. S1),
144 whereas mGFP5 inducer has a 100% match with the endogenous target gene. TBSV P19 and empty
145 expression vector were used in all assays as positive and negative controls, respectively. Co-
146 agroinfiltration of eGFP and individual flag-tagged LNYV genes showed strong GFP fluorescence
147 under UV light at 3 days post infiltration (dpi) in non-transgenic (Fig. 2A) and transgenic 16c plants

148 (Fig. 2C). At 6 dpi, GFP fluorescence after co-infiltration with constructs expressing LNYV non-
149 RSS proteins N, 4b, M and G, was significantly lower in non-transgenic plants and undetectable in
150 16c plants (Fig. 2B and D). In contrast, TBSV P19 RSS showed strong GFP expression in both non-
151 transgenic and transgenic 16c plants at 6 dpi (Fig. 2B and D). Co-infiltration of eGFP with the weak
152 LNYV P RSS led to higher GFP fluorescence in non-transgenic plants, while in 16c plants, GFP
153 fluorescence was barely detectable (compare “P” in Fig. 2B and D). Thus, the agroinfiltrated eGFP
154 appears to be silenced more quickly in transgenic 16c plants expressing mGFP than in non-
155 transgenic plants. This is likely due to agroinfiltrated eGFP and endogenous mGFP5 synergistically
156 triggering silencing of both versions of GFP in transgenic 16c plants.

157 To validate the visual suppression of local GFP silencing by LNYV P (as depicted in Fig.
158 2C-D), RNA blot analysis of infiltrated *N. benthamiana* 16c leaves was performed. At 3 dpi, RNA
159 blot analysis detected a low amount of eGFP mRNA when co-expressed with LNYV N, 4b, M and
160 G, similar to the empty vector. In contrast, co-expression of LNYV P or TBSV P19 protein with
161 eGFP resulted in increased accumulation of eGFP mRNA (Fig. 2E). However, at 6 dpi, GFP mRNA
162 was undetectable across all co-expressed LNYV proteins, including P, but remained detectable
163 when co-expressed with TBSV P19 (Fig. 2E). Lack of GFP mRNA accumulation at the later time
164 point is consistent with the observation that GFP fluorescence was nearly undetectable beyond 6 dpi
165 for each of the expressed LNYV proteins in 16c agroinfiltrated plants (Fig. 2D). We also confirmed
166 that none of the LNYV proteins, in contrast to TBSV P19, prevented the accumulation of GFP-
167 specific siRNAs at both 3 and 6 dpi (Fig. 2F). Thus, despite the faster silencing of eGFP
168 fluorescence in 16c leaves, we obtained similar RNA blot results to those seen with non-transgenic
169 plants with respect to both GFP mRNA and siRNA accumulation described previously (Mann et al.,
170 2015). Taken together, these data show i) the importance of RNA blotting to validate transient leaf
171 patch assays, and ii) that performing local GFP silencing assays in 16c plants may mask the activity
172 of weak viral RSS when only examined visually. Thus, we conclude that a weaker local RSS is
173 more reliably identified using non-transgenic *N. benthamiana* instead of transgenic 16c plants.

174 To allow comparisons between the eGFP (above) and mGFP5 silencing assay systems, we
175 carried out a local silencing assay in 16c plants using the homologous mGFP5 as the inducer of
176 silencing. Co-agroinfiltration of 16c plants with mGFP5 and LNYV N, or with CMV 2b protein
177 (weak RSS of Australian isolate 207) expression constructs, both resulted in no detectable GFP
178 fluorescence similar to the empty vector at 4 dpi (Fig. 2G). Very weak GFP fluorescence was
179 observed when mGFP5 was co-expressed with LNYV P. In contrast, TBSV P19 showed strong
180 GFP fluorescence (Fig. 2G). The induction of GFP silencing in 16c plants by exogenous mGFP5
181 was much faster than with eGFP [compare the results for the inducer/target GFP pairs eGFP/eGFP
182 (Fig. 2B), eGFP/mGFP5 (Fig. 2D) and mGFP5/mGFP5 (Fig. 2G)]. This is likely due to the mGFP5
183 inducer and endogenous target having 100% sequence identity. As a result, testing for local
184 silencing suppression activity of a weak RSS such as LNYV P or CMV-207 2b may be largely
185 masked when challenged by an assay system that generates a fast and strong GFP silencing
186 response. In summary, weaker local RSS can be more reliably detected using non-transgenic *N.*
187 *benthamiana* plants with eGFP as both inducer and target, instead of 16c plants induced by either
188 eGFP or mGFP5.

189 **3.2 Weak RSS suppress systemic silencing in *N. benthamiana* 16c plants when challenged with** 190 **eGFP but not mGFP5**

191

192 Traditionally, systemic silencing assays have been carried out in mGFP5-expressing *N.*
193 *benthamiana* 16c plants using mGFP5 as the exogenous silencing inducer. However, since we
194 observed that mGFP5 and eGFP instigate different potency and speed of local silencing induction
195 (Fig. 2), we hypothesized that a similar situation may arise during systemic silencing which may
196 have implications for detection of weak systemic RSS activity. To investigate this possibility, 16c
197 plants were co-agroinfiltrated with either mGFP5 or eGFP and individual LNYV gene expression
198 constructs. At 21 dpi, co-agroinfiltrations of empty vector with mGFP5 showed complete systemic

199 silencing (i.e. chlorophyll autofluorescence in upper non-infiltrated leaves) in three out of three 16c
200 plants (Table 1, Fig. 3A). A parallel experiment using eGFP as inducer similarly showed systemic
201 silencing in 11 out of 12 plants (Table 1, Fig. 3B). Similar results were observed for LNYV N, 4b,
202 M and G using either mGFP5 or eGFP (Table 1). In contrast, LNYV P, CMV-207 2b and TBSV
203 P19 showed suppression of systemic silencing to varying degrees (Table 1). Using mGFP5 as
204 inducer sequence, all three RSSs showed partial systemic silencing suppression (Table 1), which
205 manifested itself as incomplete GFP silencing (i.e., variegated chlorophyll autofluorescence) (Fig.
206 3A). This observation was confirmed by RNA blot analysis of total RNA which showed that RSS
207 expression facilitated accumulation of more mGFP5 mRNA than empty vector but less than the
208 mock control (Fig. 3C). On the other hand, using eGFP as inducer sequence, all three viral RSSs
209 showed strong suppression of systemic silencing as evidenced by GFP fluorescence in upper non-
210 infiltrated leaves (Fig. 3B) and target mGFP5 mRNA accumulation similar to that of the untreated
211 mock control (Fig. 3C). The efficiency of GFP silencing suppression appeared to be determined by
212 the strength of RSS activity, with TBSV P19 showing 100% and the weaker RSS LNYV P and
213 CMV-207 2b showing 83% and 74%, respectively (Table 1). These results suggest that LNYV P
214 RSS activity may also involve inhibiting systemic silencing similar to CMV-207 2b, but generally
215 in a less efficient manner compared to TBSV P19. These data provide further evidence that the
216 slower rate of silencing induced in 16c plants by eGFP compared with mGFP5, may allow weaker
217 systemic RSS such as LNYV P and CMV-207 2b to be more easily identified.

218 **3.3 LNYV P protein suppresses silencing induced by GFP-dsRNA in a dose-dependent** 219 **manner in non-transgenic *N. benthamiana* plants**

220

221 Double-stranded RNA generated by RDR6 leads to the generation of a potent secondary
222 wave of siRNA to enhance local silencing (Dalmay et al., 2000; Himber et al., 2003; Sijen et al.,
223 2001; Vaistij et al., 2002). Brosnan et al. (2007) reported two hairpin T-DNA constructs, pUQC218
224 and pUQC251, for silencing of the target *35S:eGFP* transgene (pUQC214). pUQC218 and

225 pUQC251 both express a 35S-driven GF-specific dsRNA corresponding to nucleotides 9-400 of the
226 720 nt *eGFP* coding sequence. Additionally, pUQC218 also expresses the 35S:*eGFP* transgene
227 (Fig. 1A) (Brosnan et al., 2007). To test if these constructs lead to different rates of silencing or
228 differentially affect detection of RSS activity of a weak suppressor such as LNYV P, non-transgenic
229 *N. benthamiana* plant leaves were agroinfiltrated with pUQC218 (Fig. 1B) or pUQC214 plus
230 pUQC251 (Fig. 1C). Co-agroinfiltration of *N. benthamiana* leaves with pUQC218 and LNYV P or
231 empty vector, in contrast to TBSV P19, showed no detectable GFP fluorescence as early as 2 dpi
232 (Fig. 1B). However, when the target (GFP) and inducer (GF) of silencing were expressed from
233 separate plasmids, co-agroinfiltration of pUQC214 and pUQC251 with LNYV P showed marginally
234 more GFP fluorescence than with empty vector at 2 dpi (Fig. 1C). Although the observed GFP
235 fluorescence was faint for LNYV P (Fig. 1C), this indicates that expressing GFP and GF from
236 separate plasmids, as opposed to pUQC218, which expresses these from the same T-DNA, reduces
237 the speed of local GFP silencing induction, opening the way for detection of weak suppressor
238 activity. This also suggests that LNYV P RSS activity may be characterized as inversely related to
239 the silencing induction rate.

240 To explore this further, we tested the dosage effect of serial dilutions of pUQC251 GF
241 dsRNA silencing inducer on the detection of LNYV P RSS activity. Agrobacterium cultures
242 carrying GF inducer were diluted from OD₆₀₀ of 0.8 to 0.4 (2x dilution), 0.08 (10x dilution) and
243 0.04 (20x dilution) and then co-agroinfiltrated along with equal volumes of agrobacteria harbouring
244 pUQC214 (35S:*eGFP*) (OD₆₀₀ = 0.8) and LNYV P (OD₆₀₀ = 0.8) into non-transgenic *N.*
245 *benthamiana* leaves (Fig. 4). After 6 dpi, GFP fluorescence was undetectable in the presence of
246 LNYV P similar to the empty vector when pUQC251 was diluted two-fold (Fig. 4). However,
247 stronger GFP fluorescence was observed with LNYV P in comparison to empty vector when
248 pUQC251 was diluted ten- and 20-fold (Fig. 4). Although LNYV P showed weaker GFP
249 fluorescence in comparison to the strong RSS TBSV P19 (Fig. 4), these data indicate that lower
250 doses of the dsRNA inducer may lead to a slower rate of local GFP silencing thereby allowing

weaker RSS activities to be more easily detected. Taken together, a slower rate of silencing induction (e.g., eGFP vs mGFP5 or a diluted GF-specific dsRNA construct) in a local silencing leaf patch assay allows weaker RSS activities to be identified more reliably than in a standard assay.

4. Discussion

RNA silencing in plants operates as part of the complex defence responses against invading viruses (Ding et al., 2004). We previously reported that LNYV P protein was found to weakly inhibit local silencing in non-transgenic *N. benthamiana* plants when induced with eGFP and could delay but not prevent systemic silencing in transgenic 16c *N. benthamiana* plants when induced with mGFP5 (Mann et al., 2015). In this study, we examined differences in the efficiency of silencing induction of a GFP reporter by employing two commonly used GFP variants (enhanced GFP and ER-targeting mGFP5) in *N. benthamiana* plants and delineate which silencing inducer is the better choice when studying weak viral RSS, such as LNYV P. We also examined the potential benefits of varying the dosage of a dsRNA silencing inducer to identify potential weak RSS in a local silencing assay.

For a weak viral RSS, such as LNYV P, silencing suppression efficiency appears to be a dose-dependent phenomenon that is modulated by the speed and intensity of the inducer sequence triggering silencing of GFP. We have shown that local silencing suppression by a weak RSS can be almost undetectable if mGFP5 is used as the inducer in conjunction with transgenic 16c plants (Fig. 2G). This suggests that using mGFP5 as inducer generates a silencing response too potent for LNYV P to efficiently suppress. Thus, a leaf patch assay using eGFP sequence variant as a silencing inducer in non-transgenic *N. benthamiana* plants as opposed to mGFP5 in 16c *N. benthamiana* plants can better evaluate weak local RSS activity. In addition, substitution of mGFP5 by eGFP as the inducer in transgenic 16c systemic silencing assays showed that a weak RSS has a stronger and more long-term systemic suppression activities (Fig. 3 and Table 1) and that the differing strengths of systemic RSS can be more readily differentiated using this modified assay. In further support that

277 detection of LNYV P RSS activity is contingent upon silencing dosage, LNYV P showed RSS
278 activity only when small doses of dsRNA GFP silencing inducer were applied (Fig. 4). This result
279 would have been missed for a weak RSS since strong RSS can normally prevent the potent
280 induction of silencing by dsRNA without any requirement for dilution of the silencing inducer
281 (Baumberger et al., 2007; Canizares et al., 2008; Csorba et al., 2007).

282 A potential explanation as to why inducers triggering a slower rate of silencing can be
283 beneficial for the study of weak RSS may lie in the over-expression generated from strong
284 promoters. It has been shown that a 35S promoter-driven transgene results in siRNA production 50
285 times greater than with other weaker promoters (Jefferson et al., 1987; Odell et al., 1985). Our data
286 provide evidence that current 35S-based GFP silencing assays are potentially too potent for the
287 identification and study of weak RSSs. This notion is supported by the case of the tobacco rattle
288 virus 16K RSS protein, which was shown to be a more efficient local RSS when induced with only
289 low doses of dsRNA GFP inducer (Martinez-Priego et al., 2008), similar to that observed for LNYV
290 P in this study. Such considerations will be important when testing weak viral RSS, of which only a
291 handful of studies have been reported, such as potato virus X P25 (Senshu et al., 2009), and squash
292 mosaic virus small coat protein (Yoon et al., 2012). RSS activity detection issues are likely
293 compounded by other parameters that can influence systemic silencing assays, such as temperature
294 and light conditions during maintenance of plants (Patil and Fauquet, 2014). Taken together, the
295 choice of GFP sequence variant as silencing inducer and plant genetic background in conjunction
296 with assay conditions can yield variable results, which may affect the identification and
297 characterization of both weak and strong RSSs. For instance, we previously reported that TBSV
298 P19 expression showed a reduction in systemic RSS activity (54% silencing suppression efficiency)
299 as early as 14 dpi using mGFP5 as the inducer (Mann et al., 2015), while similar assays performed
300 by other research groups showed closer to 100% efficiency at similar or even later time points (Guo
301 et al., 2013; Hamilton et al., 2002; Kong et al., 2014). We propose that weak viral RSS may be best

302 assessed for GFP silencing suppressor activity locally in non-transgenic *N. benthamiana* and
303 systemically in transgenic 16c *N. benthamiana* using eGFP instead of mGFP5 as silencing inducer.

304

305 As new plant viral RSS and their functions are to be discovered, future studies should
306 approach local and systemic silencing assays with the aim of testing both weaker and stronger
307 inducers of GFP silencing in order to firstly detect RSS activity, and secondly, to ascertain the
308 strength of this activity. Such an approach should help to strengthen and generate more valid
309 comparisons between viral RSS across different laboratories. To this end, it will be interesting to
310 see whether other plant rhabdoviruses also utilize weak RSS activity in transient assays or whether
311 they are divergent for this function as recently demonstrated by the nucleotide-binding protein of
312 carlaviruses (Deng et al., 2014). In addition, it may be possible that plant rhabdoviruses encode
313 more than one RSS, such as in the case of the rice yellow stunt virus proteins P and P6 proteins
314 (Guo et al., 2013). These multiple RSSs may potentially have different strengths and specific targets
315 in the RNA silencing pathway similar to those described for citrus tristeza virus that encodes three
316 unique RSS proteins (Lu et al., 2004). There are currently no data on the plant RNA silencing
317 defense and RSS response during a rhabdovirus infection. It is tempting to speculate in the case of
318 LNYV P, that the weak RSS activity shown by P protein in transient GFP silencing assays may be
319 similar during LNYV infection, but its potency will depend on the strength of the antiviral response,
320 rate of viral gene expression and the silencing pathway targets (Mann et al., 2016b).

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435

Figure legends

Fig. S1. Alignment of *eGFP* (GenBank accession no. U43284) and *mGFP5* (GenBank accession no. U87973) coding sequences. Regions showing >20 bp identity between *eGFP* and *mGFP*, except for one mismatch are shown in bold. Sequences in the alignment corresponding to the *GF* hairpin of pUQC218 and pUQC251 (nucleotides 9-400 of *eGFP*; Brosnan et al., 2007) are underlined.

Fig 1. GFP local silencing leaf patch assay using GFP silencing inducers from different expression vectors. (A) Schematic diagram of the vectors that encode either eGFP plus GF-specific dsRNA expressed from the same plasmid pUQC218 or eGFP and GF-specific dsRNA from separate plasmids, pUQC214 and pUQC251, respectively. *N. benthamiana* wildtype plants were co-agroinfiltrated with pUQC218 (B) or pUQC214 and pUQC251 (C) plus either vector, LNYV P or TBSV P19 (all constructs had OD₆₀₀ = 0.8). All images were taken at 2 dpi adaxially under long wavelength UV light using a Canon EOS camera equipped with a GFP filter (four seconds exposure). 35S: cauliflower mosaic virus promoter; nos: terminator; i: intron; s: sense GF (nucleotides 9-400 of eGFP coding sequence); a/s: antisense GF; ocs: octopine synthase gene; BAR: bialaphos resistance gene; nos: terminator; RB: right border; LB: left border. Dashed-white circles represent infiltration zones of vector and LNYV P (B and C).

Fig 2. Local GFP silencing suppression activity of LNYV proteins in non-transgenic and transgenic 16c *N. benthamiana* plants using eGFP and mGFP5 inducers. *N. benthamiana* non-transgenic (NT) (A-B) and transgenic (16c) (C-D) leaves were co-agroinfiltrated with eGFP (A-D) plus either LNYV N, P, 4b, M, G, TBSV P19 or empty vector (V). Leaves were photographed at 3 days post infiltration (dpi; A and C) and 6 dpi (B and D). Infiltrated 16c leaf patches from C-D were analyzed for accumulation of GFP mRNA (E), and GFP siRNA (F) at 3 and 6 dpi for all constructs. Using ~8 µg of total or low molecular weight RNA, GFP mRNA (E) and siRNA (F), respectively, were detected using a full-length eGFP-specific biotinylated probe. Loading controls for GFP mRNA (ribosomal RNA; rRNA), and siRNA (5S RNA) are shown below respective blots. All samples

462 were loaded equally with the exception of TBSV P19 in the GFP mRNA blot which was diluted by
463 a factor of ten to reduce high signal intensity. Similarly, *N. benthamiana* 16c leaves were co-
464 agroinfiltrated with mGFP5 plus either LNYV N, P, CMV-207 2b, TBSV P19 or empty vector and
465 photographed at 4 dpi (G). Mock infiltrated plants received only infiltration buffer. Empty vector
466 and CMV-207 2b or TBSV P19 were used as negative and positive controls, respectively. All
467 images were taken adaxially under long wavelength UV light using a Canon EOS camera equipped
468 with a GFP filter.

469 **Fig 3.** Systemic GFP silencing in mGFP5-expressing 16c transgenic plants using agrobacterium co-
470 infiltration of two different GFP inducers and individual LNYV genes. Vectors encoding LNYV P,
471 CMV-207 2b, TBSV P19 or empty vector (V) were co-agroinfiltrated with mGFP5 (A) or eGFP (B)
472 into *N. benthamiana* 16c leaves, and then upper non-infiltrated shoot tissue was assessed visually
473 for GFP fluorescence (A, B) and by RNA blotting for mGFP5 mRNA accumulation (C) at 21 dpi.
474 Mock infiltrated plants received only infiltration buffer. mGFP5 mRNA was detected using a full-
475 length mGFP5-specific biotinylated probe. Ribosomal RNA (denoted on the right margin) is shown
476 as a control for equal loading of total RNA (~8 µg for each sample). All plant images were taken
477 adaxially under long wavelength UV light using a Canon EOS camera equipped with a GFP filter.

478 **Fig 4.** Local eGFP silencing assay using different doses of dsRNA silencing inducer. Non-
479 transgenic *N. benthamiana* leaves were co-agroinfiltrated with pUQC214 (sense eGFP) and
480 different doses of pUQC251 (GF-specific dsRNA) (see Fig. 1A) plus either empty vector, LNYV P
481 or TBSV P19 and photographed at 6 dpi. Prior to agroinfiltration, eGFP (OD₆₀₀ = 0.8), GF and
482 experimental sample (OD₆₀₀ = 0.8) were mixed in equal volumes however GF (pUQC251) dosage
483 was reduced by a factor of 2x (OD₆₀₀ = 0.4), 10x (OD₆₀₀ = 0.08) or 20x (OD₆₀₀ = 0.04). All images
484 were taken adaxially under long wavelength UV light using a Canon EOS camera equipped with a
485 GFP filter (four seconds exposure).

486

487 **Table 1.** Efficiency of systemic silencing suppression at 21 dpi in *N. benthamiana* 16c plants co-
488 agroinfiltrated with mGFP5 or eGFP and individual viral gene expression constructs.

Expression construct	mGFP5 silencing inducer				eGFP silencing inducer			
	# silenced	# suppressed	# tested	RSS Efficiency (%)	# silenced	# suppressed	# tested	RSS Efficiency (%)
Mock	0	0	3	-	0	0	9	-
Vector	3	0	3	0	11	1	12	8
LNyV N	2	0	2	0	10	0	10	0
LNyV P	0	5*	5	100*	6	28	34	83
LNyV 4b	2	0	2	0	14	0	14	0
LNyV M	2	0	2	0	11	1	12	8
LNyV G	2	0	2	0	8	1	9	11
CMV-207 2b	0	3*	3	100*	5	14	19	74
TBSV P19	0	3*	3	100*	0	19	19	100

489 * partial/incomplete suppression of systemic silencing

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491