

1 **Systemic silencing and DNA methylation of a host reporter gene**
2 **induced by a beneficial fungal root endophyte**

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16

17 **Highlight**

18 A root-restricted, beneficial fungal endophyte can induce systemic silencing
19 and epigenetic modifications to its host plant.

20

21

22 **Abstract**

23 A growing body of evidence suggests that RNA interference (RNAi) plays a
24 pivotal role in the communication between plants and pathogenic fungi,
25 where a bi-directional cross-kingdom RNAi is established to the advantage of
26 either the host or the pathogen. Similar mechanisms acting during plant
27 association with non-pathogenic symbiotic microorganisms have been elusive
28 to this date. Here, we report on an RNAi-based mechanism of
29 communication between a beneficial fungal endophyte, *Fusarium solani* strain
30 K (FsK) and its host plants. This soil-borne endophyte that confers resistance
31 and/or tolerance to biotic and abiotic stress in tomato and, as shown in this
32 study, promotes plant growth in *Nicotiana benthamiana*, is restricted to the
33 root system in both host plants. We first showed that the fungus has a
34 functional core RNAi machinery; double stranded RNAs (dsRNAs) are
35 processed into short interfering RNAs (siRNAs) of predominantly 21-nt in
36 size, which lead to the degradation of homologous mRNAs. Importantly, by
37 using an RNAi sensor system, we demonstrated that root colonization of *N.*
38 *benthamiana* by FsK led to the induction of systemic silencing and DNA
39 methylation of a host reporter gene.. These data reflect a more general but
40 so far unrecognized mechanism wherein root endophytes systemically
41 translocate RNAi signals to the aboveground tissues of their hosts to
42 modulate gene expression during symbiosis, which may be translated to the
43 beneficial phenotypes.

44

45 **Keywords:** Endophytes, epigenetics, *Fusarium solani*, *Nicotiana*
46 *benthamiana*, RNA interference, small RNAs.

47 **Introduction**

48 RNA interference (RNAi) is a conserved eukaryotic gene regulatory
49 mechanism that is triggered by small RNAs (sRNAs) of approximately 20-25
50 nucleotides (nt) (Baulcombe, 2004; Hung and Slotkin, 2021).
51 Notwithstanding the diversity of RNAi pathways and the plethora of sRNA
52 classes, there are essentially two types of sRNAs, the small interfering RNAs
53 (siRNAs) and the microRNAs (miRNAs) (Borges and Martienssen, 2015;
54 Vaucheret, 2006). In general, Dicer and Dicer-like (DCL) endonucleases
55 cleave double stranded RNAs (dsRNAs) and stem loop hairpin RNAs (hpRNAs)
56 into 20-25-nt siRNAs and miRNAs, respectively (Paturi and Deshmukh,
57 2021). The occurring double stranded sRNA is then unzipped in an ATP-
58 dependent reaction so that only one of its two strands will eventually be
59 loaded onto an Argonaute (AGO) protein (Iwakawa and Tomari, 2022;
60 Vaucheret, 2008). Then, the AGO-loaded sRNA scans the cytoplasm for
61 complementary mRNA transcripts to cleave them or inhibit their translation
62 (Brodersen *et al.*, 2008; Hamilton and Baulcombe, 1999). At least in plants,
63 AGO-loaded sRNAs may also be transported in the nucleus, where they are
64 involved in RNA-directed DNA methylation (RdDM) of cognate sequences
65 (Wassenegger and Dalakouras, 2021; Wassenegger *et al.*, 1994). Moreover,
66 in plants, nematodes and some fungi, the presence of RNA-dependent RNA
67 polymerases (RDRs) contributes to the generation of dsRNAs from single
68 stranded transcripts, in a process termed transitivity (de Felippes and
69 Waterhouse, 2020; Sakurai *et al.*, 2021).

70 Fungal RNAi, initially described as 'quelling' in *Neurospora crassa*
71 (Romano and Macino, 1992), has essentially a two-fold role. On the one
72 hand, siRNAs generated from (usually RDR-transcribed) dsRNA precursors
73 are involved in genome defense and maintenance of genome integrity as well
74 as fighting against transposons, viruses and transgenes (Lax *et al.*, 2020;
75 Torres-Martinez and Ruiz-Vazquez, 2017). On the other hand, miRNAs (also
76 called miRNA-like, milRNAs), generated by Pol III-transcribed primary miRNA
77 transcripts, fine-tune gene expression during vegetative and sexual

78 development besides responding to various kinds of stresses (Li *et al.*, 2010;
79 Torres-Martinez and Ruiz-Vazquez, 2017). A growing body of recent evidence
80 suggests that, in addition to the aforementioned roles, RNAi also has a
81 pivotal role in the communication of fungi with their hosts. Indeed, the
82 pathogen *Botrytis cinerea* delivers sRNAs in Arabidopsis and tomato that
83 target members of the mitogen-activated protein kinases (MAPKs) that
84 function in plant immunity (Weiberg *et al.*, 2013). In reverse, plants fight
85 back; Arabidopsis and tomato deliver sRNAs in *B. cinerea* targeting the
86 fungal DCL1 and DCL2, to attenuate fungal pathogenicity and growth (Wang
87 *et al.*, 2016). Likewise, *Fusarium graminearum* translocates sRNAs to target
88 defence genes in *Hordeum vulgare* and *Brachypodium distachyon* (Werner *et al.*,
89 2021), whereas cotton plants, in response to infection with the vascular
90 pathogen *Verticillium dahliae*, export miR159 and miR166 to silence fungal
91 isotrichodermin C-15 hydroxylase and Ca(2+)-dependent cysteine protease,
92 respectively, both of which are essential for fungal virulence (Zhang *et al.*,
93 2016). However, the role of such cross-kingdom RNAi processes in
94 mutualistic interactions remains poorly understood.

95 *Fusarium solani* strain K (FsK) is an endophytic, non-pathogenic strain,
96 initially isolated from the roots of tomato plants (Kavroulakis *et al.*, 2007)
97 but other plant species serve as hosts, including legumes (Skiada *et al.*,
98 2019). FsK has been shown to protect the host against root and foliar
99 pathogens (Kavroulakis *et al.*, 2007), spider mites (Pappas *et al.*, 2018),
100 zoophytophagous insects (Garantonakis *et al.*, 2018) and to alleviate drought
101 stress (Kavroulakis *et al.*, 2018). The beneficial activity of FsK presupposes
102 an intact ethylene signaling pathway, suggesting that the fungus can induce
103 systemic responses to the plant (Kavroulakis *et al.*, 2007). However, the
104 exact molecular details governing this symbiosis remain largely elusive. In
105 this study, we characterized the core RNAi machinery of FsK and provide
106 evidence that the endophyte translocates RNAi signals to its host plant to
107 modulate expression and induce epigenetic modification of a host reporter
108 gene.

109

110

111 **Materials and Methods**

112

113 *Isolation of fungal conidia and inoculation.*

114 FsK was routinely cultured for 4 days in potato dextrose broth (PDB) (26 °C,
115 160 rpm). Following removal of mycelium fragments by sieving through
116 sterile cheesecloth, conidia were recovered from the filtrate by centrifugation
117 at 6,500 rpm, counted using a haemocytometer and suspended in an
118 appropriate volume of 0.85% NaCl to achieve the desired inoculum
119 concentration. Approximately 100 conidia were used to inoculate
120 *N.benthamiana* plants at cotyledon stage.

121

122 *Fungal RNA isolation.*

123 FsK was routinely cultured for 4 days in potato dextrose broth (PDB) (26 °C,
124 160 rpm). From the occurring mycelium total RNA was isolated with TRIzol™
125 Reagent (www.thermofisher.com) to be subsequently used in RT-qPCR

126 reactions. For small RNA sequencing, the enriched for small RNAs fraction
127 was isolation from the mycelium using mirVana™ miRNA Isolation Kit
128 (www.thermofisher.com) according to the manufacturer's instructions.

129

130 *Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).*

131 DNaseI-treated (www.thermofisher.com) RNA isolated from mycelium was
132 quantified with by Qubit Fluorometric Quantification
133 (www.thermofisher.com). The DNA-free RNA (10 ng) was then subjected to
134 RT-qPCR using the the Luna® Universal Probe One-Step RT-qPCR Kit
135 (www.neb.com) according to the manufacturer's instructions. Essentially, the
136 total volume of the reaction was reduced to 10µl and the cycling parameters
137 consisted of incubation at 55°C for 10 min for reverse transcription, 95°C for
138 1 min followed by 39 cycles of 95°C for 10 sec and 60°C for 30 sec. Analysis
139 was carried out using the geometric mean of FsK ITS and Tef-1a transcripts
140 (Skiada *et al.*, 2019). For Tef-1a (120 bp amplicon), the primers 5'-TCG AAC
141 TTC CAG AGG GCA AT-3' and 5'-CCA ACA ATA GGA AGC CGC TG-3' were
142 used. For ITS (108 bp amplicon), the primers 5'-TAG GGT AGC TGG GTC TGA
143 CT-3' and 5'-ACC AAG TCT AAC CCG CCT AC-3' were used. For GFP (133 bp
144 amplicon), the primers 5'-TCC CAG CAG CTG TTA CAA AC-3' and 5'-AAT ACT
145 CCA ATT GGC GAT GG-3' were used. The relative expression of GFP gene was
146 calculated from two to three technical replicates for every sample as
147 described in the corresponding figure legend. Data were analyzed using the
148 Student's two-tailed homoscedastic t-test.

149

150 *Plant and fungal DNA isolation.*

151 Genomic DNA from plant and fungal tissue was isolated with DNeasy Plant
152 Pro (/www.qiagen.com) according to the manufacturer's instructions.

153

154 *Phylogenetic analysis.*

155 The analyzed sequences were aligned with MUSCLE v3.7 (Edgar, 2004), and
156 informative sites were selected with Gblocks v0.91b (Talavera and

157 Castresana, 2007). The aligned selected sites were tested with the Prottest
158 v3.2 software (Darriba et al., 2011) using the Akaike information criterion
159 (AIC) values for optimal residue substitution model matrix selection. The LG
160 (Le and Gascuel, 2008) residue substitution model matrix scored best for all
161 proteins sets. The PhyML v3.0 algorithm (Guindon and Gascuel, 2003) using
162 the LG model and bootstrap testing with 100 replicates was used for
163 obtaining the best maximum likelihood tree.

164

165 *Quantification of fungal colonization by qPCR.*

166 To estimate fungal abundance within plant tissues, absolute quantification of
167 *F. solani* ITS gene was performed as previously described (Skiada *et al.*,
168 2019).

169 *Generation of constructs.*

170 For the generation of pCS-GFP, a PCR was performed using as template
171 genomic DNA from *N. benthamiana* line 16C (Voinnet and Baulcombe, 1997)
172 and the primers 5-GGT TAA CAA AGA ATG CTA ACC-3 and 5-CGA GCT CGG
173 CAA TTC CCG ATC-3 and the occurring 2017 bp amplicon was cleaved with
174 HpaI/SacI and ligated to a similarly cleaved pSilent-1, generating the
175 pSilent-GFP. Next, pSilent-GFP was cleaved with PsiI/SacI and the 6663 bp
176 fragment was ligated into the 7866 bp fragment retrieved upon ZraI/SacI
177 cleavage of pCambia1300, generating the pCS-mGFP. For the generation of
178 pCS-hpGF+GFP, a first PCR was performed using as template genomic DNA
179 from *N. benthamiana* line 16C and the primers 5-acg tct cga gAT GAA GAC
180 TAA TCT TTT TCT C-3 and 5-ACG TAA GCT TCT CTT GAA GAA GTC GTG CCG
181 C-3 and the occurring 340 bp amplicon was cleaved with XhoI/HindIII and
182 ligated to a similarly cleaved pSilent-1 vector, generating the pSilent-GF. A
183 second PCR was performed using as template genomic DNA from *N.*
184 *benthamiana* line 16C and the primers 5-acg tgg tac cAT GAA GAC TAA TCT
185 TTT TCT C-3 and 5-ACG TAG ATC TCT CTT GAA GAA GTC GTG CCG C-3 and
186 the occurring 340 bp amplicon was cleaved with KpnI/BglII and ligated to a
187 similarly cleaved pSilent-GF vector, generating the pSilent-hpGF. Next, the

188 1937 bp fragment emerging upon HpaI/SacI cleavage of the pSilent-GFP was
189 ligated into a similarly cleaved pSilent-hpGF, generating the pSilent-
190 hpGF+GFP. Finally, pSilent-hpGF+GFP was cleaved with PsiI/SacI and the
191 7277 bp fragment was ligated into the 7866 bp fragment retrieved upon
192 ZraI/SacI cleavage of pCambia1300, generating the pCS-hpGF+GFP.

193

194 *Agrobacterium-mediated fungal transformation.*

195 The binary vectors pCS-GFP and pCS-hpGF+GFP were used to transform
196 *Agrobacterium tumefaciens* AGL1 strain by electroporation using the
197 MicroPulser Electroporator (www.bio-rad.com) according to the
198 manufacturer's instructions. The AGL1-pCS-GFP and AGL1-pCS-hpGF+GFP
199 were used to transform FsK conidia as previously described (Zhang *et al.*,
200 2015).

201 *In vitro* transcription of sGFP dsRNA.

202 For the generation of the *in vitro* transcribed sGFP dsRNA, genomic DNA was
203 extracted from FsK-sGFP (Sesma and Osbourn, 2004) and used as template
204 for PCR with KAPA Taq DNA Polymerase (www.sigmaaldrich.com) with the T7
205 promoter-containing primers 5'-taa tac gac tca cta tag gga gaC GTA AAC
206 GGC CAC AAG TTC AGC-3' and 5'-taa tac gac tca cta tag gga gaG TGG CGG
207 ATC TTG AAG TTC ACC-3' (T7 promoter sequence with lowercase). The T7
208 promoter-containing 491 bp amplicon was then used as template in the
209 MEGAscript™ RNAi Kit (www.thermofisher.com) for the generation of a 445
210 bp sGFP dsRNA.

211 *In vitro* RNAi assay.

212 In 24 wells of a 96-well plate, FsK-sGFP conidia were added (in each well, 6
213 conidia diluted in 100 μ l PDB/100). In 12 wells containing these FsK-sGFP
214 conidia, *in vitro* transcribed sGFP dsRNA was added (100 μ l, 1 ng/ μ l) (dsRNA
215 application samples). In the remaining 12 wells containing FsK-sGFP conidia,
216 100 μ l water was added (control samples). The 96 well was covered with a
217 removable membrane and incubated at 28° C. At timepoints 0-24-48 hpa,

218 the plate was subjected to fluorometric analysis using the using the
219 Varioskan™ LUX multimode microplate reader (www.thermofisher.com).

220

221 *Bisulfite sequencing.*

222 Genomic DNA from the fungus (20 ng) or the plant (100 ng) was used for
223 bisulfite sequencing analysis using the EZ DNA Methylation-Gold Kit
224 (www.zymoresearch.com) according to the manufacturer's instructions and
225 as previously described (Dalakouras *et al.*, 2016) . Essentially, for the cis-
226 RdDM bisulfite analysis on FsK, the primers 5'-AAT CTC CAR TRR RTA CAC
227 TAT TC-3' and 5'-CCT CCT TRA AAT CRA TTC CCT TAA-3' were used, whereas
228 for the trans-RdDM bisulfite analysis on FsK and Nb-16C the primers 5'-AGT
229 GGA GAG GGT GAA GGT GAT G-3' and 5'-CCT CCT TRA AAT CRA TTC CCT
230 TAA-3' were used in a PCR reaction with ZymoTaq PreMix
231 (www.zymoresearch.com) according to the manufacturer's instructions. The
232 occurring 262 bp and 311 bp amplicons for cis-RdDM and trans-RdDM,
233 respectively, were cloned into pGEM®-T Easy Vector
234 (worldwide.promega.com) and for each analysis 5-10 clones were subjected
235 to Sanger sequencing.

236

237 *Small RNA sequencing.*

238 Sequencing of small RNAs from fungal RNA (small RNA fraction) was
239 performed by GenXPro (<https://genxpro.net/>) as previously described
240 (Dalakouras *et al.*, 2016).

241

242 **Results and Discussion**

243

244 *FsK colonizes the root system of Nicotiana benthamiana and stimulates plant* 245 *growth.*

246 During the colonization process of its host plants, the fungus penetrates the
247 root and grows in the root cortex and proliferates even in the vascular
248 system of root system (Skiada *et al.*, 2019). In legumes, efficient

249 colonization by Fsk is dependent on the common symbiotic signalling
250 pathway (Skiada *et al.*, 2020), typically used by rhizobia and arbuscular
251 mycorrhizal fungi. Notably, although not yet explained for, fungal growth in
252 tomato is restricted to the root system and extends only to the crown and
253 not to the stem and leaf tissues (Kavroulakis *et al.*, 2007). Here, we
254 investigated the capacity of Fsk to colonize another member of the
255 Solanaceae, *Nicotiana benthamiana*, which is a widely used model plant for
256 RNAi studies (Philips *et al.*, 2017). Similar to tomato, upon root-inoculation,
257 the fungal endophyte colonized the root system but failed to expand to the
258 shoot system (Figs 1a, 1b and S1). Interestingly, the Fsk-colonized plants
259 exhibited considerably stimulated growth, at least up to 4 weeks post
260 inoculation (wpi) when grown in both non-sterile compost (Fig. 1c) and
261 sterile sand (Fig. S2), underpinning the beneficial effect of Fsk to this host,
262 at least in terms of biomass production.

263

264 *Fsk encodes the core RNAi components.*

265 Despite being largely conserved among eukaryotes, not all fungi encode the
266 core RNAi pathway; indeed, *Saccharomyces cerevisiae* lacks DCLs, AGOs and
267 RDRs (Drinnenberg *et al.*, 2009). *Ustilago maydis* also lacks DCLs, AGOs and
268 RDRs, in contrast, surprisingly, to its close relative *U. hordei* (Laurie *et al.*,
269 2008). Furthermore, miRNAs have been identified in most fungal species but
270 not in the basal fungus *Mucor circinelloides* (Torres-Martinez and Ruiz-
271 Vazquez, 2017). Interestingly though, whereas RNAi-deficient mutants of
272 most ascomycetes and basidiomycetes are not impaired in vegetative
273 growth and development, sexual differentiation and response to stress, *M.*
274 *circinelloides* is (Ruiz-Vazquez *et al.*, 2015). These being said, the
275 mechanistic details and role of RNAi in fungal kingdom can be unusually
276 diverse.

277 To examine whether Fsk encodes the core RNAi machinery, we
278 performed transcriptome-validated genome annotation (BioProject
279 PRJNA796177, Tsiouri and Papadopoulou, unpublished results) and identified

280 two DCLs (FskDCL1 and FskDCL2), two AGOs (FskAGO1 and FskAGO2) and
281 four RDRs (FskRDR1-4) (Fig. 2a). FskDCL1 and FskDCL2 contain the Dicer-
282 like protein structures with a Dead-like helicases superfamily domain box
283 (DEXDc) box, a helicase superfamily c-terminal domain (HELICc), and two
284 ribonuclease III domains (RIBOc) responsible for the cleavage of dsRNA
285 precursors into sRNAs (Paturi and Deshmukh, 2021). Both FskAGO1 and
286 FskAGO2 proteins contain PAZ and PIWI domains; PAZ recognizes the 3' end
287 of sRNAs while PIWI exhibits an RNaseH-like endonucleolytic activity and
288 mediates target cleavage (Wu *et al.*, 2020). All four FskRDRs contain the
289 RdRP/RDR domain, which is highly conserved in fungi (Chen *et al.*, 2015).
290 FskRDR2 and FskRDR3 contain the DLDGD motif, which is often encountered
291 in plants, whereas FskRDR1 and FskRDR4 contain the DYDGD motif, which is
292 more common in fungi (Wassenegger and Krczal, 2006). To explore the
293 molecular evolution of these proteins, we performed phylogenetic analyses of
294 DCL, AGO and RDR proteins including *Fusarium graminearum* and
295 *Neurospora crassa* (Fig. 2b). Our analysis showed that FskDCL1 is related to
296 FgDCL1 and NcDCL1 that function in the meiotic silencing by unpaired DNA
297 (MSUD) pathway (Fig. 2b) (Alexander *et al.*, 2008), whereas FskDCL2 is
298 closer to FgDCL2 and NcDCL2 which have a prominent role in RNAi by
299 processing of dsRNAs into siRNAs (Chen *et al.*, 2015). FskAGO1 is closely
300 related to FgAGO1 and NcQDE2 that are loaded with dsRNA-processed
301 siRNAs during RNAi, whereas FskAGO2 is closer to FgAGO2 and the *N. crassa*
302 SMS2 that are involved in MSUD (Lee *et al.*, 2003). Of note, FskRDR4 is
303 closely related to NcQDE1 which is essential for quelling and suggested to be
304 functionally related to plant RDR6 (Wassenegger and Krczal, 2006).

305

306 *Fsk takes up RNAi molecules from its environment.*

307 In order to test the functionality of Fsk's RNAi machinery, an *in vitro*-
308 transcribed 445 bp sGFP dsRNA was applied to a sGFP-expressing Fsk, sGFP
309 being a GFP variant that contains a serine-to-threonine substitution at amino
310 acid 65, optimized for use in fungi (Sesma and Osbourn, 2004) (Figs 3a, 3b).

311 Fluorometric analysis revealed that the sGFP expression levels dropped to
312 almost 50% 24 hours post application (hpa) (Fig. 3c). These data suggested
313 that the externally applied dsRNA was processed by fungal DCLs into siRNAs
314 that were loaded onto fungal AGOs to mediate cleavage of the sGFP mRNA.
315 However, no further decrease of sGFP levels could be observed at later
316 timepoints (48 hpa), reminiscent of similar observations in *F. asiaticum*
317 (Song *et al.*, 2018) and implying the absence an active RDR-mediated self-
318 reinforcing mechanism of RNAi that could ensure ongoing RNAi even at the
319 absence/degradation of the initial dsRNA input.

320 Overall, these data suggest not only that the RNAi machinery in FsK is
321 functional but also that FsK is able to take up RNAi molecules from its
322 environment. Not all fungi are able to take up RNA molecules from their
323 environment; *Colletotrichum gloesporioides*, *Trichoderma virens* and
324 *Phytophthora infestans* being some notable examples that fail to do so (Qiao
325 *et al.*, 2021). Of note, fungi that are indeed able to receive RNAi molecules
326 from their environment are not only promising candidates for RNAi-based
327 fungicidal control (Šečić and Kogel, 2021) but also likely partners in an RNAi-
328 based cross-kingdom communication with their host (He *et al.*, 2021).

329

330 *FsK processes hairpin RNA transcripts into siRNAs that trigger mRNA*
331 *degradation but not DNA methylation in the fungal hyphae.*

332 In order to examine the mode of dsRNA processing in the endophyte, FsK
333 was transformed with a transgene comprised of a full length green
334 fluorescent protein (GFP) corresponding to mGFP5-ER (Haseloff and
335 Siemering, 2006) and a hairpin (hp) construct of the first 332 bp of GFP
336 (hpGF) (Fig. 4a). In this setup, the hpGF locus served as the RNAi-trigger
337 while the GFP locus as the RNAi-target. Small RNA sequencing (sRNA-seq) in
338 three independent FsK-hpGF+GFP transformants (#6, #7, #27) revealed the
339 accumulation of GF siRNAs (perfectly matching the GF region) having
340 variable sizes from 18-30 nt but predominantly of 21-nt, 22-nt and 24-nt
341 (Figs 4b, 4c and S3). This finding was reminiscent of the situation in plants,

342 where hpRNAs are typically processed by DCLs to 21-, 22- and 24-nt siRNAs
343 (Fusaro *et al.*, 2006). To the best of our knowledge, similar sRNA-seq studies
344 in fungi, aiming to reveal the mode of processing of a specific hpRNA/dsRNA,
345 are absent; yet, genome-wide sRNA-seq studies (identifying siRNAs, miRNAs
346 but also DCL-independent sRNAs) reveal a remarkably diverse pattern, with
347 prominent size classes ranging from 19-22-nt in *Penicillium chrysogenum*
348 (Dahlmann and Kück, 2015), to 22-25-nt in *S. pombe* (Djupedal *et al.*, 2009)
349 and 27-28-nt in *F. graminearum* (Chen *et al.*, 2015). Our analysis does not
350 allow us to identify whether all sRNA size classes are actual DCL products
351 (e.g., they could represent degradation products) or whether they all exhibit
352 biological activity. Yet, it is reasonable to assume that FskDCL2 generated
353 the bulk of sRNAs (Chen *et al.*, 2015), of which the most prominent size class
354 (21-nt) seems to undertake the major burden for RNAi activity.

355 To evaluate this RNAi activity, we measured the GF siRNA-mediated
356 downregulation of GFP mRNA in three independent Fsk-hpGF+GFP
357 transformants (#6, #7, #27) when compared to Fsk-GFP (transformed with
358 a cassette lacking the hpGF transgene) (Fig. 4a). Indeed, GFP expression
359 was virtually eliminated in all Fsk-hpGF+GFP transformants (Figure 3D). Of
360 note, we detected GF siRNAs but no or negligible P siRNAs that could had
361 potentially emerged upon the FskRDR processing on the GF siRNA-targeted
362 GFP transcript (Figure 4b). This is in contrast to the situation in plants (de
363 Felippes and Waterhouse, 2020) but in agreement with similar reports in *F.*
364 *asiaticum* (Song *et al.*, 2018), suggesting the absence of an active RDR-
365 based mechanism in Fsk.

366 Typically, the onset of RNAi and the accumulation of siRNAs leads to
367 RdDM in plants (Dalakouras and Vlachostergios, 2021). DNA methylation also
368 occurs in some, but not all, fungi, and usually in repetitive sequences
369 (Bewick *et al.*, 2019). Yet, such DNA methylation is considered to be
370 dispensable of RNAi molecules, thus fungi have been considered to lack a
371 *bona fide* RdDM mechanism (Nai *et al.*, 2020). Nevertheless, recent advances
372 challenge this assumption; indeed, sRNA-dependent RdDM-like phenomena

373 has been detected, at least in *Pleurotus tuoliensis* and *P. eryngii* var. *eryngii*
374 (Basidiomycetes) (Zhang *et al.*, 2018) and *Puccinia graminis* (Ascomycetes)
375 (Sperschneider *et al.*, 2021). Accordingly, and given the abundant
376 accumulation of GF siRNAs in FsK-hpGF+GFP, we were interested to see
377 whether they could trigger RdDM of cognate DNA sequences. To analyze cis-
378 RdDM (at the locus generating the siRNAs), we chose a 262 bp fragment of
379 the hpGF transgene (Fig. 4a). For trans-RdDM (at a locus that does not
380 generate siRNAs but is homologous to them), we chose a 311 bp fragment of
381 the GFP transgene (Fig. 4a). Whereas CG and CHG methylation can be
382 maintained in an RNAi-independent manner (Law and Jacobsen, 2010), CHH
383 methylation is the hallmark of ongoing de novo RdDM (Pelissier *et al.*, 1999),
384 and both cis and trans fragments under analysis were rich in asymmetric
385 CHH context (80% for cis and 72% for trans) (Figs 4e, 4f). However, bisulfite
386 sequencing revealed the absence of methylated cytosines in any sequence
387 context (CG, CHG, CHH), at neither cis (Fig. 4e) nor trans (Fig. 4f) loci,
388 suggesting that no RdDM takes place in FsK, at least in our experimental
389 setup. It has been suggested that fungal proteins with de novo
390 methyltransferase (DNMT) and/or helicase-like Snf2 family domains may be
391 involved in RdDM-like pathways in fungi (Nai *et al.*, 2020). However, we
392 were unable to detect such genes in the FsK genome, underpinning the
393 conclusions obtained from bisulfite sequencing about the absence of an
394 active RdDM mechanism in FsK.

395

396 *FsK translocates RNAi signals to its host to induce systemic RNAi and*
397 *epigenetic changes of a reporter gene.*

398 Establishment of mutualistic associations between fungi and their host
399 requires genetic and epigenetic reprogramming as well as metabolome
400 modulation of both by the exchange of effector molecules (Kloppholz *et al.*,
401 2011). Indeed, RdDM is essential in *Arabidopsis* to establish a beneficial
402 relationship with the root-colonizing *Trichoderma atroviride* while DNA
403 methylation and histone modifications are required for plant priming by the

404 beneficial fungus against *B. cinerea* (Rebolledo-Prudencio *et al.*, 2021).
405 Importantly, it was just recently shown that during the mutualistic interaction
406 of the ectomycorrhizal fungus *Pisolithus microcarpus* with *Eucalyptus grandis*,
407 a fungal miRNA, Pmic_miR-8, targets the host NB-ARC domain containing
408 transcripts in a cross-kingdom RNAi manner (Wong-Bajracharya *et al.*,
409 2022). Reminiscent of this, an in silico study predicted that the beneficial
410 arbuscular mycorrhizal fungi *Rhizophagus irregularis* produces sRNAs that
411 have 237 candidate targets in the host plant *Medicago truncatula*, including
412 specific mRNAs known to be modulated in roots upon AMF colonization
413 (Silvestri *et al.*, 2019). Similarly, a recent study based on transcriptome and
414 sRNA profile change analysis during the onset of the mutualistic interaction
415 between the beneficial root endophyte *Serendipita indica* with its host
416 *Brachypodium distachyon*, suggested that interaction-induced sRNAs in both
417 organisms may underlie reciprocal targeting of genes related to plant
418 development and fungal growth and nutrient acquisition (Secic *et al.*, 2021).
419 Thus, it is very likely that, similar to fungal pathogens (Cai *et al.*, 2019),
420 beneficial fungal endophytes also display an RNA-based communication with
421 their hosts. However, clear evidence of actual RNAi molecule translocation
422 and concomitant cross-kingdom RNAi between a beneficial fungal endophyte
423 and its host has been lacking to this date.

424 In order to address this question, we resorted to the GFP-expressing
425 *N. benthamiana* plant line 16C (Nb-GFP) (Voinnet and Baulcombe, 1997), as
426 an RNAi sensor system. Nb-GFP carries a 35S-driven mGF5-ER transgene
427 (Fig. 5a) and is a well-studied RNAi model plant that allows the monitoring of
428 systemic RNAi (i.e. spreading of RNAi to tissues other than those where RNAi
429 initially occurred) by observation of the presence or abolishment of GFP
430 expression under ultraviolet light. When Nb-GFP plants were inoculated with
431 FsK-hpGF+GFP (Fig. 5a), we could record the following outcomes: (i) no
432 visible RNAi (45% of the plants, 6 wpi), (ii) spot-like RNAi (45% of the
433 plants, 4 wpi), (iii) vein-restricted RNAi (5% of the plants, 4 wpi) and (iv)
434 full-tissue RNAi (5% of the plants, 4 wpi) (Fig. 5b). Colonization of Nb-GFP

435 plants with non-transformed FsK and/or FsK-sGFP failed to trigger any visible
436 RNAi phenotype even after 10 wpi, suggesting that not the mere presence of
437 the endophyte but the RNAi molecules it expresses are responsible for the
438 induction of RNAi phenotypes in its host.

439 RNAi in plants is tightly coupled to RdDM (Dalakouras and
440 Vlachostergios, 2021; Jones *et al.*, 1999). Accordingly, bisulfite sequencing
441 analysis of leaf and root tissues from the fully silenced Nb-GFP plants
442 disclosed the dense (100%) onset of DNA methylation in the GFP region (Fig.
443 5a) in every sequence context: CG, CHG and CHH (Fig. 5c). Overall, these
444 data clearly show that the endophyte triggered not only mRNA degradation
445 but also DNA methylation of a host reporter gene. We favor the scenario that
446 FsK-hpGF+GFP translocated RNAi signals (dsRNAs but most likely siRNAs) to
447 the roots of Nb-GFP initiating local RNAi of the host GFP. Importantly, once
448 present in the plant cells and upon targeting the host GFP transcript for
449 silencing, these endophyte-derived primary siRNAs culminated in the
450 generation of host-derived RDR-mediated secondary siRNAs (as implied by
451 the RdDM pattern, see below). Whether the recorded RdDM in the root
452 tissues was induced by the endophyte-derived primary or the host-derived
453 secondary siRNAs is not clear. Yet, the fact that RdDM could be detected not
454 only in the GF but also in the P region (Figure 5a, 311 bp bisulfite fragment
455 covering both GF and P regions) strongly implies in favor of transitive host-
456 derived secondary siRNAs imposing RdDM. Now, siRNAs are mobile moieties;
457 they can move cell-to-cell through the plasmodesmata and through the
458 vasculature to distant parts of the plant (Voinnet, 2022; Voinnet and
459 Baulcombe, 1997). The establishment of systemic silencing in the upper
460 parts of the plant (which FsK fails to colonize, Fig. 1) suggests that mobile
461 siRNA signals from the root entered the phloem to reach shoot tissues. Most
462 likely both endophyte-derived primary siRNAs and host-derived secondary
463 siRNAs could have played the role of the mobile systemic signal (Devers *et al.*,
464 2020). However, it is unlikely that the mere presence of endophyte-
465 derived primary siRNAs alone could trigger systemic silencing; it rather

466 seems that a certain quantitative siRNA threshold needs to be surpassed for
467 the onset of systemic silencing (Kalantidis *et al.*, 2006), rendering the
468 abundant presence of host-derived secondary siRNAs indispensable.
469 Importantly, establishment of systemic RNA in the receiving tissues requires
470 RDR6 (Schwach *et al.*, 2005). Thus, in the receiving tissues, the
471 primary/secondary siRNAs triggered a RDR6-mediated generation of (host-
472 derived) tertiary siRNAs, ensuring the efficient establishment of GFP mRNA
473 degradation and DNA methylation.

474

475 *Conclusion.*

476 Here, we have characterized the RNAi core machinery of a fungal endophyte
477 and we provide solid evidence that it translocates RNAi signals to its host to
478 trigger systemic silencing and epigenetic modifications. To prove the concept,
479 we have employed an artificial RNAi sensor system; future studies coupling
480 sRNAome, degradome and methylome analysis will be required to pinpoint
481 the nature of the endogenous fungal sRNAs (siRNAs and/or miRNAs) that are
482 translocated to the host, which host genes are targeted for transcriptional
483 and/or post-transcriptional silencing and how this process is ultimately
484 translated into a beneficial phenotype. Our data may well reflect a so far
485 unrecognized pathway according to which endophytes establish the symbiosis
486 and/or impose their beneficial impact by translocating RNA molecules that
487 modulate host gene expression and affect the epigenome's plasticity. RNAi-
488 mediated communication between plants and their interacting organisms is
489 much more widespread than previously thought and may account for the
490 improved plant performance often observed in the presence of certain
491 associated microbiota.

492 **Supplementary Data.**

493 The following supplementary data are available at JXB online.

494 Fig. S1. Colonization of FsK-sGFP in Nb-WT and stereoscopic observation of
495 sGFP fluorescence in various tissues

496 Fig. S2. Impact of FsK colonization of Nb-WT plants grown in sterile sand in
497 magenta boxes.

498 Fig. S3. Small RNA sequencing in three FsK-hpGF+GFP transformants. (a)
499 Mapping of sRNAs in GFP. (b) Size distribution of GFP sRNAs.

500 Fig. S4. Systemic silencing phenotypes upon colonization of FsK-GF+GFP in
501 Nb-GFP plants 4-6 wpi.

502

503 **Acknowledgments**

504 This work was supported by funds from the EU Horizon 2020 Marie

505 Skłodowska-Curie fellowship (RNASTIP, Grant ID 793186) and the EU

506 Horizon 2020 PRIMA program (INTOMED, Grant ID 1534).

507 **Conflict of interest**

508 *Fusarium solani* FsK is patented (20070100563/1006119, issued by the
509 Industrial Property Organization to KKP).

510 **Author Contributions**

511 A.D. and K.K.P. designed research; A.D., A.K., M.A., E.D., A.M. M.G. and

512 E.D., performed research; A.D., O.T., S.V. and KKP analyzed data; A.D. and

513 K.P.P. wrote the paper. All authors reviewed and approved the manuscript.

514 **Data availability**

515 All sequencing data supporting the findings of this study are deposited to

516 Zenodo (<https://doi.org/10.5281/zenodo.6088855>)

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770

771 **Figure Legends.**

772 **Fig. 1.** Colonization of Nb-WT by FsK. (A) Schematic representation of the
773 colonization assay. (B) Quantification of fungal colonization in shoot and root
774 system at 2 and 4 wpi. (C) Impact of the FsK in growth of Nb-WT 4 wpi.

775 **Fig. 2.** Identification of FsK RNAi core machinery. (A) Schematic
776 representation of FsK DCL, AGO and RDR proteins using DOG1.0 software
777 (Ren *et al.*, 2009). (B) Maximum likelihood phylogenies of the FsK (indicated
778 red), *Fusarium graminearum*, *Neurospora crassa* and *Arabidopsis thaliana* (as
779 an outgroup member) DCL, AGO and RDR proteins using the LG model
780 matrix and 100 bootstrap replicates for assessing branch support.

781 **Fig. 3.** In vitro RNAi in FsK-sGFP. (A) Schematic representation of the sGFP
782 transgene that is present in FsK-sGFP. PToxA: promoter for the promoter
783 from *Pyrenophora tritici-repentis* ToxA gene; sGFP: GFP variant that contains
784 a serine-to-threonine substitution at amino acid 65; TNOS: terminator for the
785 nopaline synthase gene. The 445 bp fragment chosen for in vitro
786 transcription of dsRNA is depicted. (B) Stereoscopic observation of sGFP
787 fluorescence. (C) Fluorometric analysis for in vitro RNAi in FsK-sGFP.
788 Vertical axis: RFU: relative fluorescence unit, calculated as the ratio of sGFP-
789 indicative fluorescence (excitation 488 nm, emission 515 nm) to growth-
790 indicative absorbance (wavelength 595 nm). Horizontal axis: 1-12: 12 wells
791 containing FsK-sGFP conidia. 13-24: 12 wells containing FsK-sGFP conidia
792 plus 100 ng (each well) sGFP dsRNA.

793 **Fig. 4.** Characterization of FsK RNAi machinery. (A) Schematic
794 representation of the hpGF+GFP transgene. PTrpC: promoter for the
795 Promoter for *Aspergillus nidulans* trpC gene; GF: 322 bp fragment of the
796 GFP; intron: *Magnaporthe grisea* cutinase gene intron; TTrpC: promoter for
797 the Promoter for *Aspergillus nidulans* trpC gene, P35S: Cauliflower mosaic
798 virus 35S promoter; GFP: full-length (792 bp) green fluorescent protein
799 (mGFP-ER version); TNOS: terminator for the nopaline synthase gene. FsK-

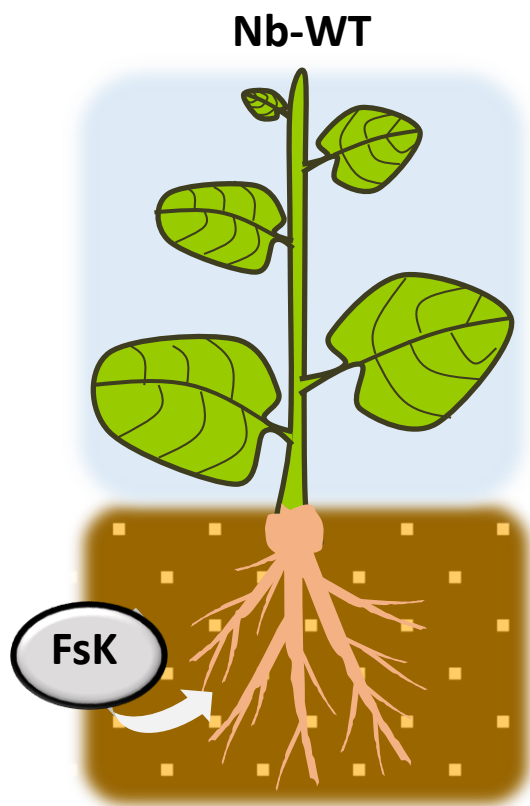
800 hpGF+GFP transformants contain the full length hpGF+GFP transgene,
801 whereas FsK-GFP transformants contain only the P35S-GFP-TNOS part of the
802 transgene. (B) SRNA-seq in FsK-hpGF+GFP #27. All sRNA reads of 18-30-nt
803 fully matching to GFP region are depicted. With light blue the siRNA reads in
804 plus polarity, with dark blue the siRNA reads in minus polarity. The Tablet
805 software (Milne *et al.*, 2013) was used for visualization of the sRNA reads.
806 (C) Pie graph of the 18-30 nt GFP siRNAs in FsK-hpGF+GFP #27. (D) RT-
807 qPCR for the estimation of GFP mRNA downregulation in FsK-hpGF+GFP
808 compared to FsK-GFP. (E) Bisulfite sequencing for cis RdDM. (F) Bisulfite
809 sequencing for trans RdDM.

810 **Fig. 5.** FsK-hpGF+GFP colonization of Nb-GFP. (A) Schematic overview of the
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812 4-6 wpi. (C) Bisulfite sequencing in the host GFP transgene in both roots and
813 leaves in silenced Nb-GFP plants.

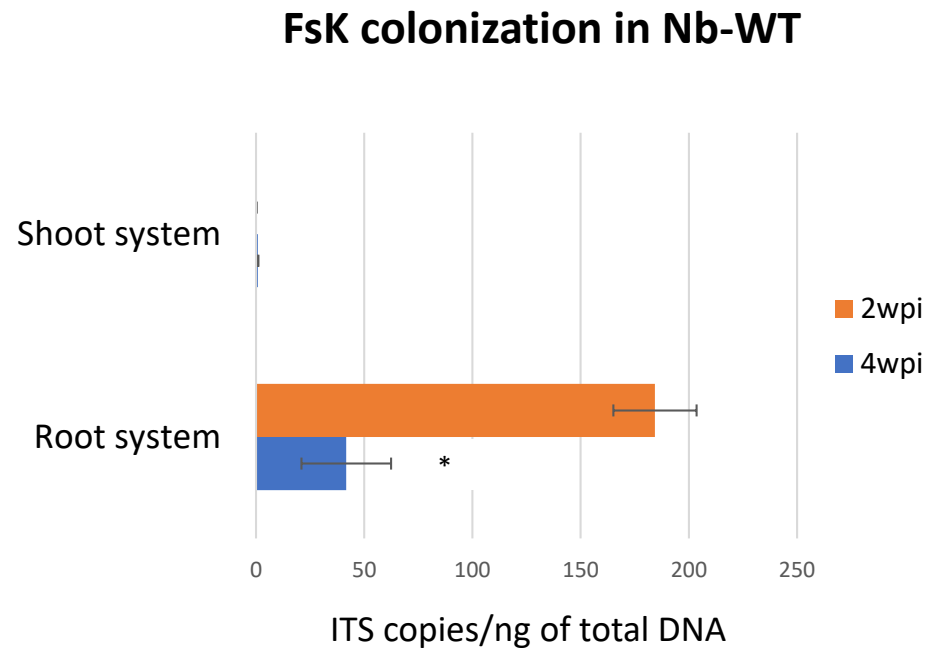
814

815

A



B



C



Fig. 1. Colonization of Nb-WT by Fsk. (A) Schematic representation of the colonization assay. **(B)** Quantification of fungal colonization in shoot and root system at 2 and 4 wpi. **(C)** Impact of the Fsk in growth of Nb-WT 4 wpi.

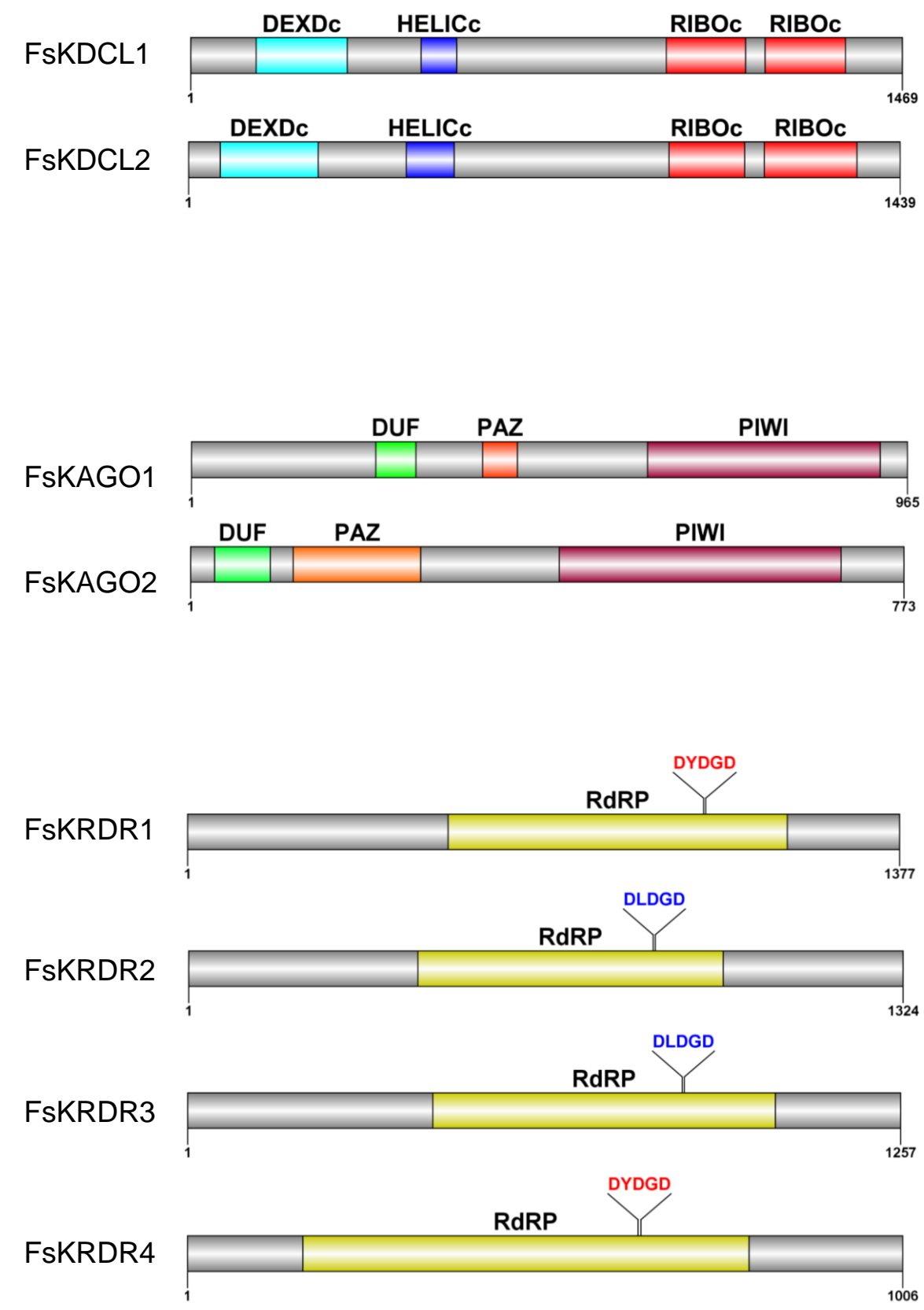
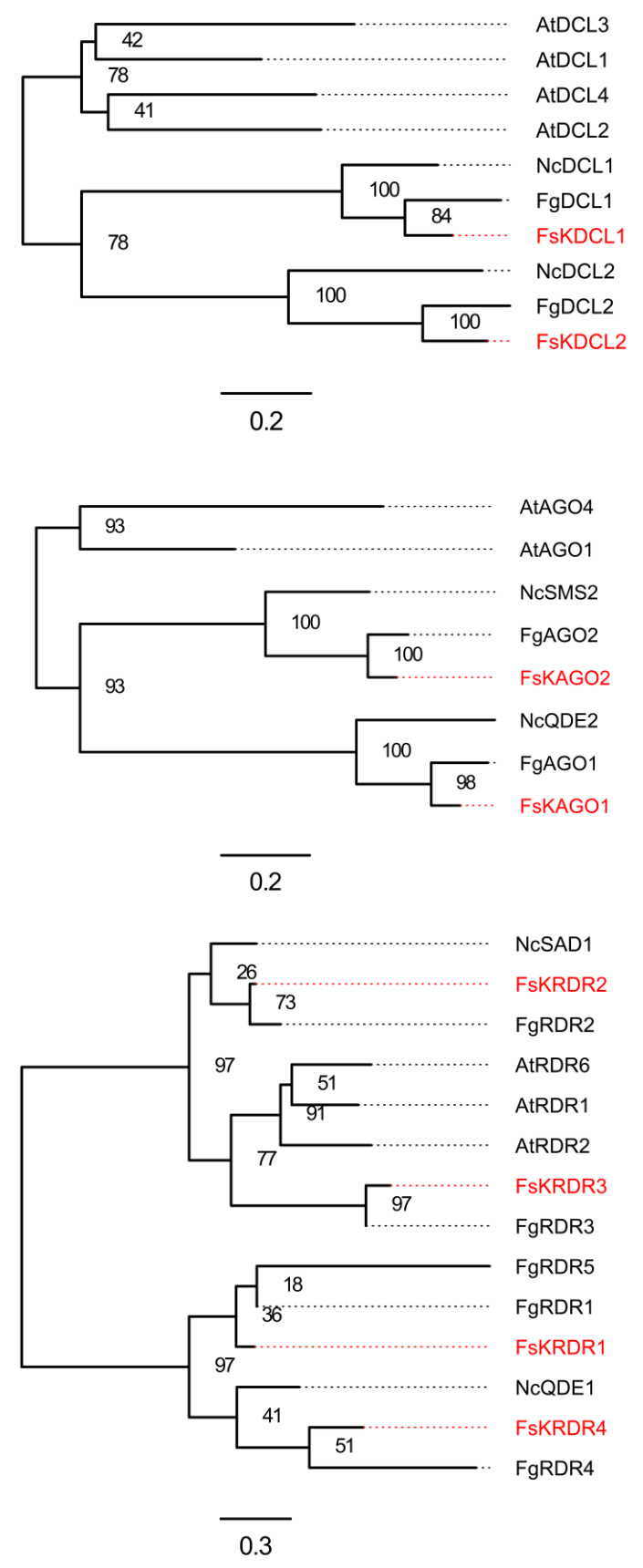
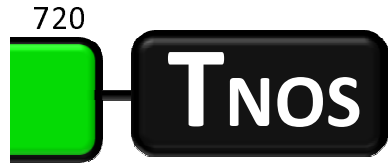
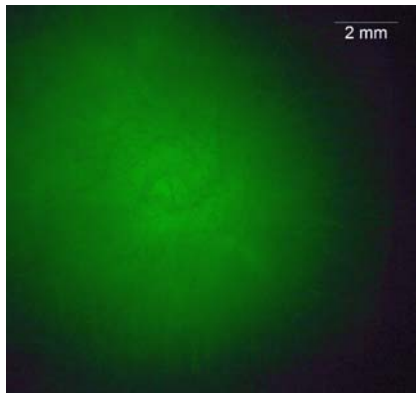
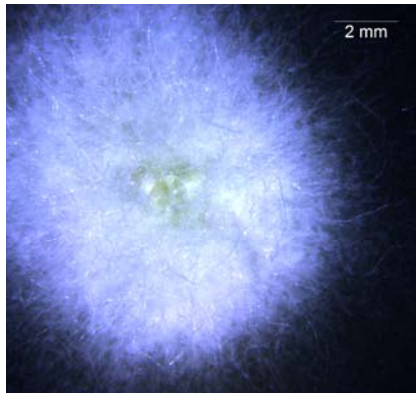
A**B**

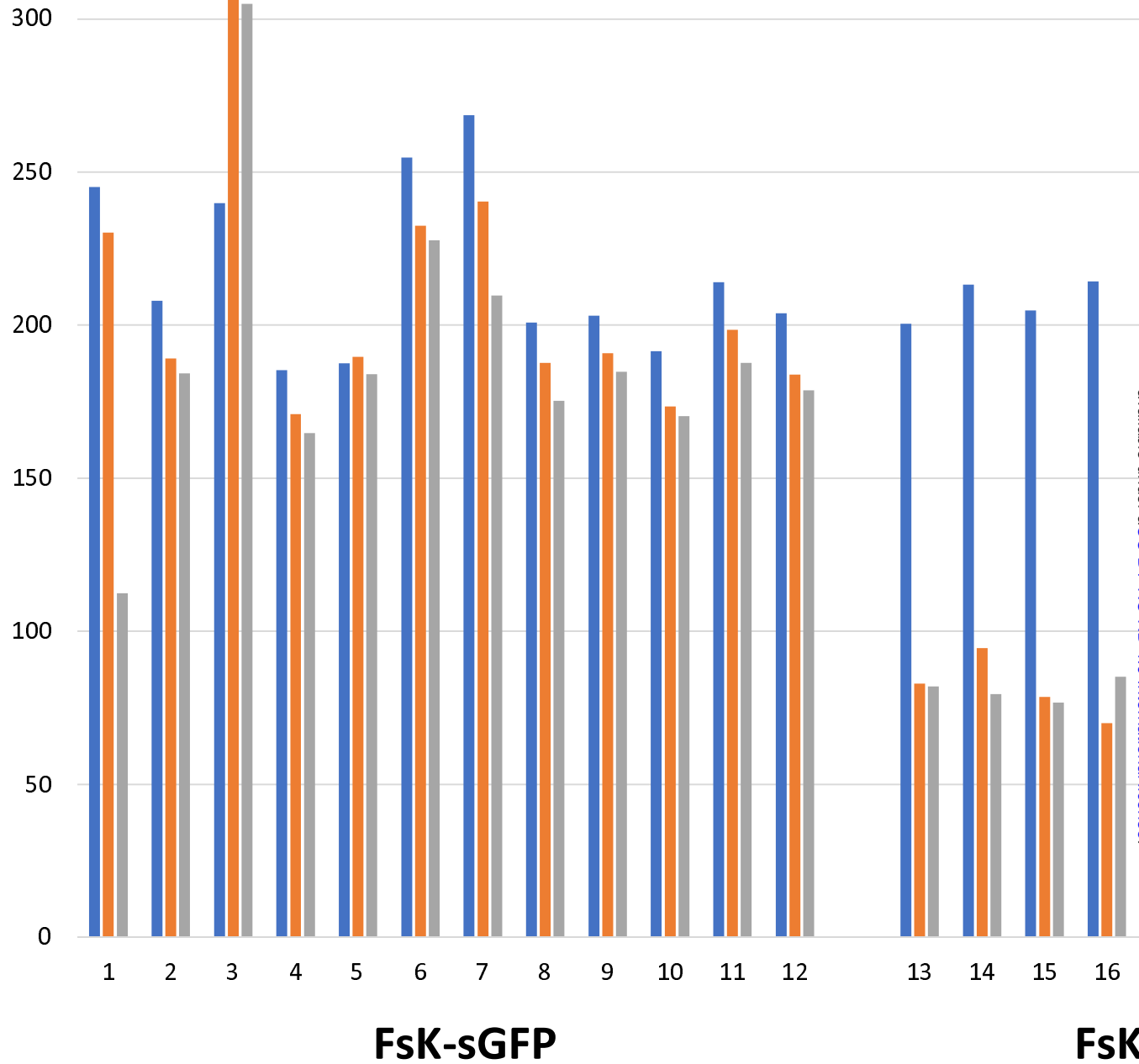
Fig. 2. Identification of Fsk RNAi core machinery. (A) Schematic representation of Fsk DCL, AGO and RDR proteins using DOG1.0 software (Ren et al., 2009). (B) Maximum likelihood phylogenies of the Fsk (indicated red), *Fusarium graminearum*, *Neurospora crassa* and *Arabidopsis thaliana* (as an outgroup member) DCL, AGO and RDR proteins using the LG model matrix and 100 bootstrap replicates for assessing branch support.



FsK-sGFP

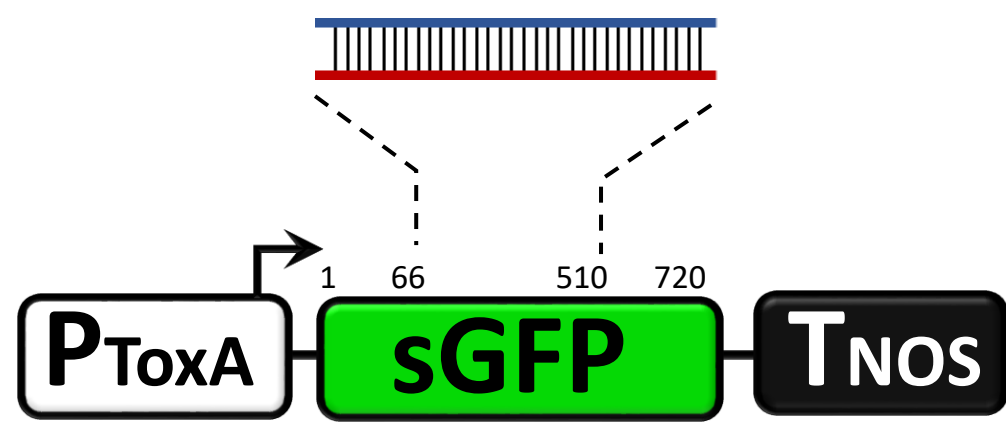


RFU



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sGFP dsRNA (445 bp)

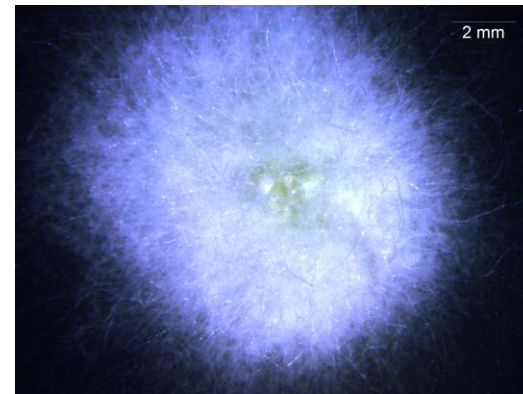
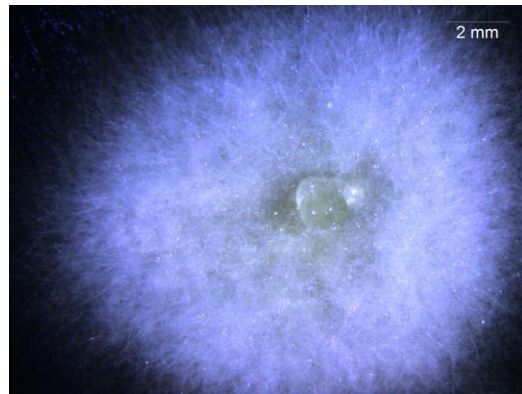


B

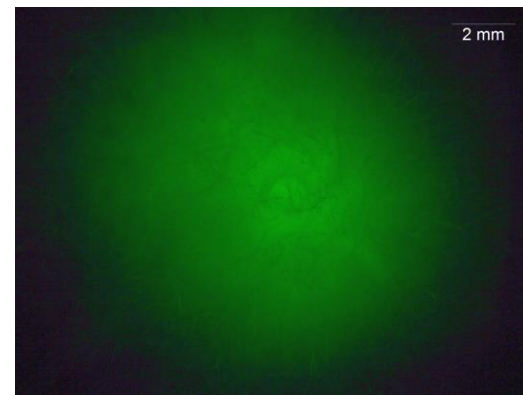
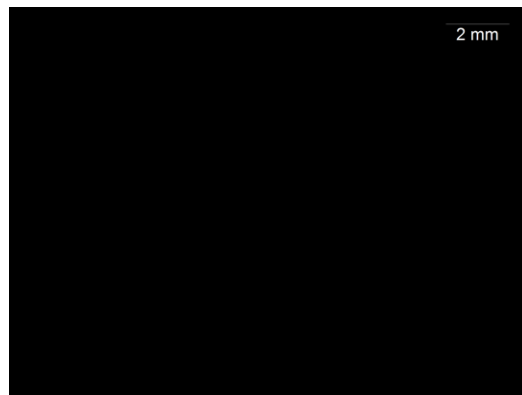
FsK

FsK-sGFP

BF



GFP



Fluorometric analysis - in vitro RNAi of FsK-sGFP

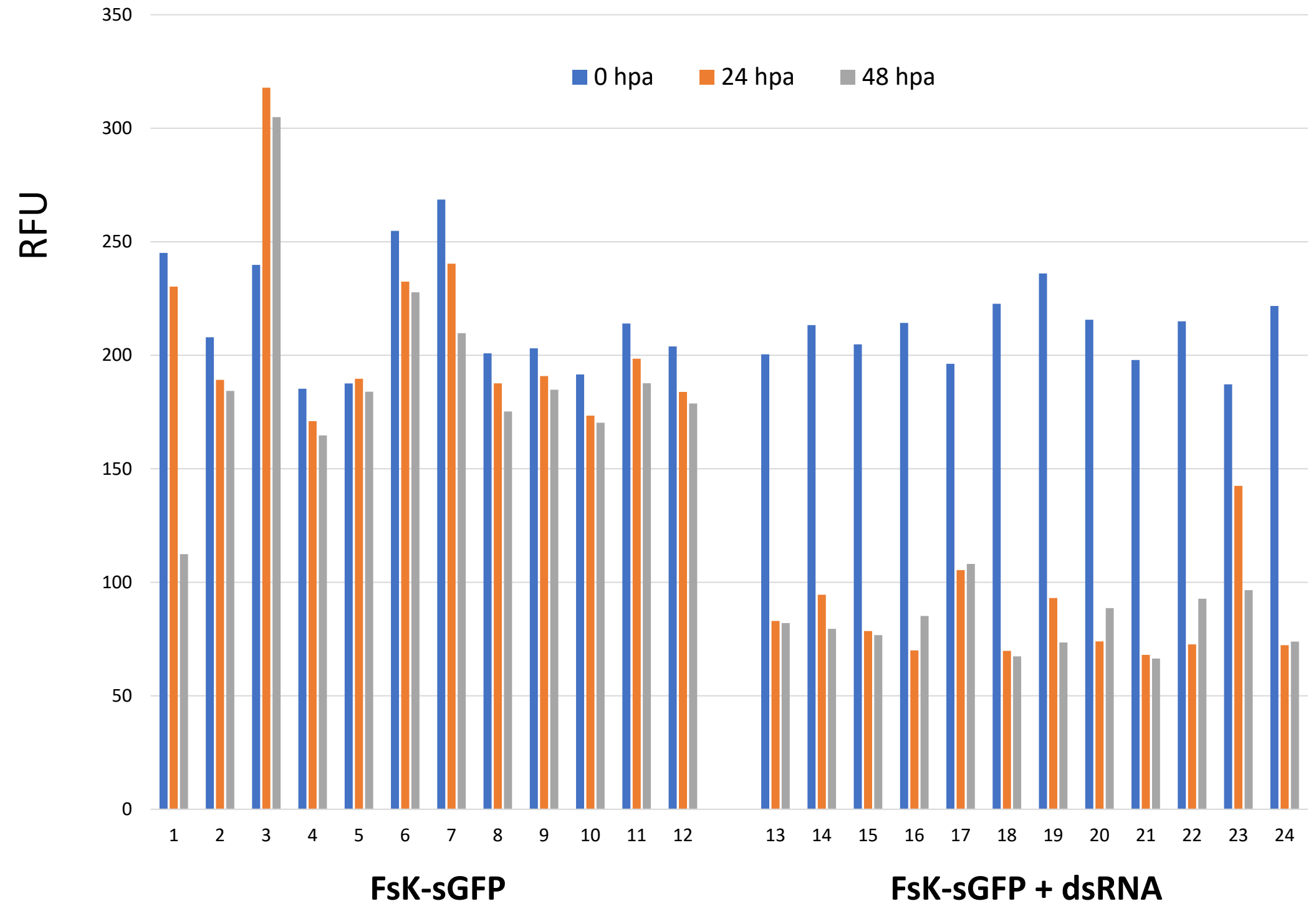


Fig. 3. In vitro RNAi in FsK-sGFP. (A) Schematic representation of the sGFP transgene that is present in FsK-sGFP. PToxA: promoter for the promoter from *Pyrenophora tritici-repentis* ToxA gene; sGFP: GFP variant that contains a serine-to-threonine substitution at amino acid 65; TNOS: terminator for the nopaline synthase gene. The 445 bp fragment chosen for in vitro transcription of dsRNA is depicted. (B) Stereoscopic observation of sGFP fluorescence. (C) Fluorometric analysis for in vitro RNAi in FsK-sGFP. Vertical axis: RFU: relative fluorescence unit, calculated as the ratio of sGFP-indicative fluorescence (excitation 488 nm, emission 515 nm) to growth-indicative absorbance (wavelength 595 nm). Horizontal axis: 1-12: 12 wells containing FsK-sGFP conidia. 13-24: 12 wells containing FsK-sGFP conidia plus 100 ng (each well) sGFP dsRNA.

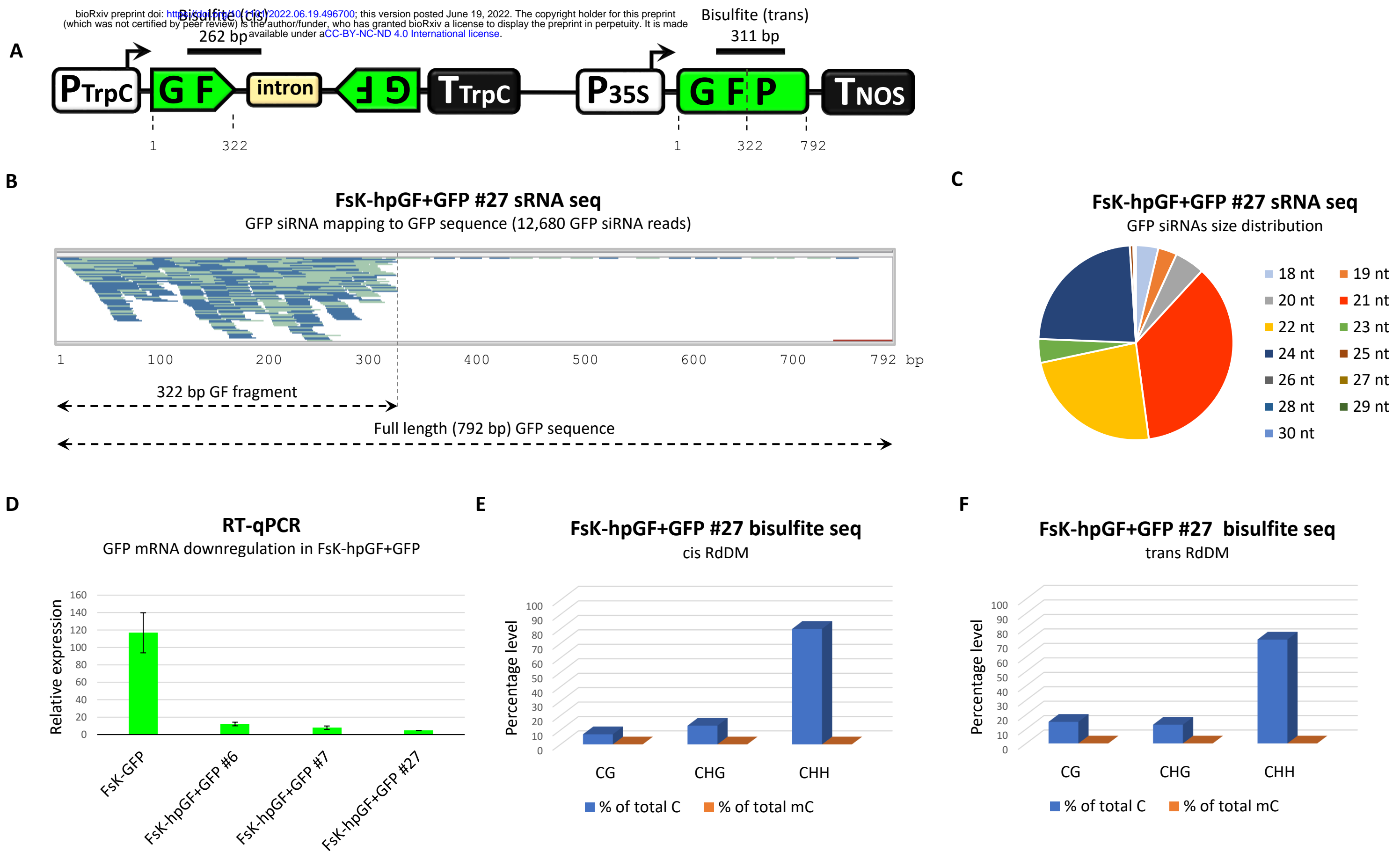


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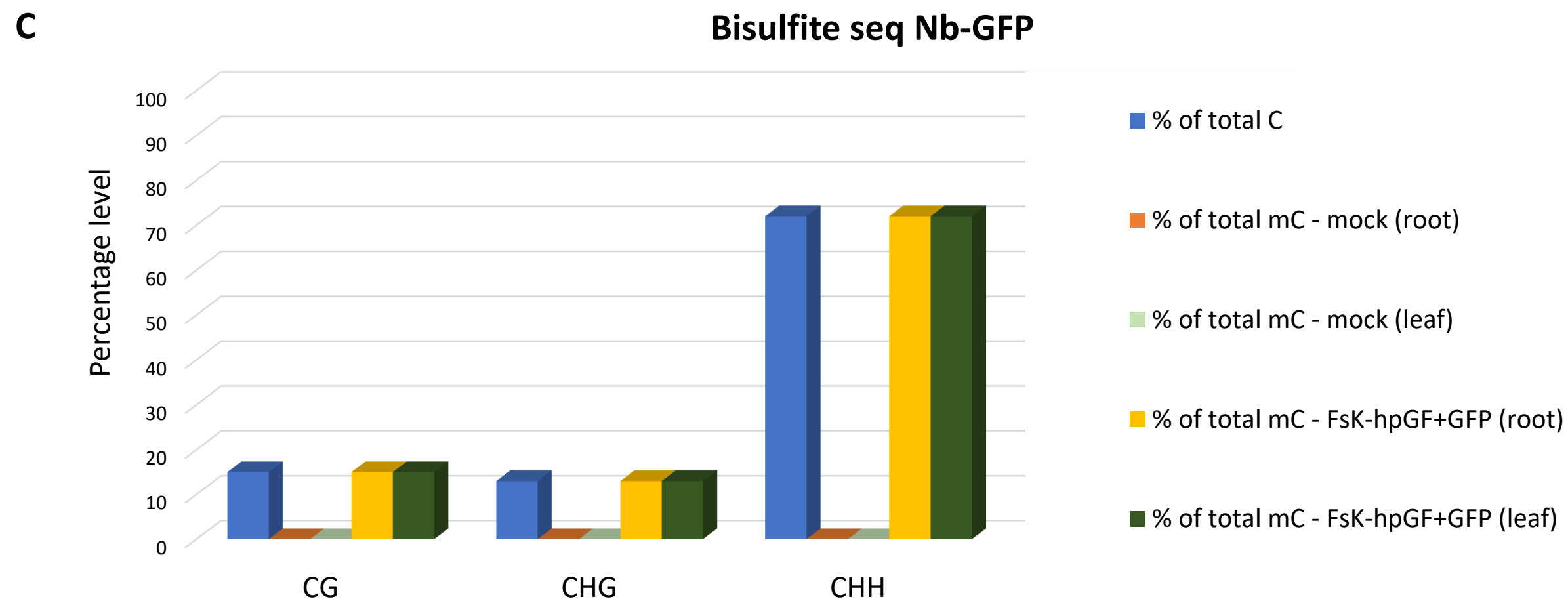
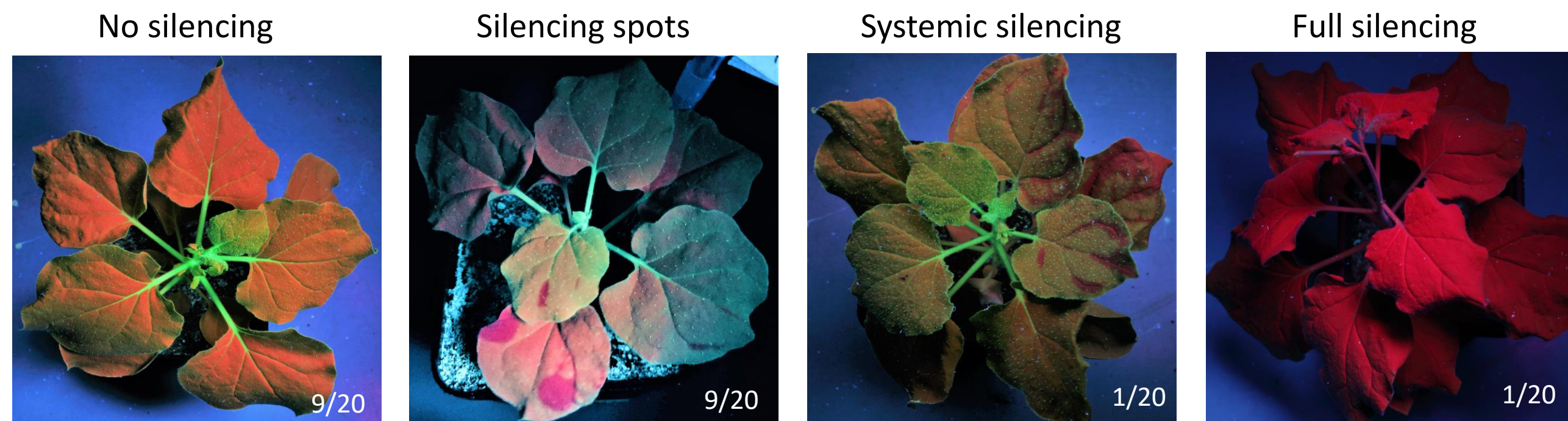
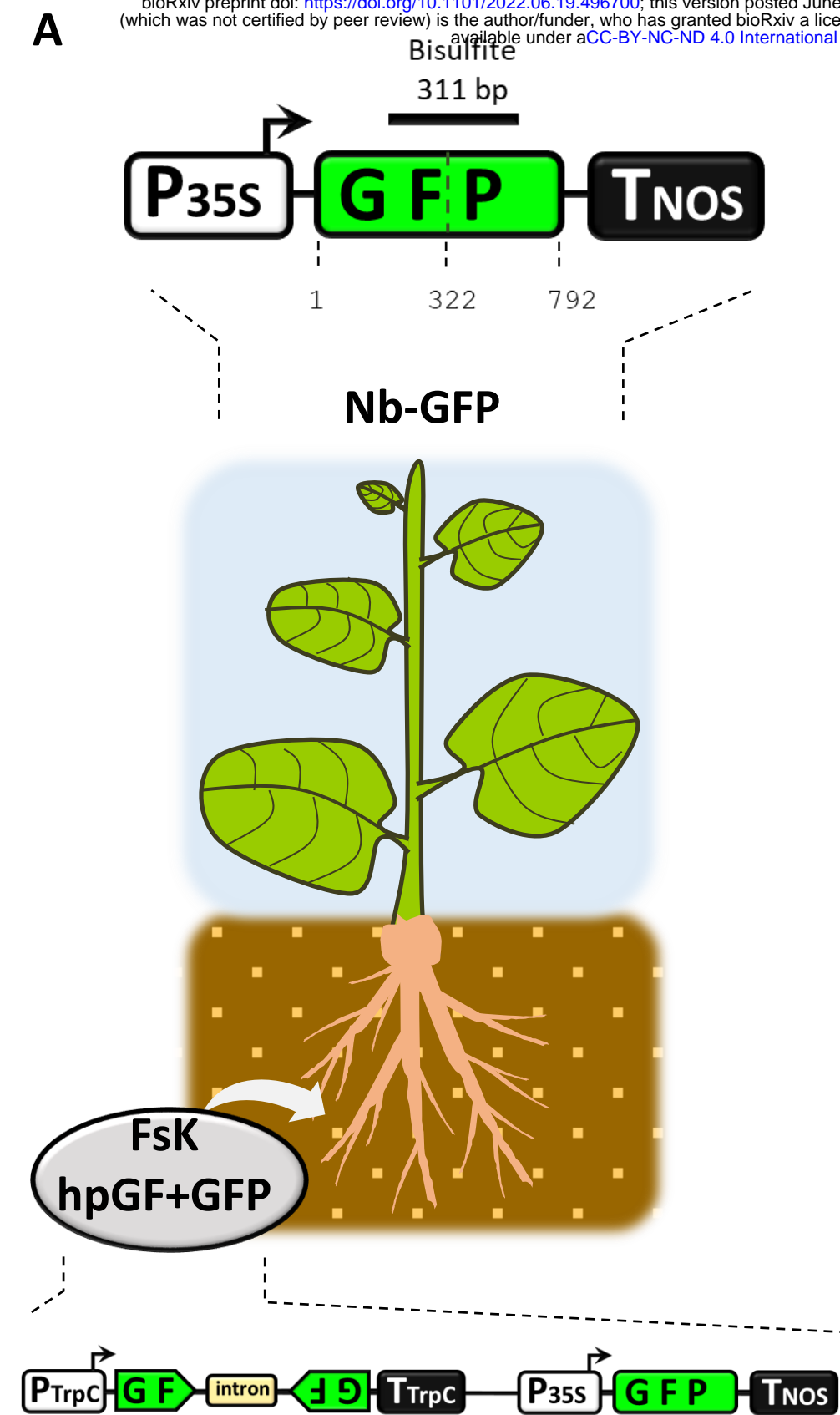


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