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1 A root-knot nematode effector Mi2G02 hijacks a host plant trihelix

2 transcription factor for nematode parasitism

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15 Running title

16 Mi2G02 effector stabilizes a transcription factor

17 Short summary

18 Root-knot nematodes establish parasitic relationships with host plants through

19 secreting effectors. In this study, we highlight the role of Mi2G02 effector and its

20 target GT-3a, a trihelix transcription factor, in plant nucleus. Mi2G02 maintains GT-

21 3a protein stabilization by inhibiting the 26S proteasome-dependent pathway,

22 leading to a suppression of *TOZ* and *RAD23C* expression, promoting *Meloidogyne*

23 *incognita* parasitism.

24 ABSTRACT

25 Root-knot nematodes (RKNs) cause huge agricultural losses every year. They

26 secrete a repertoire of effectors to facilitate parasitism through the induction of plant-

27 derived giant feeding cells, which serve as their sole source of nutrients. However,

28 the mode of action of these effectors and host targeted proteins remain largely

29 unknown. In this study, we investigated the role of the effector Mi2G02 in

30 *Meloidogyne incognita* parasitism. Host-derived *Mi2G02* RNA interference in

31 *Arabidopsis thaliana* affects giant cells development, whereas the ectopic expression

32 of Mi2G02 promotes root growth and increases plant susceptibility to *M. incognita*.

33 We used various combinations of approaches to study the specific interactions

34 between Mi2G02 and *A. thaliana* GT-3a, a trihelix transcription factor. *GT-3a*

35 knockout in *A. thaliana* affected feeding site development, resulting in the production

36 of fewer egg masses, whereas *GT-3a* overexpression in *A. thaliana* increased

37 susceptibility to *M. incognita* and also root growth. Moreover, we highlight the role of

38 Mi2G02 in maintaining GT-3a protein stabilization by inhibiting the 26S proteasome-

39 dependent pathway, leading to a suppression of *TOZ* and *RAD23C* expression,

40 promoting nematodes parasitism. Thus, this work enhances our understanding of the

41 manipulation of the role and regulation of a transcription factor by a pathogen

42 effector through interfering proteolysis pathway to reprogram genes expression for

43 nematode feeding cells development.

44 **Keywords:** *Meloidogyne incognita*, effector, giant cell, Mi2G02, transcription factor,

45 interaction

46 **INTRODUCTION**

47 Root-knot nematodes (RKNs; *Meloidogyne* spp.) can infect thousands of plant species,

48 causing huge agricultural losses every year (Abad et al., 2008; Jones et al., 2013).

49 RKN juveniles induce the redifferentiation of plant vascular cells to establish feeding

50 structures that support their development into reproductive adult females (Bartlem et

51 al., 2014). The second-stage juveniles (J2s) enter the host in the root elongation area

52 and migrate intercellularly toward the vascular tissues, where they select five to seven

53 parenchyma cells, into which they inject esophageal gland secretions through a

54 syringe-like stylet (Favery et al., 2020). These secretions contain proteinaceous

55 effectors, which reprogram the root cells to become giant cells (GCs), hypertrophied

56 multinucleate feeding cells that undergo several rounds of nuclear division without cell

57 division and extensive endoreduplication, with expansion by isotropic growth (Caillaud

58 et al., 2008). The cells surrounding the GCs simultaneously divide, to form a typical

59 root-knot or gall. The vascular tissues undergo extensive reorganization, and the

60 xylem proliferates (Baldacci-Cresp et al., 2020; Bartlem et al., 2014; Yamaguchi et al.,

61 2017). GCs are the only source of nutrients for RKNs throughout their live cycle; the

62 nematode must, therefore, maintain this parasitic interaction for several weeks, until
63 the female can lay her eggs in a gelatinous matrix on the outside of the root tissues
64 (Favery et al., 2020).

65   This intricate biotrophic interaction requires the nematode to cope with host
66 defense responses, to alter host-cell morphology and to hijack the physiology of host
67 cells for its own benefit. The nematode achieves these ends by inducing a deep
68 transcriptional reprogramming of host cells, as demonstrated by a large number of
69 transcriptomic studies (Barcala et al., 2010; Cabrera et al., 2014; Escobar et al., 2011;
70 Fuller et al., 2007; Jammes et al., 2005; Olmo et al., 2017; Portillo et al., 2009;
71 Przybylska and Spychalski, 2021; Sato et al., 2021; Shukla et al., 2018; Warmerdam
72 et al., 2018; Yamaguchi et al., 2017; Zhu et al., 2022). Genes encoding transcription
73 factors (TFs), which regulate key expression by binding to appropriate DNA elements
74 and recruiting additional proteins to initiate transcription (Strader et al., 2022), are
75 among the genes known to display differential expression in galls (Cabrera et al.,
76 2014; Przybylska and Spychalski, 2021; Yamaguchi et al., 2017; Zhu et al., 2022).
77 Many of these plant TFs are known to be key players in the regulation of plant
78 developmental processes and stress responses. They include ETHYLENE-
79 RESPONSIVE FACTORS (ERF), NO APICAL MERISTEM (NAC), AUXIN
80 RESPONSE FACTORS (ARF) and LATERAL ORGAN BOUNDARIES DOMAIN-(LBD)
81 (Shukla et al., 2018). However, very little is known about the role of these TFs in GC
82 formation and RKNs parasitism. For LBD16, inactivation led to a decrease in infection
83 or even a total absence of feeding site formation (Cabrera et al., 2014; Olmo et al.,
84 2017).

85   RKN effectors are clearly involved in modulating host transcriptional responses.
86 They may act as transcription factors, as has been shown for the *M. incognita* effector
87 7H08, which localizes to the plant cell nucleus and functions as a transcriptional
88 activator (Zhang et al., 2015). Other effectors may associate with and dysregulate host
89 transcription factors. Mi16D10 may be one such effector, as it has been shown to
90 interact with plant SCARECROW-like transcription factors known to regulate root
91 development (Huang et al., 2006). Another example is provided by MiEFF18, an
92 effector that interacts with the spliceosomal protein SmD1 to trigger alternative splicing
93 events during pre-mRNA maturation in galls, thereby increasing the diversity of host
94 transcripts (Mejias et al., 2021; Mejias et al., 2022).

95   *M. incognita* secreted protein 2 (*Mi-msp2 or Mi2G02*) was initially identified as a
96   putative parasitism gene expressed exclusively in the subventral esophageal gland
97   cells of parasitic J2s (Huang et al., 2003). It was subsequently shown to be required
98   for *M. incognita* parasitism in host-derived RNA interference experiments (Joshi et al.,
99   2022; Joshi et al., 2019). *Mj2G02,* an ortholog from *M. javanica*, has been shown to
100   suppress Gpa2/RBP-1-triggered cell death in *Nicotiana benthamiana* and jasmonate-
101   mediated plant immune responses (Song et al., 2021). 2G02 proteins are nuclear
102   effectors; they have a ShK toxin (ShKT) domain encoding a potassium channel
103   inhibitor first identified in a sea anemone (*Stichodactyla helianthus*) (Tudor et al., 1996).
104   Proteins containing ShKT domains are widely expressed in parasitic and non-parasitic
105   nematodes (Hewitson et al., 2013). There is evidence to suggest that ShkT-like
106   domains act as contact surfaces for protein interactions (Thein et al., 2009) and for
107   immune evasion (Chhabra et al., 2014; Niu et al., 2016; Song et al., 2021). However,
108   the plant target proteins by Mi2G02 are unknown.

109   In this study, we show that the Mi2G02 effector interacts with an *Arabidopsis*
110   *thaliana* trihelix transcription factor, GT-3a, and that this interaction is important for
111   nematode parasitism. We also demonstrate that GT-3a functions as a transcription
112   inhibitor, binding to the promoters of *TOZ* and *RAD23C*, thereby modulating plant cell
113   development for *M. incognita* parasitism. We found that Mi2G02 stabilized GT-3a by
114   inhibiting the 26S proteasome-dependent pathway, thereby causing a stronger
115   suppression of *TOZ* and *RAD23C* expression. Collectively, these results demonstrate
116   the involvement of a *M. incognita* effector (Mi2G02), a plant transcription factor (GT-
117   3a), and downstream regulatory genes in the formation and development of
118   multinucleate GCs for nematode parasitism.

119   **RESULTS**

120   **Mi2G02 is a nematode nuclear effector essential for giant cell development**
121   *Mi2G02/MiMSP2* gene encodes a 210-amino acid (aa) protein with an 18 aa N-
122   terminal signal peptide (SP), a ShK toxin (ShKT) domain (33–69 amino acids) and two
123   nuclear localization signals (NLS-1 and NLS-2), one of which is located in the ShKT
124   domain (Figure 1A). Transient expression assays were performed in *Nicotiana*
125   *benthamiana* leaves, to investigate the functionality of the NLSs *in planta*. Mi2G02-
126   GFP recombinant fusion proteins were detected exclusively in the plant cell nucleus,
127   exclusively in the nucleoplasm, with exclusion from the nucleolus (Figure 1B). We

128   mutated the two predicted nuclear localization signals in the effector sequence, to
129   generate Mi2G02-mu1 (mutated NLS-1), Mi2G02-mu2 (mutated NLS-2) and Mi2G02-
130   mu3 (mutated NLS-1 and NLS-2) (Figure 1A). Mi2G02-mu1-GFP, Mi2G02-mu2-GFP
131   and Mi2G02-mu3-GFP were found principally in the cytoplasm (Figure 1B).
132   Immunoblotting using cytoplasmic and nuclear fraction proteins extracted from *N.*
133   *benthamiana* plant leaves confirmed the nuclear expression of Mi2G02-GFP, but not
134   Mi2G02-mu1-GFP, Mi2G02-mu2-GFP and Mi2G02-mu3-GFP (Figure 1C). These
135   results demonstrated the requirement of both NLSs for the localization of Mi2G02 to
136   the nucleus.

137   We investigated the role of Mi2G02 in giant cell formation, by generating three
138   homozygous RNAi *A. thaliana* lines expressing the *Mi2G02* hairpin dsRNA. The
139   expression of the hairpin construct in these RNAi lines was confirmed by PCR
140   (Supplemental Figure 1A) and the transgenic plants were inoculated with nematodes.
141   The silencing of *Mi2G02* by host-derived RNAi was validated by RT-qPCR in feeding
142   nematodes recovered from the infested plants (Figure 2A and Supplemental Figure
143   1B). Consistent with previous findings (Joshi et al., 2019), *Mi2G02* silencing
144   decreased the numbers of galls and egg masses by at least 60% in the *Mi2G02* RNAi
145   lines relative to the two control lines: wild-type plants and a *GFP* RNAi line (Figure 2B
146   and Supplemental Figure 1B). We investigated the role of Mi2G02 in the formation of
147   RKN-induced feeding sites further, by analyzing the morphology of the feeding cells
148   induced in the RNAi lines. All three *Mi2G02* RNAi lines showed significantly smaller
149   (30%) GC areas than the controls (Figure 2C). These findings suggest that *Mi2G02*
150   plays a role in nematode parasitism, particularly in the development of GCs.

151   We then generated two transgenic *A. thaliana* lines with ectopic *Mi2G02*
152   expression. Semiquantitative RT-PCR (Supplemental Figure 2A) and western blot
153   (Supplemental Figure 2B) were performed to confirm the expression of *Mi2G02* in the
154   transgenic plants. Interestingly, the roots of the *Mi2G02*-expressing transgenic lines
155   were 27% and 33% longer (*n*=10) in the two independent lines than those of the wild-
156   type plants (Figure 2D and Supplemental Figure 3C). In nematode inoculation assays,
157   both transgenic lines were significantly ($P < 0.05$) more susceptible to *M. incognita*
158   infection than wild-type plants; the *Mi2G02*-expressing lines had up to 30% more galls
159   and egg masses than the wild-type plants at 35 dpi (Figure 2E and Supplemental
160   Figure 2D). Mi2G02 is, therefore, an effector essential for *M. incognita* parasitism, and
161   able to modulate root growth.

Mi2G02 interacts with the trihelix transcription factor GT-3a

We used a yeast two-hybrid (Y2H) screen to identify the proteins of *A. thaliana* targeted by Mi2G02. We used a signal peptide-deficient Mi2G02 as a bait to screen a cDNA library from *M. incognita*-infected *A. thaliana* roots. We identified 20 candidate target proteins, including six proteins annotated as predicted nuclear proteins in TAIR (Supplemental Table 1). Based on the number of captures, and predicted subcellular distributions and functions, we selected three candidate targets for further study: a trihelix transcription factor (GT-3a, AT5G01380), the LATERAL ORGAN BOUNDARIES DOMAIN PROTEIN 41 (LBD41, AT3G020550) and UBIQUITIN EXTENSION PROTEIN 1 (UBQ1, AT3G52990). A pairwise Y2H assay was performed to validate the interactions between Mi2G02 and the full-length GT-3a, LBD41 and UBQ1 proteins. GT-3a was the only protein found to interact with Mi2G02 (Figure 3A and 3B). LBD41 displayed strong auto-activation in yeast, and it was not possible to confirm any interaction between Mi2G02 and UBQ1 (Supplemental Figure 3A).

We investigated the possible involvement of the ShKT domain of Mi2G02 and the DNA-binding domain (DB) of GT-3a in the interaction, by generating two truncated versions of Mi2G02 (Mi2G02-ShKT and Mi2G02-ΔShKT) and two truncated versions of GT-3a (GT-3a-DB and GT-3a-ΔDB) (Figure 3A). Subcellular localization results showed that Mi2G02-ShKT-GFP was localized mainly in the cell nucleus (Supplemental Figure 3B). Pairwise Y2H experiments demonstrated that the ShKT domain of Mi2G02 and the DB domain of GT-3a were required for the interaction between these two proteins (Figure 3B). We also investigated the requirement of the NLSs of Mi2G02 for the interaction with GT-3a in yeast. Pairwise Y2H experiments with Mi2G02-mu1, Mi2G02-mu2 and Mi2G02-mu3 showed that both the NLSs of Mi2G02 were required for interaction with GT-3a (Figure 3C).

The co-expression of Mi2G02-GFP and mcherry-GT-3a in *N. benthamiana* leaf cells showed that the effector and its target were colocalized in the nucleoplasm of the plant cells (Figure 3D). We then investigated the interactions between Mi2G02 and GT-3a *in planta,* by performing bimolecular fluorescence complementation (BiFC) assays. The co-expression of Mi2G02 fused to the N-terminal part of YFP (Mi2G02-nEYFP) and GT-3a fused to the C-terminal part of YFP (GT-3a-cEYFP) in *N. benthamiana* epidermal leaf cells resulted in a reconstitution of YFP activity in the plant cell nucleus, whereas no YFP fluorescence was observed if Mi2G02 with mutated NLSs or an empty vector was used (Figure 3E and Supplemental Figure 3C).

196   A split luciferase complementation assay (LCA) and a co-immunoprecipitation

197   (Co-IP) assay were performed for further verification of the interaction between

198   Mi2G02 and GT-3a *in planta*. A positive luciferase signal was obtained when Mi2G02

199   was co-expressed with GT-3a in *N. benthamiana* leaves, as luciferase activity was

200   reconstituted by the interaction between Mi2G02 and GT-3a, whereas no luciferase

201   signal was observed if Mi2G02 with mutated NLSs or the GUS control was used

202   (Figure 3F). In the Co-IP assay, Mi2G02-HA, HA empty vector or GFP-HA and GT-3a-

203   GFP were co-expressed in *N. benthamiana* leaves. GT-3a-GFP coprecipitated with

204   Mi2G02-HA but not with Mi2G02-mu1-HA, Mi2G02-mu2-HA and Mi2G02-mu3-HA

205   (Figure 3G). There is, therefore, a direct interaction between the nuclear Mi2G02 and

206   *A. thaliana* GT-3a TF *in planta*.

207   **GT-3a is important for *M. incognita* parasitism**

208   GT-3a has been reported to be predominantly expressed in floral buds and roots,

209   especially at the onset of secondary root development (Ayadi et al., 2004). RNA-seq

210   data for *M. incognita*-infected *A. thaliana* galls at 3, 5 and 7 days post inoculation (dpi)

211   and for non-infected roots showed that *GT-3a* was significantly upregulated by

212   nematode infection at these early time points (Yamaguchi et al., 2017). For the

213   analysis of *GT-3a* expression in galls, we cloned a fragment of the GT-3a promoter (-

214   2023 to 0) and transformed *A. thaliana* plants with a *ProGT-3a*:*GUS* fusion. We then

215   inoculated the transformed plants with *M. incognita* and performed histochemical

216   assays. We observed a strong GUS signal in uninfected root vascular tissues and

217   lateral root initials, and in developing galls at 3, 5 and 7 dpi (Figure 4A). These results

218   suggest that *GT-3a* plays a role early in nematode feeding site development.

219   We explored the biological functions of GT-3a during gall formation in two *gt-3a*

220   T-DNA knockout (KO) mutant *A. thaliana* lines (*SALK_134703* and *SALK_040448*)

221   (Supplemental Figure 4A). We also generated transgenic *A. thaliana* lines

222   overexpressing a GT-3a-GFP fusion and GFP alone. Homozygous KO mutants were

223   verified by PCR and semiquantitative RT-PCR (Supplemental Figure 4B and 4C). Two

224   independent *GT-3a-GFP*-overexpressing lines were selected and verified by

225   semiquantitative RT-PCR, western blotting and observing GFP fluorescence

226   (Supplemental Figure 5D-5F). No macroscopic root phenotype was observed in the

227   two *gt-3a* T-DNA KO lines relative to wild-type Col-0 (Supplemental Figure 4G). As

228   observed for Mi2G02, the two independent *GT-3a-GFP*-overexpressing lines had

229   longer roots (8%; *n*=10) than the wild-type plants, and also had a larger number (92%

230 and 87%; *n*=10) of lateral roots and a greater lateral root density than the wild-type

231 plants (Figure 4B and 4C and Supplemental Figure 4H). These lines were then

232 subjected to nematode infection assays. *GT-3a*-overexpressing plants had larger

233 numbers of galls and egg masses (37% and 48%, *n*=18) than control plants (Figure

234 4D and Supplemental Figure 5A). By contrast, the two *gt-3a* mutants were significantly

235 less susceptible to *M. incognita* than control plants, as shown by their smaller numbers

236 of galls (more than 60% fewer, *n*=26) and the almost complete absence of egg

237 masses (Figure 4E and Supplemental Figure 5B). In these KO lines, the areas

238 covered by GCs were 40% (*n*=10) smaller than those in control plants (Figure 4F).

239 The GT-3a transcription factor, therefore, regulates root development and plays an

240 essential role in GC development and *M. incognita* parasitism.

241 **GT-3a targets and represses *TOZ* and *RAD23C***

242 We investigated the transcriptional activity of GT-3a, by fusing the *GT-3a* coding

243 sequence to the sequence encoding the GAL4 DNA-binding domain in the pGBKT7

244 (BD) vector, and using the resulting plasmid to transform the yeast strain AH109.

245 Yeast cells transformed with the positive control pCL-1, encoding the full-length wild-

246 type GAL4 protein, grew well on SD-Trp-His selection medium and displayed X-α-Gal

247 activity (Figure 5A). By contrast, yeast cells harboring BD-GT-3a or the empty BD

248 plasmid (negative control) were unable to grow on SD-Trp-His selection medium

249 (Figure 5A). These results suggest that GT-3a does not act as a transcriptional

250 activator and, therefore, probably acts by repressing gene expression.

251   We then sought to identify the genes for which expression was modulated by GT-

252 3a, by searching *A. thaliana* promoter sequences for 5'-GTTAC-3' DNA element,

253 which was known to be specifically targeted by GT-3a (Ayadi et al., 2004), and for the

254 5'-CACGTG-3' DNA element, with the PlantRegMap tool (Tian et al., 2019). Further,

255 we turned to the ePlant online tools and explored the expression patterns of candidate

256 genes under nematode infection and in developing roots (Waese et al., 2017). We

257 retrieved nine putative GT-3a targets (Supplemental Table 2), which were then further

258 studied with a yeast one-hybrid (Y1H) approach. The Y1H assay revealed that GT-3a

259 bound directly to the promoters of *TORMOZ* (*TOZ*; *AT5G16750*), *RADIATION*

260 *SENSITIVE 23C* (*RAD23C; AT3G02540*) and a WRKY transcription factor (*WRKY2*;

261 *AT5G56270*) (Figure 5B and Supplemental Figure 6).

262   We investigated the ability of GT-3a to repress the expression of *TOZ, RAD23C*

263 or *WRKY2* in a dual-luciferase reporter assay. A construct expressing *GT-3a* and a

264 reporter construct consisting of the *TOZ*, *RAD23C* or *WRKY2* promoter driving
265 transcription of the firefly luciferase (*LUC*) reporter gene were used for the co-
266 infiltration of *N. benthamiana* leaves. GT-3a decreased the activity of the *TOZ* and
267 *RAD23C* promoters, measured as a *firefly*-to-*Renilla* (LUC/REN) luciferase ratio, by
268 30% and 50%, respectively, relative to the GFP control, whereas it did not decrease
269 *WRKY2* promoter activity (Figure 5C). For confirmation of this result, we used RT-
270 qPCR to quantify *TOZ* and *RAD23c* expression in transgenic *A. thaliana* plants
271 overexpressing *GT-3a*. Both *TOZ* and *RAD23c* appeared to be repressed in the two
272 independent transgenic lines relative to the wild type (Figure 5D).

273   Finally, we produced a recombinant GT-3a protein, which was used in a gel
274 electrophoretic mobility shift assay (EMSA). GT-3a-His significantly decreased the
275 electrophoretic mobility of the *TOZ* and *RAD23C* promoter probes containing GTTAC
276 or CACGTG elements, but had no effect on the mobility of the mutated probes (GTTAC
277 replaced by AAAAA, CACGTG replaced by AAAAAA) (Figure 5E). This result validates
278 the binding of GT-3a to the *TOZ* and *RAD23c* promoters, and indicates that both the
279 GTTAC and CACGTG elements are important for binding.

280   These results confirm that GT-3a can bind the *TOZ* and *RAD23C* promoters
281 specifically, downregulating the expression of genes driven by these promoters.

282 **Mi2G02 promotes GT-3a function by stabilizing protein level for nematode**
283 **parasitism**

284 The *toz* mutant is not viable at postembryonic stages (Griffith et al., 2007) and could
285 not, therefore, be tested in interaction with the nematode. We investigated the role of
286 *RAD23C* in the plant response to *M. incognita* parasitism, with a *rad23c* T-DNA KO
287 mutant *A. thaliana* line (*SALK_068091*) obtained from ABRC (Supplemental Figure
288 7A). Homozygous KO plants were verified by PCR on genomic DNA (Supplemental
289 Figure 7B) and by RT-qPCR on cDNA (Supplemental Figure 7C). No difference in root
290 phenotype was observed between the *rad23c* T-DNA KO line and wild-type plants
291 (Supplemental Figure 7D), consistent with previous reports (Farmer et al., 2010).
292 Following *M. incognita* infection, the *rad23c* KO lines were significantly more
293 susceptible to the nematode than control plants, as shown by the larger numbers of
294 galls (43%, *n*=28), and egg masses (39%) observed (Figure 6A and Supplemental
295 Figure 7E). RAD23C therefore downregulates *M. incognita* parasitism.

296   We addressed the potential outcome of Mi2G02 binding to GT-3a more precisely,
297 by co-expressing Mi2G02 and GT-3a in *N. benthamiana* leaves and performing dual-

298   luciferase reporter assays. The previously observed suppression of *TOZ* and *RAD23C*

299   expression mediated by GT-3a was significantly enhanced in the presence of Mi2G02

300   (Figure 6B). We also performed transient expression assays and western blotting to

301   determine whether Mi2G02 affected the amount of GT-3a protein in *N. benthamiana*

302   agro-infiltrated leaves. The co-expression of Mi2G02 and GT-3a-GFP in *N.*

303   *benthamiana* leaves resulted in a significant higher GFP fluorescence intensity (500%

304   to 660% higher) compared with the controls using mutant Mi2G02 or MiEFF18 (Figure

305   6C and Supplemental Figure 8). Similarly, the co-expression of Mi2G02 and GT-3a in

306   *N. benthamiana* leaves resulted in high levels of GT-3a protein accumulation. No such

307   accumulation was observed with empty vector, Mi2G02 mutants or MiEFF18, used as

308   negative controls (Figure 6D and 6E). Furthermore, treatment with a proteasome

309   inhibitor, MG132, inhibited the degradation of GT-3a (Figure 6E), suggesting that

310   Mi2G02 stabilized GT-3a protein level by inhibiting the 26S proteasome-dependent

311   pathway. Together, these results suggest that the Mi2G02 effector helps to stabilize

312   the GT-3a protein, enabling GT-3a to repress the target genes *TOZ* and *RAD23C,* to

313   promote nematode parasitism (Figure 7).

314   **DISCUSSION**

315   Phytopathogen success depends on the secretion of effector proteins to reprogram

316   the host transcriptome to facilitate parasitism. Pathogens have been shown to secrete

317   effectors that can function as TFs or target TFs to manipulate host cell physiology

318   and/or immunity. In plant-nematode interactions, the 10A07 effector from the sugar

319   beet cyst nematode, *Heterodera schachtii*, is expressed in the nematode dorsal gland

320   cell and targets a plant kinase and the IAA16 transcription factor. There is also

321   evidence to suggest that the 10A07-IAA16 interaction interferes with auxin signaling

322   by modulating the expression of several auxin response factors (Hewezi et al., 2015).

323   Nevertheless, the function of nematode nuclear effectors and the ways in which they

324   manipulate their host targets for feeding site initiation and development remain largely

325   elusive. In this study, we characterized the function of a nuclear effector protein,

326   Mi2G02, and identified its plant target for giant cell formation, the nuclear trihelix

327   transcription factor GT-3a.

328   Trihelix transcription factors (GTs) are unique to plants and have been shown to

329   be involved in embryogenesis and subsequent plant growth and development and in

330   abiotic stresses (Kaplan-Levy et al., 2012). The *A. thaliana AtGT-3b* and the maize

331 (*Zea mays*) *ZmGT-3b*, of the GT-1 clade, are induced by pathogens (Park et al., 2004;

332 Zhang et al., 2021). We show here that Mi2G02 can interact with the *A. thaliana* GT-

333 1 clade protein AtGT-3a, resulting in the stabilization of this protein. *AtGT-3a* is

334 induced during the development of galls induced by *M. incognita*. Using *A. thaliana*

335 transgenic plants in which *AtGT-3a* expression was suppressed or constitutively

336 induced, we demonstrated that AtGT-3a was important for giant cell development and

337 successful RKN parasitism.

338   The *de novo* formation of new organs, such as lateral roots, rhizobium-induced

339 nodules or nematode-induced galls from one or a few root cells requires the

340 recruitment of similar developmental programs (Olmo et al., 2020; Soyano et al., 2019;

341 Yamaguchi et al., 2017). Several genes, including *ABERRANT LATERAL ROOT*

342 *FORMATION 4* (*ALF4*), a *RIBULOSE-PHOSPHATE 3-EPIMERASE* (*RPE*) and

343 *YUCCA4* (*YUC4*), have been reported to have functions associated with lateral root

344 initiation and/or development and expression regulated in nematode-induced galls;

345 they have also been shown to be required for normal feeding site formation and

346 nematode development (Favery et al., 1998; Olmo et al., 2019; Suzuki et al., 2022).

347 These genes include cell-cycle genes and TF genes, such as *LBD16* and *PUCHI,* that

348 play key roles in controlling lateral root initiation and morphogenesis (Torres-Martínez

349 et al., 2019), and are induced following nematode infection and required for feeding

350 site development and successful RKN parasitism (Cabrera et al., 2014; Suzuki et al.,

351 2021). Similarly, *Medicago truncatula LBD16* mutants display nodule initiation defects

352 on inoculation with *Sinorhizobium meliloti* (Schiessl et al., 2019). The rewiring of

353 transcriptional networks to alter root system architecture also involves changes in

354 endogenous levels of growth-related plant hormones, and the production of

355 phytohormones or deployment of hormone-mimicking strategies by symbiotic and

356 parasitic microbes (Eichmann et al., 2021; Gheysen and Mitchum, 2019). We show

357 here that the expression of *Mi2G02* in *A. thaliana* can promote root growth and the

358 development of giant cells, and that Mi2G02 acts by stabilizing AtGT-3a, with

359 mutations of the gene encoding this transcription factor also impairing giant cell

360 formation. *AtGT-3a* was found to be strongly induced at the onset of lateral root

361 development (this study and (Ayadi et al., 2004). AtGT-3a therefore seems to be one

362 of the TFs regulating both lateral root development and nematode-feeding site neo-

363 organogenesis.

364   The binding of GT-3a to the promoter of the *TOZ* and *RAD23C* genes was
365   confirmed by Y1H and EMSA assays. TOZ is a predicted WD40 repeat protein
366   involved in the regulation of cell division planes and the expression of patterning
367   genes during embryogenesis. It may, therefore be involved in plant embryogenesis
368   and organogenesis, including root development (Griffith et al., 2007), suggesting a
369   possible role in the regulation of root-knot neo-organogenesis. RAD23 probably acts
370   as a shuttle protein, delivering ubiquitinated substrates to the ubiquitin/26S
371   proteasome system for degradation. Roles in plant processes as diverse as the cell
372   cycle, cell morphogenesis, and flower development have been proposed for RAD23
373   (Farmer et al., 2010; Maclean et al., 2014). Interestingly, a role for RAD23 proteins in
374   plant immunity has been proposed, probably through interactions with stress-
375   associated proteins (SAPs) acting as ubiquitin E3 ligases (Kang et al., 2017; Liu et
376   al., 2019). Consistently, RAD23 proteins have been shown to be targeted by plant
377   pathogen effectors, possibly to modulate host protein degradation and suppress host
378   defense responses. The *A. thaliana* RAD23A was identified as a putative target of the
379   *Pseudomonas syringae* HopM1 effector (Nomura et al., 2006), and the phytoplasma
380   SAP54 effector was shown to interact with both RAD23C and RAD23D (Maclean et
381   al., 2014). Intriguingly, a previous report confirmed that RAD23 proteins associated
382   with the 26S proteasome and played an essential role in the cell cycle (Farmer et al.,
383   2010), roles of GT-3a and *RAD23C* in proteolysis and cell fate determination are
384   expected. In support of this, the transcription activity assays performed in yeast and
385   *in planta* demonstrated that GT-3a downregulated expression of the *TOZ* and
386   *RAD23C* genes, which suppression was enhanced by co-expressing with Mi2G02.
387   Moreover, Mi2G02 stabilized GT-3a protein level by inhibiting the 26S proteasome-
388   dependent pathway. Using *rad23c* KO *A. thaliana* lines, we showed that *RAD23C*
389   inactivation increased susceptibility to RKNs, demonstrating that *RAD23C* is a key
390   gene for plant-nematode interactions.

391   It has been suggested that Mj2G02 interferes with both jasmonate signaling and
392   plant immune responses (Song et al., 2021). Mj2G02 expression *in planta* resulted in
393   the accumulation of jasmonoyl-isoleucine, the endogenous bioactive form of
394   jasmonate (JA), in transgenic *A. thaliana*, and a dysregulation of the expression of
395   *JASMONATE ZIM DOMAIN* (*JAZ*) transcriptional repressors and jasmonate-
396   responsive genes (Song et al., 2021). Our data indicate that Mi2G02 could divert the
397   host plant developmental program to promote the formation of the feeding sites

398 important for nematode development and reproduction. RKNs may secrete the 2G02

399 effector to stabilize GT-3a, maintaining the concentration of this TF at a sufficiently

400 high level to repress the growth regulator genes, *TOZ* and *RAD23c*, thereby promoting

401 GC development. We hypothesise that, as previously suggested for ZmGT-3a (Zhang

402 et al., 2021), GT-3a acts at the interface between growth and immunity. Microbes can

403 interfere with central regulators of root cell identity and root growth that are also

404 involved in the response to biotic stress (Rich-Griffin et al., 2020; üstüner et al., 2022).

405 Plant hormones, the signaling pathways of which may interact at central hubs, also

406 regulate growth-immunity tradeoffs (Guo et al., 2018; Huot et al., 2014). For instance,

407 cross-talk between gibberellin (GA)-mediated growth and JA-mediated defense

408 signaling pathways participates contributes to maintaining the balance between

409 growth and immunity (Huot et al., 2014; Pieterse et al., 2014). DELLA proteins repress

410 growth-related TFs unless they are degraded in the presence of growth-promoting

411 GA. DELLA also binds to JAZs, and DELLA degradation allows JAZs to interact with

412 their cognate TFs, thereby decreasing JA-dependent signaling. Treatment with flg22

413 suppresses GA-mediated DELLA degradation, leading to an inhibition of root growth

414 dependent on salicylic acid (SA), an antagonist of the JA signaling pathway (Huot et

415 al., 2014; Pieterse et al., 2014). JA is a known growth inhibitor that stabilizes DELLA

416 and has been shown to downregulate the cyclin-dependent kinases CDKA1 and

417 CYCB1;1 required for cell-cycle progression (Qi and Zhang, 2020; Reitz et al., 2015).

418 Biotic stress may, therefore, affect cell cycle regulators, and cell division and

419 hormones influence the underlying regulatory mechanisms.

420   The plant response to pathogens is highly dependent on the interplay between

421 immunity and development. Regulators of cell identity and TFs may play a crucial

422 role in connecting the developmental and immunity gene networks to reflect

423 response specificity (Rich-Griffin et al., 2020). By modulating GT-3a TF availability in

424 plant cells, the RKN effector 2G02 can alter both the root developmental program

425 and JA-dependent signaling pathways to allow giant cell formation and successful

426 parasitism. In this respect, GT-3a constitutes a novel example of a key regulator

427 recruited by a biotrophic pathogen at the interface between growth and immunity.

428 **METHODS**

429 **Nematodes and plant materials**

430 *Meloidogyne incognita* was reproduced on tomato plants (*Solanum lycopersicum* var.

431 'Moneymaker'). Egg masses and preparasitic second-stage juveniles (pre-J2s) were

432 collected as previously described (Zhao et al., 2019). *Arabidopsis thaliana* seeds were

433 germinated on Murashige and Skoog (MS) medium (Coolaber, Cat. No. PM1012) at

434 25°C in a growth chamber and the seedlings were transplanted into pots of soil at 13

435 days. The *gt-3a* T-DNA mutant lines (*SALK_014703* and *SALK_040448*), and the

436 *At3g02540* (*rad23c*) T-DNA mutant line (*SALK_068091*) were obtained from the

437 Arabidopsis Biological Resource Center (ABRC, USA). The homozygous plants were

438 verified by PCR and semiquantitative RT-PCR. *Nicotiana benthamiana* plants and *A.*

439 *thaliana* plants were grown in pots and placed in a culture room at a temperature of

440 23°C, with a 16 h light/8 h dark photoperiod, with fluorescent bulbs used to generate

441 soft white light.

442 **Nematode infections and gall sections**

443 *A. thaliana* seedlings (one month after transplanting) were inoculated with pre-J2s. For

444 nematode susceptibility assays, *A. thaliana* roots were inoculated with 200 pre-J2s per

445 plant. The roots were collected and galls and egg masses were counted under a

446 dissecting microscope (Olympus, Japan) 35 days post inoculation (dpi). For gall

447 collection, roots were inoculated with 500 pre-J2s per plant. Galls were collected at 3

448 dpi, 5 dpi, 7 dpi, 14 dpi and 21 dpi and fixed as previously described (Gavrilovic et al.,

449 2016). At least 10 galls were fixed for each *A. thaliana* line. Gall sections were stained

450 with 0.05% toluidine blue and photographed on a Zeiss microscope (Zeiss AxioImager

451 Z2, Germany). The areas of the giant cells were measured with ImageJ software

452 (Schindelin et al., 2012). Generally, the first step is to open the program and draw a

453 line as the same with image scale, then go to 'Analyze', 'Set Scale' and enter the

454 values: 'known distance' is 100, and 'Unit of length' is 'µm', select 'Global' and click

455 'OK'. The second step is to go to 'Analyze', 'Set Measurements', select 'Area' and click

456 'OK'. The third step is to select 'Freehand selections', select giant cell area, go to

457 'Analyze', select 'Measure' and click 'OK'.

458 **DNA/RNA isolation and gene amplification**

459 *M. incognita* RNA was extracted with TRIzol reagent (Invitrogen, USA, Cat. No.

460 10296010) as previously described (Lin et al., 2013; Zhao et al., 2021). Total RNA was

461 extracted from *A. thaliana* seedlings (ten days after germination) with the RNAprep

462 Pure Plant Kit (TIANGEN, Cat. No. DP432), according to the manufacturer's

463 instructions. The RNA was then used for cDNA synthesis with the M-MLV reverse

464 transcriptase (TaKaRa, Cat. No. 2641Q) in accordance with the manufacturer's

465 instructions. DNA was extracted with the Plant Genomic DNA Kit (TIANGEN, Cat. No.

466 DP305) according to the manufacturer's instructions. Gene and promoter sequences

467 were amplified from cDNA or gDNA by PCR with specific primers. All the primers used

468 in this study are provided in Supplemental Table S3 and were synthesized by TsingKe

469 Biotechnology Co. Ltd, Beijing, China.

470 **RT-qPCR analysis**

471 RNA was extracted and cDNA was synthesized for reverse transcription-quantitative

472 PCR (RT-qPCR), with the BIO-RAD CFX96 (BIO-RAD, USA) real-time PCR system,

473 as follows: 95 °C for 5 min and 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The

474 data were analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For internal

475 controls, *M. incognita GAPDH* (*Minc12412*) or *A. thaliana UBP22* (*AT5G10790*) was

476 used for the normalization of RT-qPCR data. RT-qPCR assays were repeated three

477 times.

478 **Plasmid construction and plant transformation**

479 For RNAi experiments in *A. thaliana*, a 204-nucleotide *Mi2G02* fragment was amplified

480 and inserted upstream and downstream from the pSAT5 intron in the forward and

481 reverse orientations (Dafny-Yelin et al., 2007), and then inserted into the pSUPER

482 destination vector, to construct pSUPER-Mi2G02-RNAi.

483   A signal peptide-deficient *Mi2G02* sequence and ShKT domain were amplified by

484 PCR and inserted into the Super-GFP (C-terminal GFP) to generate Super-Mi2G02-

485 GFP and Super-Mi2G02-ShkT-GFP. The nuclear localization sequences of *Mi2G02*

486 were mutated and inserted into Super-GFP vector to generate Super-Mi2G02-mu1-

487 GFP, Super-Mi2G02-mu2-GFP and Super-Mi2G02-mu3-GFP. The ORF of *GT-3a* was

488 inserted into the pBin-mcherry (N-terminal mcherry) vector to generate pBin-mcherry-

489 GT-3a. Plasmids were checked by sequencing and used to transform *A. tumefaciens*

490 strain GV3101.

491   For ectopic expression in *A. thaliana*, the *Mi2G02* (without signal peptide

492 sequences) and *AT5G01380* (*GT-3a*) ORFs were amplified and the corresponding

493 PCR fragments were inserted into the Super-HA vector (C-terminal HA tag) and the

494 Super-GFP (C-terminal GFP tag) vector to construct Super-Mi2G02-HA and Super-

495 GT-3a-GFP, respectively. The plasmids were verified by sequencing and used to

496 transform *A. tumefaciens* GV3101, with empty vectors used as a control.

497   For the yeast two-hybrid (Y2H) screens, *Mi2G02* (lacking the signal peptide

498   sequence) was amplified and inserted into the pGBKT7 (BD) vector. For pairwise Y2H

499   verification, the coding sequences of *AT5G01380* (*GT-3a*), *AT3G52590* and

500   *AT3G02550* were inserted into the pGADT7 (AD) vector. For identification of the key

501   domains for interaction, *Mi2G02-ShKT* (Mi2G02 retaining the ShKT domain) and

502   *Mi2G02-ΔShKT* (Mi2G02 without the ShKT domain) were amplified and inserted into

503   BD vectors, and *GT-3a-DB* (GT-3a retaining the DNA-binding domain) and *GT-3a-*

504   *ΔDB* (GT-3a lacking the DNA-binding domain) were inserted into AD vectors. The

505   plasmids were verified by sequencing and used to transform Y2HGold competent

506   yeast cells.

507   For the yeast one-hybrid (Y1H) assay, the coding region of *GT-3a* was amplified

508   and inserted into the pB42AD vector. Fragments of candidate promoters were

509   amplified and inserted into the pLacZi vector.

510   For the bimolecular fluorescence complementation (BiFC) assay, *Mi2G02*

511   (lacking the signal peptide sequence) and *GT-3a* coding sequences were inserted into

512   the nE-YFP and cE-YFP vectors (Walter et al., 2004), respectively.

513   For the luciferase complementation assay (LCA), the full-length coding sequence

514   of *Mi2G02* (lacking the signal peptide sequence) or *GT-3a* was inserted into the

515   pCAMBIA1300-nLUC or pCAMBIA1300-cLUC vector. The plasmids were verified by

516   sequencing and used to transform *A. tumefaciens* GV3101.

517   For the co-immunoprecipitation (Co-IP) assay, the coding regions of *Mi2G02*

518   (lacking the signal peptide sequence) or *GT-3a* were inserted into the super1300

519   vectors with an HA-tag and a FLAG-tag, respectively, fused to the C-terminal end of

520   the sequence. Plasmids were verified by sequencing and used to transform *A.*

521   *tumefaciens* GV3101.

522   For GUS-staining assays, a 2023 bp fragment of the *GT-3a* promoter was inserted

523   into the pBI101 vector to generate *ProGT-3a*:*GUS*. The resulting plasmid was verified

524   by sequencing and used to transform *A. tumefaciens* GV3101.

525   For dual-luciferase reporter assays, the coding sequence of *GT-3a* was amplified

526   and inserted into the pCAMBIA3301 vector to generate pCAMBIA3301-GT-3a. A 623

527   bp fragment upstream from the start codon of *TOZ* and a 595 bp fragment upstream

528   from the start codon of *RAD23C* were amplified and inserted into the pro-LUC-*35S*-

529   Rluc vector to generate *TOZpro*-LUC-*35S*-Rluc and *RAD23Cpro*-LUC-*35S*-Rluc,

530   respectively. The plasmids were verified by sequencing and used to transform *A.*
531   *tumefaciens* strain GV3101.

532   A recombinant GT-3a protein was produced by amplifying the coding sequence
533   of *GT-3a* and inserting it into the pET30a vector (C-terminal His tag) to generate
534   pET30a-GT-3a. The resulting plasmid was verified by sequencing and used to
535   transform *Escherichia coli* strain BL21 (DE3) cells.

536   The primers (synthesized by Tsingke Biotechnology Co., Ltd., Beijing, China) and
537   restriction enzymes (NEB, MA, USA) used for plasmid construction are listed in
538   Supplemental Table 3.

539   **Generation of transgenic *A. thaliana* plants and nematode infection assays**
540   The sequenced pSUPER-Mi2G02-RNAi, Super1300-Mi2G02-HA, Super1300-GT-3a-
541   GFP and Super1300-GFP plasmids were used to transform *A. tumefaciens* strain
542   *GV3101*. Four-week old *A. thaliana* plants were transformed with *A. tumefaciens* by
543   the floral dip method (Clough and Bent, 1998). *A. thaliana* lines were confirmed to be
544   homozygous for the transgene by qPCR and/or western blotting. Nematode infection
545   assays were performed three times.

546   **Subcellular localization assay**
547   Four-week-old *N. benthamiana* leaves were infiltrated with *A. tumefaciens* carrying the
548   appropriate plasmids in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, and 0. 1mM AS)
549   at an OD_{600} of 0.4. Images were captured by laser confocal fluorescence microscopy
550   (Zeiss LSM700, Germany) 2 dpi, at an excitation wavelength of 488 nm for GFP and
551   561 nm for mcherry.

552   **Yeast two-hybrid (Y2H) and yeast one-hybrid (Y1H) assays**
553   A cDNA library was constructed by extracting RNA from *A. thaliana* roots infected with
554   *M. incognita* (1 dpi, 3 dpi, 5 dpi, 10 dpi, 14 dpi) and using it to screen for the target
555   proteins of Mi2G02. The Y2H assay was performed according to the Clontech protocol
556   (Clontech, USA). Interactions between Mi2G02 and candidate proteins were assessed
557   in pairwise Y2H assays. Relative BD and AD vectors were used to transform the yeast
558   strain Y2HGold for screening on SD/-Leu-Trp plates. Positive clones were verified and
559   selected for culture on SD/-Leu-Trp-His medium supplemented with 20 mg/mL 5-
560   bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal; Coolaber, Cat. No.
561   SL0940). We investigated the requirement of the nuclear localization sequences of
562   *Mi2G02* for interaction by inserting a mutated *Mi2G02* in the BD vector for pairwise
563   Y2H assays.

564   The Y1H assay was performed as previous described (Kong et al., 2023). The

565   sequenced pB42AD and pLacZi vectors were integrated into the yeast strain EGY48

566   grown on the SD/-Trp-Ura medium (Coolaber, Cat. No. PM2262). Positive

567   transformants were verified and selected for growth on medium containing raffinose

568   (Coolaber, Cat. No. SL0990), and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

569   (X-gal, Coolaber, Cat. No. CX11921) for color reactions.

570   **Bimolecular fluorescence complementation (BiFC) assay**

571   BiFC assays were performed as previously described (Zhao et al., 2019).

572   *Agrobacterium* harboring appropriate pairs of vectors was infiltrated into *N.*

573   *benthamiana* leaves for 48 h. YFP fluorescence, indicating protein interaction, was

574   captured with a confocal microscope (Zeiss LSM700, Germany) with excitation at 514

575   nm and emission at 527 nm.

576   **Luciferase complementation assay**

577   *Agrobacterium* cultures were resuspended in infiltration buffer, incubated at room

578   temperature for 3 h, and then infiltrated into four-week old *N. benthamiana* leaves.

579   Three days after agro-infiltration, 1 mM luciferin (Biovision, Cat. No. 7903) solution

580   was sprayed onto the infiltrated leaves, and luciferase activity was detected with a low-

581   light cooled CCD imaging apparatus ChemiScope 6000 (Clinx Science Instruments

582   Co., Ltd, Shanghai, China).

583   ***In vivo* co-immunoprecipitation (Co-IP) assay**

584   Total protein was extracted from four-week-old *N. benthamiana* leaves co-expressing

585   Mi2G02 and GT-3a, after 48 h of infiltration. Co-IP was performed with BeyoMag™

586   Anti-HA Magnetic Beads (Beyotime, Cat. No. P2121) as previously described (Zhao

587   et al., 2021). The eluted proteins were checked by western blotting with anti-GFP

588   (ABclonal, Cat. No. AE012) and anti-HA (Coolaber, Cat. No. AB1105) antibodies,

589   respectively.

590   **Histochemical analysis of GUS activities**

591   Transgenic *A. thaliana* was generated as described above. The homozygosity of the

592   transgenic lines was confirmed by PCR and they were inoculated with *M. incognita*.

593   Root samples were collected at 3 dpi, 5 dpi, 7 dpi, and 14 dpi. More than 10 roots were

594   collected at each sampling time. Histochemical staining for GUS enzyme activity was

595   performed with a GUS staining kit (Coolaber, Cat. No. SL7160), in accordance with

596   the manufacturer's instructions. Images were captured with a stereomicroscope (Zeiss,

597   AxioImager Z2, Germany).

Dual-luciferase reporter assay

Agrobacterium harboring pCAMBIA3301-GT-3a was infiltrated with *TOZpro*-LUC-*35S*-Rluc or *RAD23Cpro*-LUC-*35S*-Rluc, with or without Mi2G02, into four-week-old *N. benthamiana* leaves. Three days after infiltration, leaf discs with a diameter of 2 cm were harvested and ground in liquid nitrogen. Firefly and Renilla luciferase activities were measured with a Dual-Luciferase Reporter Assay System (Vazyme, Cat. No. DL101-01) according to the manufacturer's instructions, with Synergy SLXFA (BioTek, USA).

Electrophoretic mobility shift assay (EMSA)

The recombinant GT-3a-His protein was induced with 1 mM isopropyl-β-d-thiogalactoside (IPTG) and purified with the His-tag Protein Purification Kit (Beyotime, Cat. No. P2229S) according to the manufacturer's instructions (Zhao et al., 2021). The biotin-labeled and unlabeled probes for *TOZ* and *RAD23C,* containing the -GTTAC-element or the -CACGTG- element, were synthesized and purified by Sangon Biotech (Shanghai, China). EMSA was carried out with an EMSA chemiluminescence kit (Beyotime, Cat. No. GS009). Competition experiments were performed with various amounts of unlabeled oligonucleotides. The mutated competitor was generated by replacing five base pairs or six base pairs in the *TOZ* and *RAD23C* binding elements (GTTAC to AAAAA or CACGTG to AAAAAA). EMSA assays were repeated three times.

Transient expression assays in *N. benthamiana* leaves

Four-week-old *N. benthamiana* leaves were infiltrated with *A. tumefaciens* carrying the appropriate plasmids in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, and 0. 1mM AS) at an OD_{600} of 0.4. MG132 (100 μM) was added 24 h before protein extraction.

Cell fractionation, protein extraction and western blotting

Cell fractionation assay was followed as described previously (Wang et al., 2018) with some modifications. Briefly, *N. benthamiana* leaves expressing proper proteins (0.5 g) were harvested and ground in liquid nitrogen and mixed with 2 mL/g of lysis buffer (20 mM Tris-HCl, pH 7.5, 20 mM KCl, 2 mM EDTA, 2.5 mM $MgCl_2$, 25% glycerol, 250 mM sucrose, and 5mM DTT) supplemented with protease inhibitor cocktail (Beyotime, Cat. No. P1045). The homogenate was filtered through a double layer of Miracloth (Millipore, Cat. No. 475855). The flow-through was centrifuged at 1500g for 10 min at 4°C, and the supernatant, consisting of the cytoplasmic fraction, was centrifuged at 10,000g for 10 min at 4°C, and was collected as the cytoplasmic fraction. The pellet

632 from the first centrifugation was washed four times with 4 mL NRBT buffer (20 mM

633 Tris-HCl, pH7.5, 2.5 mM $MgCl_2$, 25% glycerol, and 0.2% TritonX-100) and then

634 resuspended with 500 µL of NRB2 (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.25 M

635 sucrose, 0.5% Triton X-100, and 5 mM β-mercaptoethanol) supplemented with

636 protease inhibitor cocktail and carefully overlaid on top of 500 µL of NRB3 (20 mM

637 Tris-HCl, 10 mM MgCl2, pH 7.5, 1.7 M sucrose, 0.5% Triton X-100, and 5 mM β-

638 mercaptoethanol) supplemented with protease inhibitor cocktail. The suspension was

639 centrifuged at 16,000g for 45 min at 4°C, and the pellet was collected as the nuclear

640 fraction. The 2× SDS loading buffer was then added to the cytoplasmic and nuclear

641 fractions and boiled for 5 min. Actin was detected with an anti-actin antibody (ABclonal,

642 Cat. No. AC009) as a cytoplasmic marker. H3 proteins were detected using an anti-

643 H3 antibody (ABclonal, Cat. No. A2348) as a nuclear marker. Total proteins were

644 extracted from *N. benthamiana* leaves or *A. thaliana* seedlings (ten days after

645 germination) in RIPA lysis buffer (Beyotime, Cat. No. P0031K) supplemented with

646 protease inhibitor cocktail. For tag antibodies, we used anti-His (TransGen, Cat. No.

647 HT501), anti-GFP (ABclonal, Cat. No. AE012), and anti-HA (Coolaber, Cat. No.

648 AB1105) antibodies, and a goat anti-mouse IgG (H+L)-HRP-conjugated secondary

649 antibody (Coolaber, Cat. No. AB2102). The protein was detected with the EasySee

650 Western Blot Kit (TransGen, Cat. No. DW101). Band intensity was determined by

651 ImageJ software.

652 **Statistical methods**

653 The significance of differences between two groups was assessed in two-tailed *t*-tests.

654 For multiple comparisons, the significance of differences was assessed by one-way

655 ANOVA followed by Tukey tests for multiple comparisons. All statistical analyses were

656 performed with GraphPad Prism software version 8.3.0.

657 **Accession numbers**

658 The accession numbers of major genes mentioned in this study are as follows: *Mi2G02*

659 (*AAQ10016*), *GAPDH* (*Minc12412*), *GT-3a* (*AT5G01380*), *LBD41* (*AT3G02550*),

660 *UBQ1* (*AT3G52590*), *TOZ* (*AT5G16750*), *RAD23C* (*AT3G02540*), *WRKY2*

661 (*AT5G56270*), *UBP22* (*AT5G10790*), *SRP34* (*AT1G02840*), *DVL4* (*AT1G13245*),

662 *SKP2* (*AT1G21410*), *FEI1* (*AT1G31420*), *FEI2* (*AT2G35620*), *ABS4* (*AT1G58340*).

663 **DATA AVAILABILITY**

664 All relevant data is included in the main manuscript and the Supplemental

665 Information.

AUTHOR CONTRIBUTIONS

667 J.Z., B.F., P.A., M.Q., H.J. and Z.M. conceived the project. J.Z., K.H., B.F., Y.L., J.L.

668 and Y.Y. designed and planned the experiments. J.Z., K.H., R.L., and Y.L. performed

669 the experiments and collected and analyzed the data. J.Z., B.F., P.A. and M.Q. wrote

670 the manuscript.

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COMPETING INTERESTS

686 The authors declare no competing interests.

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977

978 **FIGURE LEGENDS**

979 **Figure 1. Structure and nuclear localization of *M. incognita* effector Mi2G02.** (A)

980 Schematic diagram of the Mi2G02 and mutant Mi2G02 proteins. (B) Subcellular

981 localization of Mi2G02 and mutant Mi2G02 in plant cells. Coding sequences were

982 constructed into *ProSuper*:GFP (C-terminus GFP) vector. Mi2G02 and nuclear

983 localization signal mutants fused with GFP (Mi2G02-GFP, Mi2G02-mu1-GFP,

984 Mi2G02-mu2-GFP and Mi2G02-mu3-GFP) were co-expressed with mcherry in

985 *Nicotiana benthamiana* leaf cells. Empty vectors were used as controls. The

986 fluorescence signal was detected at 48 hours after infiltration. Mi2G02-GFP localized

987 to the nucleus. Mi2G02-mu1-GFP, Mi2G02-mu2-GFP and Mi2G02-mu3-GFP

988 primarily localized to the plasma membrane and cytoplasm. Images were captured

989 by confocal microscopy (Zeiss LSM 700, Germany). GFP, green fluorescent protein.

990 Scale bars, 20 μm. (C) The relative abundance of Mi2G02-GFP or Mi2G02-mu-GFP

991 in cytoplasmic and nuclear fractions was detected using anti-GFP antibodies after

992 transiently expressing in *N. benthamiana* leaf cells. Actin was used as an internal

993 reference for the presence of cytoplasmic proteins and Histone H3 was used as an

994 internal reference for the presence of nuclear proteins.

995 **Figure 2. Host-derived RNA interference (RNAi) and ectopic expression of**

996 ***Mi2G02* in *A. thaliana* alter plants susceptibility to *M. incognita* and root**

997 **development.** (A) *Mi2G02* expression level in three homozygous RNAi lines

998 (Mi2G02-Ri-1, Mi2G02-Ri-2 and Mi2G02-Ri-4), gfp-RNAi line (GFP-Ri) and wild type

999 (WT) were determined at 10 days post infection (dpi) of *M. incognita* by RT-qPCR.

1000 *GAPDH* was used as an internal control. The values shown are means ± SE (n = 3).

1001 Different letters indicate significant differences ($P<0.05$, one-way ANOVA). (B) Gall

1002 numbers and egg mass numbers per plant at 35 dpi. Values are presented as means

1003 ± SD (n=18). Different letters indicate significant differences ($P<0.05$, one-way

1004 ANOVA). See also Figure S1B. (C) Giant cell areas of *M. incognita*-induced galls in

1005 the *A. thaliana* Mi2G02-Ri lines were significantly reduced. Gall sections at 14 days

1006 post infection (dpi) were stained with toluidine-blue. Relatively smaller giant cells

1007 were observed in Mi2G02-Ri mature galls at 14 dpi compared with the WT and GFP-

1008 Ri controls. Data are the average surface areas ± SD (n=10) for each line. Different

1009 letters indicate significant differences ($P<0.05$, one-way ANOVA). Asterisk, giant cell;

1010 N, nematode. Bars, 100 μm. (D) *A. thaliana* phenotypes and relative root length of

31

1011 *Mi2G02* ectopic expressing *A. thaliana* lines compared with wild-type. Data

1012 represents the average length ± SD (n=10). Different letters indicate significant

1013 differences (*P*<0.05, one-way ANOVA). Scale bar, 1 cm. See also Figure S2C. (E)

1014 Expression of *Mi2G02* in *A. thaliana* increased susceptibility to *M. incognita*. Two

1015 independent Mi2G02-T3 lines were inoculated with *M. incognita* pre-J2s. Total

1016 numbers of galls and egg masses were counted at 35 dpi. *M. incognita* inoculated

1017 wild-type *A. thaliana* plant was used as control. Data are the average numbers per

1018 plant ± SD (n=16). Different letters indicate significant differences (*P*<0.05, one-way

1019 ANOVA). See also Figure S2D.

1020 **Figure 3. The Mi2G02 effector interacts with GT-3a in the nuclei.** (A) Schematic

1021 representation of the intact and truncated Mi2G02 (with or without ShKT domain)

1022 and GT-3a (with or without DNA binding domain) used for yeast two-hybrid assays

1023 (Y2Hs). (B) Pairwise Y2H tests were performed to investigate the interactions

1024 between Mi2G02 or ShKT domains and GT-3a or DNA binding (DB) domain. Left

1025 column, yeast cell growth carrying the baits in pGBKT7 vector (BD) and preys in

1026 pGADT7 (AD) grown on SD/-Trp-Leu (SD-WL) medium indicating successful

1027 transformation of the yeast with both plasmids; right column, yeast cell growth on the

1028 selective dropout medium (SD/-Trp-Leu-His, SD-WLH) following the addition of 20

1029 mg/ml X-α-gal indicating protein interaction. Yeast cells containing p53 and SV40

1030 large T-antigen were used as positive control, and yeast cells containing Lamin and

1031 SV40 large T-antigen were used as negative control. (C) Pairwise Y2H tests were

1032 performed to investigate the interactions between Mi2G02 mutants and GT-3a. (D)

1033 Mi2G02 colocalizes with GT-3a in *N. benthamiana* cell nuclei. Mi2G02 fused with

1034 GFP in C-terminus (Mi2G02-GFP) and GT-3a fused with mcherry in N-terminus

1035 (mcherry-GT-3a) were co-expressed in *N. benthamiana* leaf cells. The fluorescence

1036 signal was detected at 48 h after infiltration. Images were captured by confocal

1037 microscopy. Scale bars, 20 µm. (E) Bimolecular fluorescence complementation

1038 (BiFC) experiments demonstrate the interaction between Mi2G02 and GT-3a. *N.*

1039 *benthamiana* leaves were transformed with different combinations of nEYFP and

1040 cEYFP fused vectors. Images were obtained 48 h after co-expression. Yellow

1041 fluorescent protein (YFP) fluorescence signals were observed in the nuclei in leaves

1042 co-infiltrated with nEYFP-Mi2G02 and GT-3a-cEYFP. Scale bars, 20 µm. See also

1043 Figure S3C. (F) Determination of the interaction between Mi2G02 and GT-3a

1044 through luciferase complementation assay (LCA). *A. tumefaciens* harboring different

1045 combinations of plasmids were infiltrated into indicated regions of *N. benthamiana*

1046 leaves. The luciferase activities were recorded at 2 days post agro-infiltration by

1047 spraying 1 mM luciferin solution onto the infiltrated leaves, and the luciferase activity

1048 was detected with a low-light cooled CCD imaging apparatus. Luciferase activity was

1049 depicted with false color from low (black) to high (white). The protein levels of

1050 Mi2G02-NLuc, Mi2G02-mu1-NLuc, Mi2G02-mu2-NLuc, Mi2G02-mu3-NLuc, CLuc-

1051 GT-3a, GUS-NLuc, CLuc-GUS were determined by western blotting using anti-

1052 Luciferase antibody. Ponceau S (P) staining provided a loading control. (G) Mi2G02

1053 associates with GT-3a in a co-immunoprecipitation (Co-IP) assay. *A. tumefaciens*

1054 harboring different combinations of plasmids were infiltrated into *N. benthamiana*

1055 leaves. Co-IP was performed with BeyoMag™ Anti HA Magnetic Beads, and the

1056 eluted protein was detected using western blotting with antibody against HA and

1057 GFP. GFP, green fluorescent protein. P, ponceau staining indicates samples

1058 loading.

1059 **Figure 4. GT-3a is involved in *M. incognita* parasitism and lateral roots**

1060 **development.** (A) The activity of *GT-3a* promoter was analysed in uninfected roots

1061 and in galls induced by *M. incognita* in *A. thaliana* expressing the *ProGT-3a:GUS*

1062 construct. Scale bars, 100 μm. dpi, days-post infection. (B) *A. thaliana* phenotypes,

1063 relative root length and relative lateral root numbers of *Mi2G02* ectopic expressing *A.*

1064 *thaliana* lines compared with wild-type. Data represents the average length ± SD

1065 (n=10) and the average number ± SD (n=10). Different letters indicate significant

1066 differences (*P*<0.05, one-way ANOVA). Scale bar, 1 cm. See also Figure S4H. (C)

1067 Lateral root density calculated as the number of emerged lateral roots divided by

1068 total primary root length. Different letters indicate significant differences (*P*<0.05,

1069 one-way ANOVA). (D) Overexpression of *GT-3a* in *A. thaliana* increased

1070 susceptibility to *M. incognita*. Two independent *GT-3a* ectopic expressing T3 lines

1071 were inoculated with *M. incognita* pre-J2s. Total numbers of galls and egg masses

1072 were counted at 35 dpi. *M. incognita* inoculated wild-type *A. thaliana* plant was used

1073 as control. Data are the average number ± SD (n=18). Different letters indicate

1074 significant differences (*P*<0.05, one-way ANOVA). See also Figure S5A. (E) The *gt-*

1075 *3a* T-DNA knockout mutants (SALK_134703 and SALK_040448) were less

1076 susceptible to *M. incognita* compared with the wild-type, as indicated by the mean

1077   numbers of galls and egg masses. Data are the average number ± SD (n=26).

1078   Different letters indicate significant differences ($P<0.05$, one-way ANOVA). See also

1079   Figure S5B. (F) Giant cell areas of *M. incognita*-induced galls in the *A. thaliana gt-3a*

1080   T-DNA knockout mutant lines were significantly reduced. Gall sections at 21 dpi

1081   were stained with toluidine-blue. Relatively smaller giant cells were observed in *gt-3a*

1082   T-DNA knockout mutant lines compared with the wild-type. Data are the average

1083   surface area ± SD (n=10). Different letters indicate significant differences ($P<0.05$,

1084   one-way ANOVA). Asterisk, giant cell; N, nematode. Bars, 100 μm.

1085   **Figure 5. Targeting and suppression of *TOZ* and *RAD23C* by GT-3a and the**

1086   **susceptibility of *toz* and *rad23c* knockout mutant lines to *M. incognita*.** (A)

1087   Transcriptional activity of GT-3a in yeast cells. The yeast AH109 strain expressing

1088   pCL-1, GT-3a, GT-3a with or without DNA binding domain (GT-3a-DB or GT-3a-

1089   ΔDB) grew on Yeast Peptone Dextrose Adenine Agar (YPDA) or the selective

1090   medium SD-His-Trp with or without X-α-gal. The pCL-1 encoding the full-length

1091   GAL4 and the empty vector pGBKT7 (BD) were used as positive and negative

1092   controls, respectively. (B) Yeast one-hybrid (Y1H) experiments showed GT-3a bound

1093   to the promoter of *TOZ*, *RAD23C* and *WRKY2*. Promoter fragments containing -

1094   GTTAC- or -CACGTG- element were cloned into pLacZi vector, GT-3a was cloned

1095   into pB42AD vector, and then pLacZi vector co-transformed with pB42AD-GT-3a into

1096   yeast strain EGY48. The yeast transformants were spotted on the plate SD/-Ura-Trp

1097   with or without 20 mg/ml X-gal. pB42AD-p53 and pLacZi-p53 were used as a

1098   positive control. (C) Luciferase reporter assays of GT-3a-induced suppression of

1099   *TOZ* and *RAD23C* expression in *N. benthamiana*. LUC activity was measured by

1100   normalizing to REN signal. Values are means ± SE (n = 4). Asterisks mark significant

1101   differences according to two-tailed Student's *t* test, ***$P<0.001$. Similar results were

1102   obtained from three independent experiments (biological replicates). (D) qRT-PCR

1103   analysis of *TOZ* and *RAD23C* expression in wild type *A. thaliana* and *GT-3a*

1104   overexpressing *A. thaliana* lines. *UBP22* (*AT5G10790*) was used as an internal

1105   control. Data represent the mean of three independent experiments ± SE (n=3).

1106   Similar results were obtained from three independent experiments (biological

1107   replicates). Different letters indicate significant differences ($P<0.05$, one-way

1108   ANOVA). (E) EMSA assays confirmed GT-3a-His could direct bind to the promoter of

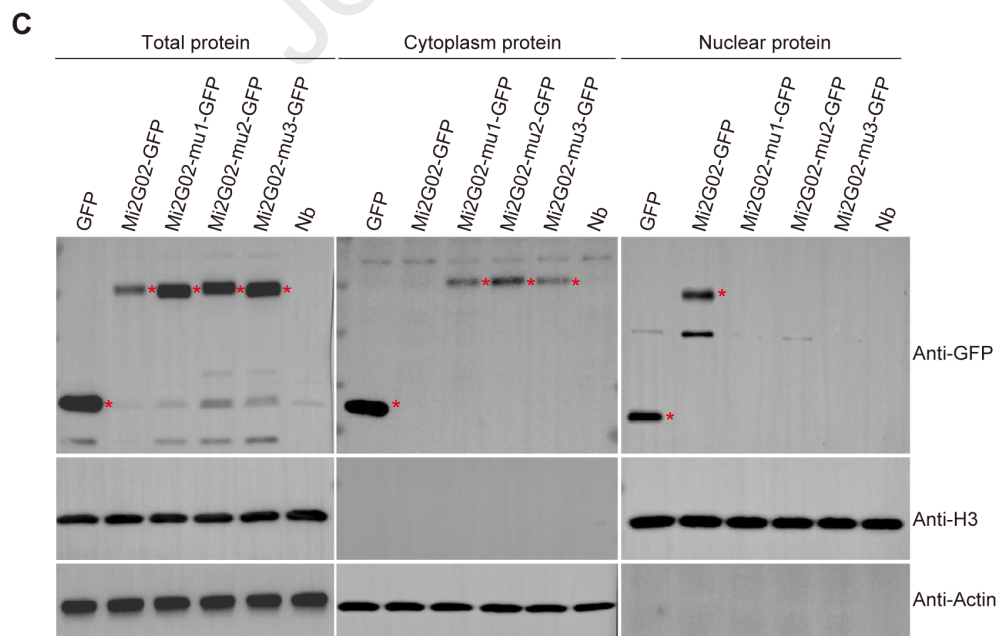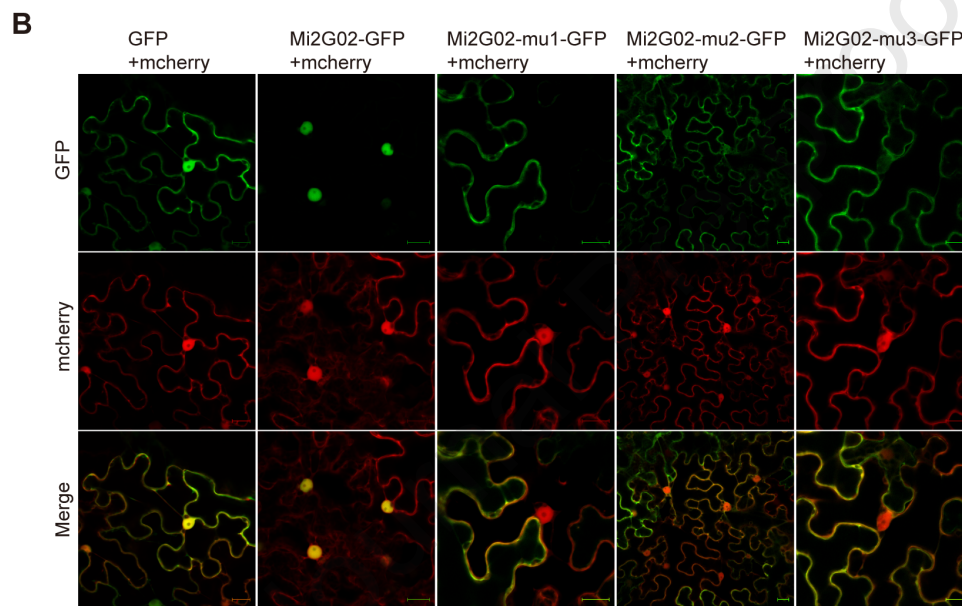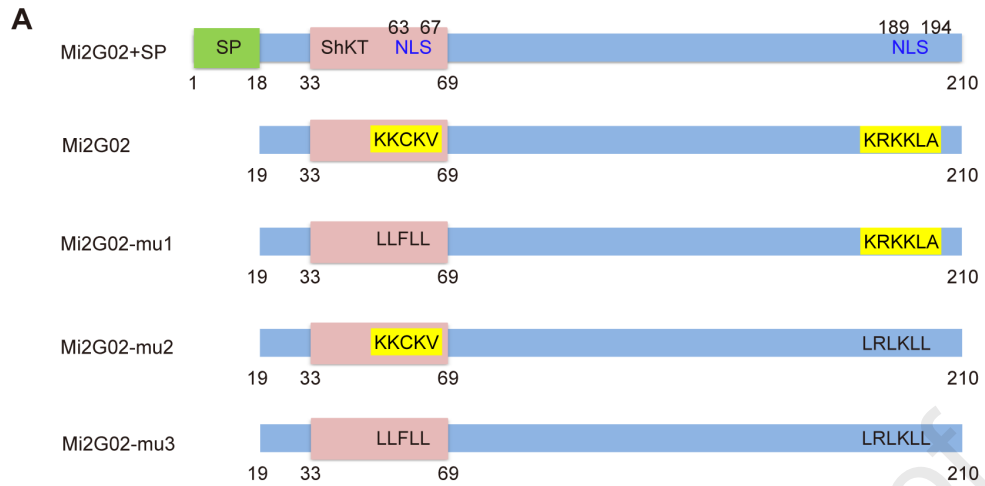1109   *TOZ* and *RAD23C*. Promoter fragments containing -GTTAC- element (P1 probe) or -

1110 CACGTG- element (P2 probe) or mutant elements (-AAAAA- or -AAAAAA-) were

1111 labeled with biotin as probes. 6×His alone served as a negative control. Unlabeled

1112 probes were used as competing probes.

1113 **Figure 6. Mi2G02 stabilizes GT-3a to promote its function in suppression of**

1114 ***TOZ* and *RAD23C* expression for nematode parasitism.** (A) The *rad23c* T-DNA

1115 knockout mutant is more susceptible than the wild type to *M. incognita*. The *rad23c*

1116 KO mutant (*SALK_068091*) was inoculated with nematodes, and the numbers of

1117 galls and egg masses were counted 35 days post-inoculation. The data presented

1118 are the mean numbers per plant ± SD (*n*=28). Similar results were obtained in three

1119 independent experiments. Asterisks indicate differences that were significant in two-

1120 tailed Student's *t* tests, **$P<0.01$. See also Figure S7E. (B) Luciferase reporter

1121 assays showed that the GT-3a-induced suppression of *TOZ* and *RAD23C*

1122 expression in *N. benthamiana* was enhanced by Mi2G02 expression. LUC activity

1123 was determined and normalized against the REN signal. The data presented are the

1124 means of three independent experiments ± SEM (*n*=4). Different letters indicate

1125 significant differences ($P<0.05$, one-way ANOVA). (C) Mi2G02 stabilizes the GT-3a-

1126 GFP fluorescence intensity. *GT-3a* was co-expressed with *Mi2G02* in *N.*

1127 *benthamiana* leaves, and Mi2G02 mutants and MiEFF18 (a nuclear *M. incognita*

1128 effector not interacted with GT-3a) were used as controls. The GT-3a-GFP

1129 fluorescence was detected with confocal microscopy (LSM700, Zeiss) 48 h after

1130 infiltration. Graphs showed the fluorescence intensity profiles across the arrows in

1131 the GFP images. Bar = 10 μm. See also Figure S8. (D) Mi2G02 stabilizes the GT-3a

1132 protein, leading to its accumulation. *GT-3a* was co-expressed with *Mi2G02* or *GFP* in

1133 *N. benthamiana* leaves. The GT-3a protein was detected with an anti-GFP antibody.

1134 Band intensity was determined with ImageJ software and is indicated below the

1135 bands. CBB, Coomassie brilliant blue staining, and P, Ponceau staining, were used

1136 to check protein sample loading. (E) Mi2G02 inhibits of the GT-3a degradation via

1137 26S proteasome pathway *in vivo*. GT-3a-GFP was co-expressed with Mi2G02-HA

1138 and Mi2G02 mutants in *N. benthamiana* leaves, respectively. The 26S proteasome

1139 inhibitor MG132 (100 μM) was infiltrated into *N. benthamiana* leaves 24 h before

1140 protein extraction. Band intensity was determined by ImageJ software and is
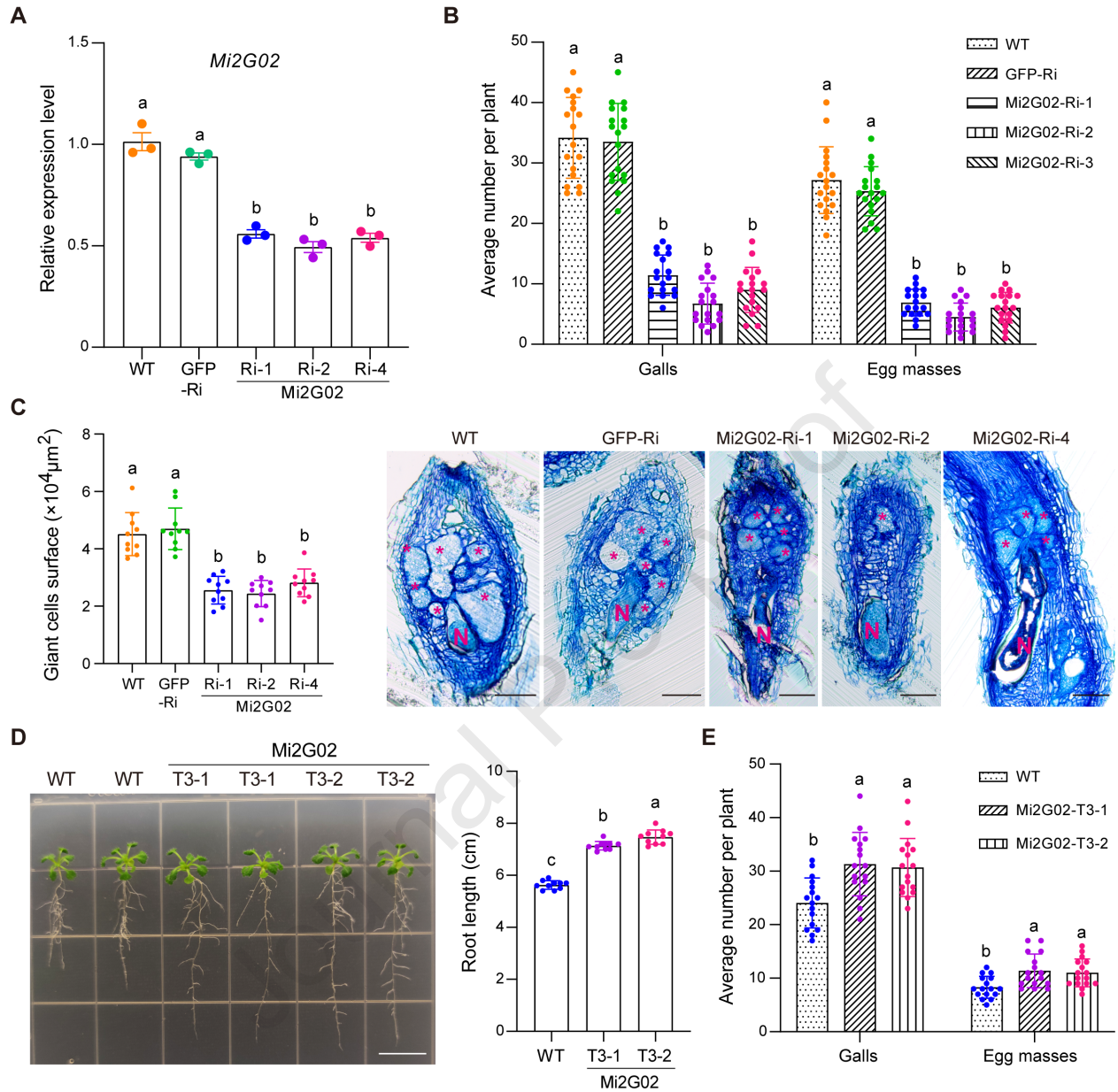
1141 indicated below the bands.

1142 **Figure 7 A proposed working model illustrating the molecular mechanism of**

1143 **the interaction among Mi2G02, GT-3a and *TOZ, RAD23C* in the nematode**

1144 **parasitism.** In the early stage of *M. incognita* parasitism, Mi2G02 effector protein is

1145 secreted into the plant cell and translocates to the plant nucleus, where targets the

1146 transcription factor GT-3a and stabilizes its proteins level by inhibiting 26S

1147 proteasome pathway, leading to the suppression of *TOZ* and *RAD23C* expression

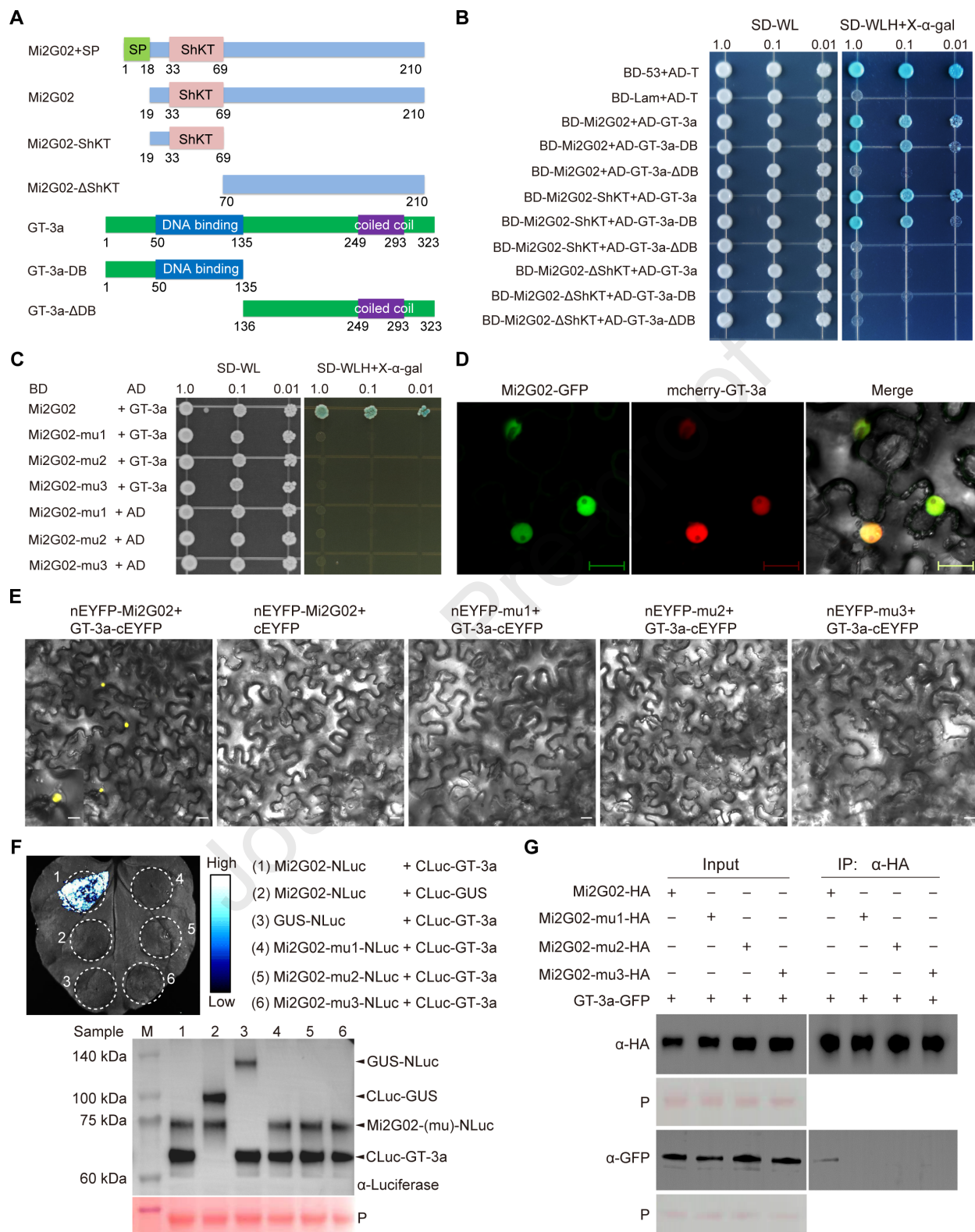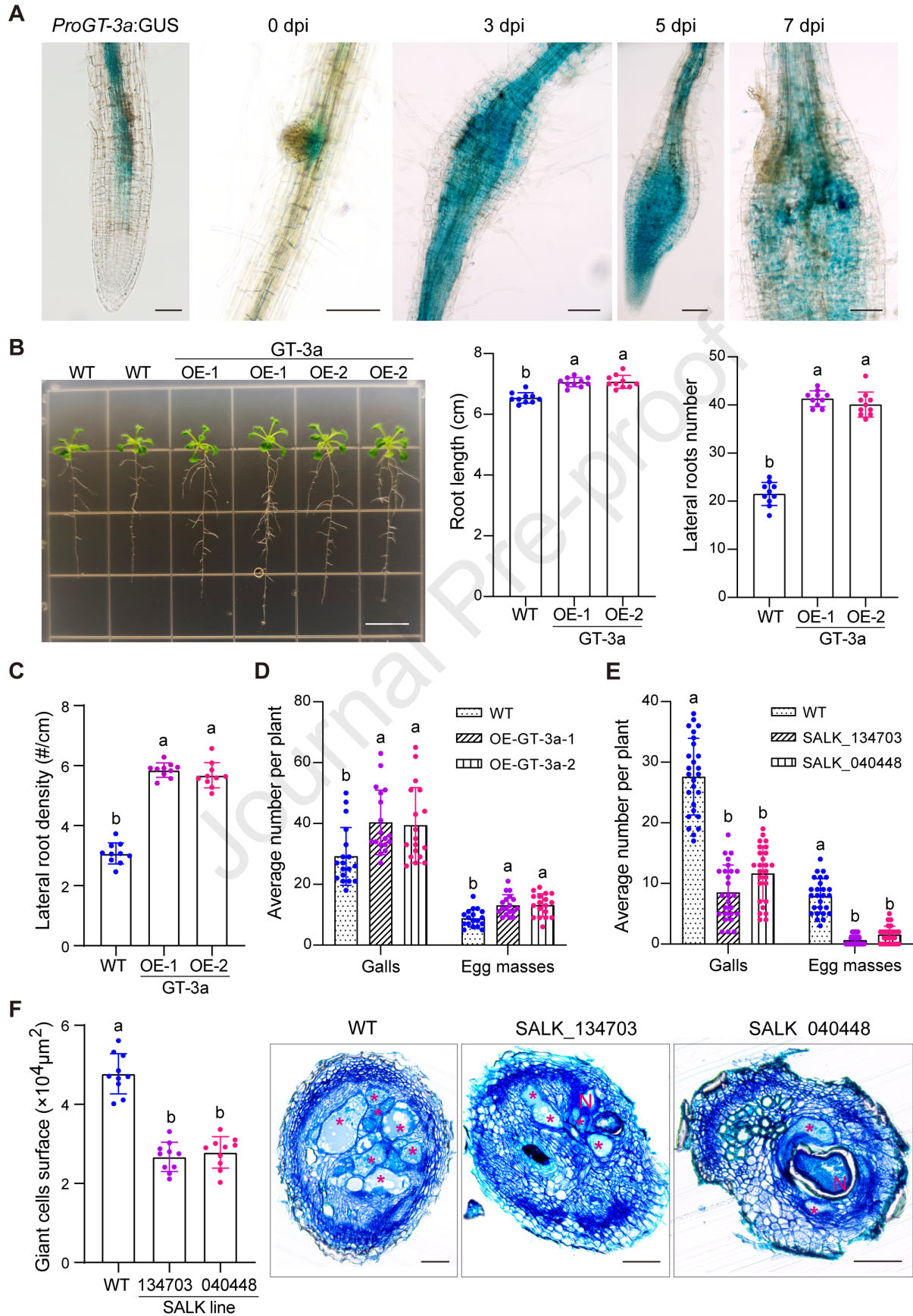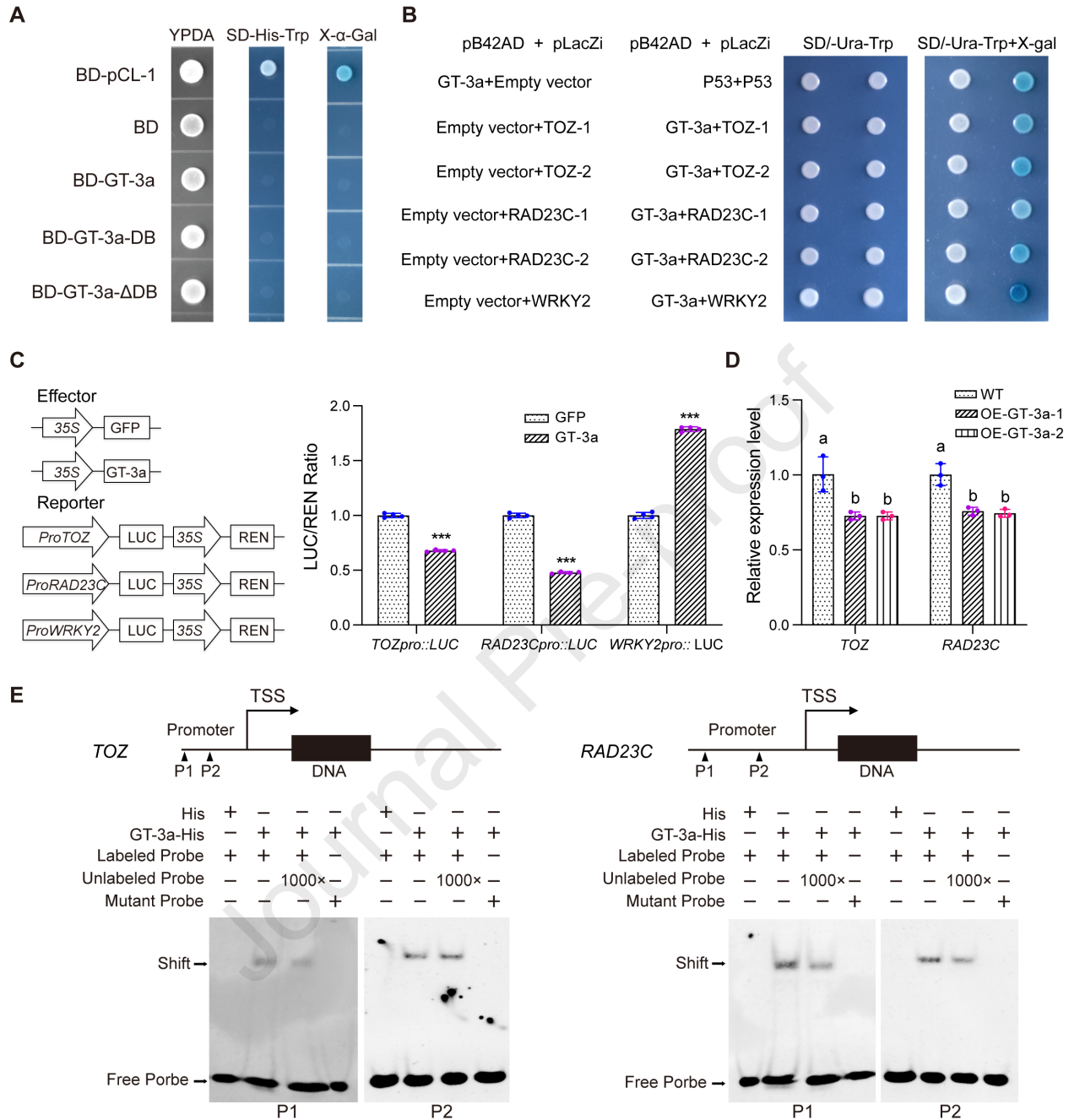1148 for nematode feeding cells formation and development.

A

Mi2G02+SP
SP | ShKT | NLS | NLS
63 67 | 189 194
1 18 33 69 210

Mi2G02
KKCKV | KRKKLA
19 33 69 210

Mi2G02-mu1
LLFLL | KRKKLA
19 33 69 210

Mi2G02-mu2
KKCKV | LRLKLL
19 33 69 210

Mi2G02-mu3
LLFLL | LRLKLL
19 33 69 210

B

	GFP +mcherry	Mi2G02-GFP +mcherry	Mi2G02-mu1-GFP +mcherry	Mi2G02-mu2-GFP +mcherry	Mi2G02-mu3-GFP +mcherry
GFP					
mcherry					
Merge					

C

Total protein	Cytoplasm protein	Nuclear protein

GFP | Mi2G02-GFP | Mi2G02-mu1-GFP | Mi2G02-mu2-GFP | Mi2G02-mu3-GFP | Nb

Anti-GFP

Anti-H3

Anti-Actin

A ProGT-3a:GUS   0 dpi   3 dpi   5 dpi   7 dpi

B GT-3a
WT   WT   OE-1   OE-1   OE-2   OE-2

C

D

E

F

A

B

C

D

E