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Copper microRNAs modulate the formation of giant feeding cells induced by the root knot nematode *Meloidogyne incognita* in *Arabidopsis thaliana*

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1

2 **Copper microRNAs modulate the formation of giant feeding cells induced by the root**
3 **knot nematode *Meloidogyne incognita* in *Arabidopsis thaliana***

4

5

6 Yara Noureddine¹, Joffrey Mejias¹, Martine da Rocha¹, Sébastien Thomine², Michaël
7 Quentin¹, Pierre Abad¹, Bruno Favery¹, and Stéphanie Jaubert-Possamai^{1*}

8 1 INRAE, Université Côte d'Azur, CNRS, ISA, Sophia Antipolis, F-06903, France

9 2 Institute for Integrative Biology of the Cell (I2BC), UMR9198 CNRS/CEA/Univ. Paris Sud,
10 Université Paris-Saclay, Gif-sur-Yvette, France.

11 Corresponding author: stephanie.jaubert@inrae.fr

12

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22

23 **Heading**

24 *Arabidopsis* copper microRNAs modulate the development of feeding site induced by root
25 knot nematodes.

26 **Twitter :**

27 @stephejaubert

28 @noureddine_yara

29 @brunofavery

30 **ORCID :**

31 Stéphanie Jaubert-Possamai : <https://orcid.org/0000-0002-3518-727X>

32 Yara Noureddine : <https://orcid.org/0000-0002-6875-5835>

33 Joffrey Mejias : <https://orcid.org/0000-0001-7663-0314>

34 Martine da Rocha : <https://orcid.org/0000-0003-0959-2295>

35 Sébastien Thomine : <https://orcid.org/0000-0003-0045-1701>

36 Michaël Quentin : <https://orcid.org/0000-0002-8030-1203>

37 Pierre Abad : <https://orcid.org/0000-0003-0062-3876>

38 Bruno Favery : <https://orcid.org/0000-0003-3323-1852>

39

40 **Summary** (161 words)

- 41
- 42 • Root-knot nematodes (RKN) are root endoparasites that induce the dedifferentiation of a
43 few root cells and the reprogramming of their gene expression to generate giant
44 hypermetabolic feeding cells.
 - 45 • We identified two microRNA families, miR408 and miR398, as upregulated in
46 *Arabidopsis thaliana* and *Solanum lycopersicum* roots infected by root-knot nematodes.
47 In plants, the expression of these two conserved microRNA families is known to be
48 activated by the SPL7 transcription factor in response to copper starvation.
 - 49 • By combining functional approaches, we deciphered the network involving these
50 microRNAs, their regulator and their targets. *MIR408* expression was located within
51 nematode-induced feeding cells like its regulator *SPL7* and was regulated by copper.
52 Moreover, infection assays with *mir408* and *spl7* KO mutants or lines expressing targets
53 rendered resistant to cleavage by miR398 demonstrated the essential role of the
54 *SPL7/MIR408/MIR398* module in the formation of giant feeding cells.
 - 55 • Our findings reveal how perturbation of plant copper homeostasis, *via* the
56 *SPL7/MIR408/MIR398* module, modulates the development of nematode-induced
57 feeding cells.

57

58

59 Key words: root-knot nematodes, microRNAs, copper, signaling, *Arabidopsis*

60 **Introduction**

61 MicroRNAs are small non-coding RNAs that regulate the expression of protein-coding genes,
62 mostly at the post-transcriptional level, in plants. They are major post-transcriptional regulators
63 of gene expression in various biological processes, including plant development (Li and Zhang,
64 2016), responses to abiotic stresses (Barciszewska-Pacak *et al.*, 2015), hormone signalling
65 (Curaba *et al.*, 2014), and responses to pathogens or symbiotic micro-organisms (Hoang *et al.*,
66 2020; Weiberg and Jin, 2015). MicroRNAs and short interfering RNAs (siRNAs) were recently
67 shown to play a key role in plant-pathogen crosstalk through trans-kingdom RNAi processes
68 (Weiberg *et al.*, 2013; Cai *et al.*, 2018; Dunker *et al.*, 2020). MicroRNAs are produced by the
69 cleavage of long double-stranded RNA precursors by the DICER RNase, generating 20-22
70 nucleotides miRNA duplexes composed of a mature (5P) and a complementary (3P) strand.
71 One of the two strands is then incorporated into the RNA-induced silencing complex (RISC) to
72 guide the major RISC protein, ARGONAUTE1 (AGO1), to the targeted messenger RNA
73 (mRNA) on the basis of sequence complementarity. The hybridization of AGO1-bound
74 miRNAs to their targets induces predominantly targeted mRNA degradation in plants, but it
75 may also lead to an inhibition of mRNA translation (Axtell, 2013).

76 The miR408 and miR398 microRNA families are conserved so-called “copper microRNAs”,
77 due to their function in the plant response to copper deficiency (Zhang *et al.*, 2015; Yamasaki
78 *et al.*, 2007). Copper is an essential nutrient for plants, due to its function as a cofactor for many
79 proteins. Copper proteins are involved in electron transport chains or function as enzymes in
80 redox reactions. In plants, copper is involved in respiration, photosynthesis, ethylene
81 perception, the metabolism of reactive oxygen species and cell wall remodelling (reviewed in
82 Burkhead *et al.*, 2009). Copper microRNAs accumulate in response to copper deficiency and
83 their synthesis is repressed when copper concentrations are sufficiently high (Yamasaki *et al.*,
84 2009). The underlying mechanism has been described in *Arabidopsis thaliana*, in which the
85 regulation of *MIR408*, *MIR398B* and *MIR398C* by copper levels was shown to be mediated by
86 the SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE7 (SPL7) transcription factor
87 (Yamasaki *et al.*, 2009). The *A. thaliana* genome contains a single copy of *MIR408*, and three
88 *MIR398* genes: *MIR398A*, *-B* and *-C*. At high copper concentrations, the DNA-binding activity
89 of the SPL7 transcription factor is repressed, preventing the induction of transcription for
90 downstream genes, such as *MIR408* or *MIR398B* and *MIR398C*, but not *MIR398A* (Sommer *et al.*
91 *et al.*, 2011; Yamasaki *et al.*, 2009). In the presence of low concentrations of copper, SPL7
92 activates the expression of copper-responsive microRNAs that target and repress the expression

93 of genes encoding copper-binding proteins. These proteins are replaced by proteins that do not
94 bind copper, to save copper resources for the functions for which this element is essential, such
95 as photosynthesis (reviewed in Burkhead *et al.*, 2009). For example, the mRNA for the cytosolic
96 COPPER/ZINC (Cu-Zn) SUPEROXIDE DISMUTASE, CSD1, which can be replaced by an
97 iron (Fe)-dependent SOD, is targeted by the copper microRNA miR398. In addition to their
98 regulation as a function of copper levels through the activity of SPL7, *MIR408* and the *MIR398*
99 family are also regulated by several environmental cues and abiotic stresses, such as light,
100 which regulates *MIR408* activity through the ELONGATED HYPOCOTYL5 (HY5) or
101 PHYTOCHROME INTERACTING FACTOR1 (PIF1) transcription factors in *A. thaliana*
102 (Jiang *et al.*, 2021), and salinity, oxidative and cold stresses, which have been shown to induce
103 *MIR408* in *A. thaliana* (Ma *et al.*, 2015), or cadmium treatment in *Brassica napus* (Fu *et al.*,
104 2019). The miR408 and miR398 families have been widely analysed in plant responses to
105 abiotic stresses, but little is known of their role in plant responses to biotic stresses. In sweet
106 potato, *MIR408* has been associated with plant defences, as it is repressed by jasmonic acid
107 (JA) and wounding, and miR408-overexpressing plants have attenuated resistance to insect
108 feeding (Kuo *et al.*, 2019). Moreover, miR398 has been shown to regulate cell death in response
109 to the causal agent of barley powdery mildew, *Blumeria graminis* (Xu *et al.*, 2014).

110 Root-knot nematodes (RKN) of the genus *Meloidogyne* are obligatory sedentary plant parasites
111 capable of infesting more than 5,000 plant species (Abad and Williamson, 2010; Blok *et al.*,
112 2008). The RKN larvae penetrate the roots, in which they induce the de-differentiation of five
113 to seven parenchyma root cells and their reprogramming into the multinucleate, hypertrophied
114 feeding cells that form the feeding site. These metabolically overactive feeding cells provide
115 the nutrients required for RKN development (Favery *et al.*, 2020). During the dedifferentiation
116 of vascular cells and their conversion into ‘giant’ feeding cells, the cells surrounding the feeding
117 site begin to divide again. The growth of the feeding cells and the division of the surrounding
118 cells lead to a root swelling known as a gall. The feeding cell induction occurs in the first three
119 days after root infection. Feeding cell formation can be split into two phases. Firstly, the cells
120 undergo successive nuclear divisions coupled with cell expansion until ten days post infection
121 (dpi) in *A. thaliana* (Caillaud *et al.*, 2008). In the second phase, from 10 to 21 dpi, the successive
122 nuclear divisions stop and the nuclei of the feeding cells undergo extensive endoreduplication
123 (Wiggers *et al.*, 1990; de Almeida Engler and Gheysen, 2013). The dedifferentiation of vascular
124 cells and their conversion into giant cells result from an extensive reprogramming of gene
125 expression in root cells, in response to RKN signals. In *A. thaliana*, the expression of

126 approximately 10% of protein-coding genes is modified in galls induced by RKN (Cabrera *et*
127 *al.*, 2014; Yamaguchi *et al.*, 2017; Jammes *et al.*, 2005; reviewed in Escobar *et al.*, 2011). The
128 sequencing of small RNAs identified 24 mature microRNAs differentially expressed (DE)
129 between *A. thaliana* galls induced by *M. incognita* and uninfected roots at 7 and 14 dpi and 62
130 microRNAs DE at 3 dpi (Medina *et al.*, 2017; Cabrera *et al.* 2015). The miR408 and miR398
131 families of copper-responsive microRNAs were found to be upregulated in galls at 7 and/or 14
132 dpi.

133 In this article, we showed a conserved upregulation of these two microRNA families in
134 *Arabidopsis* and tomato galls. Moreover, we found that the upregulation of *MIR408* in response
135 to RKN was required for successful infection. Our findings highlighted a strong activity of the
136 *MIR408* promoter (*pMIR408*) in early galls that is i) driven by the modulation of environmental
137 copper levels, ii) **is localised in the same gall tissue than the strong *SPL7* expression.**
138 Moreover, we also demonstrated that this transcription factor helps to promote giant cell
139 development. In addition, we showed that the silencing of *CSD1* and *BLUE COPPER*
140 *BINDING PROTEIN (BCBP)* transcripts by miR398 is involved in gall development. Finally,
141 the watering of *Arabidopsis* with copper sulphate solutions at concentrations below the toxicity
142 thresholds for nematode and plant development greatly decreased the RKN infection and
143 impaired feeding cell development.

144

145 **Material and Methods**

146 **Biological material, growth conditions and nematode inoculation**

147 Seeds of *A. thaliana* Col0 and mutants *mir408-1* (SALK_038860), *mir408-2*
148 (SALK_121013.28.25.n), *spl7* (SALK_093849), *mcsd1* and *mbcbp* (Beauclair *et al.*, 2010),
149 *pmiR408::GUS* (Zhang *et al.*, 2013) and *pSPL7::GUS* (Yamasaki *et al.*, 2009) were surface-
150 sterilised and sown on Gamborg B5 medium agar plates (0.5 x Gamborg, 1% sucrose, 0.8%
151 agar, pH 6.4). The plates were incubated at 4°C for two days, then transferred to a growth
152 chamber (20°C with an 8 h light/ 16 h darkness cycle). *M. incognita* strain “Morelos” was
153 multiplied on tomato plants in a growth chamber (25°C, 16 h light/8 h darkness). For the RKN
154 infection of plants in soil, two-week-old plantlets grown *in vitro* were transferred to a mixture
155 of 50% sand (Biot B5)/50% soil in a growth chamber (21°C, 8 h light/16 h darkness). For
156 studies of the effect of copper on gall development on plants *in vitro*, *Arabidopsis* plantlets were
157 sown and cultured *in vitro*, as described above, on Gamborg B5 medium supplemented with 50
158 µM CuSO₄.

159 **Root knot nematode infection assay**

160 For nematode infections *in vitro*, second-stage juveniles (J2s) were surface-sterilised with
161 HgCl₂ (0.01%) and streptomycin (0.7%), as described by Caillaud and Favery (2016). We
162 inoculated each 25-day-old seedling grown individually *in vitro* with 200 sterilised J2s
163 resuspended in Phytigel (5%). Infection assays were performed on *Arabidopsis* mutants and a
164 wild-type ecotype in soil. We inoculated 20 to 30 two-month-old plantlets with 150 J2s per
165 plant and incubated them in a growth chamber (21°C, 8 h light/16 h darkness). Seven weeks
166 after infection, the roots were collected, washed in tap water and stained with eosin (0.5 %).
167 Stained roots were weighed and galls and egg masses were counted on each root under a
168 binocular microscope. Mann and Whitney tests (2.5%) were performed to determine the
169 significance of the observed differences in the numbers of egg masses and galls per root.

170 **Small RNA sequencing from galls and uninfected tomato roots**

171 Biological material, RNA extraction, small RNA and RNA sequencing, read mapping and
172 statistical analysis are presented as supporting informations (Method S1-S6).

173 **BABB clearing**

174 Feeding site development was evaluated by the BABB clearing method described by Cabrera
175 *et al.*, (2018). Briefly, the area occupied by the giant cells was measured on galls collected
176 seven weeks post infection, cleared in benzyl alcohol/benzyl benzoate (BABB) and examined
177 under an inverted confocal microscope (model LSM 880; Zeiss). Zeiss ZEN software was used
178 to measure the area occupied by the giant cells in each gall, on two biological replicates. Data
179 were analysed in Mann and Whitney tests.

180 **Copper treatment**

181 *M. incognita* eggs were collected as previously described (Caillaud and Favery, 2016) and
182 placed on a 10 µm-mesh sieve for hatching in tap water. Free-living J2s were collected from
183 the water with a 0.5 µm-mesh sieve. We evaluated the toxicity of copper to J2s by incubating
184 freshly hatched J2s in solutions of copper sulphate of various concentrations for 24 hours. The
185 numbers of living or dead J2s were then determined by counting under a binocular microscope.
186 We investigated the effects of copper on plant-nematode interactions in *Arabidopsis* Col0
187 grown in soil. *Arabidopsis* Col0 plantlets were prepared and inoculated as previously described
188 for in-soil infection. Half the plants were watered with 50 µM CuSO₄ two days after inoculation
189 with J2s and then once per week for the next seven weeks. Control Col0 plants were watered
190 with tap water in place of copper sulphate solution, at the same frequency. Seven weeks after

191 inoculation, the plants were collected, their roots were washed and weighed, and the numbers
192 of galls and egg masses on the roots were counted, as described above.

193 **Studies of promoter-GUS fusion gene expression**

194 We localised the promoter activity of *MIR408* and *SPL7* in *A. thaliana* lines expressing various
195 fusions of the *GUS* reporter gene to promoters from these genes. We inoculated 21-day-old
196 seedlings in soil and *in vitro*, as described above. We collected inoculated roots and washed
197 them in water, 3, 7, 14 and 21 dpi. GUS staining was performed as previously described (Favery
198 *et al.*, 1998), and the roots were observed under a Zeiss Axioplan 2 microscope. Stained galls
199 were dissected, fixed by incubation in 1% glutaraldehyde and 4% formaldehyde in 50 mM
200 sodium phosphate buffer pH 7.2, dehydrated, and embedded in Technovit 7100 (Heraeus
201 Kulzer, Wehrheim, Germany), according to the manufacturer's instructions. Sections were cut
202 and mounted in DPX (VWR International Ltd, Poole, UK), and observed under a Zeiss
203 Axioplan 2 microscope (Zeiss, Jena, Germany).

204 **Bioinformatic analysis**

205 We used psRNA target with default parameters for the prediction of miR408 targets (Dai,
206 Zhuang and Zhao 2018).

207 **Quantitative PCR**

208 Total RNA was extracted from galls and uninfected roots produced in soil with the miRNeasy
209 kit (QIAGEN) according to the manufacturer's instruction. 500ng of total RNA were subjected
210 to reverse transcription with the Superscript IV reverse transcriptase (Invitrogen). qPCR
211 analyses were performed as described by Nguyen *et al.* (2018). We performed qPCR on
212 triplicate samples of each cDNA from three independent biological
213 replicates. *OXA1* (*At5g62050*) and *UBP22* (*AT5G10790*) were used for the normalization of
214 qRT-PCR data. Quantifications and statistical analyses were performed with SATqPCR
215 (Rancurel *et al.*, 2019), and the results are expressed as normalized relative quantities. Primers
216 used to amplify the premiRNAs and the transcripts are listed in Table S1.

217

218 **NBT staining of galls**

219 Biological material, and NBT staining of galls are presented as supporting information (Method
220 S7).

221 **Results**

222 ***MIR408* is induced in galls and its expression is driven by modulation of copper level**

223 Previous sequencing analyses of microRNAs expressed in *Arabidopsis* galls induced by *M.*
224 *incognita* revealed an upregulation of mature miR408 in *Arabidopsis* galls at 7 and 14 dpi,
225 whereas miR398b/c was specifically upregulated in galls at 14 dpi. None of these microRNAs
226 were shown as DE in galls at 3 dpi produced from young *in vitro* plantlets (Cabrera *et al.* 2016).
227 Sequencing of small RNAs from uninfected roots and galls of *Solanum lycopersicum* showed
228 that these two microRNA families were also upregulated in tomato galls at 7 and 14 dpi (Table
229 1 and Table S2). Therefore, these microRNAs are among the very few conserved microRNAs
230 which expression profile is conserved in *Arabidopsis* and tomato galls. We investigated the
231 induction of *MIR408* in response to nematode infection, by inoculating plants expressing
232 p*MIR408*::GUS (Zhang and Li, 2013) grown *in vitro* on Gamborg B5 with *M. incognita* J2s.
233 We observed a strong GUS signal in developing galls at 3 and 7 dpi (Fig. 1a-b). This signal had
234 decreased in intensity by 14 dpi (Fig. 1c) and disappeared completely from fully developed
235 galls at 21 and 28 dpi (Fig. S1). On gall sections, the GUS signal was localised in the giant
236 feeding cells and neighbouring cells, at the 3 dpi, 7 dpi and 14 dpi time points (Fig. 1d-f).
237 Overexpression of *MIR408* at 7 dpi was confirmed by qPCR analysis of level of premiR408 in
238 galls at 7 and 14 dpi (Fig S2). The discrepancy between mature miR408 and premiR408 level
239 in galls at 14 dpi may be explained by the delay between transcription of premiRNA and its
240 processing into mature microRNA by Dicer as it has been previously described (Thompson *et*
241 *al.*, 2006; Hutvagner *et al.*, 2001; Schulman *et al.*, 2005). MiR408 is known as a copper
242 microRNA. To investigate if copper modulation regulates *MIR408* expression in galls,
243 p*MIR408*::GUS activity was followed in plants grown *in vitro* with a high copper concentration
244 (Gamborg B5 plus 5 μ M CuSO₄). By contrast to plants grown in Gamborg B5, in plants grown
245 in the presence of high copper concentrations, the GUS signal was much weaker in galls at 3
246 and 7 dpi (Fig. 1g-h and 1j-k), and undetectable in galls at 14 dpi (Fig. 1i and 1l). The repression
247 of *MIR408* expression in galls by high copper concentrations indicates that *MIR408* expression
248 within *Arabidopsis* galls is regulated by modulation of copper levels.

249

250 **The copper microRNA miR408 is crucial for the *Arabidopsis*-*Meloidogyne* interaction**

251 We investigated the role of miR408 in gall development using two previously described
252 *Arabidopsis* knockout (KO) mutant lines: *mir408-1* and *mir408-2* (Maunoury and Vaucheret,

253 2011). The KO lines and corresponding wild-type plants were inoculated with *M. incognita* J2s
254 and their susceptibility was quantified by counting the galls and egg masses produced by adult
255 females at the root surface. The two KO lines for *MIR408* had 40 to 50% fewer galls and egg
256 masses than the wild type ($p<0.05$; Fig. 2 and Table S3). The roots of these KO lines were of
257 similar weight and global architecture to those of wild-type plants (Fig. S2 and Table S3). We
258 then investigated the effect of the *MIR408* mutation on feeding site development, by comparing
259 the area of feeding cells within galls collected from KO and wild-type plants (Fig. 2a-c and
260 Table S3). Both KO mutants had a significantly smaller feeding site area than the wild type.
261 Overall, these results demonstrate that *MIR408* helps to promote feeding cell development in
262 the *Arabidopsis*-nematode interaction, and that the lower susceptibility of the *miR408* KO lines
263 is due to defects of feeding site development.

264 We investigated the targeted transcripts by which miR408 modulates feeding cell development
265 in galls. The psRNA target algorithm (Dai *et al.*, 2018) predicted 101 genes as putative targets
266 of miR408 in *Arabidopsis* (Table S4a). The expression profiles of these genes in galls at 7 dpi
267 and 14 dpi were obtained from previous transcriptome analyses (Jammes *et al.* 2005). Only
268 seven of the 101 putative targets were differentially expressed in galls at 7 and/or 14 dpi, and
269 only two putative targets were repressed: a transcript encoding a copper-binding protein,
270 UCLACYANIN2 (*UCC2*, *At2g44790*), which is known to be cleaved by the RNA INDUCED
271 SILENCING COMPLEX (RISC) guided by miR408 in senescing leaves and siliques (Thatcher
272 *et al.*, 2015), and a transcript encoding a PHOSPHATASE 2G (*PP2CG1*, *At2g33700*) (Table
273 S4b). The repression of these two genes was confirmed in galls at 14 dpi by qPCR (Fig. S3 and
274 Table S5) supporting that mature miR408 induced the cleavage of these two targets at this time
275 point.

276

277 **SPL7 is an activator of *MIR408* transcription in galls**

278 The regulation of *MIR408* by modulation of copper levels has been shown to be mediated by
279 the *SPL7* transcription factor (Zhang *et al.*, 2014; Bernal *et al.*, 2012). Transcriptome analyses
280 from *Arabidopsis* galls showed that *SPL7* was expressed in galls at 7, 14 and 21 dpi (Jammes
281 *et al.*, 2005). We further investigated the expression of *SPL7* within galls, by inoculating a
282 *pSPL7::GUS Arabidopsis* line (Araki *et al.*, 2018) with *M. incognita* in the presence of a normal
283 copper concentration. *SPL7* promoter activity was observed within the gall from 3 to 14 dpi,
284 with lower levels at 14 dpi (Fig. 3a-c). As observed for *pMIR408::GUS*, sections of

285 *pSPL7::GUS* galls revealed a GUS signal in the giant feeding cells and neighbouring cells (Fig.
286 3d). We investigated the putative function of *SPL7* in plant responses to RKN, by inoculating
287 the *Arabidopsis spl7* KO mutant described by Zhang *et al.* (2014) with *M. incognita* J2s. *SPL7*
288 knockout led to the production of smaller numbers of galls and egg masses per plant than were
289 observed for the wild-type (Fig. 3e-f and Table S6). This knockout had no effect on root weight
290 (Fig. S4 and Table S6). Measurements of the area of the feeding site within galls revealed
291 defects of feeding site formation in the *spl7* mutants, resulting in smaller giant cells than were
292 observed in wild-type plants (Fig. 3g and Table S6). Overall, these results demonstrate the
293 requirement of miR408 and *SPL7* for the proper development of giant cells. The upregulation
294 of mature miR408 observed in galls suggests an induction of *MIR408* expression driven by
295 *SPL7* due to a decrease in copper availability within the gall.

296 **micro398, a second copper-responsive microRNA family involved in the *Arabidopsis-*** 297 ***Meloidogyne* interaction**

298 MiR408 is not the only copper-responsive microRNA reported differentially expressed in galls.
299 The expression of *MIR398B* and *MIR398C*, from the conserved miR398 family, has also been
300 shown to be induced in response to copper deficiency, via *SPL7* activity (Araki *et al.*, 2018).
301 We previously described an induction of the mature miR398b and miR398c in *Arabidopsis*
302 galls at 14 dpi by sequencing (Medina *et al.*, 2017). However, quantification of the precursors
303 by qPCR did not confirm the induction of the mature miR398s (Fig S5 and Table S7). Three
304 targets of the miR398 family have been biologically validated: the *At1g08830* and *At2g28190*
305 transcripts encoding two copper superoxide dismutases, *CSD1* and *CSD2*, respectively, and the
306 *At5g20230* transcript encoding the blue copper binding protein (BCBP), identified as a non-
307 canonical target of miR398 (Brousse *et al.*, 2014). Previous analyses of *Arabidopsis* gall
308 transcriptome showed a repression of *CSD1* at 7 dpi and of *BCBP* at 7 and 14 dpi while *CSD2*
309 was not differentially expressed (Jammes *et al.* 2005). qPCR did not confirm the repression of
310 *CSD1* and *CSD2* and showed a repression of *BCBP* at 7 dpi but with a pvalue of 0.07 (Fig. S5
311 and Table S7). Despite the lack of confirmation of sequencing and microarray results by qPCR,
312 we investigated the role of miR398 further, by infecting *Arabidopsis* lines expressing modified
313 versions of *CSD1* (*mcsd1*) or *BCBP* (*mcbcbp*) mRNAs rendered resistant to cleavage by miR398
314 (Brousse *et al.*, 2014; Beauclair *et al.*, 2010). The target mRNA levels is therefore artificially
315 increased in these plants. The prevention of *CSD1* transcript cleavage by miR398 had no effect
316 on root weight (Fig. S6), but led to lower levels of nematode infection, with the mutant having
317 less galls and egg masses than the wild type (Fig. 4 and Table S8). The *mcbcbp* line also had

318 fewer egg masses than the wild-type (Fig. 4 and Table S8). No defect of feeding cell formation,
319 such as slower feeding cell growth, was observed in either of these lines (Fig. 4 and Table S8).
320 The specific decrease in egg mass production by females provides evidence for a role for
321 miR398 in the functionality of feeding cells, although the mutations in the *mcsdl* and *mbcbp*
322 lines did not affect feeding site size (Fig. 4). These findings demonstrate that the cleavage of
323 *CSDI* and *BCBP* transcripts by the RISC guided by miR398 helps to promote parasitism.

324 **Modulation of copper levels is essential for plant-RKN interaction**

325 To study further the effect of copper on nematode infection, we analysed the direct effects of
326 copper on nematode survival and gall development. Free-living *M. incognita* J2s were
327 incubated in several concentrations of copper sulphate (50 μ M to 2 mM) used in previous
328 studies assessing the effect of copper on plant development (Schulten *et al.*, 2019). As a
329 negative control, J2s were incubated in tap water. Living J2 counts after 24 hours in the copper
330 sulphate solution showed that copper was non-toxic at a concentration of 50 μ M (Fig. S7 and
331 Table S9). By contrast, toxic effects were observed for all other concentrations tested (0.5 mM,
332 1 mM and 2 mM). We then analysed the effect of copper on gall formation in Col0 and
333 *pMIR408::GUS* plants grown in soil watered with 50 μ M CuSO₄. We also minimised J2s
334 exposure to copper in the soil, by beginning to water plants 50 μ M CuSO₄ two days after
335 inoculation, after the J2s had already penetrated the roots. Watering with 50 μ M CuSO₄
336 repressed pMIR408 activity in uninfected roots and in galls as it can be observed by the
337 reduction of the GUS signal (Fig. S8) confirming the effects of such treatment in galls. Watering
338 with 50 μ M CuSO₄ had no visible effect on root weight and architecture (Fig. S9 and Table
339 S10), but it resulted in a strong and significant decrease in the number of galls and egg masses
340 relative to control plants watered with tap water (Fig. 5). Copper is involved in many processes
341 in plants like the production of reactive oxygen species (ROS). Watering plants with CuSO₄
342 may therefore have additional effects in addition to the repression of the *pMIR408* activity. The
343 effect of watering with CuSO₄ on ROS production during the nematode infection was analysed.
344 The O²⁻ accumulation was followed by Nitro Blue Tetrazolium (NBT) staining of galls collected
345 14 dpi from plants watered with tap water or with 50 μ M CuSO₄. NBT staining showed the
346 same localisation and intensity signal in galls regardless the watering condition. A strong O²⁻
347 accumulation can be observed in cells surrounding the feeding site when the giant feeding cells
348 showed no signal (Fig. S10). Altogether, these results demonstrate that perturbation of plant
349 copper homeostasis modulates miR408 production in galls and interferes with the plant-RKN
350 interaction.

351 **Discussion**

352 RKN induce the formation of similar giant feeding cells in thousands of plant species. The
353 conservation of the ontogeny and phenotype of nematode-induced feeding cells between
354 species, strongly suggests that the plant molecular mechanisms manipulated by RKN are widely
355 conserved across the plant kingdom. Previous transcriptome analyses on various plant species
356 have shown that the development of galls in roots infected by RKN is associated with a massive
357 reprogramming of gene expression (reviewed in Escobar *et al.*, 2011). MicroRNAs are small
358 non-coding RNAs that regulate gene expression at the post-transcriptional level, and some
359 microRNA families, such as the miR156 and miR167 families, are widely conserved in plants
360 (Chavez Montes *et al.*, 2014). The role for microRNAs in controlling gene expression during
361 the formation of galls was recently reported in *Arabidopsis* (reviewed in Jaubert-Possamai *et*
362 *al.*, 2019), for the conserved microRNAs miR390, miR172 and miR159 (Cabrera *et al.* 2016;
363 Diaz-Manzano *et al.* 2018; Medina *et al.*, 2017).

364 **miR408 and miR398: two copper-responsive microRNA families activated in galls** 365 **induced by *M. incognita***

366 Sequencing of small RNAs showed that mature miR408 and miR398b/c microRNAs
367 accumulated in galls at 7 and/or 14 dpi, compared to uninfected roots, in both *A. thaliana*
368 (Medina *et al.*, 2017) and *S. lycopersicum*. A combination of *in silico* predictions of the
369 transcripts targeted by miR408 and transcriptional analyses of galls and uninfected roots
370 identified the *UCLACYANIN-2 (UCC2)* and the *PHOSPHATASE (PP2CG)* genes as two
371 putative targets of miR408 downregulated in galls. The cleavage of *UCC2* transcripts by
372 miR408 has been biologically validated in *Arabidopsis* and rice (Thatcher *et al.*, 2015; Zhang
373 *et al.*, 2017). Moreover, several targets of miR398 have been biologically validated, including
374 the cytosolic *CSD1* and chloroplastic *CSD2*, and the non-canonical target *BCBP* (Beauclair *et*
375 *al.*, 2010; Brousse *et al.*, 2014). Our analysis, thus, identified several biologically validated and
376 conserved targets that may be considered robust candidates for mediating the functions of
377 miR398b/c and miR408 in galls.

378 The inactivation of miR408 in *Arabidopsis* T-DNA mutant lines, or of miR398b/c function in
379 transgenic plants expressing mutated *CSD1* or *BCBP* resistant to miR398 cleavage, led to
380 decreases in both the parameters used to assess parasitic success (the number of galls and the
381 number of females producing egg masses per root). The smaller number of galls in the mutant
382 lines demonstrates that miR398 family and miR408 promote infection in the early plant

383 response to RKN. Moreover, the smaller feeding sites observed in the two *miR408* KO lines
384 and the *spl7* mutant demonstrate that this miR408 and SPL7 promote the development of the
385 giant cells, which are essential for nematode growth and development. Females are unable to
386 develop normally if the feeding cells are too small, as already reported in some *Arabidopsis*
387 mutants, such as lines with a knockout of *PHYTOSULFOKINE RECEPTOR1 (PSKR1)* (Rodiuc
388 *et al.*, 2016). Only a few genes and plant functions have been demonstrated to be essential for
389 the formation of giant feeding cells (Favery *et al.*, 2020). In the absence of changes in giant cell
390 size in the *mcsd1* and *mbcbp* mutants, we hypothesise that the miR398-regulated *CSD1* and
391 *BCBP* genes may play a role in giant cell functioning, potentially in reactive oxygen species
392 (ROS)-related redox regulation and signalling (Zhao *et al.*, 2020). Further studies will be
393 required to determine their precise roles in the plant-RKN interaction.

394 **SPL7 is a regulator of miR408 and miR398 in galls**

395 In *A. thaliana*, it has been shown that *MIR408*, *MIR398B* and *MIR398C* are activated by the
396 same transcription factor, SPL7, the activity of which is dependent on copper levels (Yamasaki
397 *et al.*, 2009; Araki *et al.*, 2018). We confirmed the activity of the *SPL7* promoter and *MIR408*
398 in feeding cells and neighbouring cells. The co-expression of *SPL7* and *MIR408* within
399 developing galls, and the similar nematode infection phenotype, with feeding site formation
400 defects, strongly suggest that *SPL7* is responsible for activating *MIR408* transcription in galls,
401 as already reported in leaves and the root vasculature (Yamasaki *et al.*, 2009; Araki *et al.*, 2018).
402 We therefore hypothesised that the expression of *MIR408* and *MIR398B* and *-C* is activated by
403 the *SPL7* transcription factor in response to a decrease in copper concentration within galls.
404 Other transcription factors, such as HY5 and PIF1, which are known to regulate the expression
405 of *MIR408* in response to light stress (Zhang *et al.*, 2014; Jiang *et al.*, 2021), are expressed in
406 galls and could also play a role in the regulation of *MIR408* expression. The strong repression
407 of *MIR408* by excess copper observed in galls suggests that *MIR408* upregulation in galls is
408 predominantly driven by copper and SPL7. However, we can't exclude that nematode effectors
409 may also influence directly the expression of *MIR408* and *MIR398* in addition to the regulation
410 by copper modulation. Further analyses are now required to understand how these microRNAs
411 are induced in response to nematode infection.

412

413 **Modulation of copper levels, a key conserved factor for gall formation**

414 Infection assays on *A. thaliana* plants watered with a copper sulphate solution at a concentration
415 non-toxic for plants and nematodes, showed a strong decreased RKN infection rates and
416 resulted in defective feeding site formation. Together with the upregulation in galls of two
417 microRNA families known to be induced by copper deficiency, the miR408 and miR398
418 families, and the regulation of *MIR408* expression by copper, this finding suggests that copper
419 content decreases in the galls induced by RKN infection. This hypothesis is supported by the
420 downregulation of the *COPT2* gene, encoding a copper importer, in the *Arabidopsis* gall
421 transcriptome (Jammes *et al.*, 2005). Micronutrients quantifications have also demonstrated a
422 decrease in copper concentration in RKN-infected susceptible tomato roots (Lobna *et al.*, 2017).

423 The ***SPL7/MIR408-UCC2/MIR398-CSD1* copper module** may be a key factor in gall
424 formation, conserved across the plant kingdom. MiR408 and miR398 have been identified in
425 more than 40 plant species (Griffiths-Jones *et al.*, 2007) and *SPL7* is widely conserved
426 throughout the plant kingdom (Yamasaki *et al.*, 2009). Moreover, the targeting of
427 *UCLACYANIN2* by miR408 and of *CSD1* by miR398 is conserved in both dicotyledonous and
428 monocotyledonous plants (Zhang *et al.*, 2017; Thatcher *et al.*, 2015). miR408 and miR398
429 targets are associated with lignin metabolism either directly like *UCC2* (Reyt *et al.*, 2020) or
430 indirectly like *CSD1* via the ROS. Cell wall lignin is restructured during the development of
431 the feeding cells and galls are subject to extensive changes in vascularization, resulting in the
432 giant cells being engaged within a network of *de novo* formed xylem and phloem cells (Bartlem,
433 Kones and Hammes, 2014). Therefore miR398 and miR408 may be involved in modification
434 of feeding cell wall lignin or lignin network within galls. Moreover, a role for UCLACYANINS
435 in the formation of a lignified nanodomain within the Casparian strips known to form an
436 endodermal barrier in *Arabidopsis* roots has recently been described (Reyt *et al.*, 2020).
437 Casparian strip defects have been observed in the endodermis bordering the giant cell area
438 within sorghum galls induced by *M. naasi* (Ediz and Dickerson, 1976). Moreover, *Arabidopsis*
439 mutants with disrupted Casparian strips are particularly susceptible to RKN (Holbein *et al.*,
440 2019). The infection of plants with nematodes may, therefore, provides a unique model for
441 investigating the role of copper modulation, via microRNAs and its targets, in the formation of
442 Casparian strips.

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456 **Author contribution**

457 YN, S.J.P and B.F. designed the study and wrote the manuscript. Y.N., J.M. and S.J.P. designed
458 the study and performed the experimental work. All authors analysed and discussed the data.
459 M.Q. and P.A. participated in the writing of the manuscript. S.T. participated in the copper
460 studies. MdR analysed NGS data. KM and YN performed the qPCR.

461 **Data availability**

462 The data that support the findings of this study are available from the corresponding author
463 upon reasonable request.

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629 **Supporting Information**

630 **Fig. S1** Localisation of pMIR408 activity in fully developed galls of *Arabidopsis thaliana*.

631 **Fig S2** Relative quantification of premiR408 and of *UCC2* and *PP2G* transcripts by qPCR in
632 galls in comparison to uninfected *Arabidopsis thaliana* roots at 7 and 14 dpi

633 **Fig. S3** Root phenotype of the *mir408-1* and *mir408-2* *Arabidopsis thaliana* KO lines.

634 **Fig. S4** Root phenotype of the *spl7* *Arabidopsis thaliana* KO line.

635 **Fig. S5** Relative quantification of premiR398b/c and of *CSD1*, *CSD2* and *BCBP* transcripts by
636 qPCR in galls in comparison to uninfected *Arabidopsis thaliana* roots at 7 and 14 dpi.

637 **Fig. S6.** Root phenotype and area covered by the feeding site in the *mcsd1* and *mbcbp*
638 *Arabidopsis thaliana* lines.

639 **Fig. S7** Assessment of copper toxicity in *Meloidogyne incognita* J2s.

640 **Fig. S8** Repression of pMIR408 activity in *Arabidopsis thaliana* galls by watering with
641 CuSO₄, assessed at 14 days post infection (dpi).

642 **Fig. S9** Root phenotype of *Arabidopsis thaliana* Col0 watered with 50 µM copper sulphate.

643 **Fig. S10** NBT staining of O²⁻ on 14 dpi *Arabidopsis thaliana* gall sections.

644 **Table S1** Primers used in qPCR.

645 **Table S2** Expression level of mature sequence of the miR408 and miR398 families
646 upregulated in *Arabidopsis thaliana* and tomato (*Solanum lycopersum*) galls.

647 **Table S3:** Infection assays with *mir408-1* and *mir408-2* *Arabidopsis thaliana* ko lines
648 infected with *Meloidogyne incognita*.

649 **Table S4 :** Prediction of *Arabidopsis thaliana* miR408 targets with psRNA targets and
650 expression profile of these targets in *Arabidopsis thaliana* galls.

651 **Table S5** : Relative quantification of premiR408 and of *UCC2* and *PP2G* transcripts by
652 qPCR in galls in comparison to uninfected *Arabidopsis thaliana* roots at 7 and 14 dpi.

653 **Table S6** : Infection assays with *spl7-1 Arabidopsis thaliana* lines infected with *Meloidogyne*
654 *incognita*.

655 **Table S7** : Relative quantification of premiR398b/c and of *CSD1*, *CSD2* and *BCBP*
656 transcripts by qPCR in galls in comparison to uninfected *Arabidopsis thaliana* roots at 7 and
657 14 dpi.

658 **Table S8** : Infection assays with *mbcbp* and *mcsd1 Arabidopsis thaliana* lines infected with
659 *Meloidogyne incognita*.

660 **Table S9** : *Meloidogyne incognita* second stage juvenile (J2) survival after being incubated
661 for 24 hours in a CuSO₄ solution.

662 **Table S10** : Infection assays of *Meloidogyne incognita* Col0 watered with 50 μM CuSO₄ or
663 tap water.

664 **Methods S1** Biological Materials, Growth Conditions

665 **Methods S2** RNA extraction

666 **Methods S3** RNA sequencing

667 **Methods S4** miRNAs Analysis

668 **Methods S5** mRNA sequencing

669 **Methods S6** mRNA Analysis

670 **Methods S7** NBT staining

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673

674 **Table & Figures**

675 **Table 1.** Expression profile of miRNAs of the miR408 and miR398 families in *Arabidopsis*
 676 *thaliana* and tomato galls.

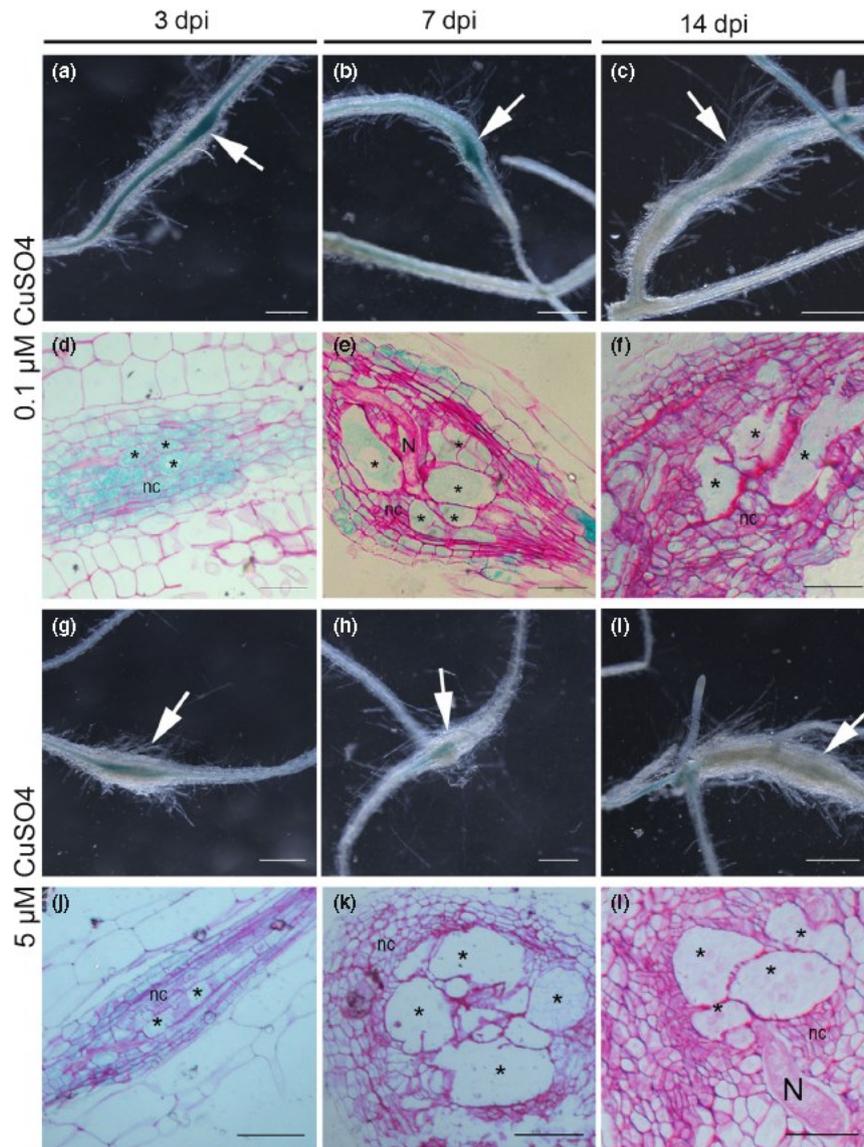
miRNA family	miRNA name	Plant species	miRNA mature sequence	logFC G/R 7dpi	adj. pvalue	logFC G/R 14dpi	adj. pvalue
mir408	SL3.0ch01_346	<i>S. lycopersicum</i>	TGCACAGCCTCTTCCCTGGCT	3,741	0,000	3,289	0,017
	SL3.0ch01_419	<i>S. lycopersicum</i>	TGCACTGCCTCTTCCCTGGCT	1,287	0,029	3,133	0,033
	Ath-miR408	<i>A. thaliana</i>	ATGCACTGCCTCTTCCCTGGC	2,042	0,042	2,411	0,001
mir398	SL3.0ch12_14_3	<i>S. lycopersicum</i>	ATGTGTTCTCAGGTTACCCCT	3,272	0,009	2,166	0,066
	SL3.0ch05_225	<i>S. lycopersicum</i>	TTGTGTTCTCAGGTCACCCCT	0,290	0,863	3,430	0,015
	SL3.0ch11_437	<i>S. lycopersicum</i>	TATGTTCTCAGGTCGCCCTG	2,144	0,112	1,759	0,067
	ath-miR398b-c	<i>A. thaliana</i>	TGTGTTCTCAGGTCACCCCT	2,464	0,119	3,287	0,000

677
 678 For each miRNA family, the reference in *Arabidopsis* (Ath-miR) and in *Solanum lycopersicum*
 679 (SL3.0ch) genomes is indicated. The sequence of the mature sequence, the log₂ of the
 680 Gall/uninfected root expression fold change (Log₂FC G/R) and statistical significance
 681 according to the Benjamini Hochberg adjustment (adj. pvalue) for the differentially expressed
 682 miRNAs at 7 and 14 days post infection (dpi) are indicated. MicroRNAs statistically
 683 upregulated in galls in comparison to uninfected roots are underlined in red.

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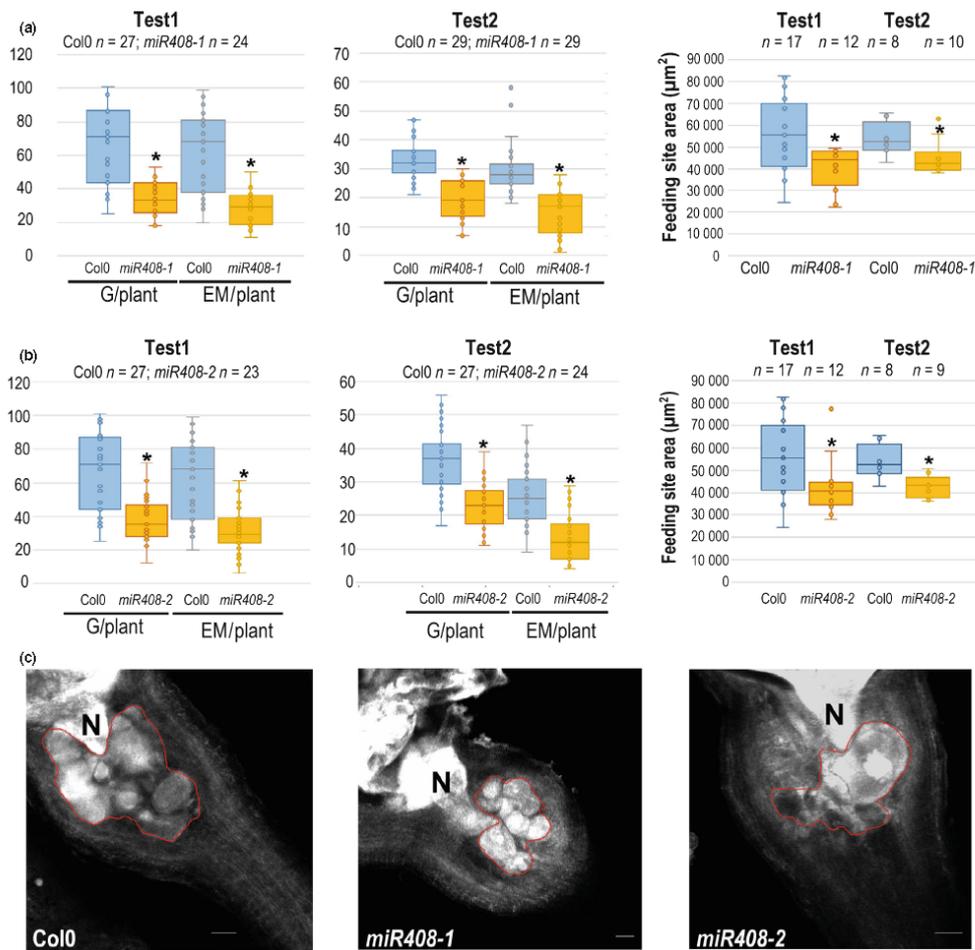
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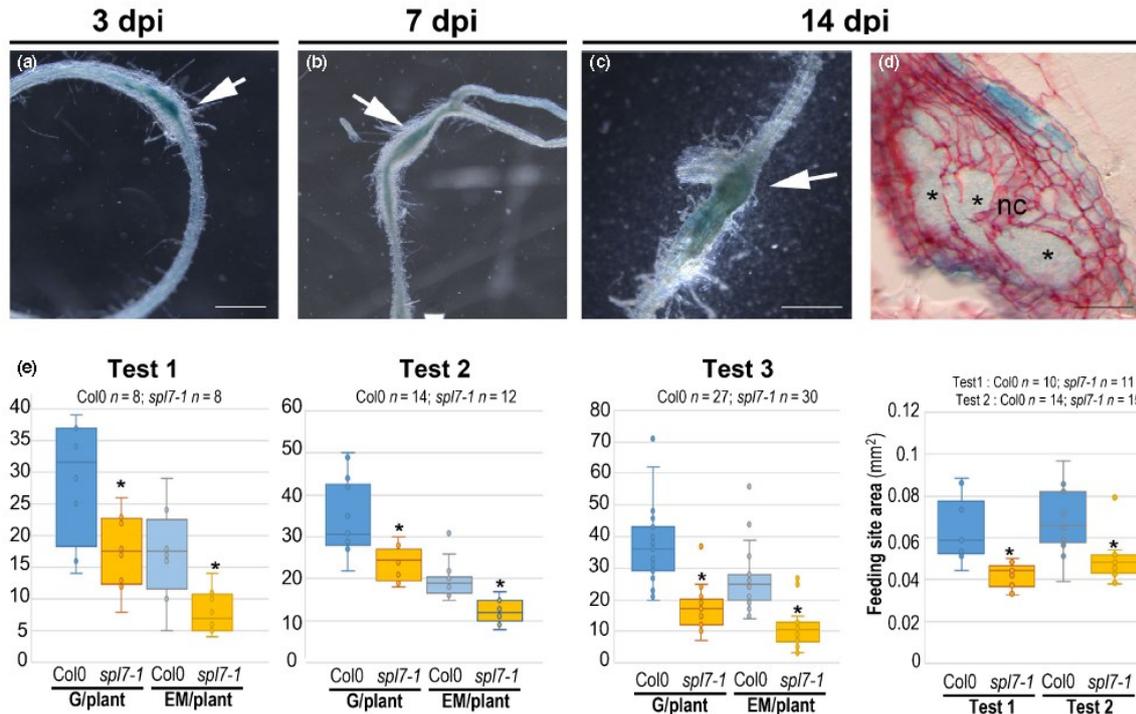
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688 **Figure 1.** Copper modulates *MIR408* promoter activity in galls. (a-h), The activity of the
689 *MIR408* promoter was analysed in galls induced by *M. incognita* in *Arabidopsis thaliana*
690 expressing the *pMIR408::GUS* construct grown in the presence of normal concentrations of
691 copper (0.1 μM CuSO_4)(a-f) or in the presence of high concentrations of copper (5.0 μM
692 CuSO_4)(g-l). (a-c), a strong blue coloration reflecting GUS activity was observed in galls 3
693 days post infection (dpi) (a), 7 dpi (b) and 14 dpi (c) in plants grown with 0.1 μM CuSO_4 . D-
694 F, Section of galls at 3 dpi (d), 7dpi (e) and 14 dpi (f) showing the GUS blue signal in giant
695 cells and in the cells surrounding the giant cells. (g-i), a weaker GUS signal was observed in
696 galls from plants grown with 5.0 μM CuSO_4 analysed at 3 dpi (g) and 7 dpi (h) and no GUS
697 signal was observed in galls at 14 dpi (i). (j-l), Section of gall at 3 dpi (j), 7dpi (k) and 14 dpi
698 (l). Galls are indicated with an arrow; N, nematode; (*) giant feeding cells; nc, neighbouring
699 cells. Bars 500 μm (a-c; g-i) or 50 μm (d-f; j-l).



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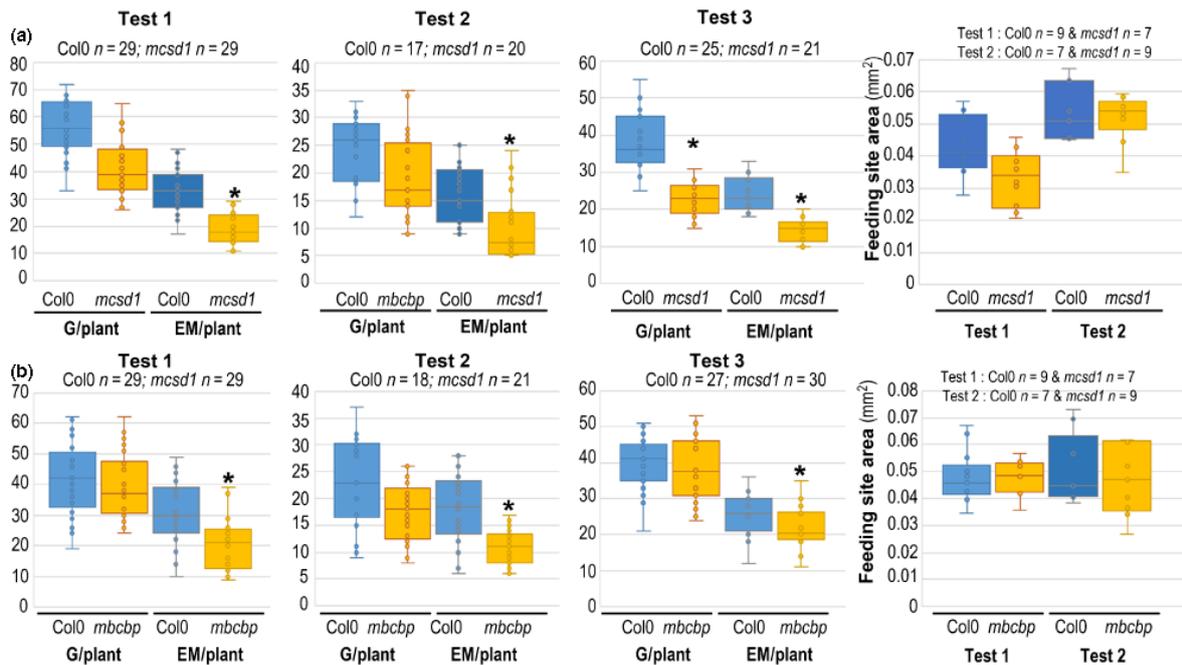
701 **Figure 2:** The *miR408* KO *Arabidopsis thaliana* lines were significantly less susceptible to
 702 *Meloidogyne incognita* than the wild type. (a-b) The susceptibility of the two *mir408* KO lines,
 703 *mir408-1* (a) & *mir408-2* (b), and Col0 wild type to *M. incognita* was evaluated by counting
 704 the number of galls and egg masses per plant (G/plant and EM/plant, respectively) in two
 705 independent infection assays in soil. (c) The effect of *MIR408* mutation on the development of
 706 giant feeding cells was further evaluated by measuring the size of the feeding site (circled in
 707 red) produced in each KO and comparing it to that in Col0. Galls were collected seven weeks
 708 post *in vitro* infection to measure the area (μm^2) covered by the giant cells by the BABB
 709 clearing method (Cabrera et al., 2018). The impact of plant genotype was analysed in Mann and
 710 Whitney tests. *, $P < 0.05$. Boxes indicate interquartile range (25th to the 75th percentile). The
 711 central lines within the boxes represent medians. Whiskers indicate the minimal and maximum
 712 of the usual values present in the data set. The circle outside the box represents outlier. n : the
 713 number of plants per line analysed in each assay for the number of galls and egg masses and
 714 the number of galls per line for each test for measurement of feeding site area. Bars 50 μm . N :
 715 nematode.



716

717 **Figure 3.** *SPL7* is induced and required for *Meloidogyne incognita* infection and giant cell
718 formation in *Arabidopsis thaliana*. (a-d), The activity of the *SPL7* promoter (*pSPL7*) was
719 studied in galls induced by *M. incognita* from *A. thaliana* expressing the *pSPL7::GUS*
720 construct, at 3 days post inoculation (dpi)(a), 7 dpi (b) and 14 dpi (c). (d), GUS blue staining
721 was observed within 5.0 μm -thick gall sections at 14 dpi. (e), the KO *spl7* line (SALK093849c)
722 was infected with *M. incognita* J2. This line was significantly less susceptible to RKN than
723 Col0, as shown by the smaller mean number of galls and egg masses per plant (G/plant and
724 EM/plant, respectively) in three independent infection assays. The effect of *spl7* mutation on
725 the development of feeding cells was further evaluated by measuring the size of the feeding site
726 area. Galls were collected seven weeks post *in vitro* infection for measurement of the area
727 (mm^2) covered by the giant cells, by the BABB clearing method (Cabrera et al., 2018). Mann–
728 Whitney tests were performed for statistical analysis in each experiment; significant differences
729 relative to Col-0: *, $P < 0.05$. Boxes indicate interquartile range (25th to the 75th percentile).
730 The central lines within the boxes represent medians. Whiskers indicate the minimal and
731 maximum of the usual values present in the data set. The circle outside the box represents
732 outlier. n : the number of plants per line analysed in each assay for the number of galls and egg
733 masses and the number of galls per line for each test for measurement of feeding site area. Galls
734 are indicated with an arrow; (*) giant feeding cells; nc, neighbouring cells. Bars 50 μm (a-c) or
735 500 μm (d).

736



737

738 **Figure 4.** The miR398-resistant *mcsd1* and *mbcbp* mutant *Arabidopsis thaliana* lines had
 739 smaller numbers of egg masses. The susceptibility of the (a) *mcsd1* and (b) *mbcbp* lines and of
 740 wild-type Col0 plants was evaluated by counting the number of galls and egg masses per plant
 741 (G/plant and EM/plant, respectively) in three independent infection assays in soil. Nematode
 742 feeding site area (in mm²) in wild-type Col0 and *mcsd1* or *mbcbp* mutants were then measured
 743 following gall clearing with BABB (Cabrera et al., 2018). Results from two independent
 744 experiments are shown. All data were analysed in Mann and Whitney statistical tests. *, P <
 745 0.05. Boxes indicate interquartile range (25th to the 75th percentile). The central lines within
 746 the boxes represent medians. Whiskers indicate the minimal and maximum of the usual values
 747 present in the data set. The circle outside the box represents outlier. n : the number of plants per
 748 line analysed in each assay for the number of galls and egg masses and the number of galls per
 749 line for each test for measurement of feeding site area.

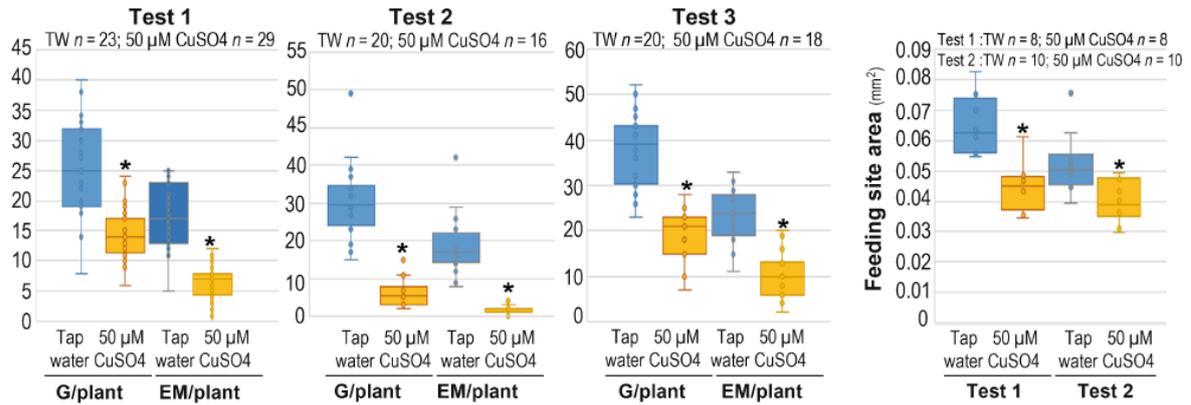
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757 **Figure 5. *Arabidopsis thaliana* Col0 watered with copper sulphate solution were**
 758 **significantly less susceptible**

759 to root-knot nematodes. The effect of physiological concentrations of copper on *Meloidogyne*
 760 *incognita* infection was evaluated by counting galls and egg masses per plant (G/plant and
 761 EM/plant, respectively) in Col0 plants watered with tap water (TW) or with a copper sulphate
 762 (CuSO₄) solution at a non-toxic concentration (50 μM) Three independent experiments were
 763 performed. Nematode feeding site area (in mm²) in wild-type Col0 cultivated on tap water or
 764 on 50 μM CuSO₄ were then measured following gall clearing with BABB (Cabrera et al., 2018).
 765 Results from two independent experiments are shown. All data were analysed in Mann and
 766 Whitney statistical tests. *, P < 0.05. Boxes indicate interquartile range (25th to the 75th
 767 percentile). The central lines within the boxes represent medians. Whiskers indicate the minimal
 768 and maximum of the usual values present in the data set. The circle outside the box represents
 769 outlier. n : the number of plants per line analysed in each assay for the number of galls and egg
 770 masses and the number of galls per line in each test for measurement of feeding site area.

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