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► To cite this version:

Yongpan Chen, Qian Liu, Xuqian Sun, Lei Liu, Jianlong Zhao, et al.. Meloidogyne enterolobii MeMSP1 effector targets the glutathione-S-transferase phi GSTF family in Arabidopsis to manipulate host metabolism and promote nematode parasitism. *New Phytologist*, In press, 10.1111/nph.19298 . hal-04239152

HAL Id: hal-04239152

<https://hal.inrae.fr/hal-04239152>

Submitted on 12 Oct 2023

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1 ***Meloidogyne enterolobii* MeMSP1 effector targets the glutathione-S-transferase**
2 **phi GSTF family in Arabidopsis to manipulate host metabolism and promote**
3 **nematode parasitism**

4

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Total word count (excluding summary, references and legends):	6233	Acknowledgements:	104
Summary:	188	Author contributions:	68
Introduction:	900	No. of figures:	8 (all Figs in colour)
Materials and Methods:	1986	No. of Tables:	1
Results:	1758	No of Supporting Information files:	25 (Fig S1-S16, Tables S1-S9)
Discussion:	1417		

- 38 **Brief heading:** Root-knot nematode secretes MeMSP1 effector to manipulate host
- 39 metabolism and promote nematode parasitism
- 40 Twitter @brunofavery
- 41

42 **Summary**

43 ● *Meloidogyne enterolobii* is an emerging root-knot nematode species that
44 overcomes most of the nematode resistance genes in crops. Nematode effector
45 proteins secreted in planta are key elements in the molecular dialogue of
46 parasitism. Here, we show the MeMSP1 effector is secreted into giant cells and
47 promotes *M. enterolobii* parasitism.

48 ● Using Co-IP and BiFC assays we identified glutathione-S-transferase phi GSTFs
49 as host targets of the MeMSP1 effector. This protein family plays important roles
50 in plant responses to abiotic and biotic stresses. We demonstrate that MeMSP1
51 interacts with all *Arabidopsis* GSTF. Moreover, we confirmed that the N-terminal
52 region of AtGSTF9 is critical for its interaction, and *atgstf9* mutant lines are more
53 susceptible to RKN infection.

54 ● Combined transcriptome and metabolome analyses showed that MeMSP1 affects
55 the metabolic pathways of *Arabidopsis thaliana*, resulting in the accumulation of
56 amino acids, nucleic acids, and their metabolites, and organic acids and the down-
57 regulation of flavonoids.

58 ● Our study has shed light on a novel effector mechanism that targets plant
59 metabolism, reducing the production of plant-defence related compounds while
60 favouring the accumulation of metabolites beneficial to the nematode, and thereby
61 promoting parasitism.

62

63 **Keywords:** *Arabidopsis thaliana*, Effector, Glutathione-S-transferase, *Meloidogyne*
64 *enterolobii*, Pathogen, Plant metabolism, Root-knot nematode.

65

66 **Introduction**

67 Plant parasitic nematodes (PPNs) are one of the most economically important plant
68 pathogens, causing more than 100 billion dollars loss annually worldwide (Abad *et al.*,
69 2008). Root-knot nematodes (RKNs; *Meloidogyne* spp.) are one of the greatest threats
70 to agriculture production. RKNs infect more than 5,500 plant species and cause a global
71 yield loss of 70 billion dollars every year (Blok *et al.*, 2008; Caboni *et al.*, 2012; Chen,
72 J *et al.*, 2017). RKNs are sedentary endoparasitic nematodes that induce the formation
73 of complex feeding cells in the vascular cylinder of host root, known as giant cells,
74 which serve as the sole nutrient source for the development and reproduction of RKNs
75 (Favery *et al.*, 2016; Rutter *et al.*, 2022). In order to establish parasitism successfully,
76 RKNs need to suppress plant defence, induce and maintain the giant cells. They have
77 evolved numerous secreted effectors that originate from the three oesophageal glands
78 or other organs like amphids and hypodermis (Favery *et al.*, 2020; Zhao *et al.*, 2019;
79 Haegeman *et al.*, 2012). Although some effectors have predicted functions, e.g. redox-
80 regulated proteins such as thioredoxins, glutathione peroxidases, glutathione-S-
81 transferases (GST) and protein disulphide isomerases (PDI) (Bellafiore *et al.*, 2008;
82 Tian *et al.*, 2019, Zhao *et al.* 2021), a large majority of these effectors are pioneers
83 without known functional domains (Jagdale *et al.*, 2021; Goverse & Mitchum, 2022).
84 The identification of host targets of these RKN pioneer effectors is one of the strategies
85 to understand their roles in the parasitism.

86 Only a few plant targets of RKN effectors have been identified (reviewed by Mejias *et*
87 *al.*, 2019 and Rutter *et al.*, 2022) and recently several targets involved in defence or
88 stress response have been characterised. A stress-associated protein from *Solanaceae*
89 and *Arabidopsis* has been shown to be targeted by *M. incognita* MiPDI1 to establish
90 disease (Zhao *et al.*, 2020). The C-type lectin effector MiCTL1a interacts with plant
91 catalases to regulate the redox state in the host (Zhao *et al.*, 2021). Another example is
92 the *M. graminicola* MgMO289 effector that interacts with the rice heavy metal-
93 associated plant protein 04 (OsHPP04) to modulate host superoxide dismutase (SOD)
94 activity and scavenge reactive oxygen species (Song *et al.*, 2021). In addition, the *M.*
95 *enterolobii* translationally controlled tumour protein (MeTCTP) is able to

96 homodimerise to bind calcium and prevent cytosolic calcium rise in order to suppress
97 plant immunity (Guo et al., 2022). However, the targets and functions of RKN effectors
98 are still largely unknown.

99 Plant metabolism play a central role in the molecular dialogue during plant-pathogen
100 interactions. Giant cells are a metabolic sink that act as the nutrient source for the
101 developing nematode. During giant cell formation, RKNs modified physiological and
102 transport processes of host cells, resulting in altered metabolite production and transport
103 (Bartlem *et al.*, 2014). Studying the changes in host plant metabolites caused by
104 nematodes allows a better understanding of the giant cell. An untargeted proton Nuclear
105 Magnetic Resonance (1H-NMR) analysis of primary metabolites in roots and galls 21
106 days post infection (dpi) with *M. incognita* of normal and (homo) glutathione (h)GSH
107 depleted *Medicago truncatula* identified 15 metabolites (sugars, organic acids, and
108 amino acids) significantly different between galls and uninfected roots (Baldacci-Cresp
109 et al, 2012). This study showed that (h)GSH depletion affects gall metabolism
110 (Baldacci-Cresp *et al.*, 2012). The metabolite concentration is higher in galls than in
111 roots at 35 dpi resulting in water potential and osmotic pressure modification in galls
112 (Baldacci-Cresp *et al.*, 2015). Gas chromatography coupled to mass spectrometry
113 untargeted fingerprint analysis was also used to analyse metabolites in tomato leaves
114 and stem upon *M. incognita* infection (Eloh *et al.*, 2016). However, the effects of RKN
115 effectors on host metabolism is limited.

116 *Meloidogyne enterolobii* (syn. *M. mayaguensis*) is one emergent species, first reported
117 in 1983, that can overcome RKN resistance genes such as the *Mi-1.2* gene (tomatoes),
118 *Mh* gene (potato), *Mir1* gene (soybean), *N* gene (bell pepper), *Tabasco* gene (sweet
119 pepper) and *Rk* gene (cowpea) (Yang & Eisenback, 1983; Castagnone-Sereno, 2012;
120 Philbrick *et al.*, 2020). Utilizing RNA interference (RNAi) to silence *M. enterolobii*
121 specific effector genes or the effector targets may be an opportunity to enhance plant
122 resistance to this uncontrolled RKN. MeTCTP silencing resulted in reduced parasitism
123 of *M. enterolobii* on tomato (Zhuo *et al.*, 2017). The EFFECTOR18 (EFF18) protein is
124 a conserved RKN effector, and both *M. incognita* and *M. enterolobii* EFF18 have been
125 shown to interact with the spliceosomal small nuclear ribonucleoprotein D1 (SmD1)

126 from *A. thaliana*, *S. lycopersicum*, and *N. benthamiana* (Mejias *et al.*, 2021; Mejias *et*
127 *al.*, 2022). Virus-induced gene silencing of *SmDI* in tomato affected giant cell
128 formation and nematode development (Mejias *et al.*, 2022). Thus, this evidence shows
129 that modifying the expression of RKN effectors or their target genes to improve plant
130 resistance to *M. enterolobii* a promising approach.

131 In this study, we characterised a novel nematode effector mechanism in *M. enterolobii*.
132 *M. incognita* MiMSP1 has been reported as a potential effector, as it is expressed in the
133 dorsal gland of parasitic RKNs, although there are no further reports on its functional
134 analysis (Huang *et al.*, 2003). We demonstrated that its ortholog MeMSP1 is secreted
135 into the host through the stylet, is capable of physically interacting with all *Arabidopsis*
136 glutathione-S-transferase phi proteins (AtGSTF), and found that the knockout (ko)
137 mutant lines of *AtGSTF9* were more susceptible to RKN infection. Combined
138 transcriptome and metabolome analyses revealed that ectopic expression of MeMSP1
139 in *Arabidopsis* affected host metabolic pathways and secondary metabolite biosynthesis.

140

141 **Materials and Methods**

142 **Nematode and Plant materials and growth conditions**

143 *M. enterolobii* were propagated on the susceptible tomato cultivar (*Solanum*
144 *lycopersicum* L. cv. “Baiguo Qiangfeng”) in a greenhouse starting from a single egg
145 mass. Egg masses were collected and hatched according to Niu *et al* (Niu *et al.*, 2016).
146 All seeds of *A. thaliana* (L.) Heynh were surface-sterilized and then grown on solidified
147 half-strength Murashige and Skoog (MS) medium with 2% sucrose. The T-DNA
148 insertion mutant lines of *AtGSTF9* (SALK_001519C and SALK_148672C) were
149 obtained from the *Arabidopsis* Biological Resource Center (ABRC, USA). *Nicotiana*
150 *benthamiana* plants were grown in pots under long-day conditions (16h light/8 h dark)
151 at 25°C.

152

153 **Sequence analysis, alignment and phylogenetic tree**

154 MSP1 sequences were obtained from WormBase Parasite
155 (<https://parasite.wormbase.org/index.html>) and *Meloidogyne* genomic resources

156 (http://www6.inra.fr/meloidogyne_incognita/) by blastp against the *Meloidogyne*
157 predicted protein database, the protein sequences with a higher identity than 85.5%
158 were selected. Multiple amino acid sequence alignment analyses of MSP1 proteins
159 were conducted using DNAMAN V6 (Lynnon Biosof, USA). These MSP1 protein
160 sequences were aligned with the MAFFT tool on the EBI server
161 (<https://www.ebi.ac.uk/Tools/msa/mafft/>). The alignment was then used as input for the
162 IQTree Web server (<http://iqtree.cibiv.univie.ac.at/>) to generate the maximum
163 likelihood phylogenetic tree. The model chosen by the inbuilt model test was Flu+I.
164 Support for the nodes was calculated with 100 bootstrap replicates. MiMSP12 was used
165 as the outgroup in the phylogenetic tree for MeMSP1 putative orthologs. The tree was
166 visualized in iTOL (<https://itol.embl.de/>).

167

168 **RNA isolation and gene amplification**

169 The mRNA of *M. enterolobii* was extracted using an RNAPrep pure Micro Kit (Tiangen
170 Biotech Co., Ltd Beijing), and total RNA of *Arabidopsis* was isolated from seedlings
171 using TRIzol Reagent (Invitrogen, USA). Complementary DNA (cDNA) was
172 synthesized using M-MLV reverse transcriptase (TaKaRa, Japan). *MeMSP1* genes
173 were amplified from cDNA of *M. enterolobii* by PCR using specific primers (Table
174 S1). The PCR products were cloned into pMD18-T vector (TaKaRa, Japan) and
175 sequenced. All primers used in this study are listed in Supplementary Table S1 and
176 were synthesized by TsingKe Biotechnology Co. Ltd, Beijing, China.

177

178 **Developmental expression analysis and *in situ* hybridisation**

179 mRNA samples of *M. enterolobii* at different life stages were obtained as above. The
180 cDNA was synthesized using M-MLV reverse transcriptase (TaKaRa, Japan). qRT-
181 PCR was conducted using the SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara,
182 Japan). *GAPDH* gene of *M. enterolobii* was used as a control. The results were
183 determined using the $2^{-\Delta\Delta CT}$ method. Three technical replicates for each reaction were
184 performed in all experiments, and three independent experiments were conducted.

185 For *in situ* hybridisation, freshly hatched *M. enterolobii* pre-J2s were collected on a 0.5

186 μm sieve. The primers ISH-MeMSP1-F and ISH-MeMSP1-R were used to amplify the
187 179 - 491 bp (313 bp) of the MeMSP1 coding sequences (CDS) from cDNA, and the
188 DIG-labelled sense (negative control) and antisense probes were synthesised by
189 asymmetric PCR. The hybridisation were performed as described previously (Niu *et*
190 *al.*, 2016) and examined using a BX51 microscope (Olympus, Japan).

191

192 **Anti-MeMSP1 antibodies production and immunolocalisation analysis**

193 The CDS of MeMSP1 without signal peptide was inserted into the pET-28a (+) vector
194 and expressed in BL21 (DE3) cells. The purified recombinant MeMSP1 protein was
195 used to produce polyclonal antibodies in rabbits at ABclonal Company (Wuhan, China).
196 For immunolocalisation, galls of tomato (*Solanum lycopersicum* var. 'Baiguo') were
197 harvested at 14 dpi, and were then fixed, dehydrated, embedded and sectioned as
198 previously described (Vieira *et al.*, 2011). Anti-MeMSP1 antibodies and Goat anti-
199 Rabbit Alexa Fluor 488 conjugated antibodies (Thermo Fisher Scientific, San Jose, CA,
200 USA) were diluted 100- and 500-fold in blocking solution of 1% BSA in 50 mM
201 piperazine-N, N'-bis (ethanesulphonic acid) (PIPES) buffer (pH 6.9), respectively.
202 Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI, 1 $\mu\text{g}/\text{mL}$ in water).
203 Finally, slides were mounted with ProLong anti fade medium (Invitrogen, USA) and
204 observed under confocal microscope at an excitation wavelength of 488 nm (Leica SP8,
205 Germany).

206

207 **Protein extraction and western blot analysis.**

208 Total proteins of *N. benthamiana* leaves or *A. thaliana* seedlings were extracted using
209 a protein extraction kit (CW0885, Beijing ComWin Biotech Co., Ltd., China). For
210 western blot, total protein samples were separated on a 10% polyacrylamide gel and
211 transferred onto a polyvinylidene difluoride (PVDF) membrane using a semi-dry
212 transfer system (BioRad, USA). After blocked in 5% skimmed milk (in PBS, pH 7.2)
213 for 1 hour, the membrane was incubated with horseradish peroxidase (HRP) tag-
214 conjugated antibodies (anti-Flag or anti-HA, MBL, China) diluted (1 : 5000) with PBS
215 (pH 7.2) containing 1% skimmed milk for 1 hour. After washes, the membrane was

216 detected using an EasySee Western Blot Kit (DW101, TransGen Biotech, China) and
217 imaged with a multifunctional molecular imaging system through automatic exposure
218 (C600, AZURE).

219

220 **Generation of transgenic *Arabidopsis* and infection assays**

221 For *in planta* RNAi, a specific fragment (300 bp) of MeMSP1 was amplified and then
222 inserted into a pSAT5 RNAi plasmid (Dafny-Yelin *et al.*, 2007). For ectopic expression
223 of MeMSP1 in *Arabidopsis*, the ORF without signal peptide of MeMSP1 was cloned
224 and inserted into Super1300-FLAG. These constructs were transformed into
225 *Agrobacterium tumefaciens* GV3101 and used for transformation of *A. thaliana* via the
226 floral dip method (Clough & Bent, 1998). Homozygous single insertion T3 plants were
227 used for the susceptibility experiment to nematode. The plants were grown in pots
228 (length: 5.5cm, width: 5.5cm, and height: 5cm) with soil (nutrient soil, Fangjie
229 Company, China) and vermiculite (1: 1) under a growth chamber condition (22°C, 16
230 h: 8 h, light: dark photoperiod). Three-week-old *Arabidopsis* were inoculated with 300
231 freshly hatched J2s per plant. 14 days after infection, roots from MeMSP1-Ri lines and
232 WT plants were collected and digested in a mixture of pectinase (P2611, Sigma) and
233 cellulose (C2730, Sigma) at 28 °C and 160 rpm overnight. Parasitic nematodes were
234 collected under a stereomicroscope and used to measure the expression level of
235 *MeMSP1* by qRT-PCR. Roots were collected 30 days after infection and stained using
236 the sodium hypochlorite-acid fuchsin method (Bybd *et al.*, 1983). The numbers of galls,
237 females and other stage nematodes were counted under a stereomicroscope microscope
238 (SZ61, Olympus, Tokyo, Japan). At least 15 plants of each transgenic line or wild-type
239 (Col-0; WT) were used for each experiment and three independent experiments were
240 performed.

241

242 **Interaction analysis**

243 For identifying the target of MeMSP1 in *Arabidopsis*, total proteins were extracted from
244 MeMSP1-expressing lines (MeMSP1-OE-1 and MeMSP1-OE-3) and WT plants.
245 Immunoprecipitation (IP) was performed using anti-FLAG M2 affinity gel resin

246 (Sigma-Aldrich, USA). Proteins were eluted with competitive 3X FLAG peptide
247 (F4799, Sigma-Aldrich, USA). Q Exactive (Thermo Q-Exactive nanospray ESI-MS
248 mass spectrometer, USA) was used for liquid chromatography-tandem mass
249 spectrometry (LC-MS/MS) at China Agricultural University Functional Genomics
250 Platform. The acquired MS data were pre-analysed using Mascot Distiller 2.4 (UK) and
251 then anatomized to search a NCBI non-redundant protein database and Swiss-prot
252 database.

253 For bimolecular fluorescence complementation (BiFC) analysis, coding sequences
254 (CDS) of *MeMSP1* without its signal peptide and *AtGSTFs* were cloned into the
255 pUC_SPYCE or the pUC_SPYNE vector (Walter *et al.*, 2004), respectively.
256 *AtGSTU19* (AT1G78380) and empty vectors were used as negative control. Mixtures
257 of *A. tumefaciens* cells (OD₆₀₀=0.5, respectively) containing each pair were co-
258 infiltrated into the leaves of *N. benthamiana* and observed using a laser confocal
259 fluorescence microscope (Leica SP8, Germany).

260 For co-immunoprecipitation (Co-IP) assays, the *AtGSTF* CDS were cloned into
261 pGR107_flag vector and *MeMSP1* CDS without signal peptide was cloned into
262 pGR107_HA. EGFP was cloned into pGR107_Flag as a negative control. All
263 constructs were sequenced and introduced into *A. tumefaciens* GV3101. Co-
264 infiltrations and immunoprecipitation were conducted as previously described (Zhao *et*
265 *al.*, 2019).

266

267 **Transcriptome Analysis of transgenic *Arabidopsis* lines**

268 Total RNA of *MeMSP1*-OE and WT lines were extracted from the 14 days *Arabidopsis*
269 seedlings with Spin Column Plant total RNA Purification Kit following the
270 manufacturer's protocol (Sangon Biotech, Shanghai, China). The cDNA libraries were
271 carried out as previously described (Chen, C *et al.*, 2017) and sequenced on the Illumina
272 HiSeq platform (Illumina Inc., San Diego, USA) by Wuhan MetWare Biotechnology
273 Co., Ltd. (www.metware.cn, Wuhan, China). The sequenced reads were compared with
274 the unigene library using Bowtie (Langmead *et al.*, 2009), and the expression level was
275 estimated in combination with RSEM (Li & Dewey, 2011). The gene expression level

276 was determined according to the FPKM. DESeq2 package (Love *et al.*, 2014; Varet *et*
277 *al.*, 2016) was used to identify the differentially expressed genes (DEGs) between WT
278 and MeMSP1-OE, with the $|\log_2\text{Fold Change}| \geq 1$ and FDR (False Discovery Rate) $<$
279 0.05. KOBAS2.0 was used for the KEGG pathway enrichment analysis of the DEGs
280 (Xie *et al.*, 2011). Two biological repeats were used for the Col-0 line and three
281 biological repeats were used for the MeMSP1-OE-1 and MeMSP1-OE-3 lines in this
282 transcriptome analysis, respectively. The DEGs shared by MeMSP1-OE-1 and
283 MeMSP1-OE-3 *Arabidopsis* lines were analysed with NEMATIC, AgriGO (version 2,
284 <http://systemsbiology.cau.edu.cn/agriGOv2/>) and compared with previous RNAseq
285 data (Cabrera *et al.*, 2014; Tian *et al.*, 2017; Yamaguchi *et al.*, 2017).

286

287 **Metabolome Analysis of transgenic *Arabidopsis* lines**

288 Three biological repeats for transgenic lines (MeMSP1-OE-1 and MeMSP1-OE-3) and
289 Col-0 were used for metabolome analysis. The samples were extracted from frozen 14-
290 days-old seedlings overnight at 4°C with 70% aqueous methanol. All procedures related
291 to metabolomics analysis were performed at Wuhan MetWare Biotechnology Co., Ltd.
292 (www.metware.cn) following their standard procedures (Zhang *et al.*, 2019). The data
293 acquisition instrument system included Ultra Performance Liquid Chromatography
294 (UPLC) (Shim-pack UFLC SHIMADZU CBM30A, Tokyo, Japan) and tandem mass
295 spectrometry (MS/MS) (Applied Biosystems 6500 QTRAP). Based on the self-built
296 database MWDB (Metware database), the metabolites were characterized according to
297 the secondary spectrum information. Metabolite quantification was performed using
298 multiple reaction monitoring (MRM) in triple quadrupole mass spectrometry (Fraga *et*
299 *al.*, 2010).

300 Data matrices with the intensity of metabolite feature under-treated and control
301 conditions were uploaded to the Analyst 1.6.1 software (AB SCIEX, Ontario, Canada).
302 For statistical analysis, missing values were assumed to be below the limits of detection,
303 and these values were imputed with a minimum compound value (Chen *et al.*, 2013).
304 Orthogonal partial least squares-discriminant analysis (OPLS-DA) was used to
305 maximize the metabolome difference between the control and treated samples. The

306 relative importance of each metabolite to the OPLS-DA model was checked using a
307 parameter variable importance in projection (VIP). Metabolites with fold change ≥ 2 or
308 fold change ≤ 0.5 and VIP ≥ 1 were considered as differential metabolites for group
309 discrimination. KEGG pathway analysis was performed in the R software ([www.r-](http://www.r-project.org)
310 [project.org](http://www.r-project.org)).

311

312 **Bio-assay of the lethal effect of metabolites on *M. enterolobii* J2s**

313 All tested compounds were purchased from Alphabio life science company
314 (www.51alphabio.com). Compounds were dissolved in dimethyl sulfoxide to prepare a
315 stock solution of 50 mg/ml. Four concentrations (10, 25, 50, 100 $\mu\text{g/ml}$) for butin and
316 three concentrations for naringenin (10, 25, 50 $\mu\text{g/ml}$; due to the low solubility in water,
317 naringenin recrystallized in the tested solution when we increased the concentration to
318 100 $\mu\text{g/ml}$) were tested for the nematode lethal effect assay. Controls (CK) consisted of
319 the corresponding concentration of DMSO in water. Around 100 freshly hatched
320 second-stage juveniles (J2s) were used for each repetitions. The dead nematode was
321 counted every 24 h for 3 days (24 h, 48 h, and 72 h) under stereomicroscope microscope
322 (SZ61, Olympus, Japan). All this experiment were done in 24-well plate, and the total
323 volume is 1 ml each well. For each metabolites 3 independent experiments were
324 performed, with six replicates for each treatment in each experiment.

325

326 **Statistical analysis**

327 The data were analysed with Dunnett's multiple comparisons test of one-way ANOVA.
328 Statistical computations were carried out with GraphPad Prism (GraphPad Software
329 Inc., La Jolla, CA, USA).

330

331 **Accession numbers**

332 Sequence data from this article can be found in the *Arabidopsis* Information Resource
333 (<https://www.arabidopsis.org>), WormBase Parasite and GenBank/EMBL databases
334 under the following accession numbers: MeMSP1 (OQ256232), *MiMSP12* (AY134431),
335 *AtGSTF2* (At4g02520), *AtGSTF3* (At2g02930), *AtGSTF4* (At1g02950), *AtGSTF5*

336 (*At1g02940*), *AtGSTF6* (*At1g0293*), *AtGSTF7* (*At1g02920*), *AtGSTF8* (*At2g47730*),
337 *AtGSTF9* (*At2g30860*), *AtGSTF10* (*At2g30870*), *AtGSTF11* (*At3g03190*), *AtGSTF12*
338 (*At5g17220*), *AtGSTF13* (*At3g62760*), *AtGSTF14* (*At1g49860*), *MjMSP1*
339 (*M.Javanica_Scaff11723g062660*), *MiMSP1* (*Minc3s00173g06738*), *MaMSP1a*
340 (*M.Arenaria_Scaff41g001480*), *MaMSP1b* (*M.Arenaria_Scaff1390g022660*) and
341 *MfMSP1* (*M.fscf7180000424015.g12030*). The transcriptome data are available at the
342 Sequence Read Archive (SRA) via accession number PRJNA933796. The metabolomic
343 data are available at MetaboLights via accession number MTBLS7145.

344

345 RESULTS

346 ***MeMSP1* is highly upregulated during *M. enterolobii* parasitism**

347 A homolog of *M. incognita* dorsal oesophageal gland cell secretory protein 1 (*MiMSP1*)
348 was identified in the *M. enterolobii* transcriptome (*isotig 10924*) and was designated
349 *MeMSP1* (Li *et al.*, 2016). The *MeMSP1* gene contains an open reading frame (ORF)
350 of 525 bp that encodes a 174-amino-acid (aa) polypeptide that had no known functional
351 domain except a secretion signal peptide of 20 amino acids at its N-terminus according
352 to SignalP 5.0. This indicates *MeMSP1* may be secreted from gland cells. According
353 to previous report, there is a Mel-DOG box (TGCACTT) motif in the 346 bp upstream
354 of the CDS of *MeMSP1* (Fig. S1) suggesting it may be specifically expressed in the
355 dorsal gland of RKNs (da Rocha *et al.*, 2021). Five *MSP1* homologues were obtained
356 from the genome sequences of *Meloidogyne* spp. by blast against all protein databases.
357 The *MeMSP1* protein shared approximately 85.7% aa sequence identity with the
358 other *Meloidogyne* species homologs (Fig. 1a). The alignment and a maximum
359 likelihood phylogenetic tree showed with six *MSP1* protein sequences from *M.*
360 *enterolobii*, *M. incognita*, *M. arenaria*, *M. floridensis* and *M. javanica* grouped together
361 (Fig. 1b).

362 The developmental expression level of the *MeMSP1* gene in different stages of *M.*
363 *enterolobii* was analysed by quantitative real-time PCR (qRT-PCR). Using the
364 expression level of *MeMSP1* at the egg stage as a reference for calculating the relative
365 fold changes in the other stages, *MeMSP1* was more strongly expressed in the parasitic

366 juveniles and females from 10 to 30 dpi, with a maximum at 10 dpi (Fig. S2a).
367 Interestingly, the RNAseq data of *M. incognita* showed *MiMSP1* upregulated in
368 parasitic stage (Fig. S2b) (Rocha *et al.*, 2021), we hypothesise the *MeMSP1* is also
369 upregulated in the parasitic stage. These results indicate that *MeMSP1* is highly
370 upregulated during giant cell formation *in planta*.

371

372 **MeMSP1 is expressed in the dorsal gland and secreted into the nematode feeding** 373 **site**

374 *In situ* hybridisation was used to investigate the tissue localisation of *MeMSP1* in *M.*
375 *enterolobii* J2s. Signals were observed in the dorsal gland cell of J2s (n=14) after
376 hybridisation with the digoxigenin-labelled antisense probe (Fig. 2a). No signal was
377 observed when using the sense probe as a negative control (Fig. 2b). A polyclonal
378 antibodies was raised against MeMSP1 to analyse the localisation of MeMSP1 in the
379 nematode and plant tissue. Western blot showed a clear hybridising band in the total
380 protein samples from *M. enterolobii* J2s and in the recombinant MeMSP1 extract. No
381 band was observed in the protein sample from healthy tomato roots (Fig. S3a), nor with
382 the pre-immune serum in all the three protein samples (Fig. S3b). These results
383 illustrated that the anti-MeMSP1 polyclonal antibodies can specifically recognize
384 MeMSP1. Immunolocalisations performed on parasitic juveniles of *M. enterolobii*
385 showed signals in the dorsal gland cell and in the oesophagus (Fig. 2c-d, Fig. S4). No
386 signal was observed when the pre-immune serum was used as a negative control (Fig.
387 2e). To determine whether MeMSP1 is actually secreted into host plants,
388 immunolocalisation was performed on sections of tomato galls collected 14 dpi. Signals
389 were consistently observed in the cytoplasm of giant cells (Fig. 2f-h, Fig. S5). No signal
390 was observed in the gall sections incubated with pre-immune serum (Fig. 2i). These
391 results demonstrated that MeMSP1 is produced in the dorsal gland and secreted into
392 the feeding cells in the host plant roots.

393

394 **MeMSP1 is involved in nematode parasitism**

395 *In planta* RNA silencing was used to investigate the role of *MeMSP1* gene in the

396 parasitism of *M. enterolobii*. The RNAi construct targeting *MeMSP1* was developed
397 and transferred into *Arabidopsis*, and three homozygous MeMSP1-RNAi lines
398 (MeMSP1-Ri) were used for RKN infection assays. One homozygous RNAi transgenic
399 *Arabidopsis* line targeting GFP (GFP-Ri) and the wild-type Col *Arabidopsis* were used
400 as controls. At 14 dpi, nematodes from all tested *Arabidopsis* lines were extracted to
401 measure the *MeMSP1* transcript abundance. We found a strong reduction of the
402 transcript level of *MeMSP1* in the nematodes recovered from MeMSP1-Ri lines (Fig.
403 3a). Infection assays showed that the numbers of galls, parasitic juveniles and nematode
404 females were significantly decreased in MeMSP1-Ri lines, compared to the controls at
405 30 dpi (Fig. 3b; Fig. S6).

406 Moreover, two independent homozygous transgenic *Arabidopsis* lines expressing
407 *MeMSP1* (MeMSP1-OE) were generated (Fig. S7). Infection assays showed these
408 transgenic lines were both significantly ($P < 0.05$) more susceptible to *M. enterolobii*
409 infection, with 30% more galls and parasitic nematodes at 30 dpi than the controls (COL
410 and plants transformed with an empty super1300 vector) (Fig. 3c; Fig. S6). These
411 results demonstrated that MeMSP1 play important roles in *M. enterolobii* parasitism.

412

413 **MeMSP1 interacts with all the GSTF family members of *Arabidopsis***

414 To identify the plant targets of MeMSP1, we performed *in planta* immunoprecipitation
415 (IP) followed by liquid chromatography-tandem mass spectrometry (LC-MS) on
416 MeMSP1-OE-1, MeMSP1-OE-3 plants and WT *Arabidopsis*. Among the candidate
417 proteins that were pulled down in both MeMSP1-OE plants (Table S2), but not in WT
418 plants, three *Arabidopsis* glutathione-S-transferase phi (AtGSTF2, AtGSTF9 and
419 AtGSTF10) proteins were identified that are localised in the cytosol as MeMSP1 (Fig.
420 S8) (Dixon *et al.*, 2009). The AtGSTFs is a large plant-specific class of proteins with
421 13 members in *Arabidopsis*. Only a few members appear differentially expressed in the
422 galls, either overexpressed and/or repressed (Table S3). We then investigated the
423 interactions between MeMSP1 and the 13 AtGSTFs (AtGSTF2 to AtGSTF14) using
424 BiFC and Co-IP assays.

425 For Co-IP, the Flag-AtGSTFs and MeMSP1-HA expression constructs were co-

426 expressed in *N. benthamiana* leaves. Flag-eGFP was used as a negative control. All the
427 proteins were correctly expressed in tobacco leaves as evidenced by their detection with
428 anti-Flag and anti-HA antibodies (Fig. 4a). Analysis of the immunoprecipitated protein
429 samples with anti-HA antibodies showed that under the same conditions, MeMSP1-HA
430 was specifically pulled down by Flag-AtGSTFs, but not by Flag-eGFP (Fig. 4a).
431 Furthermore, BiFC assays in *N. benthamiana* leaf cells showed that the YFP
432 fluorescence signals were observed in the cytoplasm of *N. benthamiana* epidermal cells
433 that co-expressed the MeMSP1-YFPn fusion and each of the 13 AtGSTF-YFPc
434 construct (Fig. 4b, Fig. S9), but not with the controls. These results demonstrated that
435 MeMSP1 specifically interacts *in planta* with the AtGSTF family members.

436

437 **AtGSTF9 is involved in plant immunity to nematodes**

438 MS data showed that AtGSTF9 had the highest score of all identified GSTF proteins
439 (Table S2). Moreover, AtGSTF9 has been shown to be involved in plant immunity
440 (Horváth *et al.*, 2015; Gong *et al.*, 2018). We therefore focused on this gene for further
441 analysis of its interaction with MeMSP1 and function in nematode parasitism. Typical
442 of AtGSTFs, the AtGSTF9 protein contains a conserved N-terminal thioredoxin-fold
443 domain (1-75 aa) for the conjugation of reduced glutathione and a C-terminal alpha-
444 helical domain (90-208 aa) for the conjugation of hydrophobic substrate. To investigate
445 which domain is important to the interaction, three mutant structures of AtGSTF9 were
446 constructed, AtGSTF9-N-mu, AtGSTF9-C-mu and AtGSTF9-NC-mu (Fig. S10). BiFC
447 assays showed fluorescence only in the positive control and in the leaves infiltrated
448 with AtGSTF9-C-mu-YFPc construct (Fig. 5a). These results showed that the N-
449 terminal part of AtGSTF9 is essential for the interaction between MeMSP1 and
450 AtGSTF9.

451 To investigate the role of AtGSTF9 in plant response to RKN, we obtained two T-DNA
452 insertional alleles of *AtGSTF9* (Salk_001519C, Salk148672C). No *AtGSTF9* transcripts
453 were detected in plants homozygous for the insertions (Fig. S11). These *gstf9* ko lines
454 were significantly more susceptible to *M. enterolobii* than control plants, illustrated by
455 a higher number of galls and nematodes inside roots (Fig. 5b; Fig. S12). This result

456 showed that AtGSTF9 has a role in plant immunity to *M. enterolobii*.

457

458 **Expression of *MeMSP1* affects metabolic pathways and the biosynthesis of**
459 **secondary metabolites in *Arabidopsis***

460 To understand the mode of action of MeMSP1, we investigated genes and metabolites
461 differentially expressed/accumulated in MeMSP1-OE lines compared to WT seedlings,
462 by combining transcriptomic and metabolomic analyses. Transcriptome data identified
463 696 differentially expressed genes (DEG) in both MeMSP1-OE- lines compared to WT.
464 288 DEGs are downregulated and 407 upregulated (Fig. 6a, b; Table S4-S6). Eight
465 DEGs (four upregulated and four down-regulated) were selected to validate the
466 transcriptome data through qPCR, and the results of three biological replicates were
467 consistent with the transcriptome results (Fig. S13). Among these downregulated DEGs,
468 we identified *AtGSTF3* (*At2g02930*), one of the targets of MeMSP1, *TPI* (*At1g73260*),
469 a gene downregulated in galls that have been validated by qRT-PCR (Jammes *et al.*,
470 2005). Interestingly *CCS52B* (*At5g13840*), strongly induced in galls (de Almeida *et al.*,
471 2012), was identified in the upregulated DEGs. The KEGG analysis of shared DEGs
472 revealed that the “metabolic pathways” and “biosynthesis of secondary metabolites”
473 were the two most significantly enriched pathways in the two MeMSP1-OE lines (Fig.
474 6c). Gene ontology (GO) analysis of the common DEGs showed enrichment in the GO
475 terms “oxidation-reduction process” and “oxidoreductase activity”, indicating that
476 *MeMSP1* expression affects the oxidation-reduction balance in *Arabidopsis* (Fig. S14).
477 Metabolome data identified differently accumulated metabolites (DAMs), 13 with a
478 lower content and 25 with a higher content, in both MeMSP1-OE lines (Fig. 7a, b, Table
479 S7 to S9). Among these, amino acid derivatives, nucleic acids and their metabolites,
480 and organic acids all showed upregulated accumulation, while most flavonoids like
481 flavanone, flavone, and flavonolignan showed downregulated accumulation (Table 1).
482 Three metabolites, GSH, L-CYS-GLY, and γ -Glu-Cys involved in the glutathione cycle
483 were significantly accumulated (Table 1). Most DAMs are involved in metabolic
484 pathways by KEGG classification analysis (Fig. 7c). These combined analyses
485 demonstrated that MeMSP1 modulates the metabolic pathway and biosynthesis of

486 secondary metabolite pathways in *Arabidopsis*.

487

488 **Down-regulated metabolites show a harmful effect on nematodes**

489 Of the 13 DAMs with lower levels, 11 belong to the flavonoid family, a well-known
490 group of plant defence compounds. To investigate their roles against RKN, we first
491 tested the mortality of five compounds at 50 mg/L by soaking *M. enterolobii* J2s for 48
492 hours. Three of these had significant and reproducible paralysis activity, namely
493 naringenin, N', N''-di-p-coumaroyl spermidine and butin (Fig. S15). Different
494 concentrations of butin and naringenin and different treatment times were tested on
495 nematodes. The results showed that no significant effect on nematodes was observed
496 when the 10 µg/ml concentration of butin and naringenin was used to treat nematodes
497 for 24 h. With the increase in treatment time and the substances concentration, both
498 butin and naringenin showed a lethal effect on nematodes compared with the control.
499 And when nematodes were soaked with 50 µg/ml concentration for 72 hours, the
500 mortality rate of butin and naringenin to nematodes reached 24.7% and 21.4%
501 respectively. (Fig. 8; Fig. S16). This finding indicated that the expression of MeMSP1
502 in *A. thaliana* results in reduced accumulation of compounds that are harmful to
503 nematodes.

504

505 **Discussion**

506 *Meloidogyne* spp. are obligate plant parasites with a worldwide distribution; they are
507 considered the most devastating of plant-parasitic nematodes (Jones *et al.*, 2013). *M.*
508 *enterolobii* is an emerging RKN species capable of overcoming most of the nematode
509 resistance genes in crops (Sikandar *et al.*, 2022). One of the specific features of RKN
510 interactions with plants is their ability to reprogram root cells to form specialised giant,
511 hypertrophied, multinucleate feeding cells, which serve as their sole source of nutrients
512 (Favery *et al.*, 2020). RKN parasitism is facilitated by the secretion of a large number
513 of effector proteins. The RKN effectors described to date have three major functions:
514 (1) the degradation and modification of plant cell walls; (2) the suppression of host
515 defences; (3) modulation of the physiology of the host plant to allow the formation and

516 the functioning of the permanent feeding site (Mitchum *et al.*, 2013). Here we identified
517 a RKN effector that manipulate plant metabolism.

518

519 **MeMSP1 is a conserved RKN-specific effector secreted into giant cells to promote**
520 **parasitism**

521 MeMSP1 orthologs have been found in five of the eight RKNs for which genome
522 sequences are available (*M. incognita*, *M. javanica*, *M. arenaria*, *M. enterolobii* and *M.*
523 *floridensis*). No such orthologs have been found in cyst nematodes and free-living
524 nematodes (Li *et al.*, 2016). We performed immunolocalisation experiments, which
525 confirmed the biosynthesis of MeMSP1 in the dorsal oesophageal gland of parasitic
526 juveniles and the secretion of this molecule *in planta*. The host-induced gene silencing
527 (HIGS) of *MeMSP1* in nematodes feeding on transgenic plants producing dsRNA and
528 the overexpression *in planta* of the *MeMSP1* gene confirmed the role of this molecule
529 as an effector involved in parasitism. The immunolocalisation of MeMSP1 in giant cells
530 and the results of the HIGS experiments suggest that MeMSP1 acts during later stages
531 of parasitism, after the nematode has become sedentary.

532

533 **MeMSP1 interacts with glutathione-S-transferase phi GSTF proteins**

534 Using biochemical and cell biology approaches, we were able to identify and validate
535 the GST phi (GSTF) class of proteins as targets of MeMSP1 within the plant. Indeed,
536 we found that MeMSP1 interacted with the 13 *Arabidopsis* GSTF proteins. GSTF
537 proteins constitute a large plant-specific class of GSTs proteins. GSTs display
538 significant sequence divergence, but crystallographic and biophysical studies have
539 shown that their protein structure is conserved with a G-site at the N-terminus that
540 specifically binds to GSH and an H-site that binds to the electrophilic substrate (Oakley,
541 2011). We found that several amino acids at the N-terminus of AtGSTF9 were essential
542 for its interaction with MeMSP1, suggesting that MeMSP1 targets the G site, which is
543 critical for GSTF function.

544 Most previous functional analyses of plant GSTFs have focused on tolerance to heavy
545 metals, herbicides, drought, extreme temperatures, or salinity, but there is now evidence

546 to suggest that GSTFs are also involved in plant responses to biotic stresses (Sappl *et*
547 *al.*, 2009; Gullner *et al.*, 2018). Plant GSTFs are generally induced by treatment with
548 the defence-related plant hormone salicylic acid (Sappl *et al.*, 2009; Gong *et al.* 2018),
549 and it has been shown that some *AtGSTFs* are induced by fungal or bacterial pathogens
550 (Sappl *et al.*, 2009). GSTF11 overexpression in oilseed rape (*Brassica napus*) increases
551 resistance to the causal agent of powdery mildew, *Erysiphe cruciferarum* resulting in
552 impaired mycelial growth (Mikhaylova *et al.*, 2021). Interestingly, GSTF9 has been
553 implicated in the responses of cotton (*Gossypium arboreum*) and *A. thaliana* to the
554 fungal pathogen *Verticillium dahliae* (Gong *et al.*, 2018). Cotton in which *GaGSTF9* is
555 silenced and *Arabidopsis atgstf9* mutants have been shown to be more susceptible to *V.*
556 *dahliae*, consistent with our finding for the interaction with *M. enterolobii*.

557 GSTFs are involved in anti-microbial metabolite synthesis and transport. AtGSTF6 has
558 been implicated in the biosynthesis of the phytoalexin camalexin, whereas, AtGSTF2
559 is involved in the transport of defence-associated secondary metabolites such as
560 camalexin and the flavonol quercetin-3-O-rhamnoside (Kumar, 2014). AtGSTF9,
561 AtGSTF10 and AtGSTF11 have been shown to be involved in the biosynthesis of
562 glucosinolates, a group of plant secondary metabolites with relevant nematicidal
563 activity (Sonderby *et al.*, 2010; Eugui *et al.* 2022). We hypothesized that RKNs secrete
564 MeMSP1 into the plant to hijack the functions of GSTFs to modulate host
565 metabolism for their own benefit.

566

567 **Combined analyses of transcriptomic and metabolomic analyses show that** 568 **MeMSP1 modulates host metabolic pathways**

569 We investigated the changes to plant responses induced by the effector through a
570 combination of transcriptomics and metabolomics analysis. Such combined analyses
571 have proved a powerful tool for deciphering plant responses to pathogens (Chen *et al.*,
572 2019; Duan *et al.*, 2022). Such analyses of the vascular tissues of *Eucalyptus urophylla*
573 infected with *Ralstonia solanacearum* have revealed an activation of plant hormone
574 signal transduction, flavonoid production, mitogen-activated protein kinase (MAPK)
575 signalling, and amino-acid metabolism (Yang *et al.*, 2022). Many transcriptomic

576 studies have shown that most of the DEGs involved in the transcriptional
577 reprogramming associated with the development of galls or giant cells are related to
578 metabolism (Jammes *et al.*, 2005; Barcala *et al.*, 2010; Ji *et al.*, 2013; Portillo *et al.*,
579 2013). However, a few reports have focused on the changes in the host transcriptome
580 induced by a single RKN effector (Shi *et al.*, 2018a and 2018b; Mejias *et al.*, 2021;
581 Song *et al.*, 2021). A transcriptomic analysis of *A. thaliana* lines ectopically expressing
582 MiMIF-2 and treated with flg22 revealed effects on metabolic pathways (Zhao *et al.*,
583 2020). We compared the transcriptomes and metabolomes of MeMSP1-OE
584 *Arabidopsis* lines with those of the WT. The DEGs and DAMs identified were enriched
585 in metabolic pathways and secondary metabolite biosynthesis pathway.

586 We show here that primary metabolites, such as organic acids, amino acids and their
587 derivatives, including GSH accumulate following the expression of a single effector in
588 the plant, as previously reported in mature *Medicago truncatula* galls after RKN
589 infection (Baldacci-Cresp *et al.*, 2012). GSH is involved in plant responses to pathogens,
590 and, particularly, in the protection of plants against oxidized stress (Foyer & Noctor,
591 2009; Baldacci-Cresp *et al.*, 2012). Glutathione-deficient *Arabidopsis* mutants have an
592 impaired activation of defence marker genes and of genes encoding proteins involved
593 in the biosynthesis of the antimicrobial compound camalexin early in cyst nematode
594 infection (Hasan *et al.*, 2022). Based on these findings, we hypothesise that the
595 accumulation of GSH in MeMSP1-OE lines would help protect the giant cells from
596 oxidative stress, enabling them to nourish the RKN successfully. The higher level of
597 GSH in MeMSP1-OE lines at least partly accounts for their greater susceptibility to *M.*
598 *enterolobii*. MeMSP1 expression affects plant metabolism, leading to the accumulation
599 of primary metabolites. This may result in both the provision of more nutrients for
600 nematode development and a decrease in the resources of the plant available for the
601 production of defence compounds

602

603 **MeMSP1 expression reduces the production of plant-defence related flavonoid** 604 **compounds**

605 Plants produce diverse secondary metabolites in response to infection with plant-

606 parasitic nematodes (Sato *et al.*, 2019; Chen, *et al.*, 2021). Certain metabolites may
607 inhibit egg hatching or nematode motility, or even kill nematodes. Flavonoids are
608 important members of this group of metabolites. Most of the metabolites downregulated
609 in MeMSP1-OE lines are flavonoids. This group of specialised plant metabolites
610 includes more than 10,000 different compounds. Plant GSTFs are involved in flavonoid
611 biosynthesis (Shao *et al.*, 2021; Aktar *et al.*, 2022). Our understanding of the functions
612 of almost all these substances remains poor, but there is increasing evidence to suggest
613 a role for flavonoids in plant stress resistance (Sugiyama & Yazaki, 2014). There have
614 been reports of flavonoid antimicrobial activity. For example, the maackiain produced
615 by alfalfa and pea inhibits the growth of *Pythium graminicola* (Jiménez-González *et al.*,
616 2007) and *Rhizoctonia solani* (Guenoune *et al.*, 2001). Several flavonoids have
617 recently been reported to have nematicidal activity, suggesting a possible role in plant-
618 nematode interactions (Bano *et al.*, 2020). Various flavonols, including kaempferol and
619 quercetin, have been shown to inhibit the chemotaxis and motility of nematodes (Wuyts
620 *et al.*, 2006). Naringenin is a flavonoid that have been shown to have both antiviral and
621 antifungal activity, including the inhibition of *Xanthomonas oryzae* growth and
622 *Magnaporthe grisea* spore germination, and activity against *Fusarium* spp. (Den
623 Hartogh & Tsiani, 2019). We show here that two flavonoids downregulated by
624 MeMSP1, butin and naringenin, have lethal activity against *M. enterolobii*. This finding
625 suggests that flavonoids play an important role in anti-nematode defences, and that
626 nematodes may target their biosynthesis to promote parasitism.

This work sheds light on a novel effector mechanism targeting plant metabolism that
decreases the production of plant defence-related compounds while favouring the
accumulation of metabolites beneficial to the nematode, thereby promoting parasitism.

627

628

629 **Acknowledgments**

630 We thank Profs. Zejian Guo and Wenxian Sun, China Agricultural University, for their
631 valuable suggestions. This research supported by the National Natural Science
632 Foundation of China (no. 31772138), Sanya Yazhou Bay Science and Technology City

633 (SYND-2021-11), the National Key Research and Development Program of China (no.
634 2017YFD0200601). YC received scholarships from China Scholarship Council (nos.
635 201806350108) to study at INRA, France. PA, BF and MQ were supported by INRAE
636 and by the French Government (National Research Agency, ANR) through the
637 ‘Investments for the Future’ LabEx SIGNALIFE: programme reference#ANR-11-
638 LABX-0028-01 and IDEX UCAJedi ANR-15-IDEX-0 and by the French-Chinese
639 bilateral collaboration programme PHC XU GUANGQI (2020-2021).

640

641 **Author contributions**

642 YC designed, performed experiments and analysed the data. XS propagated the
643 transgenic *Arabidopsis* lines. LL cloned the *MeMSPI* gene. SY and JZ constructed
644 several vectors. YC and MQ analysed the RNA-seq data. QL and XW supervised some
645 of this work and provided expertise. HJ, BF and PA were responsible for the
646 development and management of the project. YC, PA, MQ, BF and HJ wrote the
647 manuscript.

648

649 **Competing interests**

650 None declared.

651

652 **Data availability**

653 The data that support the findings of this study are available within the paper and
654 within the supporting information of this article. The accession numbers of the genes
655 mentioned in this study are shown in Accession numbers section of Materials and
656 Methods. The transcriptome data are available at the Sequence Read Archive (SRA,
657 <https://www.ncbi.nlm.nih.gov/sra>) via accession number PRJNA933796. The
658 metabolomic data are available at MetaboLights
659 (<https://www.ebi.ac.uk/metabolights/>) via accession number MTBLS7145.

660

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926 **Supporting Information**

927 Additional Supporting Information may be found online in the Supporting Information
928 section at the end of the article.

929

930 **Fig. S1** *MeMSP1* is a putative dorsal gland effector gene containing the Mel-DOG
931 motif.

932 **Fig. S2** Both *MeMSP1* and *MiMSP1* are upregulated in the parasitic stage of root-knot
933 nematodes.

934 **Fig. S3** Western blot analysis verified specificity of MeMSP1 antibodies.

935 **Fig. S4** Localisation of MeMSP1 in parasitic stages of *M. enterolobii*.

936 **Fig. S5** Localisation of MeMSP1 in tomato root gall sections of *M. enterolobii*.

937 **Fig. S6** *In planta* RNA interference (RNAi) and ectopic expression of MeMSP1 in
938 *Arabidopsis* shows MeMSP1 participated in the parasitism of *M. enterolobii*.

939 **Fig. S7** Verification of the MeMSP1 expression in *MeMSP1* overexpressing
940 *Arabidopsis* lines by western blot.

941 **Fig. S8** eGFP fused MeMSP1 localised in the cytosol of *Nicotiana benthamiana*
942 epidermal leaf cells.

943 **Fig. S9** Bimolecular fluorescent complementation (BiFC) shows that MeMSP1
944 interact with all the members of AtGSTFs, but not with the negative controls
945 (AtGSTU19 and empty vector).

946 **Fig. S10** Alignment of AtGSTF9 and its mutant protein sequences.

947 **Fig. S11** Verification of homozygous T-DNA insertion mutants of the *Arabidopsis*
948 *AtGSTF9*.

949 **Fig. S12** The *Arabidopsis gsf9* mutant is more susceptible to *M. enterolobii* than the
950 WT.

951 **Fig. S13** Validation of eight differentially expressed genes (DEGs) identified in the
952 MeMSP1-OE *Arabidopsis* lines through RT-qPCR.

953 **Fig. S14** The expression of *MeMSP1* affects the oxidation-reduction balance in
954 *Arabidopsis*.

955 **Fig. S15** Three of five down-regulated metabolites shows a paralysis effect on
956 nematodes.

957 **Fig. S16** Down-regulated metabolites butin and naringenin in MeMSP1-OE
958 *Arabidopsis* lines shows a paralysis effect on nematodes.

959

960 **Table S1** Primers used in this study.

961 **Table S2** Potential targets of MeMSP1 in *Arabidopsis* identified by
962 immunoprecipitation (IP) followed by liquid chromatography-tandem mass
963 spectrometry (LC-MS) in both MeMSP-OE lines and not in control WT line.

964 **Table S3** Expression of AtGSTFs in giant cells and galls induced by root-knot
965 nematodes.

966 **Table S4** Differentially expressed genes (DEGs) in MeMSP1-OE-1 *Arabidopsis* line.

967 **Table S5** Differentially expressed genes (DEGs) in MeMSP1-OE-3 *Arabidopsis* line.

968 **Table S6** Differentially expressed genes (DEGs) in both MeMSP1-OE *Arabidopsis*
969 lines and their expression pattern in root-knot nematode-infected tissues

970 **Table S7** Differently accumulated metabolites (DAMs) in MeMSP1-OE-1
971 *Arabidopsis* line.

972 **Table S8** Differently accumulated metabolites (DAMs) in MeMSP1-OE-3
973 *Arabidopsis* line.

974 **Table S9** Amino acids detected in MeMSP1-OE *Arabidopsis* lines.

975

Figure legends

Table 1 Differentially accumulated metabolites shared by MeMSP1-OE-1 and MeMSP1-OE-3 *Arabidopsis* lines

Class	Compounds	LogFC(MeMSP1-OE-1)	LogFC(MeMSP1-OE-3)	Type
Amino acid derivatives	3-Chloro-L-tyrosine	1.13	1.01	up
	Glutathione reduced form (GSH)	1.49	3.00	up
	S-(methyl)glutathione	1.42	1.61	up
	γ -Glu-Cys	1.55	2.60	up
	CYS-GLY	2.53	3.26	up
	L-Alanine	1.67	1.31	up
Nucleotide and its derivatives	N-Methylnicotinamide	-1.35	-1.43	down
	Adenosine 3'-monophosphate	2.71	1.49	up
	Inosine 5'-monophosphate	2.18	1.47	up
	Adenosine 5'-monophosphate	1.95	1.07	up
Organic acids	Argininosuccinate	1.37	1.72	up
	Diethyl phosphate	1.72	1.40	up
Quinate and its derivatives	O-Sinapoyl quinic acid	1.65	1.31	up
Tryptamine derivatives	N-Feruloyl serotonin	2.15	1.06	up
	6-Hydroxymelatonin	-1.23	-2.47	down
Vitamins	Pyridoxal 5'-phosphate	1.47	1.88	up
Hydroxycinnamoyl derivatives	1-O-beta-D-Glucopyranosyl sinapate	1.44	1.11	up
Lipids Glycerophospholipids	LysoPC 16:2	1.23	2.25	up
	Others	O-Phosphorylethanolamine	1.11	1.29
Anthocyanins	Pelargonin	2.11	1.44	up
Proanthocyanidins	Procyanidin A3	1.34	1.13	up
Catechin derivatives	(+)-Gallocatechin (GC)	1.25	1.14	up
Coumarins	N-sinapoyl hydroxycoumarin	1.35	1.26	up
	O-Feruloyl 4-hydroxycoumarin	1.44	1.60	up
Flavanone	Naringenin	-2.10	-3.90	down
	Naringenin chalcone	-15.85	-15.85	down
Flavone	Chrysoeriol O-rhamnosyl-O-glucuronic acid	-2.34	-4.11	down
	Acacetin	15.16	15.52	up
	Butin	-15.80	-15.80	down
Flavone C-glycosides	Chrysin C-hexoside	-2.29	-2.31	down
	di-C,C-hexosyl-apigenin	-2.22	-2.33	down
	8-C-hexosyl-luteolin O-hexoside	-1.59	-1.15	down
Flavonol	Quercetin 3-alpha-L-arabinofuranoside (Avicularin)	2.30	2.65	up
Flavonolignan	Tricin 4'-O-(β -guaiaacylglyceryl) ether O-hexoside	-2.72	-2.36	down
	Tricin 4'-O-syringyl alcohol	-1.29	-11.44	down
Phenolamides	N', N''-di-p-coumaroyl spermidine	-14.85	-14.85	down
	N-sinapoyl cadaverine	-1.23	-14.31	down
	N-Sinapoyl agmatine	1.46	1.34	up
Magenta background	Significantly upregulated accumulation			
Green background	Significantly downregulated accumulation			

Fig. 1 *MSP1* is a conserved effector in root-knot nematodes. (a) Alignment of MeSP1-like proteins from different *Meloidogyne* species using ClustalW2. Identical amino acids residues are highlighted against black background shading, highly similar (>75%) amino acid residues are shown in pink background shading. (b) Maximum likelihood phylogenetic tree of MSP1 sequences. Numbers at tree nodes represent bootstrap support values. Support for the nodes was calculated with a hundred bootstrap replicates. MiMSP12 was used as outgroup.

Fig. 2 MeMSP1 is a dorsal gland protein secreted in the giant cells. (a) Localisation of MeMSP1 in the dorsal glands of *M. enterolobii* parasitic J2s through *in situ* hybridisation. Fixed J2s were hybridised with antisense cDNA probes from MeMSP1 (Figure S1). (b) No signal has been observed when the sense probe was used as a negative control. (c-e) Immunolocalisation on parasitic nematodes extracted from infected root at 14 dpi. The use of anti-MeMSP1 antibodies showed the specific production of MeMSP1 in the dorsal gland of parasitic nematodes (c) and its delivery in the oesophagus (d) of parasitic nematodes. No signal was observed when pre-immune serum was used as a negative control (e). (f-i) Immunolocalisation in sectioned tomato root galls at 14 dpi. Secreted MeMSP1 protein signal were detected in the cytoplasm of the giant cells (f, g, h). No signal was observed when pre-immune serum was used as a negative control (i). Micrographs (c-e) are overlay of images of the bright and Alexa Fluor 488 fluorescence. Micrographs (f-i) are merge of images of the Alexa Fluor 488-conjugated secondary antibodies, DAPI-stained nuclei and differential interference contrast. N, nematode; Asterisks *, giant cells; Red arrow, signals of Alexa Fluor 488 fluorescence; M, metacarpus. The dashed white line marks the outline of a giant cell. Scale bars: (a and, b) 20 μm , (c, d, e and i) 100 μm , (f and h) 75 μm or (g) 50 μm .

Fig. 3 *In planta* RNA interference (RNAi) and ectopic expression of *MeMSP1* in *Arabidopsis* shows MeMSP1 participated in the parasitism of *M. enterolobii*. (a) The relative expression level of *MeMSP1* in *M. enterolobii* collected from three

independent, homozygous *MeMSP1-RNAi* lines (MeMSP1-Ri-1, -2 and -3), *GFP-RNAi* line (GFP-Ri) and wild type (COL) Arabidopsis. Error bars represent +/- SD. (b) *In planta* RNAi of *MeMSP1* reduced *M. enterolobii* infection. Galls, nematodes (all stages *in planta*) and adult females (n=15) were counted at 30 dpi. The experiments were performed three times with similar results. (c) *MeMSP1* overexpression in Arabidopsis increased susceptibility to *M. enterolobii*. Galls, nematodes (all stages *in planta*) and adult females (n=15) were counted at 30 dpi in two independent, homozygous *MeMSP1* overexpressing lines (MeMSP1-OE-1 and -3), empty vector control line (super1300) and wild type (COL) Arabidopsis. The experiments were performed three times with similar results and the results of the other two tests were shown in Fig. S6. (b, c, d) Boxes indicate interquartile range (25–75th percentile). The central lines within the boxes represent medians. Whiskers represent extreme values that are not outliers. The “+” in the boxes represent average values. The black dots outside the box represents outliers. Different letters indicate statistically significant difference in one-way ANOVA with Dunnett's multiple comparisons test ($P < 0.05$).

Fig. 4 MeMSP1 interacts with all the AtGSTF members from Arabidopsis *in vivo* and *in planta*. (a) Co-immunoprecipitation was used to verify the interaction between AtGSTF family members and MeMSP1. WB (western blotting) assay confirmed expressions of input proteins: Flag-AtGSTFs, Flag-GFP (anti-Flag antibodies) and MeMSP-HA (anti-HA antibodies). In the samples after immunoprecipitation, MeMSP1-HA was detected when co-expressing with Flag -AtGSTFs, but not when co-expressing with Flag-GFP. (b) Bimolecular fluorescence complementation (BiFC) visualization of the interaction between some AtGSTFs and MeMSP1. Images were obtained 48 h after co-infiltration in *N. benthamiana* leaves. YFP, yellow fluorescent protein. Scale bar, 100 μ m. BiFC images of the interaction between MeMSP1 and all the members of AtGSTF were provided in supplement Fig. S9.

Fig. 5 The N-terminal of AtGSTF9 is essential to their interaction and AtGSTF9 has a role in plant immunity to nematodes. (a) BiFC visualization of the interactions between

the mutated AtGSTF9 and MeMSP1. The corresponding proteins were co-expressed in tobacco leaves. Images were obtained 48 h after co-expression. Scale bar, 100 μm . (b) The *Arabidopsis gstf9* mutants are more susceptible to *M. enterolobii* than the WT. Galls and nematodes ($n \geq 22$) were counted at 30 dpi. The experiments were performed two times with similar results and the result of the other test was shown in Fig. S10. Boxes indicate interquartile range (25–75th percentile). The central lines within the boxes represent medians. Whiskers represent extreme values that are not outliers. The “+” in the boxes represent average values. Different letters indicate statistically significant difference in one-way ANOVA with Dunnett's multiple comparisons test ($P < 0.05$).

Fig. 6 MeMSP1 affects the expression of metabolic-related genes in *Arabidopsis*. (a, b) Venn diagram showing overlap between two MeMSP1 transgenic lines differentially expressed genes compared to Col-0. (c) KEGG classification of differentially expressed genes shared by two MeMSP1 transgenic lines. The ordinate represents the KEGG pathway, and the abscissa represents number of genes.

Fig. 7 MeMSP1 affect the metabolic pathway and biosynthesis of secondary metabolites pathway in *Arabidopsis*. (a, b) Venn diagram showing overlap between two MeMSP1 transgenic lines differentially expressed metabolites compared to Col-0. (c) KEGG classification of differentially expressed metabolites shared by two MeMSP1 transgenic lines. The ordinate represents the KEGG pathway, and the abscissa represents number of metabolites.

Fig. 8 Down-regulated metabolites butin and naringenin in MeMSP1-OE *Arabidopsis* lines shows a paralysis effect on nematodes. (a) Butin and naringenin show a paralysis effect on nematodes at different concentration and time. CK, control check which consisted of the corresponding concentration of DMSO. Boxes indicate interquartile range (25–75th percentile). The central lines within the boxes represent medians. Whiskers represent extreme values that are not outliers. The “+” in the boxes represent

average values. * indicate statistically significant difference in one-way ANOVA with Dunnett's multiple comparisons test ($P < 0.05$). The experiments were performed three times with similar results and the results of the other two tests were shown in Fig. S16. (b) The nematodes pictures from CK treatment at 48 h. (c) Picture of nematode treated with 50 μ g/ml butin for 48 hours. (d) Picture of nematodes treated with 50 μ g/ml naringenin for 48 hours. (c, d) Red arrow, paralysed/dead nematode. Scale bars: (b, c, d) 1000 μ m.

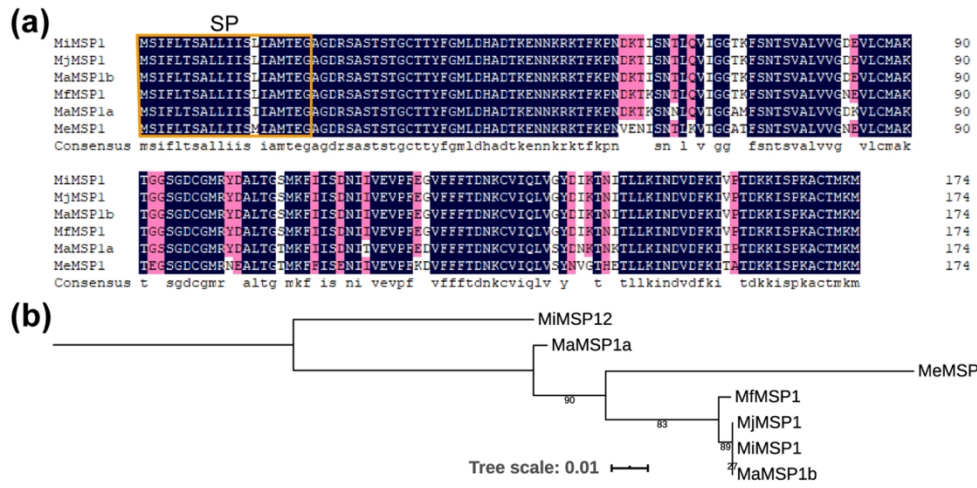


Fig 1

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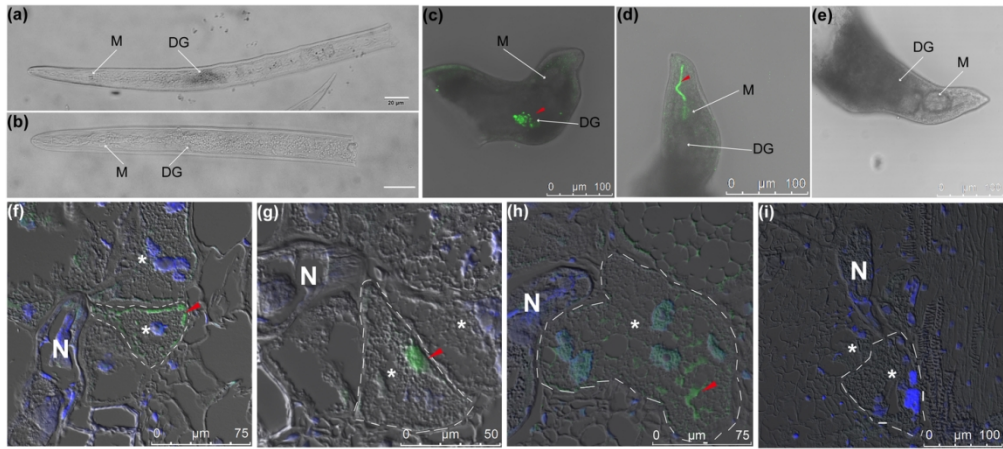


Fig 2_new_R3

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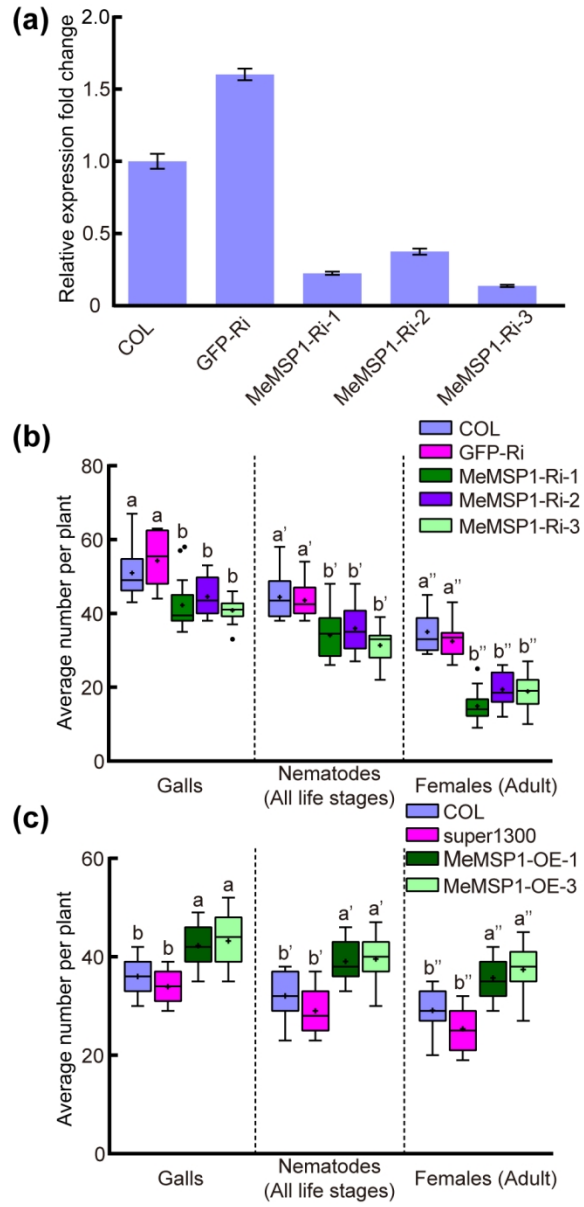


Fig 3

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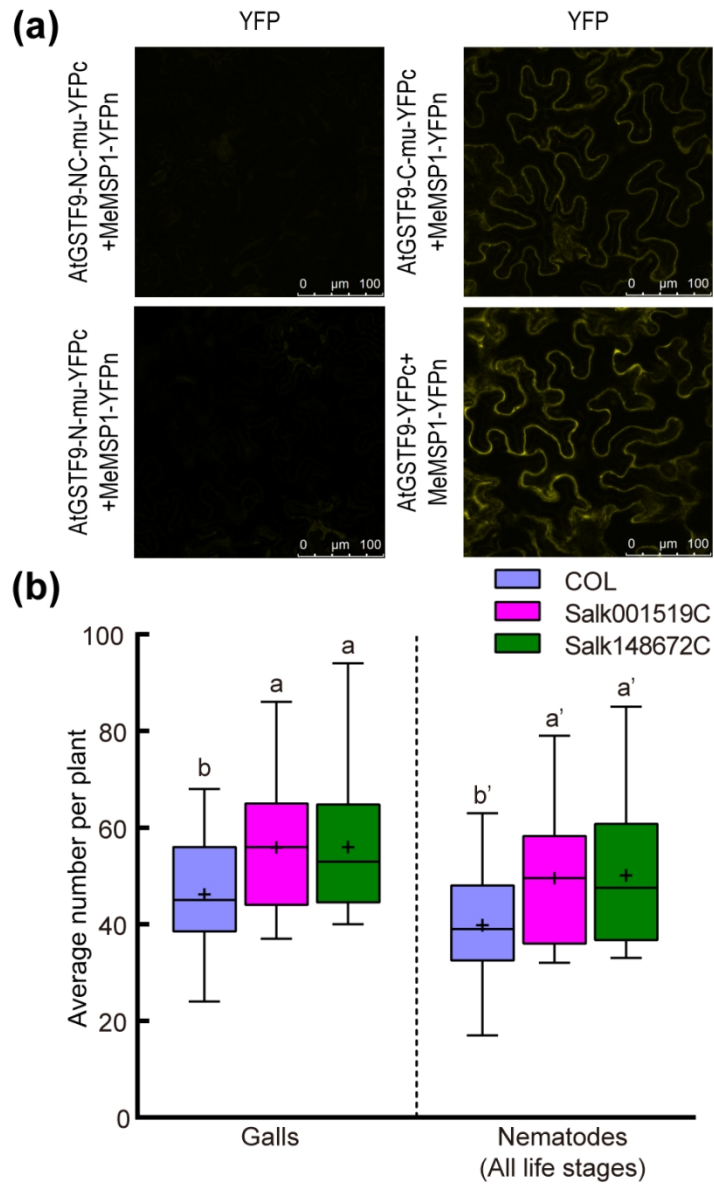


Fig 5

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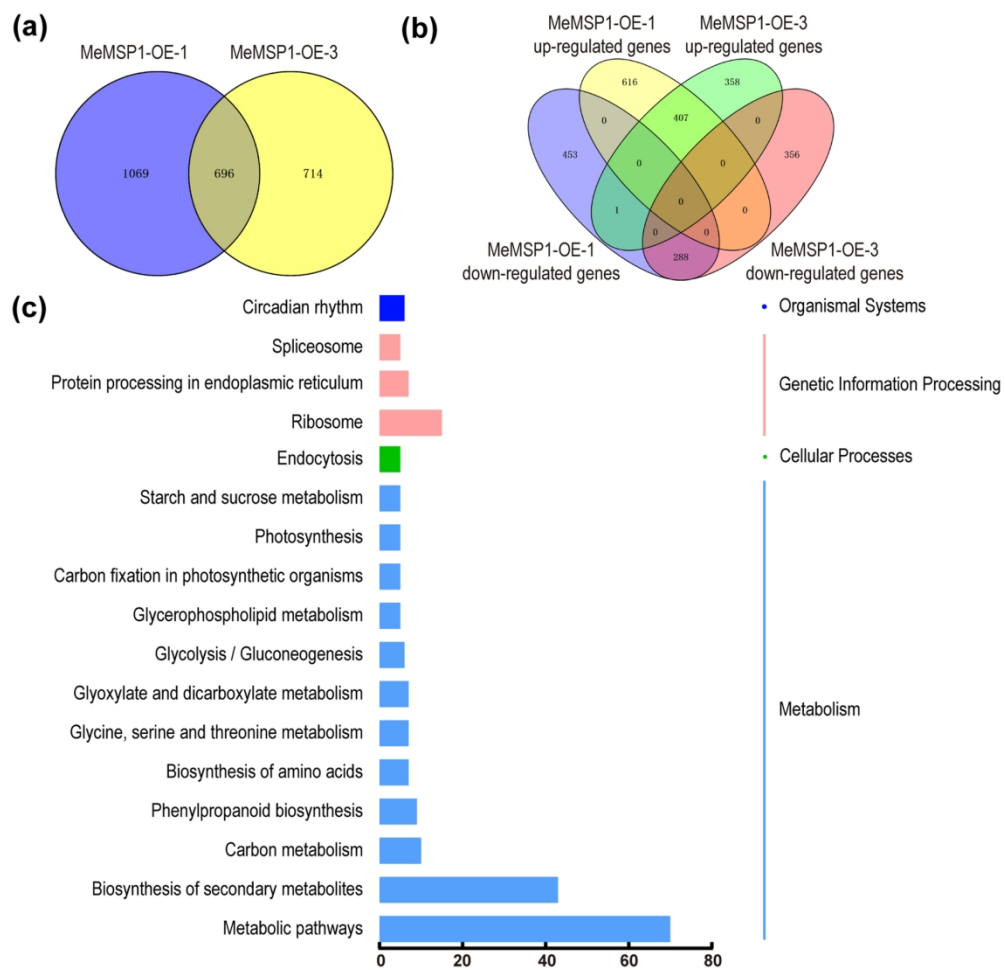


Fig 6

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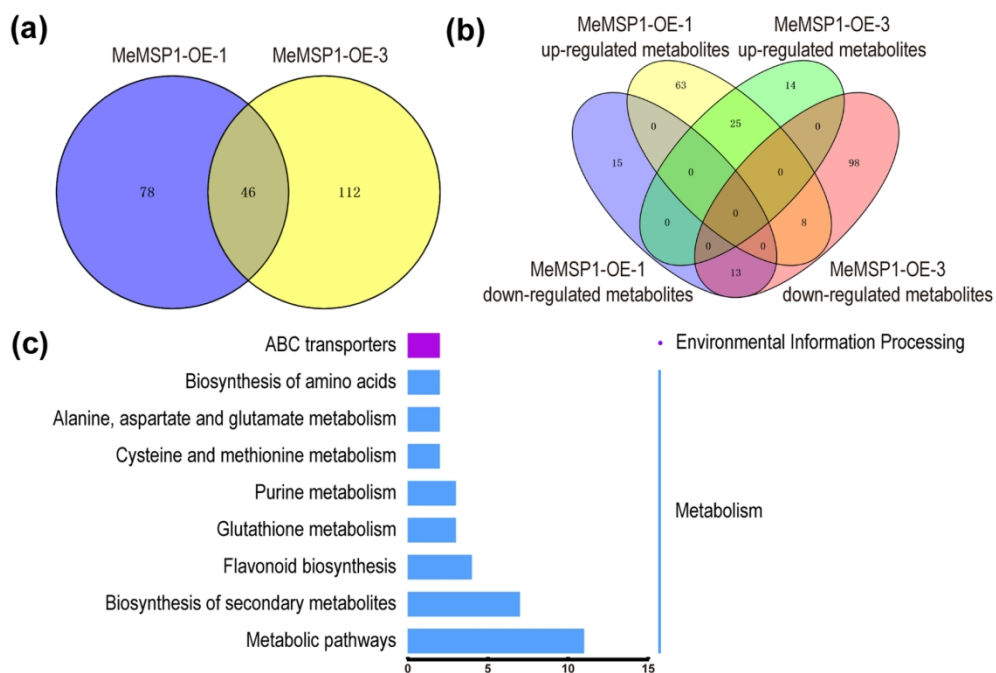


Fig 7

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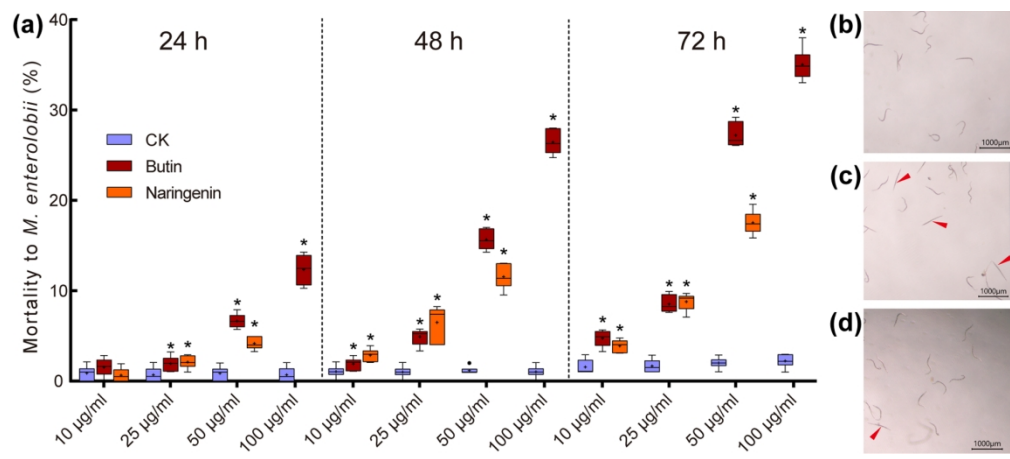


Fig 8

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