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1 **The root-knot nematode effector MiPDI1 targets a stress-associated protein, SAP, to**
2 **establish disease in Solanaceae and *Arabidopsis***

3

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43 **Summary**

- 44 ● Large amounts of effectors are secreted by the oesophageal glands of plant-parasitic
45 nematodes, but their molecular mode of action remains largely unknown. We characterised
46 a *Meloidogyne incognita* protein disulphide isomerase (PDI)-like effector protein
47 (MiPDI1) that facilitates nematode parasitism.
- 48 ● *In situ* hybridisation showed that *MiPDI1* was expressed specifically in the subventral
49 glands of *M. incognita*. It was significantly upregulated during parasitic stages.
50 Immunolocalisation demonstrated MiPDI1 secretion *in planta* during nematode migration
51 and within the feeding cells. Host-induced silencing of the *MiPDI1* gene affected the
52 ability of the nematode to infect the host, whereas *MiPDI1* expression in *Arabidopsis*
53 increased susceptibility to *M. incognita*, providing evidence for a key role of MiPDI1 in *M.*
54 *incognita* parasitism.
- 55 ● Yeast two-hybrid assays, BiFC and Co-IP showed that MiPDI1 interacted with a tomato
56 stress-associated protein (SISAP12) orthologous to the redox-regulated AtSAP12, which
57 plays an important role in plant responses to abiotic and biotic stresses. *SAP12* silencing or
58 knocking out in *N. benthamiana* and *Arabidopsis* increased susceptibility to *M. incognita*.
- 59 ● Our results suggest that MiPDI1 acts as a pathogenicity factor promoting disease by fine-
60 tuning SAP-mediated responses at the interface of redox signalling, defence and stress
61 acclimation in Solanaceae and *Arabidopsis*.

62

63

64 **Key words:** plant-parasitic nematodes, effector, stress, redox, giant cells

66 **Introduction**

67 Plant parasitic nematodes are among the most economically devastating plant pathogens,
68 causing global yield losses of more than 100 billion dollars each year (Abad *et al.*, 2008). The
69 obligate sedentary endoparasitic nematodes causing the most severe problems are root-knot
70 nematodes (RKNs) and cyst nematodes (CNs). The RKNs, *Meloidogyne* spp., can infest more
71 than 5,500 crop species (Blok *et al.*, 2008), and therefore represent a huge threat to agricultural
72 production. After hatching, the infective second-stage juveniles (J2s) are attracted to the tip of
73 plant roots. They penetrate the root elongation zone and migrate between cells to reach the
74 vascular cylinder of the plant. There, they become sedentary, and construct a feeding site. This
75 feeding site consists of around seven multinucleate giant cells, resulting from nuclear divisions
76 and isotropic growth, surrounded by cells that divide and initiate vascular differentiation. The
77 giant cells are the sole source of nutrients for the developing nematode (Favery *et al.*, 2016).
78 RKNs ensure that parasitism is successful by secreting a large number of effectors that help
79 juveniles to invade roots, suppress plant defence mechanisms, and induce and maintain giant
80 cells. These effectors are mostly produced in the three oesophageal glands, but they may also be
81 secreted by other organs, such as the amphids and hypodermis (Mejias *et al.*, 2019; Vieira &
82 Gleason, 2019). The precise localisation of effectors and the identification of their host targets
83 are essential for a better understanding of their biological functions. Only a few host target
84 proteins of RKN effectors have been identified to date (Mejias *et al.*, 2019). The *M. incognita*
85 effector Mi16D10 has been shown to interact with *Arabidopsis* SCARECROW-like
86 transcription factors (Huang *et al.*, 2006). The *M. graminicola* effector MgMO237 has been
87 shown to interact with three rice defense-related proteins (OsGSC, OsCRRSP55 and OsBetv1)
88 (Chen *et al.*, 2018) and the Mg16820 and *M. chitwoodi* Mc01194 effectors interact with rice
89 dehydration-stress inducible protein 1 (OsDIP1) and *Arabidopsis* papain-like cysteine protease
90 (RD21A), respectively (Davies *et al.*, 2015; Naalden *et al.*, 2018). A MACROPHAGE
91 MIGRATION INHIBITORY FACTOR-like effector, MiMIF-2, was recently shown to interact
92 with two *Arabidopsis* annexins, mediating plant immune responses (Zhao *et al.*, 2019).
93 However, the functions of a large number of RKN effectors, their plant targets and working
94 mechanisms remain unknown and in need of clarification.

95 Protein disulphide isomerase (PDI) is a protein thiol oxidoreductase located in the eukaryotic
96 endoplasmic reticulum. PDI has both protein disulphide isomerase and protein-glutamine
97 gamma-glutamyltransferase activities. It is involved in the oxidoreduction and isomerisation of
98 protein disulphide bonds, peptidyl-proline hydroxylation to 4-hydroxy-L-proline, and protein
99 deglutathionylation (Ali Khan & Mutus, 2014). More than 50 PDI-like proteins have been
100 identified in fungi, plants, animals and humans. The free-living nematode *Caenorhabditis*
101 *elegans* has three conserved *PDI*-like genes, *PDI-1*, *PDI-2*, and *PDI-3*, and *PDI-2* has been
102 shown to be expressed in the hypodermis and cuticle collagen (Winter & Page, 2000). Many
103 animal parasites, including protozoans in particular, have PDI-like proteins that play major roles
104 in parasitism (Achour *et al.*, 2002; Han *et al.*, 2014). Three PDI-like proteins in the tick
105 *Haemaphysalis longicornis* have been shown to be expressed predominantly in the salivary
106 glands, and blood feeding significantly increases the expression of *HIPDI-1* and *HIPDI-3* (Liao
107 *et al.*, 2007). There are also many PDI-like proteins in malaria parasites. *Plasmodium*
108 *falciparum* PfPDI-8 has specific enzyme activity and facilitates the disulphide-dependent
109 conformational folding of a malaria protein (Mahajan *et al.*, 2006).

110 Secreted PDIs have also recently been characterised in plant pathogens. The PDI1 protein of
111 *Phytophthora parasitica* (PpPDI1) is associated with haustoria-like structures and contributes to
112 plant infection (Meng *et al.*, 2015). Two PDI-like effectors have been identified in RKNs and
113 CNs, shown to be expressed in oesophageal glands and upregulated in late parasitic J2s or J3. A
114 functional analysis of *Heterodera schachtii* HsPDI showed that this protein promoted
115 parasitism by regulating the plant ROS burst (Habash *et al.*, 2017). Recombinant *M.*
116 *graminicola* MgPDI protein had oxidase and isomerase activities. The expression of both
117 *MgPDI* and *HsPDI* was induced by exogenous H₂O₂, suggesting that PDI may protect
118 nematodes from oxidative stress (Tian *et al.*, 2019). Interestingly, PDI-like proteins were
119 identified in the secretome of *M. incognita* juveniles (Bellafiore *et al.*, 2008) and the proteome
120 of oesophageal gland cells from *M. incognita* females (Wang *et al.*, 2012), demonstrating that
121 these proteins are secreted by *M. incognita*. However, the detailed mode of action and plant
122 targets of PDI-like effectors remain unknown.

123 In this study, we characterised a new *PDI-like* gene from *M. incognita* cDNA (*MiPDI1*) that is
124 expressed in the subventral oesophageal glands. Immunohistochemical staining of tomato root
125 sections with an anti-MiPDI1 antibody showed that this protein was secreted into infected roots.
126 Our findings also showed that MiPDI1 was important for nematode infection. Yeast two-hybrid
127 assays, BiFC and Co-IP showed that MiPDI1 interacted physically with a stress-associated
128 protein (SISAP12) from tomato. Virus-induced gene silencing (VIGS) of orthologues in
129 knockout lines of *N. benthamiana* and *Arabidopsis* resulted in higher levels of *M. incognita*
130 infection. Moreover, MiPDI1 expression *in planta* affects the expression of *Arabidopsis*
131 defence-associated genes. Our data suggest that MiPDI1 may act as a novel effector, promoting
132 *M. incognita* parasitism by fine-tuning SAP-mediated host responses.

133

134 **Materials and methods**

135 **Nematodes and plant materials**

136 Egg masses of *M. incognita* (Morelos strain) were collected from tomato plants (*Solanum*
137 *lycopersicum* cv St Pierre). The hatched preparasitic second-stage juveniles (pre-J2s) were
138 collected for plant inoculation. Various *M. incognita* stages were isolated from digested tomato
139 roots, as previously described (Zhao *et al.*, 2019). Surface-sterilised *Arabidopsis thaliana* seeds
140 (ecotype Col-0) were sown on Murashige and Skoog (MS) medium in sterile conditions. After
141 germination, the plantlets were transplanted into pots containing soil and sand (1:1) and grown
142 at 21°C. The T-DNA mutant line for *AtSAP12* (*At3g28210*) (*SALK_014706*) was obtained from
143 the *Arabidopsis* Biological Resource Center (ABRC, USA). *Nicotiana benthamiana*, *Solanum*
144 *lycopersicum* and *N. tabacum* plants were grown at 24°C (photoperiod, 16 h : 8 h, light : dark).

145

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

147 MiPDI sequences were obtained from *Meloidogyne* genomic resources
148 (http://www6.inra.fr/meloidogyne_incognita/). Sequences were aligned with the MAFFT tool
149 on the EBI server (<https://www.ebi.ac.uk/Tools/msa/mafft/>). The alignment obtained was used
150 as input for the IQTree Web server (<http://iqtree.cibiv.univie.ac.at/>) (Trifinopoulos *et al.*, 2016),

151 to generate the maximum likelihood phylogenetic tree. The model chosen by the inbuilt model
152 test was LG+F+I+G4. Support for the nodes was calculated with a hundred bootstrap replicates.
153 PpPDI was used as the outgroup. The tree was visualised in iTOL (<https://itol.embl.de/>).

154

155 **RNA extraction and real-time quantitative RT-qPCR**

156 A Dynabeads® mRNA DIRECT™ kit (Invitrogen, USA) was used to extract mRNA from *M.*
157 *incognita*. Arabidopsis, tomato and tobacco total RNA were extracted with TRIzol Reagent
158 (Invitrogen, USA). We synthesised cDNA with the reverse transcriptase SuperScript III
159 (Invitrogen, USA), in accordance with the manufacturer's instructions. RT-qPCR was
160 performed with an ABI Prism 7000 (Applied Biosystems, USA) real-time PCR system (the
161 primers are shown in Table S1). *M. incognita* *GAPDH* (*Minc12412*) and *HK14* (*Minc18753*) or
162 *A. thaliana* *OXA1* (AT5G62050) and *UBP22* (AT5G10790) were used as internal controls for
163 the normalisation of RT-qPCR data. PCR was performed with SYBR Premix Ex Taq (TaKaRa,
164 Japan), as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 31 s, and
165 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. Data were analysed by the $2^{-\Delta\Delta C_t}$ method (Livak
166 & Schmittgen, 2001). Three technical replicates for two to four independent biological
167 experiments were performed.

168

169 ***In situ* hybridisation (ISH) of *MiPDI1* and immunolocalisation in preparasitic J2s**

170 ISH was performed on freshly hatched *M. incognita* preparasitic J2s (pre-J2s), as previously
171 described (Jaouannet *et al.*, 2018). The *MiPDI1* was amplified with the specific primers ISH-
172 PDI-F and -R (Table S1). Immunolocalisation was performed as previously described (Jaubert
173 *et al.*, 2002; Jaubert *et al.*, 2005). A polyclonal antibody against MiPDI1 was produced with a
174 specific peptide (KEGSPPSENSLDDLVE). Western blotting was performed to check the
175 specificity of this antibody. Immunolocalisation was performed directly on *M. incognita* pre-
176 parasitic J2s with the anti-MiPDI1 antibody (1:300) and the Alexa Fluor 488-conjugated goat
177 anti-rabbit antibody (1:500) (Molecular Probes, Eugene). As a negative control, nematodes
178 were incubated with pre-immune serum. Images were collected with a confocal microscope

179 (Zeiss LSM880, Germany).

180

181 **Immunohistochemistry on tomato gall sections**

182 Tomato galls were collected 5 and 10 days post infection (dpi) with *M. incognita*, cut into small
183 pieces with a razor blade in a Petri dish and fixed in 8% formaldehyde in 50 mM piperazine-N,
184 N'-bis (ethanesulphonic acid) (PIPES) buffer (pH 6.9). The immunolocalisation procedure was
185 performed essentially as previously described (de Almeida Engler *et al.*, 2004). Galls were
186 dehydrated and embedded in butyl-methylmethacrylate and sections were incubated in acetone
187 for 30 min to remove the butyl-methylmethacrylate. Slides containing 5 μ m-sectioned galls
188 were then treated with a series of ethanol solutions and incubated in a blocking solution of 1%
189 BSA. Sections were subsequently incubated with anti-MiPDI1 antibody (1:300) at 4 °C
190 overnight and then at 37 °C for 2 hours in a damp box. As a negative control, gall sections were
191 incubated with pre-immune serum. Finally, slides were incubated with Alexa Fluor 488-
192 conjugated goat anti-rabbit antibody (Molecular Probes, Eugene) and cell nuclei were stained
193 with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma, USA). Images were captured at an
194 excitation wavelength of 488 nm by confocal microscopy (Zeiss LSM880, Germany).

195

196 ***In planta* RNAi, DEX-induced *MiPDII* expressing Arabidopsis lines and RKN infection** 197 **assay**

198 *MiPDII* fragments (forward and reverse) were amplified with primers (Table S1) inserted
199 separately into the forward and backward sequences of the pSAT5 intron of the pSuper-RNAi
200 vector (Dafny-Yelin *et al.*, 2007). The *MiPDII* coding sequence (cds) without the signal
201 peptide sequence for secretion (MiPDI1 woSP) was amplified (Table S1) and inserted into the
202 PTA7001 vector (a gift from Professor Wenxian Sun, China Agricultural University) to
203 generate PTA7001-MiPDI1-FLAG. These vectors were used to transform *Agrobacterium*
204 *tumefaciens* GV3101, which was in turn used to transform *A. thaliana* Col-0 (WT) by the floral
205 dip method (Zhang *et al.*, 2006). Homozygous T3 plants from three pdi-Ri lines and two
206 MiPDI-FLAG lines were used. Lines were verified by western blotting after induction with 30

207 μM dexamethasone (DEX). The homozygous *gfp*-Ri T3 lines have been described elsewhere
208 (Zhao *et al.*, 2019). *Arabidopsis* plants were inoculated with 300 pre-J2s of *M. incognita*. The
209 RNAi effect was assessed 10 dpi by RT-qPCR. *Arabidopsis* roots were then collected and
210 washed carefully at 35 dpi. Nematodes (parasitic juveniles at any stages and females) were
211 stained by the sodium hypochlorite-acid fuchsin method (Bybd *et al.*, 1983). Nematodes, galls
212 and egg masses were counted under a stereomicroscope microscope (Olympus, Japan). Three
213 independent replicates were performed for each experiment, with counting on 30 plants of each
214 line in each replicate.

215

216 **Subcellular localisation and bimolecular fluorescence complementation (BiFC)**

217 *MiPDI1* woSP was amplified by PCR with specific Gateway primers (Table S1) and inserted
218 into the pDONR207 donor vector. It was inserted into the pK7WGF2 (N-terminus eGFP)
219 vector by recombination, with Gateway technology (Invitrogen). The *SICYP* woSP and
220 *SISAP12* ORFs were inserted into the pK7WGR2 (N-terminus RFP) vector by recombination.
221 For the BiFC assay, *MiPDI1* was inserted into YFPn-, and *SISAP12* and *SICYP* were inserted
222 into YFPc-vectors (Walter *et al.*, 2004). The constructs were used to transform *A. tumefaciens*
223 strain GV3101 or GV3301. Leaves from three- to four-week-old *N. tabacum* plants were
224 subjected to agroinfiltration with recombinant strains of *A. tumefaciens*, as described by Zhao
225 *et al.* (2019). Images were captured by confocal microscopy (Zeiss LSM880, Germany) at an
226 excitation wavelength of 488 nm for GFP or YFP and 561 nm for RFP.

227

228 **Yeast two-hybrid (Y2H) and co-immunoprecipitation (Co-IP) assay**

229 For the Y2H screens, *MiPDI1* woSP or a mutated version, *MiPDI1*-mu (the first CGHC
230 activation site mutated to CGHG), was amplified (Table S1) and inserted into pB27 as a C-
231 terminal fusion to LexA. The constructs were verified by sequencing and used to transform a
232 L40 Δ Gal4 (*mata*) yeast strain. These baits were used to screen a random-primed cDNA library
233 made from tomato roots infected with *M. incognita* and *Ralstonia solanacearum* in the Y187
234 (*mata*) yeast strain (Hybrigenics Services, Paris, France), by a mating approach. Diploids

235 displaying interactions were selected on a medium lacking tryptophan, leucine and histidine.
 236 The prey fragments of the positive clones were amplified by PCR and sequenced. The resulting
 237 sequences were used to identify the tomato interacting proteins with the Sol Genomics Network
 238 (<https://solgenomics.net/>) blast analysis tools. Pairwise Y2H assays were conducted following
 239 the instruction of Clontech protocol (Clontech, USA). Briefly, the *MiPDII* coding region
 240 without the predicted signal peptide sequence was cloned into the pGBKT7 vector as the bait.
 241 The sequences encoding the SAP12 (SISAP12, NbSAP12 and AtSAP12) were cloned into the
 242 pGADT7 vector as the preys. After co-transformation of yeast (Y2HGOLD) and screening on
 243 SD/-Leu-Trp plates, positive clones were verified and selected to grow on SD/-Leu-Trp-His
 244 medium with 0.5 mM 3-Amino-1,2,4-Triazole (3AT).

245 For Co-IP assays, the *MiPDII*, *SISAP12*, and *SICYP* sequences were inserted into the PVX
 246 vector pGR107 with a FLAG-tag fused at the N-terminus (FLAG-MiPDII) or an HA-tag fused
 247 at the C-terminus (SISAP12-HA and SICYP-HA) (Zhao *et al.*, 2019) and the resulting
 248 constructs were used to transform *A. tumefaciens* GV3101, which was then used to transform
 249 *N. tabacum* leaves. Total proteins were extracted from *N. tabacum* leaves. Co-IP was
 250 performed with anti-FLAG M2 affinity gel resin (Sigma-Aldrich, USA), according to the
 251 manufacturer's instructions. Briefly, the gel resin was washed twice with 1 ml ice-cold PBS.
 252 We then immediately added 1 ml of protein solution and incubated the mixture at 4°C for 4 h.
 253 The resin was thoroughly washed 5 times with 1 ml of ice-cold PBS, and then the proteins were
 254 eluted for western blot analysis. Anti-FLAG antibody (1:5000) and anti-HA (1:5000)
 255 antibodies (MBL, Japan) were incubated with the blot for protein detection.

256

257 **Virus-induced gene silencing (VIGS)**

258 VIGS assays were performed on *N. benthamiana*. The *N. benthamiana* orthologues of *SISAP12*
 259 and *SICYP* were identified in https://solgenomics.net. Regions for targeted gene silencing of
 260 *NbSAP12_Niben101Scf06280g06001/Niben101Scf06013g06013* and
 261 *NbCYP_Niben101Scf12813g00004* were identified with the Sol Genomics Network VIGS-Tool
 262 (Fernandez-Pozo *et al.*, 2015). Specific fragments were amplified by PCR with the primer pairs

263 TRV2-SAP12-F/TRV2-SAP12-R, and TRV2-CYP-F/TRV2-CYP-R (Table S1). The PCR
264 products were digested with *EcoRI* and *XhoI*, and ligated to the tobacco rattle virus RNA 2
265 vector (TRV2) for transformation of the *A. tumefaciens* strain GV3101. VIGS assays were
266 performed as previously described, by the infiltration of agrobacterial strains containing RNA 1
267 vector (TRV1) or TRV2 into leaves (Velasquez *et al.*, 2009; Lange *et al.*, 2013). The phytoene
268 desaturase (*PDS*) gene was used as a positive control to check for successful gene silencing,
269 which results in typical photobleaching symptoms on young growing leaves. Empty TRV2 and
270 TRV1 were used as negative controls. One-week post-agroinfiltration, TRV-infected plants
271 were inoculated with 200 *M. incognita* J2s. One-week later, five plants were used for RT-qPCR
272 to assess the efficacy of gene silencing. Galls and females producing egg masses were counted
273 50 dpi.

274

275 **Protein extraction, SDS-PAGE and western blotting**

276 Total protein was extracted from agro-infiltrated *N. tabacum* leaves 2 dpi, with a protein
277 extraction kit (Beijing ComWin Biotech Co., Ltd., China), according to the manufacturer's
278 instructions. Briefly, 0.1 g of plant material was ground in liquid nitrogen and added to 0.5 ml
279 of protein extraction buffer supplemented with the protease inhibitor PMSF (Sigma). Samples
280 were kept on ice for 30 min, centrifuged at 12,000 rpm for 20 min at 4°C, and the protein was
281 obtained in the supernatant. Proteins were fractionated by sodium dodecyl sulphate
282 polyacrylamide gel electrophoresis (SDS-PAGE). For western blotting, an anti-MiPDI1 primary
283 antibody was added to TBST (TBS with 0.1% Tween 20, 1% nonfat dry milk) at a ratio of 1:
284 6000. The membrane was then incubated with a secondary antibody directed against GFP
285 (ABclonal, China), a FLAG-HRP antibody or an HA-HRP antibody (MBL, Japan), at a dilution
286 of 1: 10000. Proteins were detected with the eECL Western Blot Kit (Beijing ComWin Biotech
287 Co., Ltd., China).

288

289 **Statistical analysis**

290 The data were subjected to one-way ANOVA with Dunnett's multiple comparisons test or two-

291 way ANOVA with Tukey's multiple comparisons test. Statistical computations were carried out
 292 using GraphPad Prism (GraphPad Software Inc, La Jolla, CA, USA).

293

294 **Accession numbers**

295 All accession numbers are provided in Table S2. The GenBank accession numbers of *M.*
 296 *incognita* PDI sequences are MT370326 (MiPDI1), MT370324 (MiPDI2a) and MT370327
 297 (MiPDI2b).

298

299 **Results**

300 **Identification of secreted *M. incognita* PDIs**

301 Using *M. incognita* genome and transcriptome datasets (Abad *et al.*, 2008; Nguyen *et al.*, 2018),
 302 we identified MiPDI1 (Minc07853a / MT370326) and MiPDI2 (MiPDI2a / Minc12006 /
 303 MT370324 and MiPDI2b / Minc15047 / MT370327, which are 99% identical). Sequence
 304 analysis showed that MiPDI1 and MiPDI2s contained the PDI structures common to other PDI-
 305 like proteins: four thioredoxin (Trx) domains (two activation sites in the first and fourth
 306 domains) and a N-terminal signal peptide (Fig. 1a). MiPDI1 was identified in previous *M.*
 307 *incognita* J2 secretome and female proteome studies, whereas MiPDI2 was detected only
 308 among female oesophageal gland proteins (Bellafiore *et al.*, 2008; Wang *et al.*, 2012).

309 Multiple sequence alignment and phylogenetic analysis were performed with PDI protein
 310 sequences from *M. incognita*, *M. graminicola* (MgPDI), *H. schachtii* (HsPDI), the free-living
 311 nematode *Caenorhabditis elegans* (CePDI1, CePDI2 and CePDI3), and the animal parasites
 312 *Brugia malayi* (BmPDI) and *Phytophthora parasitica* PpPDI1 (Fig. 1b, c). Two subgroups were
 313 identified in the tree; MiPDI2a/b was found to be more similar to the recently described HsPDI
 314 and MgPDI, whereas MiPDI1 formed a subgroup with CePDI1, CePDI2 and BmPDI (Fig. 1c).
 315 These results suggest that MiPDI1 is a new PDI-like protein that could potentially act as an
 316 effector of *M. incognita*.

317

318 ***MiPDI1* is expressed in the subventral oesophageal glands and upregulated in the parasitic**

319 **stages of *M. incognita***

320 The distribution of *MiPDII* transcripts was investigated by ISH in preparasitic *M. incognita* J2s
321 (pre-J2s). A specific signal was detected in subventral oesophageal gland cells after
322 hybridisation with the digoxigenin-labelled *MiPDII* antisense probe (Fig. 2a; Fig. S1). No
323 signal was detected in pre-J2s with sense negative controls (Fig. 2b).

324 The transcription of *MiPDII* was analysed by RT-qPCR at various stages of *M. incognita*
325 development. *MiPDII* was significantly more strongly expressed in parasitic juveniles (Par-J 5
326 and 15 dpi) and females (Fig. 2c) than in pre-J2 and eggs. Together, these findings suggest that
327 *MiPDII* may play an important role in nematode parasitism in the plant.

328

329 ***MiPDII* is secreted into plant tissues during parasitism**

330 We performed immunolocalisation to determine whether and where *MiPDII* was secreted in
331 host roots. A specific anti-*MiPDII* antibody was produced and a single band was detected in
332 total protein samples from female pre-J2s, at the expected size of 57 kDa, corresponding to
333 *MiPDII* without its signal peptide, and from *N. tabacum* leaves expressing *MiPDII*-FLAG (Fig.
334 S2). No signal was detected in protein samples from uninfected tomato roots, tomato leaves, *N.*
335 *tabacum* and *N. benthamiana* leaves (Fig. S2). Immunolocalisation showed that the *MiPDII*
336 protein was present in the subventral glands of pre-J2s (Fig. 2d,e), consistent with the ISH
337 results. Immunohistochemistry on tomato gall sections at 5 and 10 dpi showed that *MiPDII* was
338 present in the parasitic juveniles, at the stylet tip, and in the apoplast at early stages of infection
339 (Fig. 2f,g; Fig. S3). In a number of gall sections containing nematodes, *MiPDII* was detected in
340 both the anterior part of the nematode, along the cell wall of adjacent giant cells and in the giant
341 cells (Fig. 2h,i,j; Fig. S3). No signal was observed in tomato gall sections incubated with pre-
342 immune serum (Fig. S3). These results provide evidence for the secretion of the *MiPDII*
343 effector *in planta* during parasitism.

344

345 **Host-derived RNAi silencing and ectopic expression of *MiPDII* shows the importance for** 346 ***M. incognita* parasitism**

347 We investigated the role of *MiPDII* in *M. incognita* parasitism, through the use of host-derived
348 RNAi to silence *MiPDII* in feeding nematodes. Three T3 homozygous *pdi-Ri Arabidopsis* lines
349 (*pdi-1*, *pdi-3* and *pdi-5*) transformed with the *MiPDII* hairpin dsRNA were obtained and
350 infected with *M. incognita*. RT-qPCR analysis of *MiPDII* in parasitic juveniles (10 dpi) showed
351 much lower levels of *MiPDII* expression (about 60%) in the three T3 *pdi-Ri* lines than in the
352 control (WT and *gfp-Ri*) lines (Fig. 3a). Four weeks post infection, *pdi-Ri* lines presented
353 significantly smaller numbers of galls and parasitic nematodes in the roots (Fig. 3b,c).

354 Two independent *Arabidopsis* lines ectopically expressing *MiPDII* were generated, in which
355 expression was induced by 30 μ M dexamethasone (DEX). *MiPDII*-expressing leaves showed
356 some necrosis spots after they were sprayed with DEX (Fig. S4). The susceptibility to nematode
357 infection of these two transgenic *Arabidopsis* lines was then determined. Both these transgenic
358 lines were more susceptible ($P < 0.05$) to *M. incognita* infection than wild-type *Arabidopsis*
359 (WT) after 35 dpi (Fig. 3d). Thus, the data for *MiPDII* silencing and overexpression provide
360 evidence for a key role of *MiPDII* in *M. incognita* parasitism.

361

362 **MiPDII interacts with SISAP12, a stress-associated AN1-type zinc finger protein**

363 We searched for host proteins interacting with *MiPDII* in two independent yeast two-hybrid
364 analyses. We used the *MiPDII* CDS without the signal peptide, or a form with a mutated first
365 active site as baits to screen a tomato root cDNA library. In total, 7.5 and 26.7 million
366 interactions were tested, respectively, leading to the identification of 13 proteins interacting
367 with *MiPDII* and six proteins interacting with the mutated form (Table S3). The preys captured
368 several times included two zinc finger proteins, a RING-type protein (Solyc03g026060), the
369 AN1-type Stress-Associated Protein 12 (SISAP12; Solyc02g087210), and two eukaryotic thiol
370 proteases or cysteine proteases displaying 72.4% identity (Solyc04g078540 and
371 Solyc12g088670). Interestingly, the SISAP12 protein and one cysteine protease
372 (Solyc04g078540, referred to hereafter as SICYP) were identified in both independent screens,
373 and were thus studied further. Reciprocal BLASTP showed that Solyc02g087210 was an
374 orthologue of AtSAP12. Both these small cysteine-rich proteins (10%) are AN1-type SAPs,

375 containing only two AN1 zinc-finger domains, and 16 conserved cysteine residues (Fig. S5).
376 Pairwise yeast two-hybrid assays with full-length proteins confirmed the interaction between
377 SISAP12 or SICYP and MiPDI1 in yeast (Fig. 4a). Subcellular localisation studies involving
378 agroinfiltration in *N. tabacum* showed a cytoplasmic localisation of RFP-SICYP and MiPDI1-
379 GFP and a nucleo-cytoplasmic localisation of RFP-SISAP12 in epidermal cells (Fig. 4b). We
380 also investigated the interaction of MiPDI1 with SICYP and SISAP12 *in planta* in bimolecular
381 fluorescence complementation (BiFC) assays. The co-expression of YFPn-MiPDI1 and
382 SISAP12-YFPc reconstituted YFP fluorescence signals in the cytoplasm of *N. tabacum*
383 epidermal cells (Fig. 4c, Fig. S6). We also performed co-immunoprecipitation (Co-IP)
384 experiments to check the interaction between MiPDI1 and SICYP or SISAP12. MiPDI1
385 interacted with SISAP12 in *N. tabacum*, further confirming the association of these two proteins
386 *in planta* (Fig. 4d). By contrast, no interaction was detected between MiPDI1 and SICYP, by
387 either BiFC or Co-IP (Fig. 4c,d). These results confirm the specific interaction of MiPDI1 with
388 SISAP12 in plant cells.

389

390 **SAP12 affects *M. incognita* parasitism**

391 We further investigated the role of SAP12 in mediating the response to *M. incognita*, by
392 silencing the *N. benthamiana* SAP12 genes (*Niben101Scf06280g06001* and
393 *Niben101Scf06013g06013* named *NbSAP12s*) or *CYP* (*Niben101Scf12813g00004*, named
394 *NbCYP*) by virus-induced gene silencing (VIGS) (Table S2, Fig. S7). Quantitative RT-PCR
395 analysis of the expression of the targeted homologous genes showed that *NbSAP12s* and
396 *NbCYP* were downregulated (55-60%) in the plants subjected to VIGS relative to control plants
397 (Fig. 5a; Fig. S7). *NbSAP12* silencing led to an increase in the number of females producing
398 egg masses relative to the empty TRV2 control, whereas no significant effect was observed
399 with *NbCYP* silencing (Fig. 5b). Finally, we tested a knockout T-DNA insertional mutant for
400 *AtSAP12* (*AT3G28210*) (Fig. S8). The number of females producing egg masses and the
401 number of galls were larger in homozygous *Arabidopsis* knockout (KO) *sap12* plants
402 (*Salk_014706*) than in wild-type plants (Fig. 5c). These results indicate that SAP12 proteins

403 may play a role in plant - *M. incognita* interaction in Solanaceae and *Arabidopsis*.

404

405 **MiPDI1 expression in *Arabidopsis* affects stress- and defence-associated gene expression**

406 We investigated the effects of *MiPDI1* expression and *sap12* mutation on stress tolerance in
 407 *Arabidopsis*. We analysed genes involved in antioxidative functions (*AtCSD1* and *AtCSD2*; (Ma
 408 *et al.*, 2015), and responses to abiotic (*AtCOR47*, *AtRAB18* and *AtDHI*; (Kothari *et al.*, 2016)
 409 and biotic (*AtEM6*, *AtPR1a*, *AtPDF1.2a*, *AtPR4* and *AtPR1a*; (Qiu *et al.*, 2008; Aslam *et al.*,
 410 2009; Rodiuc *et al.*, 2016; Kang *et al.*, 2017; Chang *et al.*, 2018) stresses in *Arabidopsis*. Four
 411 genes (*AtCSD2*, *AtEM6*, *AtRAB18* and the salicylic acid (SA) marker *AtPR1a*) were expressed
 412 less strongly in *MiPDI1*-overexpressing *Arabidopsis* plants, whereas the levels of expression of
 413 jasmonate (JA) and the ethylene markers PDF1.2a and PR4 were much higher in these plants
 414 (Fig. 5d). The expression of six genes (*AtCSD1*, *AtCSD2*, *AtDHI*, *AtPDF1.2a*, *AtPR4* and
 415 *AtPR1a*) was upregulated in the *sap12* KO line, whereas the stress-related genes *AtCOR47*,
 416 *AtRAB18* and *AtEM6* were downregulated (Fig. 5d). These results suggest that both *MiPDI* and
 417 its target may be responsible for regulating the expression of genes involved in stress responses
 418 to RKNs.

419

420 **Discussion**

421 The repertoire of putative nematode effectors is extremely large, and these molecules have been
 422 shown to manipulate many host plant functions to orchestrate the suppression of plant defences
 423 and the formation of specialised feeding cells (Mejias *et al.*, 2019). However, few data are
 424 available concerning the functions of effectors and few plant targets have been characterised,
 425 particularly for RKN effectors. Analyses of the secretomes of plant-parasitic nematodes and
 426 animal-parasitic nematodes have provided compelling evidence for the secretion of redox-
 427 regulated proteins, such as Trx, glutathione peroxidases, glutathione-S-transferases and PDIs
 428 (Bellafiore *et al.*, 2008; Hewitson *et al.*, 2008). PDIs are involved in the oxidoreduction and
 429 isomerisation of protein disulphide bonds, hydroxylation and protein deglutathionylation (Selles
 430 *et al.*, 2011; Ali Khan & Mutus, 2014). Recent studies have shown that *H. schachtii* and *M.*

431 *graminicola* PDI genes are expressed in the subventral glands of preparasitic J2s and
432 upregulated in parasitic J2s. Functional studies have shown that these PDIs, which belong to the
433 same subgroup as MiPDI2, play important roles in nematode parasitism through ROS
434 detoxification (Habash *et al.*, 2017; Tian *et al.*, 2019). We characterised the role of a secreted
435 *M. incognita* MiPDI1 effector identified in J2 secretome (Bellafiore *et al.*, 2008; Hewitson *et*
436 *al.*, 2008) and identified its target in plant-RKN interactions, a stress-associated protein.

437

438 **MiPDI1 is secreted throughout parasitism and targets the giant cells *in planta***

439 *MiPDI1* transcript abundance increased significantly throughout parasitic stages *in planta* (from
440 juveniles to females). We demonstrated that MiPDI1 was produced in the subventral
441 oesophageal gland and secreted *in planta*, both in the apoplast during nematode migration, but
442 also within the giant cells. Although the SvGs have been shown to be more active in the early
443 stage of parasitism (Davis *et al.*, 2000), SvGs remain active *in planta* and produce effectors
444 showing an increase in transcript abundance in parasitic juvenile stages compared to
445 preparasitic J2s (Nguyen *et al.*, 2018). The secretion of MiPDI1 by SvGs would thus allow its
446 production from the migration step to the formation of giant cells. Recent studies showed two
447 PDI-like proteins of PPNs, MgPDI and HsPDI, were localised to the apoplast when GFP
448 fusions were transiently expressed in *N. benthamiana* (Habash *et al.*, 2017; Tian *et al.*, 2019),
449 indicating their different functional mechanism during nematode parasitism. Despite the
450 hundreds of effectors characterised (Mejias *et al.*, 2019), few RKN effectors have been
451 demonstrated to be secreted *in planta* and to target giant cells. Example of effectors are the *M.*
452 *incognita* Mi-EFF1 (Jaouannet *et al.*, 2012) and MiMIF-2 (Zhao *et al.*, 2019), the *M. javanica*
453 MjNULG1a (Lin *et al.*, 2016) and the *M. graminicola* MgGPP (Chen *et al.*, 2017) and
454 Mg16820 (Naalden *et al.*, 2018). We also showed that the silencing of MiPDI1 *in planta*
455 affected the number of galls and egg masses obtained and delayed nematode development.
456 Accordingly, ectopic *MiPDI1* expression in *Arabidopsis* increased susceptibility to *M.*
457 *incognita*. These results provide evidence that MiPDI1 is a novel plant-parasitic effector
458 playing an essential role in nematode parasitism. In light of essential roles of PDI-like proteins

459 in redox regulation and mediating pathogens entry in infectious disease (Parakh & Atkin, 2015),
460 MiPDI1 may protect parasitic nematode stages and feeding cells from oxidative stress. Indeed,
461 studies of antioxidant molecule depletion have shown that the control of plant cell redox status
462 is a key regulator of giant cell effectiveness (Baldacci-Cresp *et al.*, 2012).

463

464 **MiPDI1 targets the redox-regulated stress-associated SAP12 proteins in *Arabidopsis* and** 465 **Solanaceae**

466 We further investigated the function of MiPDI1 in host cells, by searching for the proteins
467 interacting with MiPDI1 in tomato. The putative targets identified included cysteine proteases
468 and stress-associated zinc finger proteins, the activities of which are regulated by
469 thiols/cysteines. Interestingly, the two cysteine proteases (SICYPs) displaying high levels of
470 sequence identity captured in our Y2H screen are orthologous to *Arabidopsis* RD21a, a known
471 target of the *M. chitwoodi* effector Mc01194 (Davies *et al.*, 2015). Cysteine proteases have a
472 thiol group in the active site of the enzyme and are known to interact with Trx proteins
473 (Montrichard *et al.*, 2009). PDI-like proteins have been shown to regulate RD21a activity in
474 *Arabidopsis* (Andeme Ondzighi *et al.*, 2008), but we were unable to confirm the interaction of
475 MiPDI1 with SICYP in plant cells. The silencing of NbCYP in *N. benthamiana* did not affect
476 plant susceptibility to *M. incognita*, suggesting that these molecules are not functional targets of
477 MiPDI1.

478 The interaction of MiPDI1 with the tomato stress-associated protein SISAP12, an AN1-type
479 zinc finger protein was confirmed by Y2H, BiFC *in planta* and co-immunoprecipitation
480 experiments. *SISAP12* has been shown to be upregulated at later stages of gall formation in
481 tomato, in response to *M. incognita* attack (Shukla *et al.*, 2018). The cysteine residues of zinc
482 finger proteins are involved in zinc binding. The association of cysteine residues with zinc may
483 therefore be affected by Trx, with consequences for protein activity (Carter & Ragsdale, 2014).
484 AtSAP12 is a protein that undergoes major reversible redox-dependent conformational changes,
485 facilitating a rapid response to changing environmental conditions (Stroher *et al.*, 2009). Under
486 oxidising conditions (H₂O₂), oxidised SAP12 forms high-molecular mass aggregates. By

487 contrast, DTT and Trx reduce the oligomeric/dimeric form of SAP12 to the monomeric form
488 lacking intermolecular disulphide bridges. SAP12 acts as a redox sensor capable of undergoing
489 changes in its oligomeric conformation as a function of cellular redox potential, thereby
490 transmitting redox information to other cell components (Stroher *et al.*, 2009). MiPDI1, which
491 contains two Trx domains, could potentially regulate the activity of SAP12 by controlling its
492 oligomerisation state *in planta*.

493

494 **SAP proteins play important roles in plant responses to abiotic and biotic stresses**

495 SAPs, which contain the AN1 and/or A20 zinc-finger domains in rice, are known to respond
496 rapidly to diverse abiotic stresses and to play important roles in plant responses to these stresses
497 (Krishna *et al.*, 2003; Vij & Tyagi, 2008; Solanke *et al.*, 2009; Stroher *et al.*, 2009; Dixit *et al.*,
498 2018). Thirteen *SAP* genes have been described in tomato (Solanke *et al.*, 2009), 14 in
499 *Arabidopsis* (Stroher *et al.*, 2009), 18 in rice (Vij & Tyagi, 2006) and 57 in *Brassica napus* (He
500 *et al.*, 2019). Levels of *AtSAP12* and *SISAP12* expression increase immediately in response to
501 various abiotic stresses (Solanke *et al.*, 2009). Interestingly, the *Arabidopsis* microRNA
502 miR408, a key component of abiotic stress responses, is upregulated at 7 and 14 dpi in galls
503 induced by *M. incognita* (Medina *et al.*, 2018). Higher levels of miR408 expression are
504 associated with better tolerance to oxidative stress (Ma *et al.*, 2015). Cellular antioxidant
505 capacity is enhanced in plants with high levels of miR408 expression, as demonstrated by the
506 lower levels of reactive oxygen species and the induction of genes associated with antioxidative
507 functions, such as *SAP12* (Ma *et al.*, 2015). In rice, *OsiSAP8* confers tolerance to abiotic
508 stresses (Kanneganti & Gupta, 2008). Likewise, *OsSAP1* plays important roles in the responses
509 to both abiotic and biotic stresses, by interacting with aminotransferase (*OsAMTR1*) and the
510 Pathogenesis-Related 1a Protein (*OsSCP*) (Tyagi *et al.*, 2014; Kothari *et al.*, 2016). *AtSAP9* has
511 been shown to mediate ABA signalling in response to biotic and abiotic stresses, possibly via
512 the proteasome pathway (Kang *et al.*, 2017). Meanwhile, the plant A20-AN1 protein acts as a
513 key hub, mediating antiviral immunity (Chang *et al.*, 2018). However, the modes of action of
514 AN1-type proteins in plant pathogen responses remain largely unknown.

515 SISAP3 and SISAP4 have recently been shown to be positive regulators of immunity, to
516 *Pseudomonas syringae* pv. tomato (Goldberger *et al.*) and *Botrytis cinerea* (Liu *et al.*, 2019a;
517 Liu *et al.*, 2019b). SISAP3 silencing decreased the Pst DC3000-induced expression of SA
518 signaling and defense genes and attenuated immunity to Pst DC3000, whereas SISAP3
519 overexpression in transgenic tomato increased them. We show here that *SAP12* silencing or
520 knocking out *SAP12*, in *N. benthamiana* and in the *sap12* Arabidopsis mutant, respectively
521 increased susceptibility to *M. incognita*. Moreover, the expression of some stress-associated
522 marker genes was decreased in *MiPDI1*-expressing lines, whereas most of the genes
523 investigated were upregulated in the *sap12* mutant. Thus, a dual function of *MiPDI1* can
524 be hypothesized during plant–nematode interaction. *MiPDI1* may contribute to *M. incognita*
525 parasitism by protecting nematodes from oxidative stress during migration *in planta* and by
526 interacting with *SAP12* in the giant cells to fine-tune *SAP12*-mediated responses at the interface
527 of redox signalling, defence and stress acclimation. One of the challenges for the future will be
528 the establishment of assays for investigating the regulatory mechanism and showing how
529 *MiPDI1* and *SAP12* orchestrate downstream responses.

530

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544

545 **Author contributions**

546 JZ, JM and MQ designed, performed experiments and analysed the data. YC, ZM, QS, QL, BX
547 performed experiments. JAE gave guidance for immunostaining. MQ, BX and PA supervised
548 some of this work and provided input and expertise. HJ and BF were responsible for the
549 development and guidance of the project. JZ, MQ, PA, BF and HJ wrote the manuscript with
550 input from all co-authors.

551

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Fig. 5 Effect on susceptibility to *M. incognita* of virus-induced gene silencing (VIGS) of *NbSAP12s* and *NbCYP* in *N. benthamiana*, and of the *A. thaliana sap12* knockout mutant. (a) Levels of *NbSAP12s* and *NbCYP* transcripts in *N. benthamiana* following silencing, as assessed by real-time quantitative PCR (RT-qPCR). Error bars represent the standard errors for 10 biological replicates, and the results of two independent experiments were presented. (b) *N. benthamiana* plants in which *NbSAP12s* was silenced were more susceptible to *M. incognita*, whereas those in which *NbCYP* was silenced were not significantly different from the wild type

in terms of susceptibility, as indicated by the mean numbers of egg masses on plant roots. Error bars represent the mean \pm SD ($n \geq 15$). All experiments were performed twice, and at least 15 plants were analysed per treatment. (c) The *sap12* mutant line (*SALK_014706*) was more susceptible to *M. incognita*, as shown by the mean numbers of egg masses and galls in roots. Error bars represent the mean \pm SD ($n \geq 20$). Two independent experiments were conducted and yielded similar results, with at least 30 plants analysed per treatment. (a, b, c) Different letters indicate statistically significant difference in two-way ANOVA with Tukey's multiple comparisons test ($P < 0.05$). (d) Levels of expression for stress- and defence-related genes in MiPDI1-expressing lines (*MiPDI1-1* and *MiPDI1-2*), the *sap12* mutant line (*SALK_014706*) and the wild type (WT). The genes considered were *AtCSD1* (cytosolic Cu/Zn superoxide dismutase), *AtCSD2* (chloroplastic Cu/Zn superoxide dismutase), *AtCOR47* and *AtRAB18* (from the dehydrin protein family), *AtADHI* (catalysing the reduction of acetaldehyde with NADH as reductant), *AtEM6* (stress-induced protein), *AtNPR1* and *AtPR1a* (SA-mediated defence response marker gene), *AtPDF1.2a* (encoding ethylene- and jasmonate-responsive plant defences), *AtPR4* (ethylene-responsive pathogenesis-related protein). *AtOXAI* (AT5G62050) and *AtUBP22* (AT5G10790) were used as internal controls. Expression levels were measured by real-time quantitative PCR (RT-qPCR) and the data shown are means \pm SD ($n = 4$). Asterisks indicate significant differences for single stress- and defence-related gene among different plant lines by one-way ANOVA with Dunnett's multiple comparisons test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Supporting information

- 768 **Table S1** Primers used in this study.
- 769 **Table S2** Accession numbers used in this study.
- 770 **Table S3** Yeast-two hybrid clones obtained using MiPDI1-mu or MiPDI1 as bait and
- 771 corresponding gene expression in galls induced by *M. incognita*.
- 772 **Fig. S1** MiPDI1 probe used for *in situ* hybridization (ISH).
- 773 **Fig. S2** Western blot analysis verified specificity of MiPDI1 antibody.

- 774 **Fig. S3** Localization of MiPDI1 in tomato root gall sections during *M. incognita* parasitism.
- 775 **Fig. S4** Verification of homozygous *MiPDII* ectopic expressing Arabidopsis lines by RT-PCR
776 and western blot.
- 777 **Fig. S5** Structure of AtSAP12, NbSAP12 and SISAP12 proteins.
- 778 **Fig. S6** MiPDI1 could not interact with SICYP *in planta* by using BiFC.
- 779 **Fig. S7** Characteristics of *SAP12* and *CYP* genes, and VIGS phenotypes.
- Fig. S8** Verification of homozygous T-DNA insertion mutants of *AtSAP12*.

For Peer Review

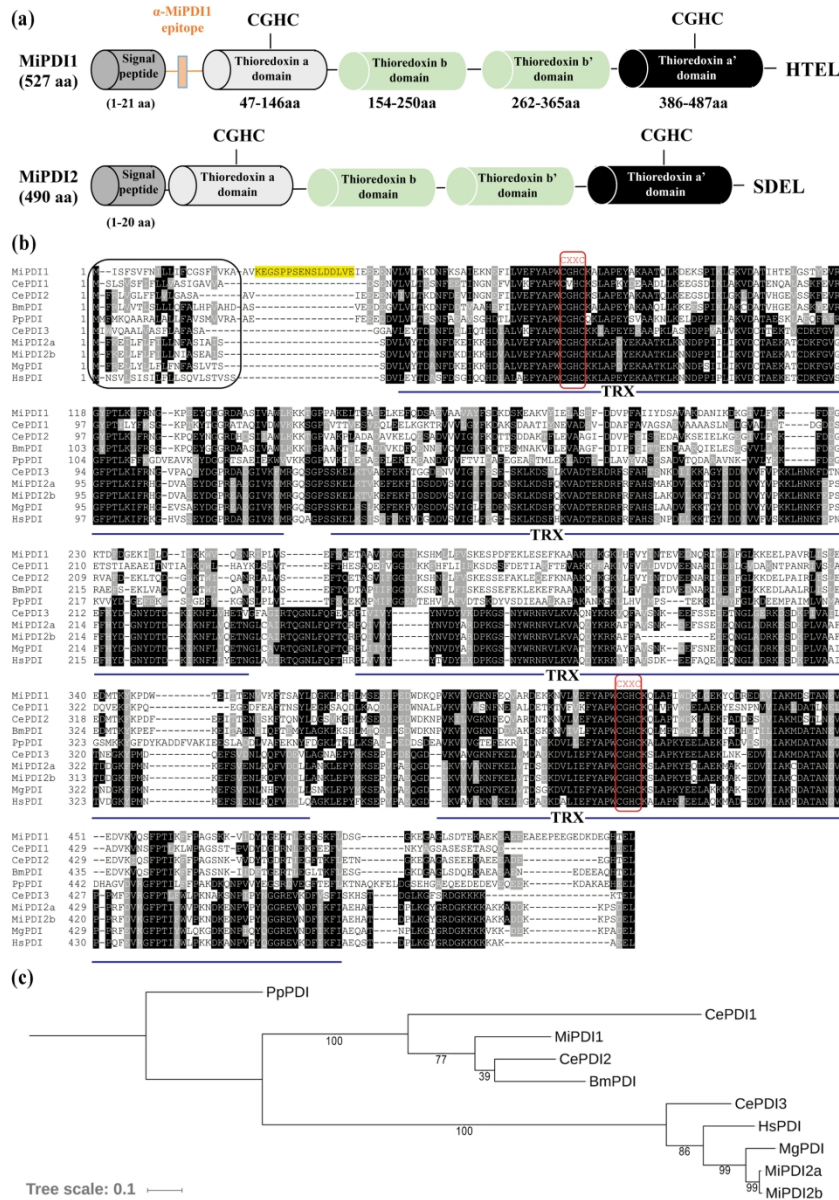


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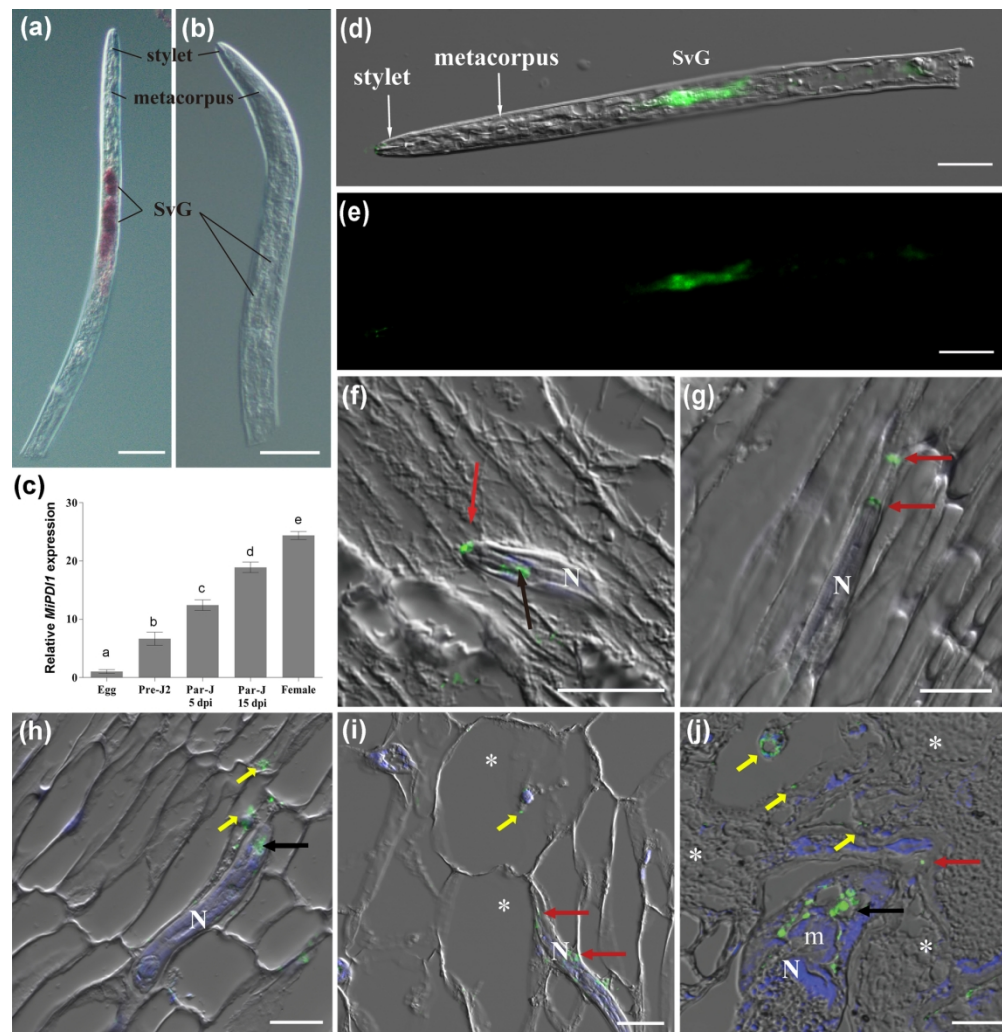


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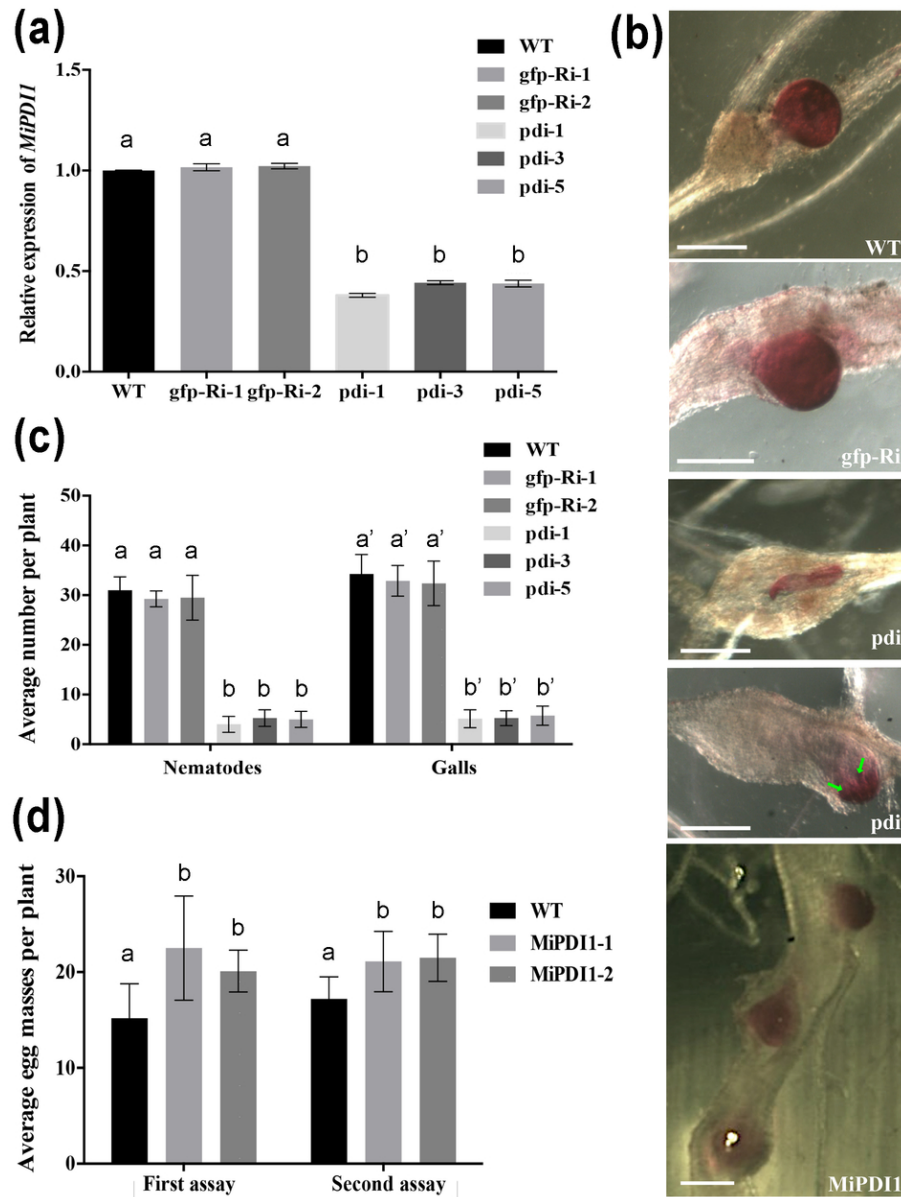


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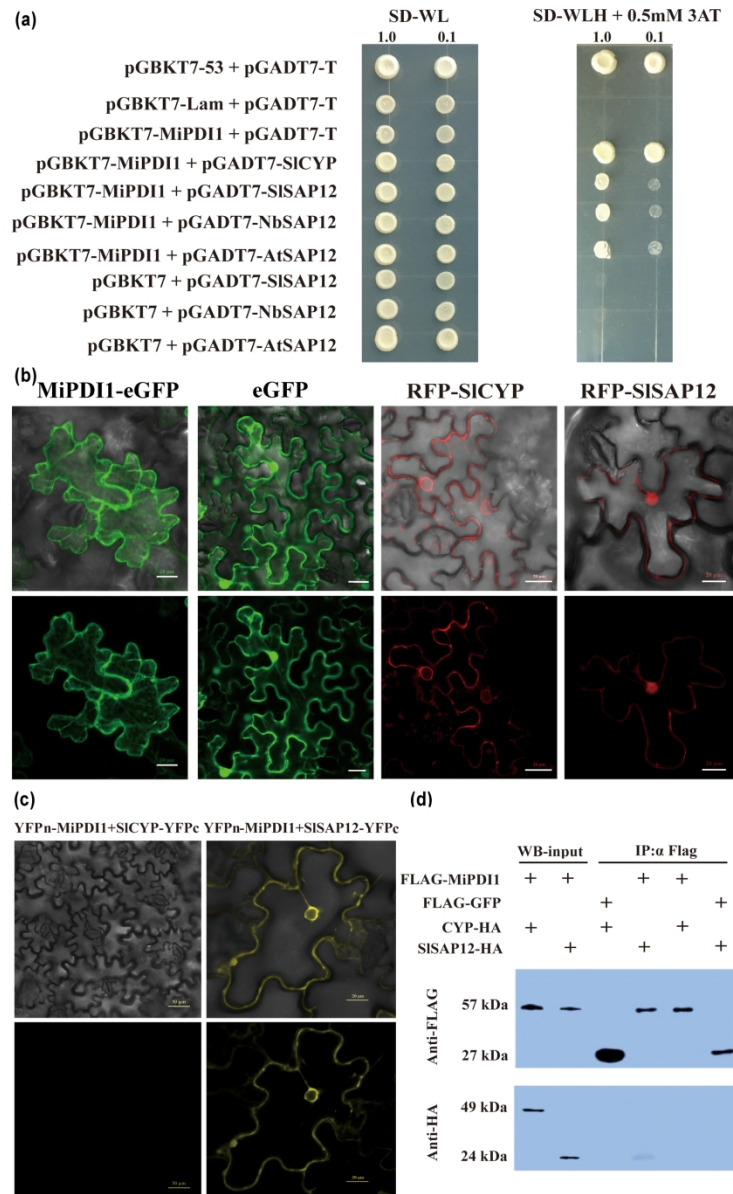


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YFPc. (b, c) At least ten cells from three leaves of three different plants were observed with similar results. YFP, yellow fluorescent protein. Scale bar, 20 μm or 50 μm . (d) Co-immunoprecipitation (Co-IP) analysis of MiPDI1 interacting with SISAP12. FLAG-MiPDI1 or FLAG-GFP was transiently co-expressed with SISAP12-HA or SICYP-HA in tobacco leaves. Co-IP was performed with anti-FLAG M2 affinity gel resin (Sigma-Aldrich), and the isolated protein was detected by western blotting with an anti-FLAG antibody to detect MiPDI1 or eGFP, and an anti-HA antibody to detect SISAP12 or SICYP. eGFP, enhanced green fluorescent protein.

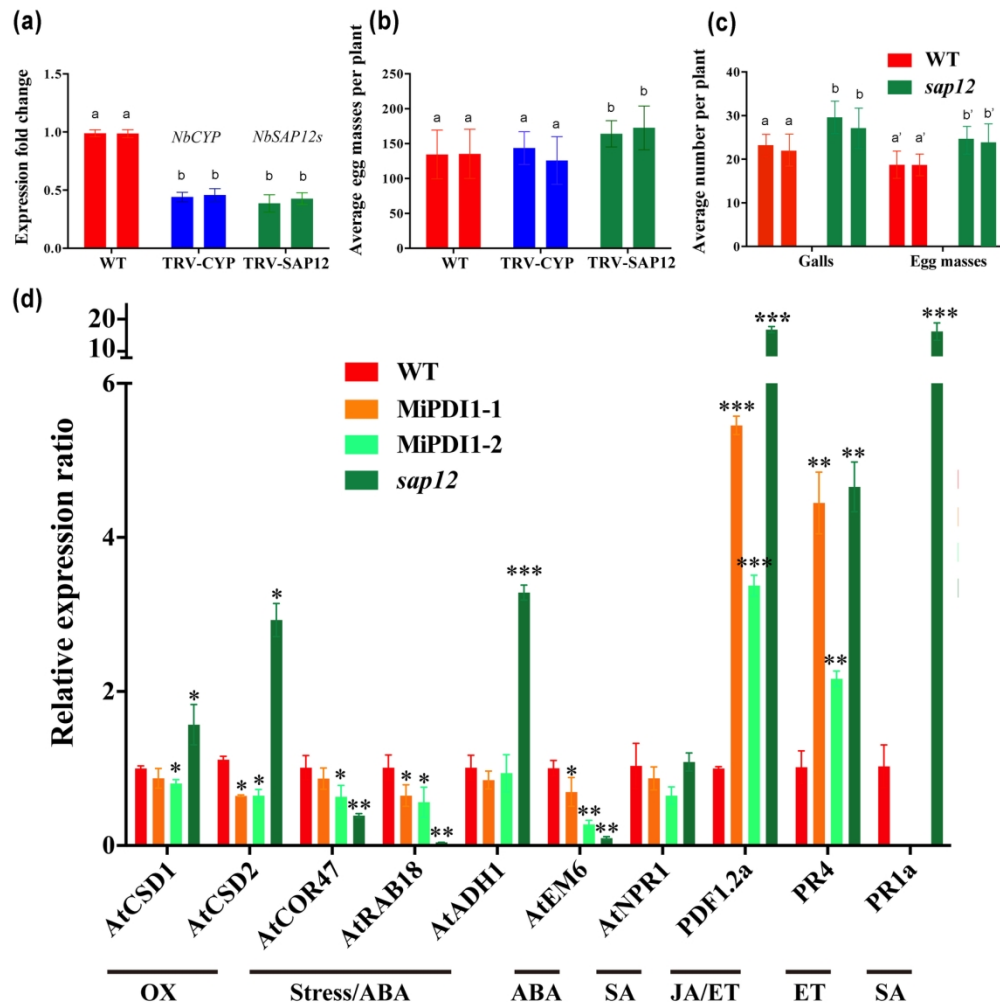


Fig. 5 Effect on susceptibility to *M. incognita* of virus-induced gene silencing (VIGS) of *NbSAP12s* and *NbCYP* in *N. benthamiana*, and of the *A. thaliana sap12* knockout mutant. (a) Levels of *NbSAP12s* and *NbCYP* transcripts in *N. benthamiana* following silencing, as assessed by real-time quantitative PCR (RT-qPCR). Error bars represent the standard errors for 10 biological replicates, and the results of two independent experiments were presented. (b) *N. benthamiana* plants in which *NbSAP12s* was silenced were more susceptible to *M. incognita*, whereas those in which *NbCYP* was silenced were not significantly different from the wild type in terms of susceptibility, as indicated by the mean numbers of egg masses on plant roots. Error bars represent the mean \pm SD ($n \geq 15$). All experiments were performed twice, and at least 15 plants were analysed per treatment. (c) The *sap12* mutant line (SALK_014706) was more susceptible to *M. incognita*, as shown by the mean numbers of egg masses and galls in roots. Error bars represent the mean \pm SD ($n \geq 20$). Two independent experiments were conducted and yielded similar results, with at least 30 plants analysed per treatment. (a, b, c) Different letters indicate statistically significant difference in two-way ANOVA with Tukey's multiple comparisons test ($P < 0.05$). (d) Levels of expression for stress- and defence-related genes in *MiPDI1*-expressing lines (*MiPDI1-1* and *MiPDI1-2*), the *sap12* mutant line (SALK_014706) and the wild type (WT). The genes considered were *AtCSD1* (cytosolic Cu/Zn superoxide dismutase), *AtCSD2* (chloroplastic Cu/Zn superoxide dismutase), *AtCOR47* and *AtRAB18* (from the dehydrin protein family), *AtADH1* (catalysing the reduction of acetaldehyde with NADH as reductant), *AtEM6* (stress-induced protein), *AtNPR1* and *AtPR1a* (SA-mediated defence response marker gene), *AtPDF1.2a* (encoding ethylene- and jasmonate-responsive plant defences), *AtPR4* (ethylene-responsive pathogenesis-related protein). *AtOXA1* (AT5G62050) and *AtUBP22* (AT5G10790) were used as internal controls. Expression levels were measured by real-time quantitative PCR (RT-qPCR) and the data shown are means \pm SD ($n = 4$).

Asterisks indicate significant differences for single stress- and defence-related gene among different plant lines by one-way ANOVA with Dunnett's multiple comparisons test (*P < 0.05, ** P<0.01, *** P <0.001).

160x160mm (300 x 300 DPI)