A MIF-like effector suppresses plant immunity and facilitates nematode parasitism by interacting with plant annexins

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Abstract

Plant-parasitic nematodes secrete numerous effectors to facilitate parasitism, but detailed functions of nematode effectors and their plant targets remain largely unknown. Here, we characterized four macrophage migration inhibitory factors (MIFs) in Meloidogyne incognita resembling the MIFs secreted by human and animal parasites. Transcriptional data showed MiMIFs are up-regulated in parasitism. Immunolocalization provided evidence that MiMIF proteins are secreted from the nematode hypodermis to the parasite surface, detected in plant tissues and giant cells. In planta MiMIF RNA interference in Arabidopsis decreased infection and nematode reproduction. Transient expression of MiMIF-2 could suppress Bax- and RBP1/Gpa2-induced cell death. MiMIF-2 ectopic expression led to higher levels of Arabidopsis susceptibility, suppressed immune responses triggered by flg22, and impaired [Ca²⁺]cyt influx induced by H₂O₂. The immunoprecipitation of MiMIF-2-interacting proteins, followed by co-immunoprecipitation and bimolecular fluorescence complementation validations, revealed specific interactions between MiMIF-2 and two Arabidopsis annexins, AnnAt1 and AnnAt4, involved in the transport of calcium ions, stress responses, and signal transduction. Suppression of expression or overexpression of these annexins modified nematode infection. Our results provide functional evidence that nematode effectors secreted from hypodermis to the parasite cuticle surface target host proteins and M. incognita uses MiMIFs to promote parasitism by interfering with the annexin-mediated plant immune responses.

Keywords: Annexin, cuticle, immune response, interaction, macrophage migration inhibitory factor (MIF), Meloidogyne incognita, parasitism, plant immunity, root-knot nematode, secretion.

Introduction

Root-knot nematodes (RKNs; Meloidogyne spp.) are among the most devastating obligate parasites of plants, causing billions of dollars of agricultural losses worldwide annually (Ibrahim et al., 2011; Jones et al., 2013). The apomictic RKN species M. incognita, M. javanica, and M. arenaria can infect thousands of plant species (Teixeira et al., 2016). Disease development requires the motile RKN pre-parasitic second-stage juveniles (J2s) to penetrate the host root apex and migrate towards the vasculature, where they select root cells and transform them into specialized feeding structures known as ‘giant
MIF is involved in inflammation by interacting with a NOD-like receptor (NLRP3) and mediating the interaction between NLRP3 and the intermediate filament protein vimentin (Lang et al., 2018).

MIF-like proteins from invertebrate species have been shown to be involved in immune escape from hosts. The first nematode MIF homolog was characterized in the filamentous parasite *Brugia malayi*, which secretes a MIF-like protein (BmMIF) that can be detected in somatic extracts and in the excretory–secretory products of the parasite, providing the first demonstration that BmMIF could promote parasite survival by modifying host immune responses (Pastrana et al., 1998).

A MIF-like protein produced by the hookworm *Ancylostoma ceylanicum*, AceMIF, was shown to bind to the human MIF receptor CD74 and to facilitate infection of larval stages and adult worm survival within the intestine; these findings indicate that AceMIF may act as a virulence factor regulating the host immune response (Cho et al., 2007). Similarly, the filarial parasite *Onchocerca volvulus* secretes MIF-like proteins into the outer cellular covering of the adult worm body, the syncytial hypodermis, and the uterine wall (Ajonina-Ekoti et al., 2013).

Recently, a MIF-like protein was reported to be secreted by aphids; this protein acts as a cytokine, modulating host immune responses (Naessens et al., 2015), in a new mechanism of interaction between parasites and plant hosts. However, it remains largely unknown whether and how MIF-like proteins regulate host immune responses or signal transduction during plant–pathogen interactions. Interestingly, plant MIF-like proteins (MDLs) were recently identified, but no functional data are available to date (Panstruga et al., 2015).

Plants have evolved an innate immune system to protect them against various types of attack, in which pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) play essential roles (Jones and Dangl, 2006; Dangl et al., 2013). The model plant *Arabidopsis* displays induced immune response–related marker gene expression as part of a response to pathogens (Pieterse et al., 2009; Jaouannet et al., 2013), involving local callose deposition at the site of penetration (Elliger et al., 2013), and the generation of ROS (Tripathy and Oelmüller, 2012). Many *Arabidopsis* genes have been implicated in the plant immune system, including those encoding annexins, which act as important regulators of various abiotic and biotic stress responses (Clark et al., 2010; Laohavisit and Davies, 2011). Interestingly, cyst nematodes were reported to secrete annexins to assist parasitism. The best cyst nematode effector Hs4F01 is an annexin-like protein that interacts with an *Arabidopsis* oxido-reductase member of the 2-oxoglutarate (2OG)–Fe(II) oxygenase family (Patel et al., 2010). Another cyst nematode annexin-like effector is Ha-ANNEXIN secreted by *Heterodera avenae*, which could suppress host immune responses (Chen et al., 2015). These results highlight the importance of annexins for plant–nematode interactions.

In this study, we characterized MIF-like genes from *M. incognita* that are up-regulated upon infection. We showed that MiMIF proteins are secreted into plant tissues and giant cells, thereby promoting parasitism. We chose MiMIF–2 as a representative and found that this protein engaged in physical interactions...
with two Arabidopsis annexins, which suppressed host immune responses. Based on these results, we suggest that MiMIF-2 may act as a novel effector interacting with plant annexins to manipulate host immune responses and signal transduction, to promote the survival of parasitic stages of *M. incognita*.

**Materials and methods**

**Nematode culture, plant materials and growth conditions**

*Meloidogyne* *incognita* strains were reproduced initially from a single egg mass on tomato plants (*Solanum lycopersicum* var. ‘Bagnou’) in a greenhouse at 25 °C. Infective J2s were collected with an improved Baermann funnel for 48–72 h. Different parasitic life stages of the nematode were collected from roots as previously described (Chen et al., 2015). Infected tomato roots were collected at different days after inoculation, cut into 1 cm sections and digested in a mixture of pectase and cellulase (Sinopharm Chemical Reagent Beijing Co., Ltd, China) at 28 °C and 160 rpm overnight. Nematodes were separated under a stereomicroscope.

Surface-sterilized Arabidopsis ecotype Columbia (Col-0) was sown on MS medium (Sigma–Aldrich, USA) as previously described (Quentin et al., 2016). The *AnnAt1* and *AnnAt4* T-DNA mutant lines (*SALK_015426* and *SALK_096465*) were obtained from the Arabidopsis Biological Resource Center (ABRC, USA). Tobacco (*Nicotiana benthamiana*) seeds were grown in pots under conditions of 16 h light and 8 h dark at 23 °C with 60–75% relative humidity.

For *in vivo* nematode infection, Arabidopsis or tomato seedlings were inoculated in soil with 300 J2s per plant. Roots were collected at 35 d post-infection (dpi) and stained using the sodium hypochlorite–acid fuchsine method (Bybd et al., 1983). Egg masses and galls were counted under a dissecting microscope (Olympus, Japan). Juveniles inside roots were isolated by digestion with cellulase. Each experiment was performed three times independently, and 30 plants were counted each time. Data were analysed using Statgraphics Centurion 17 software (Statgraphics Technologies, USA). One-way ANOVA followed by Duncan’s *post hoc* test was run to identify significant differences between treatments.

**RNA isolation and gene amplification**

*Meloidogyne* *incognita* miRNA was extracted using a Dynabeads® miRNA DIRECT™ Kit (Invitrogen, USA), and complementary DNA (cDNA) was synthesized using reverse transcriptase SuperScript III (Invitrogen). Arabidopsis total RNA was isolated from seedlings using TRIzol Reagent (Invitrogen) and cDNA was synthesized using M-MLV reverse transcriptase (TaKaRa, Japan). MiF-like genes were amplified from cDNA or gDNA of *M. incognita* by PCR, using specific primers. The PCR products were cloned into the pMD18-T vector (TaKaRa, Japan) and sequenced. All primers used in this study are listed in Supplementary Table S1 at JXB online and were synthesized by TsingKe Biotechnology Co. Ltd, Beijing, China.

**Accession numbers and databases used**

The predicted nucleotide sequences of *MiMIFs* were obtained from the National Center for Biotechnology Information (NCBI) EST database (http://www.ncbi.nlm.nih.gov/nuccore/), and from *Meloidogyne* genomic resources (http://www6.inra.fr/meloidogyne_incognita/). Multiple amino acid sequence alignment analyses of MiF-like proteins from *B. malayi, Arabidopsis, Homo sapiens, M. hapla, M. incognita, A. thaliana, O. velutinus, Caenorhabditis elegans*, and *Plasmodium falciparum* were conducted using DNAMAN V6 (Lynnon Biosoft, USA). All accession numbers are listed in Supplementary Table S2.

**Developmental expression analysis and RT-qPCR analysis**

Illumina transcriptomic data obtained during developmental stages (eggs, pre-parasitic J2s, parasitic juveniles, and adult females) were described recently (Blanc-Mathieu et al., 2017). Real-time quantitative PCR (RT-qPCR) was performed on an ABI Prism 7000 real-time PCR system (Applied Biosystems, USA). *Meloidogyne incognita* tubulin was used as an internal control. RT-qPCR was conducted using a SYBR Premix Ex Taq (TaKaRa, Japan) under standard conditions. The results were analysed using the 2^ΔΔCt method (Livak and Schmittgen, 2001).

**Anti-MiMIF-2 polyclonal antibody production and immunolocalisation**

Recombinant MiMIF-2 (rMiMIF-2, with an N-terminal His tag) was purified using QIAexpress® Ni–NTA Fast Start (Qiagen, Germany). Protein concentration and purity were determined by the BCA Protein Assay Kit (Beijing ComWin Biotech Co., Ltd, China) and by SDS-PAGE. The anti-MiMIF-2 polyclonal serum was purified from immunized rabbits (Beijing Protein Innovation Co., Ltd, Beijing, China). Western blotting was conducted to verify the antibody specificity. The *MiMIF-2*-mutant (rMiMIF-2-mu; N-terminal Pro1→Gly1) was generated by PCR.

Ultrastructural immunocytochemistry was performed as previously described (Dubreuil et al., 2011). Freshly hatched J2s were fixed with 4% paraformaldehyde (PAF) or 4% PAF and 0.2% glutaraldehyde (PG) in 0.1 M phosphate buffer (pH 7.4). J2s were dehydrated in an ethanol series and embedded in acrylic resin (LR–White) before sectioning and immunogold labelling. The anti-MiMIF-2 antibody (1:200) and protein A–gold (PAG) of 15 nm were used. For controls, sections were processed according to the same procedure except that the primary antibody was omitted. Eighty-nanometer ultrathin sections were contrasted with 4% uranyl acetate in water and visualized using a JEM 1400 electron microscope operating at 100 kV equipped with a Morada SIS camera. For immunohistochemistry on sections of tomato galls, we used the protocol previously described (de Almeida Engler et al., 2004). Tomato galls were fixed in 8% formaldehyde, dehydrated and embedded in butyl-methylmethacrylate. MiMIF-2 antibody and secondary antibody (goat anti-rabbit Alexa Fluor 488 conjugate antibody, Molecular Probes) were diluted 250- and 300-fold in blocking solution, respectively. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich). Images were captured by confocal microscopy (Zeiss LSM880, Germany).

**In planta RNAi and generation of transgenic Arabidopsis plants**

For RNAi experiments, two *MiMIF-2* fragments (1–339 bp) were amplified and cloned in the forward and backward pSAT1 intron, and then inserted into pSuper-RNAi (Dafny–Yelin et al., 2007). For expression, the open reading frames (ORFs) of *MiMIF-2, AnnAt1* (At1g353720) and *AnnAt4* (At2g38750) were amplified and inserted into Super1300-FLAG. The vectors were transformed into *Agrobacterium tumefaciens* GV3101 and used for transformation of Arabidopsis via the floral dip method (Zhang et al., 2006). Homozygous T3 plants were confirmed by RT-PCR and western blotting.

**Cell-death suppression assay in N. benthamiana, callose staining and defense gene expression analyses**

For RNAi experiments, two *MiMIF-2* fragments (1–339 bp) were amplified and cloned into PVX vector pGR107 with a FLAG-tag fused at the N-terminus or a HA-tag fused at the C-terminus and transformed into *A. tumefaciens* GV3101. *Agrobacterium tumefaciens* cells carrying BAX or RBP1/Gpa2 were used to trigger cell death in *N. benthamiana* leaves (Sacco et al., 2009; Jing et al., 2016). Agro-infiltrations into 4-week-old *N. benthamiana* leaves were performed as described in Nguyen et al. (2018). For callose deposition assay, Arabidopsis leaves from 4-week-old *MiMIF-2*-lines and wild type (WT, Col-0) were treated with 1 µM flg22 or buffer for 24 h. Samples were stained with aniline blue for 3 h and then examined under a photomicroscope.

Defense suppression test was conducted as described previously (Jouannet et al., 2013). To analyse defense gene expression, 10-day-old seedlings of *MiMIF-2*-expressing line and WT were treated with 1 µM flg22. The expression of the defense marker genes *PR1, PR3, FRK1, CYP81, RbohD,* and *RbohD* were examined under a photomicroscope.
and RbohF was determined by RT-qPCR. Arabidopsis ubiquitin carboxyl terminal hydrolase 22 (UBP22) was used as an internal control.

**Arabidopsis protein immunoprecipitation (IP) experiments and LC-MS/MS analysis**

For the IP experiments, Arabidopsis total protein was extracted from MiMIF-2-FLAG-expressing lines (three different lines) and WT1 plants. IP was performed using the anti-FLAG M2 affinity gel resin (Sigma-Aldrich). The beads were washed twice with 1 ml of ice-cold phosphate-buffered saline (PBS), followed by the immediate addition of 1 ml protein solution and incubation at 4 °C for 4 h. The resin was thoroughly washed five times with 1 ml of ice-cold PBS, and then the proteins were eluted for analysis. A Q-Exactive nanospray ESI-MS mass spectrometer (Thermo, USA) was used for liquid chromatography–tandem mass spectrometry (LC-MS/MS) at China Agricultural University Functional Genomics Platform. The acquired mass spectrometric data were pre-analysed using Mascot Distiller 2.4 (UK) and then annotated to search an NCBI non-redundant protein database and the Swiss-prot database.

**Subcellular localization, bimolecular fluorescence complementation in planta and aequorin-based [Ca\(^{2+}\)]\(_{cyt}\) measurement**

The open reading frame of MiMIF-2 without stop codon was amplified and cloned into the p35S-eGFP vector with an enhanced green fluorescent protein (eGFP) fused at the C-terminus. Ten-day-old *N. benthamiana* leaves were agro-infiltrated and images were captured by laser confocal fluorescence microscopy (Leica SP8, Germany) at an excitation wavelength of 515 nm.

**Aequorin/MiMIF-2 transgenic lines were generated from a cross between the aequorin line (Laohavisit et al., 2009) and MiMIF-2 transgenic lines. The aequorin-annAt1 and aequorin-annAt4 seeds were gifts from Yan Guo’s lab. Ten-day-old aequorin-expressing seedlings were used for measurements of root [Ca\(^{2+}\)]\(_{cyt}\) according to a previously described method (Laohavisit et al., 2012). Roots were suspended in aequorin buffer (5 mM KCl, 2 mM CaCl\(_2\), and 2 mM Tris/MES, pH 5.7, with 10 µM coelenterazine) for 5 h in the dark (25 °C), washed with osmotically adjusted aequorin buffer without coelenterazine, and resuspended in this buffer (100 µl), and left in the dark for 30 min. The baseline luminescence was recorded for 60 s, followed by the addition of 100 µL of buffer (20 mM H\(_2\)O\(_2\)). Luminescence was recorded every second for 120 s. Discharge buffer was then injected (final concentration: 10% (v/v) ethanol, 1 M CaCl\(_2\)), and the luminescence was recorded for an additional 120 s.

**In vivo co-immunoprecipitation assay**

For a co-immunoprecipitation (Co-IP) assay, the coding regions of *AnnAt1*, *AnnAt4*, or *MiMIF-2* were cloned into the PVX vector pGR107 with a FLAG-tag fused at the N-terminus and a HA-tag fused at the C-terminus, respectively, and then transformed into *A. tumefaciens* strain EHA105. Four-week-old *N. benthamiana* leaves were agro-infiltrated and images were captured by laser confocal fluorescence microscopy (Leica SP8, Germany) at an excitation wavelength of 515 nm. For a co-immunoprecipitation (Co-IP) assay, the coding regions of *AnnAt1*, *AnnAt4*, or *MiMIF-2* were cloned into the PVX vector pGR107 with a FLAG-tag fused at the N-terminus and a HA-tag fused at the C-terminus, respectively, and then transformed into *A. tumefaciens* strain EHA105. For immunoblot analysis of MAPK activation, 10-day-old Arabidopsis seedlings were used. The protein was detected with the eECL Western Blot Kit (Beijing ComWin Biotech Co., Ltd, China).

**Results**

**Identification of *M. incognita* MiMIF-like genes and their expression in different life stages**

Our searches of the NCBI EST database and *M. incognita* genome sequences (Abad et al., 2008; Blanc-Mathieu et al., 2017) identified four genes encoding MiMIF-like proteins: MiMIF-1 (Vermeire et al., 2008), MiMIF-2, MiMIF-3, and MiMIF-4. These genes are organized in tandem arrays on two scaffolds. MiMIF-1 and MiMIF-2, and MiMIF-3 and MiMIF-4 share 97–98% nucleotide sequence identity, whereas MiMIF-1/MiMIF-2 and MiMIF-3/MiMIF-4 only share about 82% sequence identity (Fig. 1A; Supplementary Fig. S1A). The MiMIF-1 gene has a single base-pair deletion in position 260 (Supplementary Fig. S1A, indicated by black arrow), resulting in the premature termination of translation at bp 285 (Supplementary Fig. S1A, indicated by red frame), leading to a protein truncated by 19 amino acids (aa). Interestingly, MiMIF-3 and MiMIF-1 form an operon and are transcribed together to generate a single mRNA strand (Supplementary Fig. S1B, C). Sequence alignment showed that MiMIF-1 (94 aa), MiMIF-2 (113 aa), MiMIF-3 (112 aa), and MiMIF-4 (112 aa) were 75–99% identical (Fig. 1A; Supplementary Fig. S1D). They all contain three conserved residues (Pro1, Lys32, and Ile64) of the active sites for tautomerase activity identified in human MIF (Fig. 1B, black triangle), but MiMIF sequences lacked the Val106 residue (Fig. 1B, red triangle) for tautomerase activity and the C-X-X-C motif found in the thioredoxin superfamily of thiol-protein oxidoreductases (Fig. 1B).

The nucleotide sequences of the four *MiMIFs* displayed a high level of identity, and MiMIF-3 and MiMIF-4 were found to be cotranscribed (Supplementary Fig. S1A, B), making it difficult to study individual *MiMIF* expression profiles. However, Illumina transcriptomic data obtained during developmental stages (eggs; pre-parasitic J2s; parasitic juveniles and adult females) (Blanc-Mathieu et al., 2017) revealed higher levels of *MiMIFs* expression in parasitic juveniles than in pre-parasitic J2s (Fig. 1C).

**MiMIFs are localized in the hypodermis and cuticle, and secreted into plants and accumulated in giant cells**

To analyse the tissue localization of MiMIFs and demonstrate that MiMIFs are secreted into host-plant tissues, we produced a polyclonal antibody against rMiMIF-2. On western blots, this polyclonal antibody specifically detected rMiMIF-2 and MiMIFs in total proteins from pre-parasitic J2s at the expected size of ~13 kDa (Supplementary Fig. S2). No signal was detected in total proteins from tomato roots or leaves. Ultrastructural immunocytochemistry analyses with the polyclonal antibody were performed on pre-parasitic J2s. MiMIF proteins were
mostly detected in the hypodermis in contact with the cuticle, in the cuticle, in the pseudocoelom surrounding the stylet, and barely in the muscle (Fig. 2A, B; Supplementary Fig. S3). No gold particles (black dots) were observed in control treatments (Fig. 2C). Immunohistochemistry analyses were performed on gall sections from tomato plants 15 d post-infection (dpi) with M. incognita. Fluorescence was observed around the nematode head and cuticle adjacent to the surrounding host cells (Fig. 2D, E; Supplementary Fig. S4). This result indicates secretion into and accumulation of MiMIFs in the intercellular space between the nematode body and the host cells. Moreover, several giant cells showed a clear signal at the cytoplasmic level and in particular near the plasma membrane (Fig. 2G, H). In control experiments with secondary antibody only, no signal was observed on sections of infected roots at similar time points (Fig. 2F, I). Thus, MiMIFs are secreted in planta from M. incognita hypodermis to the parasite surface during parasitism and are able to target the giant cells.

In planta RNAi of MiMIFs affects nematode parasitism

We assessed the role of MiMIF genes during parasitism by silencing MiMIFs in planta through host-derived RNAi. Four homozygous Ri-T3 transgenic Arabidopsis lines expressing the MiMIF-2 full-length coding sequence (CDS) hairpin double-stranded RNA (dsRNA; 339 bp in pSuper vector; the identical phenotypes of different lines are shown in Supplementary Fig. S5) and two GFP hairpin dsRNA lines were obtained. MiMIFs transcript abundance was assessed in parasitic nematodes extracted at 14 dpi, and was found to be strongly reduced (64% to 80%) in the four Ri-T3 lines compared with control lines (Fig. 3A), demonstrating the efficacy of host-derived RNAi-mediated gene silencing. At 35 dpi, the numbers of galls and nematodes were about 60% lower (P<0.05) in Ri-T3 plants than in plants treated with the GFP control lines (Fig. 3B). Similar results were obtained in three independent trials. These findings suggest that MiMIFs play a key role in root-knot nematode parasitism.

MiMIF-2 expression in Arabidopsis increases susceptibility to M. incognita

We showed that native MiMIFs were indeed delivered into the cytoplasm of host cells and we then expressed MiMIF-2 ectopically in transformed Arabidopsis lines. Transgenic plants were verified by RT-PCR; the phenotype of three independent
homzygous T3 lines expressing MiMIF-2-FLAG (lines OE-T3-3, OE-T3-4, and OE-T3-5) showed no obvious difference compared with WT plant, and the presence of a 13 kDa MiMIF-2-FLAG protein was confirmed by western blotting (Supplementary Figs S5, S6). Infection assays with M. incognita showed that all three transgenic lines were significantly (P<0.05) more susceptible than WT to M. incognita infection (Fig. 3C). The transgenic lines had up to 30% more galls and parasitic nematodes at 35 dpi. Similar results were obtained in three independent biological tests, each with 30 plants.

**Fig. 2.** Localization of MiMIF proteins in nematodes and in planta. (A, B) Ultrastructural immunocytochemistry of M. incognita MIF proteins. Section of the head and anterior part of a pre-J2 fixed in 4% paraformaldehyde (PAF) or 4% PAF and 0.2% glutaraldehyde (PG) showing MIF protein accumulations in the hypodermis (h) and in the cuticle (c), and also detected in the body wall muscle (bwm) and in the pseudocoelom surrounding the stylet (s). ad, amphid. n=9 indicates the number of similar results. (C) Control sections of J2s fixed in PG or PAF without anti-MiMIF-2 antibody. (D–I) Immunodetection of the MiMIF proteins in tomato gall sections. Tomato roots were fixed into methylacrylate after infection with M. incognita for 15 d. The anti-MiMIF-2 antibody (1:250) was applied on gall sections. Alexa Fluor 488-conjugated secondary antibody was used to detect the fluorescence. (D, E) MiMIF proteins accumulated along the nematode cuticle and adjacent plant tissues surrounding the nematode head. (F) Galls containing parasitic J2 nematodes incubated only with secondary antibody as a negative control, showing no signal. (G) Signal was observed around nematode head and in giant cells near the plasma membrane. (H) Signal was detected around the giant cell and nucleus. (I) Galls containing nematode and giant cells incubated only with secondary antibody as a negative control, showing no signal. Micrographs (E, F, G, H) are overlays of images of the differential interference contrast, Alexa Fluor 488-conjugated secondary antibody and DAPI-stained nuclei. Individual images are listed in Supplementary Fig. S3. N, nematode; *, giant cell. Scale bars 20 μm for images (D–I). White arrows indicate the MiMIF-2 fluorescence.

**MiMIF-2 suppresses defense responses in plants**

As the expression of MiMIF-2 in Arabidopsis enhanced M. incognita infection, we investigated whether MiMIF-2 may regulate plant immune responses. We first tested whether MiMIF-2 has the ability to suppress programmed cell death (PCD) induced by proapoptotic BAX or Globodera RBP-1/Gpa2 resistance protein. BAX and RBP-1/Gpa2 constructs were agroinfiltrated 24 h after tagged MiMIF-2 or controls into N. benthamiana leaves, respectively. BAX-induced PCD was completely suppressed when the leaves were infiltrated with
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A. tumefaciens cells carrying FLAG–MiMIF-2 but not with FLAG–GFP (Fig. 4A, B). Moreover, we found that MiMIF-2–HA could suppress cell death triggered by Gpa2/RBP-1 (Fig. 4C, D). Msp40, a M. incognita effector, was used as negative control (Niu et al., 2016) and did not suppress cell death triggered by Gpa2/RBP-1.

We next investigated whether MiMIF-2 affected PAMP-triggered immunity (PTI). PTI requires signal transduction from receptors of downstream components via MAPK cascades. We compared the MAPK cascades of WT Arabidopsis and MiMIF-2-OE-T3 plants, by monitoring levels of activated phosphorylated MAPKs, MPK3 and MPK6, after treatment with the bacterial PAMP flg22 (flagellin-derived peptide), with a polyclonal α-phospho-44/42 MAPK (ERK1/2) antibody. The activation of MPK3 and MPK6 by flg22 was significantly weaker in MiMIF-2-OE-T3 seedlings than in WT seedlings (Fig. 5A). We analysed the expression of six defense marker genes (PR1, PR3, FRK1, CYP81, RbohD, and RbohF) after treatment with flg22. As expected, flg22 significantly induced the expression of five defense marker genes (PR1, PR3, FRK1, CYP81, and RbohD) in WT plants, but not RbohF. By contrast, these five marker genes were repressed in MiMIF-2-OE-T3 plants, and this repression was particularly strong for CYP81 (Fig. 5B). Moreover, flg22 induced almost no callose deposition in MiMIF-2-OE plants, contrasting with its effects on WT cotyledons (P<0.001, Fig. 5C, D).

MiMIF-2 interacts with AnnAt1 and AnnAt4 in vivo

We identified the target proteins of MiMIF-2 by performing liquid in planta immunoprecipitation (IP) followed by LC-MS/MS analysis on the three MiMIF-2-OE lines and WT Arabidopsis. We focused on candidate proteins that specifically pulled down in the three MiMIF-2-OE plants but not in the WT control (Supplementary Table S3). We then examined the interactions between these candidates and MiMIF-2 using BiFC assays and validated annexin AnnAt1 (AT1G35720) as a MiMIF-2 target. The co-expression of MiMIF-2–nEYFP and AnnAt1–cEYFP in tobacco leaves reconstituted yellow fluorescent protein (YFP) activity in the cytoplasm of transformed cells (Fig. 6A; Supplementary Fig. S7), whereas no YFP fluorescence was observed in YFP plasmids containing control constructs. These results constitute the first validation of AnnAt1 and MiMIF-2 interaction.

Eight annexins have been described in Arabidopsis (Clark et al., 2001; Cantero et al., 2006). We investigated whether MiMIF-2 could interact with other annexins that are strongly expressed in roots (AnnAt2, AnnAt3, AnnAt4, AnnAt5, AnnAt8) by using BiFC. We found that only AnnAt4 (AT1G35720) was a MiMIF-2 target. The co-expression of MiMIF-2–nEYFP and AnnAt1–cEYFP in tobacco leaves reconstituted yellow fluorescent protein (YFP) activity in the cytoplasm of transformed cells (Fig. 6A; Supplementary Fig. S7), whereas no YFP fluorescence was observed in YFP plasmids containing control constructs. These results constitute the first validation of AnnAt1 and MiMIF-2 interaction.

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To validate these interactions, Co-IP was performed in N. benthamiana. FLAG–AnnAt1 or FLAG–AnnAt4 and MiMIF-2–HA were co-expressed in plants. As a negative control, tobacco leaves were co-infiltrated with FLAG–GFP

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Fig. 3. The effect of MiMIF-2 silencing and expression in planta on M. incognita parasitism. (A) RT-qPCR assays showed the level of MiMIF expression in M. incognita collected from Arabidopsis lines (GFP RNAi lines and MiMIF-2 RNAi lines) treated by host-derived RNAi in planta, 14 days post-inoculation (dpi). The tubulin gene was used as an internal control. (B) The total numbers of galls and nematodes were counted at 35 dpi in the MiMIF-2 RNAi line T3-3 (T3-2, -5, -7, and -8). (C) MiMIF-2 expression in Arabidopsis increases susceptibility to M. incognita. The total numbers of galls and nematodes were counted at 35 dpi in MiMIF-2 ectopically expressing lines (MiMIF-2-OE-3, -4, and -5). Data are presented as means ±SD (n=30). Asterisks denote significant differences (*P<0.05, **P<0.001, one-way ANOVA). All experiments were performed three times with similar results, and at least 30 plants were analysed per line. (This figure is available in color at JXB online.)
and MiMIF-2–HA. An anti-FLAG antibody clearly detected single target bands at 37 and 27 kDa, corresponding to the expected sizes of AnnAt1 and AnnAt4, and eGFP, respectively. Analyses of the immunoprecipitated proteins with HA antibody showed that MiMIF-2 (13 kDa) was specifically pulled down by AnnAt1 and AnnAt4, but not by eGFP (Fig. 6B).

The entire MiMIF-2 structure and the Ca\(^{2+}\)-binding sites of annexins are required for interaction

The amino-terminal proline residue of MIF is crucial for its biochemical activities (Calandra and Roger, 2003). Likewise, annexins contain Ca\(^{2+}\)-binding sites of functional importance (Gerke and Moss, 2002; Laohavisit and Davies, 2009). We investigated the possible involvement of these sites in protein–protein interactions, by generating a series of mutations in the protein sequences of MiMIF-2, AnnAt1, and AnnAt4 (Fig. 7A). A missense mutant (P2/G, MiMIF-2-mut) and a deletion mutant (MiMIF-2–113aa) were generated in the BiFC and Co-IP vectors. Four missense mutations with amino-acid substitutions affecting the Ca\(^{2+}\)-binding sites of AnnAt1 (AnnAt1E68A, AnnAt1D299A, and AnnAt1E68A/D299A) and AnnAt4 (AnnAt4E72A) were also constructed. The BiFC and Co-IP assays clearly showed that the entire structure of MiMIF-2, including, in particular, the amino-terminal proline residue, was required for interaction with AnnAt1 and AnnAt4 (Fig. 7B, D). The Ca\(^{2+}\)-binding sites of AnnAt1 and AnnAt4 were also essential for interaction with MiMIF-2 (Fig. 7C, D).

AnnAt1 and AnnAt4 are involved in Arabidopsis response to M. incognita

We investigated the biological functions of AnnAt1 and AnnAt4 in mediating the response to M. incognita by manipulating the
Fig. 5. MiMIF-2 suppresses the defense triggered by flg22 in Arabidopsis. (A) MiMIF-2 suppresses the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) response induced by flg22 in Arabidopsis. Twelve-day-old seedlings of wild-type plants (WT) and plants expressing MiMIF-2 (MiMIF-2-OE-3 and MiMIF-2-OE-4) were treated with 1 µM flg22. MAPK activation was analysed by western blotting with an α-phospho-p44/42 MAPK antibody (Cell Signaling Technology, USA). P, Ponceau S staining of the membrane, demonstrating equal protein loading. (B) WT and MiMIF-2 (MiMIF-2-OE-3 and MiMIF-2-OE-4) seedlings were grown for 10 d on MS agar and treated with 1 µM flg22. Induction of the defense marker genes PR1, PR3, FRK1, CYP81, RbohD, and RbohF in response to flg22 was assessed by RT-qPCR and comparison with plants grown without flg22 treatment. (C, D) Callose deposition in the cotyledons of 10-day-old Arabidopsis seedlings from WT and MiMIF-2-expressing plants (MiMIF-2-OE-3 and MiMIF-2-OE-4) 24 h after mock or 1 µM flg22 treatment. Callose deposition was analysed by staining with aniline blue, images were captured by UV epifluorescence microscopy, and ImageJ was used to determine the number of fluorescent spots. Error bars represent the mean ±SD (n=12), and asterisks denote significant differences (**P<0.001, one-way ANOVA). All these experiments were repeated three times with similar results. (This figure is available in color at JXB online.)
levels of AnnAt1 and AnnAt4 expression via overexpression and knockout approaches in Arabidopsis. Homozygous T3 lines overexpressing AnnAt1 and AnnAt4 under the control of the Super promoter (Supplementary Fig. S6C) were less susceptible to *M. incognita* than control plants (Fig. 8A), illustrated by a reduced number of galls and nematodes inside roots. In contrast, T-DNA insertional alleles of *annAt1* (Salk_015426) or *annAt4* (Salk_096465) resulted in the opposite phenotype, a higher number of galls and nematodes (Fig. 8B; Supplementary Fig. S11). These results showed the importance of annexin 1 and annexin 4 for restricting *M. incognita* infection.

The increase in [Ca^{2+}]_{cyt} triggered by H_{2}O_{2} is impaired in MiMIF-2-expressing Arabidopsis

Annexin 1 and annexin 4 have been shown to be involved in Ca^{2+} signature in Arabidopsis roots (Richards et al., 2014; Ma et al., 2019). To identify the roles of MiMIF-2 in Ca^{2+} influx, MiMIF-2-OE-T3 Arabidopsis and two mutants of AnnAt1 and AnnAt4 with a reporter gene encoding aequorin (Supplementary Fig. S12) were challenged with H_{2}O_{2}. A transient increase in [Ca^{2+}]_{cyt} was observed in all genotypes (WT, MiMIF-2-OE-T3, *annAt1*, and *annAt4*). However, there was a significant (P<0.05) difference between the maximum [Ca^{2+}]_{cyt} in aequorin-WT (0.819±0.033 μM) versus aequorin-MiMIF-2 (aequorin-MiMIF-2-OE-3: 0.579±0.055 μM; aequorin-MiMIF-2-OE-4: 0.592±0.011 μM), aequorin-AnnAt1 (0.568±0.013 μM), and aequorin-AnnAt4 (0.558±0.015 μM) (mean ±SEM; Fig. 9). Together, these results highlight the key role of MiMIF-2 in modulating plant immune responses and Ca^{2+} signal transduction.

**Discussion**

MIFs have been shown to be an important endocrine immune factor in animals (Twu et al., 2014; Sparkes et al., 2017), and some animal parasites, including nematodes, ticks and protozoa,
A nematode MIF-like protein targets plant annexins

have been reported to secrete MIFs as a mechanism of host immune system evasion (Pastrana et al., 1998; Falcone et al., 2001). Nevertheless, only a few studies have investigated the role of MIFs in plant parasites. Aphid MIFs were secreted from *Myzus persicae* saliva and were crucial for aphid survival, fecundity, and feeding on a host plant. The ectopic expression of aphid MIFs in leaf tissues inhibits the major plant immune response (Dubreuil et al., 2014; Naessens et al., 2015). Interestingly, MIFs have been identified in many plant-parasitic nematodes, including cyst nematodes and RKNs (Vermeire et al., 2008). We characterized MIF-like proteins of *M. incognita*, and explored their functions in plant–nematode interaction.

Secreted effectors play essential roles in nematode–host interactions. Research efforts in recent decades have focused largely on effectors from the esophageal gland cells of plant-parasitic nematodes (Hussey, 1989; Davis et al., 2008; Hewezi, 2015), and there have been a few reports relating to effectors secreted onto the parasite surface. The majority of known plant nematode effectors are synthesized in specialized secretory cells, the esophageal/salivary glands. So far only a few RKN effectors have been localized inside giant cells, all in nuclei (Favery et al., 2016; Chen et al., 2017). In planta immunocytological analyses showed that the giant cell apoplasm is an important compartment of nematode stylet-secreted proteins among which are proteins that are predicted to act intracellularly such as a calreticulin (Vieira et al., 2011). No mechanism of nematode effector translocation has been described and the minute amount of secreted products might prevent their detection in hypertrophied RKN-induced giant cell cytoplasm. Here we demonstrated that MiMIF-2 secreted from the hypodermis into the plant apoplast finally targets the giant cell cytoplasm (Fig. 2). This result highlights a process by which an apoplastic effector enters into plant cells.

In mammals, MIF has been shown to mediate its biological effects via two signaling mechanisms: the classical receptor-mediated pathway and a non-classical endocytic pathway (Donn and Ray, 2004). The first human MIF receptor to be identified was a cell surface class II major histocompatibility
chaperone, CD74 (Leng et al., 2003). MIF has also been shown to be directly taken up into target cells and to interact with intracellular signaling molecules, including the human Jun activation domain-binding protein JAB-1 (Kleemann et al., 2000).

The characterization of the pathway(s) used by MiMIFs, and/or endogenous plant MDLs, to enter into the plant cells would represent a breakthrough in our knowledge of the mode of action of small proteins in plants.

Host-derived RNAi silencing or ectopic expression of MiMIFs in planta significantly decreased or increased, respectively, the infectiousness of *M. incognita*, as indicated by the mean numbers of galls and nematodes at 35 dpi in plant roots. The two annexin mutant lines were more susceptible to *M. incognita*, as indicated by the mean numbers of nematodes and galls in plant roots. Error bars represent the mean ± SD (n=30), and asterisks denote significant differences (*P<0.05, one-way ANOVA). All experiments were repeated three times with similar results, and each line counted at least 30 plants. (This figure is available in color at JXB online.)

 defenses at this stage. To achieve this, RKNs deliver several effectors into host cells to suppress immune responses including PCD (Niu et al., 2016; Chen et al., 2017; Naalden et al., 2018; Nguyen et al., 2018; Shi et al., 2018). We showed that transient expression of MiMIF-2 could suppress PCD triggered by BAX or RBP1/Gpa2 (Fig. 4). In addition, the use of flg22 to stimulate the defense responses of Arabidopsis (Gómez-Gómez and Boller, 2000; Asai et al., 2002; Navarro et al., 2004; Lee and Back, 2016) revealed low levels of MPK3 and MPK6 phosphorylation, suppression of defense marker gene expression and low levels of callose deposition in MiMIF-2-expressing lines (Fig. 5). MPK3 and MPK6 are crucial positive regulators of plant defense responses; they are involved in the biosynthesis of both ethylene (Meng and Zhang, 2013) and jasmonate (Schweighofer and Meskiene, 2008). MAPK signaling in Arabidopsis roots has also been demonstrated to be activated following infection by the cyst nematode *Heterodera schachtii* (Sidonskaya et al., 2016). Thus, MiMIF-2 expression may interfere with host signal transduction and immune responses as described in mammalian cells.

In an attempt to understand how MiMIFs interfere with these plant pathways, we used IP-LC-MS to identify proteins that interact with MiMIF-2. BiFC and Co-IP demonstrated two annexins were targeted by MiMIF-2 in Arabidopsis (Fig. 6). MiMIF-2–AnnAt1 and MiMIF-2–AnnAt4 complexes were found to localize within the cytosol. Previous studies have indicated that AnnAt1 not only rescues a mutant *E. coli* strain lacking OxyR from H$_2$O$_2$ stress (Gidrol et al., 1996), but also may protect (i) human tumor cells against tumor necrosis factor and (ii) mammalian cells against oxidative stress (Jänicke et al., 1998; Kush and Sabapathy, 2001). AnnAt1 levels
have been shown to increase significantly during osmotic stress and powdery mildew infection (Lee et al., 2004; Cantero et al., 2006; Chandran et al., 2010). The annexin from Brassica juncea (AnnBJ1) gave resistance to numerous abiotic and biotic stresses in tobacco (Jami et al., 2008). There is also evidence to suggest that annexins are involved in plant wound responses through their role in membrane repair (Schapire et al., 2009; Clark et al., 2012). Interestingly, cyst nematodes induce expression of ANNEXIN genes in Arabidopsis (Puthoff et al., 2003) and soybean roots (Klink et al., 2007) and secrete annexin-like effectors to interfere with plant immune responses. Hs4F01 could complement the function of Arabidopsis annexin 1, indicating a mimicry of plant annexins by nematode annexin. Moreover, Hs4F01 may modulate defense responses through interacting with the 2OG-Fe(II) oxygenase (DMR6) (Patel et al., 2010). These results suggest dual roles of Hs4F01 during cyst nematode parasitism. However, previous studies on transcript changes of RKN-induced galls showed that the expression of Arabidopsis annexin 1 and annexin 4 were both repressed (Jammes et al., 2005; Barcala et al., 2010). Here, we showed that AnnAt1- or AnnAt4-overexpressing lines are more resistant to M. incognita and that, on the contrary, the AnnAt1 or AnnAt4 knockout lines are more sensitive (Fig. 8). This is in accordance with the role of annexins in plant defense. While no annexins have been shown to be secreted by root-knot nematodes, we can propose that MiMIF-2 is involved in protection of nematodes from oxidative stress by fine-tuning the plant annexin function.

Annexins are Ca\(^{2+}\)- and phospholipid-binding proteins widespread throughout the animal and plant kingdoms (Gerke and Moss, 2002; Laohavisit and Davies, 2011). Ca\(^{2+}\) is widely seen as a common and simple messenger that acts by coupling extracellular signals to cellular responses and integrating biotic and abiotic information (Dodd et al., 2010; Newton et al., 2016; Ranty et al., 2016). Moreover, the Ca\(^{2+}\) signals act in synergy with ROS and activate MAPK cascades (Gilroy et al., 2014; Lee and Back, 2016). Annexins regulate abiotic stress-induced calcium signal transduction and mediate biotic stress responses to environmental stimuli (Gorecka et al., 2007; Vandeputte et al., 2007; Davies, 2014; Yang et al., 2014). Calcium signatures also activate various plant enzymes, even altering gene expression, and can, ultimately, modify cells and the calcium signals corresponding to their physiological and biochemical reactions (Tuteja and Mahajan, 2007; Kim et al., 2009). Peroxide-induced increases in [Ca\(^{2+}\)]\(_{cyt}\) were recently reported to be aberrant in roots and root epidermal protoplasts from an annAt1-knockout mutant, indicating an essential role for AnnAt1 in the [Ca\(^{2+}\)]\(_{cyt}\) signature and downstream signaling (Richards et al., 2014). Moreover, AnnAt1 interacts with AnnAt4, which is modulated by Ca\(^{2+}\) binding (Huh et al., 2010), and recent studies confirmed AnnAt4 interacted with the calcium sensor SCaBP8, which regulated calcium transients and signatures in Arabidopsis (Ma et al., 2019). These results inspire the possibility that plant annexins with other proteins may form complexes modulating calcium signaling. It’s possible that MiMIF-2 is involved in the complexes that fine-tune annexin-mediated signal transduction and immune responses. Evidence showed that in rat testicular peritubular cells, MIF signal transduction was based on mobilizing intracellular calcium (Wennemuth et al., 2000). In human neutrophils, MIF induced calcium influx by acting on two chemokine receptors, CXCR2 and CXCR4 (Bernhagen et al., 2007). Here, we found that the Ca\(^{2+}\) binding sites of annexins were required for interaction between MiMIF-2 and annexins (Fig. 7). This led us to hypothesize that Ca\(^{2+}\) signal transduction might be impaired in MiMIF-2 ectopically expressing Arabidopsis plants. Consistent with this hypothesis, Arabidopsis expressing MiMIF-2 displayed smaller increases in [Ca\(^{2+}\)]\(_{cyt}\) following stimulation with H\(_2\)O\(_2\), i.e. a decrease Ca\(^{2+}\) influx (Fig. 9), as previously observed in annAt1 and annAt4 mutants (Richards et al., 2014; Ma et al., 2019). Our findings suggest a new model for Ca\(^{2+}\), annexins, and pathogen effectors: the combination of annexins with effectors modulates calcium signaling, interfering with the immune response. Ultimately, our discovery further improves our understanding of effector proteins in M. incognita and identifies new avenues of exploration for studies investigating mechanism by which MIF-like proteins function.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Characteristics of MiMIF sequences.

Fig. S2. Western blot analysis detected specificity of MiMIF-2 antibody.

Fig. S3. Ultrastructural immunocytochemistry of M. incognita MIF proteins.

Fig. S4. Localization of MiMIF proteins in tomato gall sections.

Fig. S5. Phenotypes of MiMIF-2 ectopically expressing Arabidopsis and Mimif-2-RNAi lines.

Fig. S6. Verification, by RT-PCR and/or western blotting, of the presence of MiMIF-2 transcripts, MiMIF-2 protein, and annexin proteins in transgenic Arabidopsis.

Fig. S7. MiMIF-2 could not interact with a chloroplasts chaperonin (AT3G13470), heat shock protein 70 (AT5G2490) and heat shock protein 9 (AT5G56010).

Fig. S8. MiMIF-2 could not interact with AnnAt2, AnnAt3, AnnAt5, and AnnAt8.

Fig. S9. Subcellular localization of AnnAt1, AnnAt4, AnnAt1E68A, AnnAt1D299A, AnnAt1E68A/D299A, and AnnAt4E72A.

Fig. S10. Subcellular localization of MiMIF-2, MiMIF-2-mu, and MiMIF-2-113aa.

Fig. S11. Verification of homozygous T-DNA insertion mutants of annAt1 and annAt4.

Fig. S12. RT-PCR verification of gene expression in transgenic Arabidopsis.

Table S1. Primers used in this study.

Table S2. Accession numbers of genes or proteins used in this study.

Table S3. Candidate proteins that interact with MiMIF-2 identified by LC-MS.

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