Original Article

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A novel *Meloidogyne incognita* chorismate mutase effector suppresses plant immunity by manipulating the salicylic acid pathway and functions mainly during the early stages of nematode parasitism

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Abstract

Meloidogyne incognita is the most economically important plant-parasitic nematode. *M. incognita* manipulates plant cell development and metabolism by injecting effectors from the esophageal glands into the plant host. Chorismate mutase (CM) is one such effector that may contribute to successful parasitism by *M. incognita*. In this study, we identified and functionally characterized a novel CM effector, Mi-CM-3, which is more similar to CMs from bacteria than to those previously characterised from other phytoparasitic nematodes. Spatial and temporal expression assays showed that *Mi-cm-3* mRNA accumulates specifically in the subventral esophageal glands, and that transcription is up-regulated during the early parasitic stages of the nematode. *In planta* gene silencing of *Mi-cm-3* attenuated nematode parasitism. In addition, *Mi-cm-3* could fully restore the full virulence phenotype of the pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* by complementation when it was introduced into a mutant strain carrying a deletion in the CM gene. Transient expression of Mi-CM-3 caused a reduction of SA and *PR1* levels in *Nicotiana benthamiana* in response to oomycete pathogen *Phytophthora capsici* infection, while confocal observations showed that Mi-CM-3 was localized within the cytoplasm and the nucleus, but not the plastids, of transfected *N. benthamiana* leaf cells. Constitutive expression of Mi-CM-3 in *N. benthamiana* plants inhibited root growth and increased susceptibility to *M. incognita* infection. These results provide direct experimental evidence to show that Mi-CM-3 may play an important role in suppressing plant immunity by regulating the SA pathway during the early parasitic stage of *M. incognita* so as to promote nematode parasitism.

Keywords: Meloidogyne incognita, RNAi, complementation, *in planta* expression, localization, salicylic acid

Introduction

Root-knot nematodes (RKNs; *Meloidogyne* spp.) are some of the most devastating plant-parasitic nematodes (PPNs). These sedentary endoparasites can infect more than 3,000 plant species and cause extensive annual crop yield losses worldwide (Sasser, 1980). The second-stage juvenile (J2), the infectious stage of the RKN, penetrates the root and migrates intercellularly through the plant tissue towards the developing vascular cylinder, where approximately four to eight plant cells are selected to establish a feeding structure, known as giant cells, which provide a continuous nutrient supply for the nematode throughout the rest of its life cycle. Proteins synthesized in the oesophageal glands and injected through the stylet into plant tissue (so-called effectors) play essential roles in this process of nematode parasitism.

Recent studies have identified a suite of effectors from RKNs, as well as the cyst nematode species *Globodera pallida*, *G. rostochiensis*, *Heterodera glycines*, and *H. schachtii* (Rehman et al., 2014; Thorpe et al., 2014; Eves-van den Akker et al., 2016), and their functions in the plant hosts during nematode parasitism have been elucidated gradually. Among these proteins, cell wall-modifying enzymes, such as cellulases, pectate lyases, and cellulose-binding proteins, have been characterized as essential weapons involved in the invasion of plant roots and migration within root tissues by J2s (Popeijus et al., 2000). In addition, some nematode effectors have been shown to mimic plant peptides so as to manipulate growth of the host when secreted into plant cells (Patel

et al., 2010; Wang et al., 2011). Moreover, several effectors which can suppress programmed cell death (PCD) in host plants induced by various elicitors have also been identified (Lozano-Torres et al., 2012; Zhuo et al., 2017).

Chorismate mutase (CM) is an effector that has previously been identified in different sedentary endoparasitic nematode species and has been shown to play important roles in plant-nematode interactions (Lambert et al., 1999; Huang et al., 2005; Vanholme et al., 2009). Prior to its description from plant-parasitic nematodes, CM had been studied extensively in many other organisms, including bacteria, fungi, plants, and protists (Schmid and Amrhein, 1995; Roberts et al., 1998; Djamei ea al., 2011). CM catalyses the conversion of chorismate formed in the last step of the shikimate pathway to prephenate, which is a precursor for the biosynthesis of the aromatic amino acids phenylalanine and tyrosine (Dewick, 1998). The first animal-derived CM gene, *Mj-cm-1*, was identified from the root-knot nematode *M. javanica*; the spatial and temporal expression patterns and phenotypic assays of transgenic plants expressing *Mj-cm-1* revealed that the gene may participate in the compatible interaction between nematode and host plant (Lambert et al., 1999). Further research has shown that CMs are widely distributed in phytonematodes, but are absent in the free-living nematode *Caenorhabditis elegans* and various animal parasites, suggesting that this enzyme plays a role in plant parasitism (Parkinson et al., 2001).

Herein we report a novel CM effector (designated Mi-CM-3) that was identified through analysis of genome sequencing data from *M. incognita.* Mi-CM-3 shares only 29-31% identity with the amino acid sequences of the two paralogs *Mi-cm-1* and *Mi-cm-2* reported previously (Huang et al., 2005). In order to investigate whether Mi-CM-3 is a functional chorismate mutase and to understand its role during the interaction between *M.*

incognita and its host, phylogenetic analysis, spatial and temporal expression assays, a complementation test in a CM-deletion mutant of the pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), subcellular localization, and both transient and constitutive expression of *Mi-CM-3* in plants were carried out in the present study. Our results suggest that Mi-CM-3 may suppress plant immunity by manipulating the salicylic acid (SA) pathway, and that it functions mainly at the early stage of nematode parasitism.

Materials and methods

Nematodes and plants

The population of *M. incognita* isolated from Jiangsu, China, was maintained on susceptible tomato plants (variety Sufen No. 8) in a greenhouse at 25°C. Eggs, pre-parasitic J2s (preJ2s), parasitic-stage juveniles (parJ2s), and females were collected as previously described (Huang et al., 2005). Seedlings of *Nicotiana benthamiana* were grown a greenhouse at 25°C, and were used at the four-leaf stage for nematode inoculation and virus infiltration. Plants of the bacterial blight-susceptible rice cultivar IR24 were grown in a greenhouse at 28°C day and 25°C night and were used for inoculation with strains of *X. oryzae* pv. *Oryzae* (*Xoo*).

Sequence and phylogeny analysis

The whole genomic sequences including proteins and coding sequence of *M. incognita* were downloaded from the wormbase database, and local BLAST searches using *Mi-cm-1* and *Mi-cm-2* as queries were conducted with an E-value cutoff of 1e-10

using Seqhunter2 (Ye et al., 2010). The signal peptide and its cleavage site were predicted using SignalP 4.0 (Petersen et al., 2011). The protein molecular mass and theoretical isoelectric point were predicted through ExPASy available at the Bioinformatics Resource Portal (http://www.expasy.ch/tools/). Motif analyses were performed using Motif Scan (Pagni et al., 2007). BLASTP and BLASTX were used for sequence similarity searches (Altschul et al., 1997). Sequence alignments were conducted using CLUSTALW 1.82 (Thompson et al., 1994). The phylogenetic analysis of CMs was performed with the PHYLIP package (version 3.696) using the neighbor-joining method (Felsenstein et al., 1989). Statistical support of the inferred clusters was evaluated with 1,000 bootstrap replicates.

Nucleic acid extraction and gene cloning

Total RNA was isolated from *M. incognita* preJ2s by flash freezing the nematodes in liquid nitrogen and grinding them with Trizol reagent (Invitrogen) in a sterile 1.5 ml microfuge tube. First strand cDNAs were synthesized using the SMART RACE cDNA Amplification Kit (Takara) following the manufacturer's instructions. The 5'- and 3'-RACE were performed using anchor and gene-specific primers designed from the sequence of a coding sequence (CDS) (Minc10536) deposited in the wormbase database (http://www.wormbase.org). PCR amplification products were purified using a universal DNA purification kit and cloned into the pMD19-T vector (TaKaRa) for transformation. The genomic clone of *Mi-cm-3* gene was amplified from DNA with primers cm3-F and cm3-R. The positive clones that were confirmed by colony PCR were then sent to Sunbiotech (Beijing, China) for DNA sequencing. The full length cDNA sequence of

Mi-cm-3 was assembled from the overlapping sequences of both 5'- and 3'-RACE products using BioEdit Version 7.0.1 (T.A. Hall, North Carolina State University, USA). All primers used in this study are listed in Table S1.

Developmental expression analysis and *In situ* hybridization

Total RNA was extracted separately from eggs and nematodes at different life stages as described above. Transcripts of *Mi-cm-3* and the actin reference gene were detected by the real-time quantitative reverse transcription PCR (RT-qPCR) with primer pairs cm3RT-F/cm3RT-R and MiactF/MiactR, respectively. The RT-qPCR assays were performed in the Applied Biosystems 7500 System (Applied Biosystems) using SYBR Premix ExTaq (TaKaRa). The transcript abundance of *Mi-cm-3* in each sample was normalized using the actin gene (Minc06769), and relative transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). Three independent biological replicates were conducted while each RT-qPCR amplification was run in triplicate.

A 350 bp fragment of the *Mi-cm-3* gene was amplified from *M. incognita* pre-J2 cDNA using the primers cm3IH-F and cm3IH-R. The amplicon was used as a template to produce digoxigenin (DIG)-labelled sense and antisense cDNA probes using the PCR DIG Probe Synthesis Kit (Roche) by asymmetric PCR. *In situ* hybridization was performed as previously described (Huang et al., 2005) with minor modifications. Briefly, the pre-parasitic juveniles and mixed parasitic stage nematodes of *M. incognita* were first fixed in 2% formaldehyde solution at 5°C for 18 h. The nematodes were then sectioned by hand with a razor blade on a glass slide and subjected to proteinase-K digestion at room temperature for 30 min. Hybridization was performed with the

DIG-labelled sense and antisense cDNA probes at 50°C overnight. Signals were detected using anti-DIG antibody conjugated to alkaline phosphatase and its substrate. The nematode sections were observed with an Olympus BX51 microscope and images were captured with a DP72 Digital Camera (Olympus).

In planta RNAi

Tobacco rattle virus (TRV)-mediated gene silencing was used to knock down the expression of *Mi-cm-3* and for further inoculation assays. A fragment of the target gene *Mi-cm-3* was first amplified from the cDNA of *M. incognita* pre-J2s with the primer pair cm3TRV-F/ cm3TRV-R, and a DNA fragment derived from green fluorescent protein (GFP) was also amplified and used as a negative control. The PCR products were then cloned into the pTRV2 vector by digestion with *BamH*I and *Xma*I and transformed into *Agrobacterium tumefaciens* GV3101. The *cp* gene encoding the TRV coat protein was detected in total RNA extracted from TRV-infected *N. benthamiana* seedlings by reverse transcription PCR (RT-PCR) using primers TRV2-F and TRV2-R, confirming the spread of the virus from leaves to roots. TRV-inoculated and -uninoculated seedlings were then each inoculated with 400 *M. incognita* J2s at day 7 post agroinfiltration. The parasitic juveniles collected from seedlings at 7 dpi from both the TRV-inoculated and control plants were used to test the effects of silencing *Mi-cm-3*. RNA extraction, first-strand cDNA synthesis, and RT-qPCR were performed as described above. Three biological replicates were conducted for each treatment, and each RT-qPCR reaction was performed in triplicate. Seedlings of *N. benthamiana* that had been inoculated with J2 nematodes were kept at 25°C for 45 days, the roots were carefully washed free of soil and the fresh

weight recorded. The numbers of females produced on the roots of each plant were then counted following staining with acid fuchsin, The results are presented as females per gram of root fresh weight.

Construction of a Xoo CM-deletion mutant and genetic complementation

Two DNA fragments flanking the *Xoo-cm* gene, *Xoo*-cm-L (400 bp) and *Xoo*-cm-R (380 bp), were first amplified from strain PXO99^{A} genomic DNA using the primer pairs XooLB-F/XooLB-R and XooRB-F/XooRB-R, respectively. The amplicons were then ligated into the pMD-T vector and their identities confirmed by sequencing. The pMD-T-L and pMD-T-R plastids were separately digested with the restriction enzymes *BamH*I and *EcoR*I, and the R fragment was ligated into pMD-L to form pMD-T-LR. The fused fragment LR was then subcloned into the pEASYT3 vector, generating pEASY-LR, followed by the introduction of a SacB cassette at the SpeI site. The resulting plasmid, pEASY-LR-SacB, was introduced into strain PXO99 ^{A} by electroporation to produce the deletion mutant Δ*Xoo-cm*. Transformed cells were then screened successively on NA medium without and with 10% sucrose. The individual colonies that grew on NAS plates were confirmed by PCR with the primer pair XooMut-F/XooMut-R. Those clonies that had a genomic deletion of ~500 bp were considered to be *Xoo-cm* mutants and were used for further study.

To construct plasmids for complementation of the CM-defective deletion mutant, DNA fragments containing the region deleted from *Xoo-cm* and/or the coding region of *Mi-cm-3* minus the signal peptide were amplified using primer pairs XooC-F/XooC-R and cm3C-F/cm3C-R, respectively. After being confirmed by sequencing, the DNA

fragments were ligated into pEASY-LR-SacB between the *BamH*I and *Sac*I sites to produce the complementation plasmids pEASY-LR-*Xoo-cm*-SacB and pEASY-LR-*Mi-cm*-SacB, respectively. The recombinant plasmids were electroporated into the CM-deletion mutant Δ*Xoo-cm* and screened as described above.

Southern blotting

Southern blot analysis was performed as previously described (Sambrook et al., 1989) with minor modifications. Briefly, genomic DNA was isolated from *Xoo* strains using the Wizard Genomic DNA Purification Kit (Promega) and then digested with the restriction enzymes *Hind*III. After separation by electrophoresis in a 0.8% agarose gel, the DNA fragments were transferred to nylon membranes and hybridized with DIG-labelled probes generated with the primer sets XooC-F/XooC-F and cm3C-F/cm3C-F, respectively. Hybridization and signal detection were carried out using a DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) according to the manufacturer's instructions.

Pathogenicity assays in rice

Rice plants grown for six weeks were used for bacterial inoculation using the leaf-clip method (Kauffman et al., 1973). *Xoo* strains were cultured in NB medium at 28°C with shaking at 200 rpm for 24–48 hours until they reached the late logarithmic phase. The bacterial inocula were then adjusted to an optical density OD_{600} = 1.0. The leaves of the rice plants were clipped ~2 cm from the tip with scissors that had been immersed in the

inoculum solutions. At least ten leaves were inoculated for each strain, and the experiment was repeated three times. The lengths of the disease lesions on the treated leaves were measured 15 d after bacterial inoculation.

Transient expression and subcellular localization

The *Mi-cm-3* coding sequence minus the signal peptide sequence was amplified using gene-specific primers cm3gw-F and cm3gw-R. The resulting PCR product was purified and cloned into the pCR8/GW/TOPO vector (Invitrogen) followed by DNA sequencing. The DNA fragment was then recombined into the destination vector pK7WGF2 (GFP fusion) using the Gateway LR Clonase Enzyme Mix (Invitrogen), after which it was transformed into *A. tumefaciens* GV3101. To investigate the effect of transient expression of Mi-CM-3 on pathogen infection, the *Mi-cm-3* strains as well as the controls expressing GFP were infiltrated into *N. benthamiana* leaves 24 h prior to inoculation with *P. capsici*. The concentrations of free SA and conjugated salicylate glucoside (SAG) were examined at 12 hours post-inoculation (hpi) in infected leaves using HPLC as described by Lee et al. (2011), and the relative levels of *PR1* mRNA were also measured at 12 hpi using RT-qPCR with primer pair NbPR1-F/ NbPR1-R. The actin gene of *N. benthamiana* (*Nbactin*) was used as an internal reference to normalize gene expression levels. In addition, the lesion diameters caused by *P. capsici* infection were measured at 48 hpi. For subcellular localization, the GFP:Mi-CM-3 fusion protein was expressed together with the mCherry plastid localization marker (pt-rk CD3-999) in *N. benthamiana* leaves by co-agroinfiltration. The fluorescence signals in the *N. benthamiana* leaf cells were observed using a ZEISS LSM 710 confocal microscope (Zeiss Microsystems).

Generation of transgenic *N. benthamiana* plants and phenotypic assays

For constitutive expression of *Mi-cm-3*, a binary vector pBI121 containing the coding sequence of Mi-CM-3 without the signal peptide was generated and transformed into *A. tumefaciens* strain LBA4404. *N. benthamiana* transformation was performed using the leaf disk method as described previously (Horsch et al., 1985). The T_3-generation lines selected by segregation analysis of kanamycin resistance were used for further experiments. To detect the expression of *Mi-cm-3* transcript and its abundance, RT-qPCR analysis was performed using the primer pair cm3qRT-F/cm3qRT-R with the *Nbactin* gene as an internal reference as described above. Seeds of the transgenic and wild type (WT) lines were germinated on MS agar medium at 22 °C under a 16 h/8 h light-dark cycle. Seedlings were grown vertically on Petri dishes for two weeks, and the root lengths was then measured manually. The transgenic and WT lines were also used in a nematode infection assay to evaluate the effect of Mi-CM-3 ectopic expression on parasitic susceptibility. The J2s inoculation as well as counting the number of females were performed as described above. Statistically significant differences between the different treatments were determined by Student's t-test using the statistical package SPSS.

Western blotting

The leaves of *N. benthamiana* were harvested 2 days after *Agrobacterium* infiltration, and proteins were extracted from 300 mg of ground frozen tissue mixed with 1 ml of extraction buffer (50 mM HEPES, 150 mM KCL, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, pH 7.5) supplemented with 1× protease inhibitor cocktail (Roche). The extracts were centrifuged at 12,000 g for 15 min at 4°C, and the supernatants were recovered.

Samples were fractionated by SDS-PAGE and then transferred to a PVDF membrane using a semi-dry transfer system (BioRad). The membrane was then blocked in 1× PBST containing 5% skim milk (in PBS, pH 7.2) for 1 h at room temperature, followed by an incubation with the anti-GFP monoclonal antibody (Sigma-Aldrich) solution overnight at 4°C. After washing 3 times with 1× PBST, the membrane was transferred to the HRP-conjugated anti-mouse IgG secondary antibody solution diluted with 1% skim milk and incubated for 1 h at room temperature. The hybridization signals were detected using SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer's instructions (Pierce).

Results

Identification and characterization of *Mi-cm-3*

BLAST searches against the *M. incognita* genome database (http://www6.inra.fr/meloidogyne_incognita; http://www.wormbase.org) with the *M. incognita Mi-cm-1* and *Mi-cm-2* gene sequences (GenBank accessions AY422834 and AY422835) revealed the presence of a 600-bp CDS (Minc10536) predicted to encode a CM paralog, designated as *Mi-cm-3*, which was further confirmed by 3′- and 5′-RACE-PCR using cDNA extracted from *M. incognita*. The 5′ untranslated region (UTR) of the *Mi-cm-3* was 89 bp long and included the spliced leader SL1 sequence, while the 146-bp 3′ UTR contained two polyadenylation signals (attaaa) followed by a polyA tail. The genomic clone of *Mi-cm-3* was also amplified and sequenced, and the exon/intron boundaries of the genomic sequences were determined by aligning the genomic sequence with the CDS.

The alignment showed that *Mi-cm-3* contains two introns of 46 and 281 bp, and all intron sequences follow the 'GU-AG rule' for *cis*-splicing (Blumenthal and Steward, 1997; Fig. S1). *Mi-cm-3* encodes a predicted polypeptide of 199 amino acids. SignalP analysis indicated that the deduced protein contains a predicted signal peptide with a cleavage site between Ala21 and Asp22, resulting in a mature protein with a theoretical molecular weight of 20.1 kDa and an isoelectric point of 5.86. A motif scan identified four conserved N-glycosylation sites in Mi-CM-3 at amino acids Asn75, Asn97, Asn111 and Asn117 as well as a CM_2 domain (residues 30–106) (http://pfam.xfam.org/family/PF01817). In addition, two Casein kinase II phosphorylation sites at residues Thr77 and Thr119, as well as two Protein kinase C phosphorylation sites at residues Thr159 and Ser193 were also predicted (Fig. S2).

Homology search and phylogenetic analysis

To study the phylogenetic relationships between the predicted amino acid sequences of *Mi-cm-3* and other CMs from phytoparasitic nematodes, plants, fungi, and bacteria, we performed a BLASTP search with the default e-value threshold against the NCBI databases (www.ncbi.nlm.nih.gov) using the Mi-CM-3 amino acid sequence minus the signal peptide as a query. The highest predicted amino acid identity were observed between Mi-CM-3 and a homologue from *M. arenaria* (Ma-CM-1, ABB02655) with 98% sequence identity and 100% similarity, followed by a CM from *Hirschmanniella oryzae*, which shows 41% identity and 92% similarity. Other than for these two nematode homologues, Mi-CM-3 was more similar to CMs from Gram-negative bacteria in the genus *Burkholderia* than to those from the majority of phytoparasitic nematodes, with

sequence identities ranging from 32% to 34%. Mi-CM-3 showed only limited sequence similarity with the fungal and plant CMs.

The sequences of these CM genes were used to conduct a phylogenetic analysis, which resulted in six distinct, well-supported clades. Most of the CMs, except the one from *Ustilago maydis* and those from phytoparasitc nematodes, were grouped into three clades for genes from plants, fungi and bacteria. The distribution of CM-specific sequences from plant-parasitic nematodes was more complicated. All CMs from cyst nematodes were included in a clade with two well supported subclades that is well separated from the root-knot nematode CMs. The RKN CM sequences were distributed between two separate clades; 4 RKN CMs grouped together in one clade, while Mi-CM-3, along with Ma-CM-1 and Ho-CM-1, grouped in another clade with CMs from bacteria in the genus *Burkholderia* (Fig. 1).

Spatial and temporal expression assays

In situ mRNA hybridization was carried out to examine the tissue-specific expression of *Mi-cm-3* in *M. incognita*. The hybridization signals of the transcripts were observed mainly in the subventral esophageal gland cells of the nematode (Fig. 2a), whereas no signal was observed in the control treatment using the DIG-labelled sense-strand cDNA probe (Fig. 2b). The expression pattern of *Mi-cm-3* during development of *M. incognita* was quantified using RT-qPCR. The lowest level of *Mi-cm-3* expression was detected within eggs, after which the mRNA level increased 29.8-fold within preJ2, and *Mi-cm-3* transcription peaked in the parJ2, in which the relative amount of mRNA increased approximately 72.6-fold when compared with that in the eggs. Then the relative

expression of *Mi-cm-3* showed sharp reductions in J3/J4 stage and females to around 1/7 and 1/3 the level in the parJ2, respectively (Fig. 3). These results indicate that *Mi-cm-3* is most highly expressed during the early parasitic stages of *M. incognita*.

Influence of *Mi-cm-3* silencing on nematode infectivity

To determine the role of CM in *M. incognita* pathogenicity, the TRV-mediated gene silencing technique was utilized to knock down *Mi-cm-3* expression, and the effects on nematode parasitism were evaluated. The expression of *cp*, the TRV coat protein gene, was detected by RT-PCR on the 7th day post agroinfiltration, which confirmed that virus particles had already spread to the roots from the leaves of *N. benthamiana* inoculated with the TRV silencing constructs (Fig. S3). The *Mi-cm-3* mRNA levels were analyzed by RT-qPCR in juveniles at 7 days post-inoculation (dpi), a time when the parasitic juveniles of *M. incognita* have already initiated nutrient acquisition from root cells and when *Mi-cm-3* transcript abundance should be at a high level based on the results of developmental expression assays.

The results showed that the juvenile nematodes collected from *N. benthamiana* plants that had been agroinfiltrated with TRV::*Mi-cm-3* showed a 65.2% reduction in *Mi-cm-3*-specific mRNA compared with the control (Fig. 4a). No significant difference was observed in the expression of *Mi-cm-3* in the juveniles collected from control *N. benthamiana* and plants infected with the TRV::*GFP* recombinant virus. The pathogenicity of nematodes on the TRV::*Mi-cm-3* plants also exhibited an obvious reduction, and the numbers of females were significantly reduced by 51.6% at 45 days after inoculation compared with that on uninfected *N. benthamiana*. There was no

significant difference in the number of females on *N. benthamiana* plants infected with the *TRV::GFP* virus and uninfected plants (Fig. 4b).

Complementation assay of *Xoo* cm-deletion mutant

In order to determine whether CM is a functional chorismate mutase, a mutant of the bacterial blight pathogen of rice, *X. oryzae* pv. *oryzae* (*Xoo*) was used. The single-copy gene *Xoo-cm*, which was confirmed by analysis of the sequenced genome, was first knocked out by homologous suicide plasmid integration as described in the Methods. Complemented bacterial strains were then generated by introducing the *cm* gene from strain PXO99^{A}, as well as *Mi-cm-3* from *M. incognita*, into the mutant Δ*Xoo-cm*. The deletion- and complementation of *Xoo-cm* were subsequently verified by PCR and Southern blot hybridization (Fig. S4).

The virulence of these *Xoo* strains was tested on the leaves of susceptible rice cultivar IR24 using the scissor-clip inoculation method. All strains were able to induce obvious symptoms of bacterial leaf blight at 15 days post infiltration (dpi). However, the symptoms produced by the deletion mutant Δ*Xoo-cm* were less severe than those caused by the wild-type strain PXO99^{A} (Fig. 5a); the mean length of lesions caused by Δ*Xoo-cm* was significantly different from that caused by the wild-type strain, with a reduction of 39.7%. No obvious differences were observed between the lengths of lesions caused by the complemented strain Δ*Xoo-cm(Xoo-cm)* and the wild-type strain. As expected, the complemented strain Δ*Xoo-cm(Mi-cm-3)* containing the CM gene from *M. incognita*, was also able to restore the virulence of the deletion mutant to a level comparable to

that of the wild-type strain (Fig. 5b). These results suggest that *Xoo-cm* is essential for the pathogenicity of *Xoo* strain PXO99 ^{A}, and that *Mi-cm-3* could functionally replace *Xoo-cm*.

Transient expression of Mi-CM-3 on plant immunity

To investigate whether the action of Mi-CM-3 within plant cells leads to compromised immunity, the nematode effector without signal peptide was transiently expressed in *N. benthamiana* leaves via agroinfiltration. A compatible pathogen, *P. capsici*, was then inoculated onto the leaves of *N. benthamiana* 12 h after infiltration with agrobacterium strains carrying the Mi-CM-3 construct and the negative control GFP expression construct, as well as the un-infiltrated control. Western blotting with an anti-GFP antibody confirmed the expression of proteins of the expected lengths (Fig. 6a). We found that the diameters of the lesions caused by *P. capsici* were significantly larger following transient expression of Mi-CM-3 compared with the two controls (Fig. 6b, c). In addition, the accumulation of free SA and SAG in the Mi-CM-3 infiltrated sites were significantly reduced by 45.2% and 42.1%, respectively, compared with those in wild type plants (Fig. 6d, e). Furthermore, the expression levels of the SA-associated gene *PR1* also showed a significant reduction of 39.4% compared with the control (Fig. 6f). Meanwhile, no evidently phenotypic difference was observed between GFP treatment and un-infiltrated control. These results provide evidence that Mi-CM-3 can promote *P. capsici* infection in *N. benthamiana* and its action is correlated with the SA-mediated defense signaling pathways.

Localization of Mi-CM-3 in leaf cells of *N. benthamiana*

To examine the distribution of Mi-CM-3 in plant cells, the coding sequence of *Mi-cm-3* without the native signal peptide fused with GFP was transiently expressed in *N. benthamiana* leaves via agroinfiltration and its localization was monitored by confocal microscopy. Fluorescence observation revealed that GFP signals were localized within the cytoplasm and nucleus of transfected *N. benthamiana* leaf cells, similar to the pattern observed for native GFP alone, while no signal co-localized with the mCherry-tagged plastid marker pt-rk CD3-999 (Fig. 7). These results suggest that the Mi-CM-3 effector probably functions within the cytoplasm of the host plant cell during nematode parasitism.

Effect of ectopic Mi-CM-3 on root growth and nematode infection

To further understand the biological roles of Mi-CM-3, we constitutively expressed the coding sequence of the gene minus the signal peptide driven by the CaMV 35S promoter in *N. benthamiana* plants. The transgenic plants were identified by selection for antibiotic resistance and three independent homozygous T_3-generation lines were chosen for further analysis. The Mi-CM-3 overexpressing lines displayed a slow main root growth phenotype when the seedlings were grown on MS agar medium (Fig. 8a), and the line cm-3-40 showed a increased production of lateral roots. The average root lengths of transgenic lines were reduced by 25.8-33.3% compared with the empty vector-transfected line, while there was no significant difference in the length of the empty vector control and wild type (Fig. 8b). RT-qPCR demonstrated that the *Mi-cm-3* transcript was detectable in all three transgenic lines and the line cm-3-09 showed

significantly higher mRNA expression than the other two lines. The *Mi-cm-3* transcript could not be detected in the WT or the empty vector control lines (Fig. 8c). We next performed nematode susceptibility assays to assess the virulence function of Mi-CM-3. All three transgenic lines expressing *Mi-cm-3* were more susceptible to *M. incognita* parasitism than were the wild-type controls, and the line cm-3-09 and cm-3-40 showed statistically significant increases of 49.4%and 39.5% in the average number of females at 45 dpi compared to the empty vector control. No significant difference was found between the empty vector control line and WT line. (Fig. 8d). These data indicate that constitutive expression of Mi-CM-3 in plants affects normal root growth and promotes nematode parasitism.

Discussion

Chorismate mutase has been identified from many plant-parasitic nematode species, including *Globodera rostochiensis*, *G. tabacum*, *G. pallida*, *G. aellingtonae*, *Heterodera glycine*, *H.schachtii*, *Hirschmanniella oryzae*, *Meloidogyne javanica*, *M. arenaria*, as well as *M. incognita*, from which two CM genes, *Mi-cm-1* and *Mi-cm-2*, were reported previously (Lambert et al., 1999; Huang et al., 2005; Vanholme et al., 2009). In this study, we cloned and characterized a new nematode CM gene (*Mi-cm-3*) from *M. incognita*. Sequence alignment showed that the decuced amino acid sequence of *Mi-cm-3* is more similar to CMs from bacteria than to most CMs from phytoparasitic nematodes. It has been proposed that there is a suite of nematode genes that may have been acquired from bacteria via horizontal gene transfer (HGT), including

cell-wall-degrading enzymes (Davis et al., 2000), expansins (Kudla et al., 2005), invertases (Danchin et al., 2016), as well as CMs (Vanholme et al., 2009).

Our phylogenetic analysis showed that all nematode CMs were more closely related to bacterial CMs than to CMs from plants and fungi. The nematode CMs were clustered into three separate clades, two of which in particular contained CMs from cyst nematodes and root-knot nematodes, and a third clade that consisted of Mi-CM-3, two CM homologues from *M. arenaria* and *H. oryzae*, as well as several bacterial CMs. It is not clear here whether these nematode CMs that clustered dispersedly in phylogeny tree were acquired separately from bacteria by multiple HGT events or were diverged from a common precursor of nematode under strong selective pressure.

Chorismate mutases are widely distributed in prokaryotes, and secreted CMs may play a role in bacterial pathogenesis (Qamra et al., 2006). In the present study, we generated a CM deletion mutant of the pathogenic bacterium *Xoo* strain POX99^{A}. Virulence tests on the leaves of susceptible rice cultivar IR24 showed that significantly reduced disease symptoms were induced by infection with the *Xoo* mutant. To confirm that the phenotype resulted from the deletion of CM, we complemented the mutant strain (Δ*Xoo-cm*) with the CM gene from strain POX99^{A}, which restored full virulence to the mutant, similar to the WT strain. Interestingly, the nematode-derived CM could also fully restore the pathogenic phenotype of Δ*Xoo-cm* when it was introduced into the cm deletion mutant. From this indirect evidence, combined with the TRV-mediated gene silencing assay which resulted in fewer nematode infections, we concluded that Mi-CM-3 may play an important role in virulence during *M. incognita* parasitism. This conclusion was also supported by the results of spatial and temporal expression assays, which showed that *Mi-cm-3* is up-regulated at the parasitic J2 stage of *M. incognita* and that its

mRNA accumulates specifically in the subvental gland cells. These data also suggest that MI-CM-3 probably functions in the early stages of parasitism as the subventral glands are thought to play main roles for the early stages of parasitism and the dorsal gland for the development and maintenance of feeding site (Hussey, 1989).

Chorismate mutase is an enzyme in the shikimate pathway of higher plants, in which there are both plastidic and cytosolic forms of CM, resulting in a competition between the plastid and cytoplasmic CMs for chorismate synthesized specifically in the plastid (Eberhard et al., 1996). The nematode CMs are thought to be secreted into the plant cytosol, and may tip the balance in favor of the cytosolic metabolism pathway (Doyle et al., 2003). As expected, our localization results provide evidence that Mi-CM-3 is localized in the cytoplasm, but could not co-localize with the plastid marker pt-rk CD3-999 when they were both transiently expressed in the leaves of *N. benthamiana.*

Normal metabolism of chorismate in the plastid as well as the cytoplasm is vital in plants, because chorismate-derived compounds (CDCs) are converted into a vast array of secondary metabolites, including aromatic amino acids (phenylalanine, tyrosine, and tryptophan), plant hormones (indole-3 acetic acid), and numerous compounds (phytoalexins and salicylic acid) (Dewick, 1998).

Salicylic acid (SA) has been recognized as a critical signaling molecule in the defense response in many plant species to attack by various pathogens. It has been reported that the bacterial pathogen *Pseudomonas syringae* and the the oomycete pathogen *P. capsici* can be inhibited by SA-dependent defence responses (Liu et al., 2010; Wang et al. 2013). Likewise, activation of SA biosynthesis and signaling pathways could also reduce the infection of root-knot nematode (Kyndt et al., 2014). A ‘chorismate competition model’

has been proposed to explain how nematode CMs could regulate localized concentrations of SA so as to suppress defense responses during infection (Doyle et al., 2003). A similar mechanism has already been reported for *U. maydis* CMU1 (Djamei et al., 2011), while a similar model for the function of *P. sojae* and *Verticillium dahliae* secreted isochorismatases (PsIsc1 and VdIsc1, respectively), which are proposed to act on plant isochorismate (Liu et al., 2014). In the current study, we demonstrate experimentally for the first time that a nematode CM can disrupt SA biosynthesis and SA-mediated defense when transiently expressed in *N. benthamiana* leaf tissue using agroinfiltration.

Auxin is an important CDC and plays a major role in plant root development. Disruption of auxin biosynthesis could be the reason why constitutive expression of Mi-CM-3 in plants inhibited root growth in our study. A similar result has been reported by Doyle et al. (2003), in which the expression of MJ-CM-1 in transformed soybean hairy roots gave an abnormal root development phenotype. Moreover, auxin has also been demonstrated to be involved in the induction and formation of plant-parasitc nematode feeding site, of which may be by manipulating the balance between auxin transport and synthesis (Goverse et al., 2000; Kyndt et al., 2016), howerver, it is not clear that whether the alteration of auxin accumulation caused by ectopic exprssion of Mi-CM-3 in transgenic line of *N. benthamiana* may also contribute to the parasitsm of *M. incognita* in the present work, therefore, more work is needed in future research to investigate the exact mechanism during the interaction between the nematode CM effector and plant.

In summary, we have characterized a novel CM effector from the root-knot nematode *M. incognita*, and found that it is more similar to CMs from bacteria than to its paralogs previously identified in *M. incognita*. This effector, Mi-CM-3, can restore full pathogenicity when expressed in a CM-deficient strain of the plant pathogenic bacterium

Xoo. Our results suggest that Mi-CM-3 may play an important role in suppressing plant immunity by regulating the SA pathway to promote nematode parasitism. Further studies are required to understand how gene expression of *N. benthamiana* is manipulated in Mi-CM-3 overexpression line, and this will increase our understanding of the molecular mechanisms of plant-nematode interactions.

Disclosure

The authors have declared that no competing interests exist.

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Supporting Information legends

Figure S1. Nucleoide sequence of *Mi-cm-3.* The 5'- and 3'-UTR are shadowed; SL1 is underlined; open reading fragment (ORF) is shown in uppercase and the predicted start and stop codons are presented in bold; two introns are presented in lower-case letters; the poly-adenylation signal are boxed.

Figure S2. Multiple sequence alignment of Mi-CM-3 with chorismate mutases (CMs) of other plant-parasitic nematodes. Gr-CM-1 (ABR19887) from *Globodera rostochiensis*; Gt-CM-1 (AEA07501) from *G. tabacum*; Gp-CM-1A (AEA07498), Gp-CM-1B (AEA07500) and Gp-CM-1B (CAD29887) from *G. pallida*; Ge-CM-1 (AIE45298) from *G. ellingtonae*; Hg-CM-1A (AAY42590) and Hg-CM-1B (AAO19577) from *Heterodera glycines*; Hs-CM-1 (ABA06538) and Hs-CM-2 (ABA06539) from *H. schachtii*; Ho-CM-1 (ALI53581) from *Hirschmanniella oryzae*; Mj-CM-1 (AAD42163) from *Meloidogyne javanica*; Ma-CM-1 (ABB02655) and Ma-CM-2 (ABB86550) from *M. arenaria*; Mi-CM-1 (AAR37372), Mi-CM-2 (AAR37373) and Mi-CM-3 (Minc10536) from *M. incognita*. The putative signal sequence of all CMs is underlined. Shading indicates identical (black) or similar (grey) amino acids. Conserved CM_2 domain is marked with overbar. Putative N-glycosylation sites, Casein kinase II phosphorylation site and Protein kinase C phosphorylation sites of Mi-CM-3 are marked with triangles, rhombuses and squares, respectively.

Figure S3. Reverse transcription PCR detection of the *cp* (coat protein) gene in two tobacco rattle virus lines at 1, 3, 7 and 14 days (d) post Agrobacterium inoculation; the transcripts of the actin gene of *N. benthamiana* (*Nbactin*) were used as control.

Figure S4. Deletion- and complementation of *Xoo-cm*. (A) Schematic drawing of *Xoo-cm* deletion construct. (B) Confirmation of *Xoo-cm* deletion mutants by PCR analysis. M: DNA ladder. (C) Southern blot of the *Xoo-cm* deletion mutant and its complementation transformant.

Table S1. Primers used in this study.

Figure legends

Figure 1 Phylogenetic analysis of Mi-CM-3 to CMs from plants, fungi and bacteria. The unrooted consensus tree was generated by PHYLIP package and bootstrap values from 1,000 replicates are shown at each node. Gr-CM-1 (ABR19887) from *Globodera rostochiensis*; Gt-CM-1 (AEA07501) from *G. tabacum*; Gp-CM-1A (AEA07498), Gp-CM-1B (AEA07500) and Gp-CM-1B (CAD29887) from *G. pallida*; Ge-CM-1 (AIE45298) from *G. ellingtonae*; Hg-CM-1A (AAY42590) and Hg-CM-1B (AAO19577) from *Heterodera glycines*; Hs-CM-1 (ABA06538) and Hs-CM-2 (ABA06539) from *H. schachtii*; Ho-CM-1 (ALI53581) from *Hirschmanniella oryzae*; Mj-CM-1 (AAD42163) from *Meloidogyne javanica*; Ma-CM-1 (ABB02655) and Ma-CM-2 (ABB86550) from *M. arenaria*; Mi-CM-1 (AAR37372), Mi-CM-2 (AAR37373) and Mi-CM-3 (Minc10536) from *M. incognita*; *Burkholderia multivorans* (SAK23723); *B. oklahomensis* EO147 (KEO71221); *B. cepacia* (KVW02555); *B. mallei* (AJX54512); *B. ambifaria* AMMD(ABI86426); *Escherichia coli* 53638 (ZP_00735647); *Ralstonia solanacearum* GMI1000 (CAD14606); *Xanthomonas campestris* (WP_080565796); *X. oryzae* (WP_011259051); *Magnaporthe oryzae* 70-15 (XP_363784); *Aspergillus nidulans* FGSC A4 (XP_664470); *Neurospora crassa* OR74A (XP_961975); *Penicillium subrubescens* (OKP10745); *Umbilicaria pustulata* (SLM40300); *Histoplasma capsulatum* H88 (EGC46822); *Trichophyton interdigitale* H6 (EZF28730); *Ustilago maydis* (XP_011391476); *Arabidopsis thaliana* (CAB54518); *Acacia koa* (AOX49229); *Nicotiana attenuate* (OIT28362); *Solanum lycopersicum* (AAD48923); *Medicago truncatula* (ABE94587).

Figure 2 *In situ* hybridization of the *Mi-cm-3* transcript using digoxigenin (DIG)-labelled probes. Hybridization with antisense (A) and sense (B) cDNA probes showing that transcripts of the gene are mainly detectable in the subventral oesophageal gland cells in the second-stage juveniles of *Meloidogyne incognita*. S: stylet; M: metacorpus; SvG, sbuventral gland cell. Bar =100 µm.

Figure 3 Developmental expression analysis of *Mi-cm-3* through *Meloidogyne incognita* life stages. The transcript abundance fold change of *Mi-cm-3* were normalized with the actin internal standard and are presented as the ratio relative to the amount in preJ2s. Each column represents the mean relative expression and standard error obtained from three independent biological experiments which were repeated three times with similar results. Statistically significant differences using a Student's t-test ($P < 0.01$) are indicated with different letters. preJ2: preparasitic second-stage juvenile; parJ2: parasitic J2; J3: third-stage juvenile; J4: fourth-stage juvenile.

Figure 4 Effect of *in planta* RNAi of *Mi-cm-3* on the pathogenicity of *Meloidogyne incognita*. (A) Relative expression level of *Mi-cm-3* in *M. incognita* collected from control and virus infected lines of *N. benthamiana*. (B) The females of *M. incognita* produced on different lines of *N. benthamiana* at 45 days post-infection. Data represent the mean and standard deviations from 5-8 plants and the independent experiments were repeated three times with similar results. Statistically significant differences using a Student's t-test ($P < 0.01$) are indicated with different letters.

Figure 5 Virulence test of various *Xanthomonas oryzae* pv. *oryzae* strains in rice. (A) Wild type, Δ*Xoo-cm*, Δ*Xoo-cm(Xoo-cm)*, Δ*Xoo-cm(Mi-cm-3)* strains were inoculated on the rice leaves (6 weeks old) by using the leaf-clipping method, respectively. The disease symptoms were observed at 15 days post-inoculation. (B) The lesion lengths were recorded from 10 inoculated leaves for each strain. Data represent the mean and standard deviations of three independent experiments, and different letters above the bars denote statistically significant differences ($P < 0.05$, Student's t test).

Figure 6 Analysis of transient expression of Mi-CM-3 on plant immunity against *Phytophthora capsici* infection. (A) Western blotting of the fusion expression proteins in *N. benthamiana* using anti-GFP antibodies. (B) Phenotypes of leaves transiently expressing Mi-CM-3 and controls upon *P. capsici* infection. (C) Lesion diameters of *N. benthamiana* leaves at 48 hpi, averaged from at least 10 independent biological replicates. (D) SA and (E) SAG levels at 12 hpi in *P. capsici* inoculated leaves. (F) Relative expression levels of *PR1* gene at 12 hpi in *P. capsici* inoculated leaves. The independent experiments were repeated three times with similar results. Statistically significant differences using a Student's t-test ($P < 0.05$) are indicated with different letters.

Figure 7 Subcellular localization of Mi-CM-3 in plant cells. *N. benthamiana* leaves expressing GFP fused to the Mi-CM-3 as well as GFP alone together with the mCherry-labeled plastid localization marker were observed using confocal microscopy.

Figure 8 Phenotype and susceptibility assays of transgenic tobacco lines expressing Mi-CM-3.(A) Root growth of wild type and transgenic seedlings on MS media.(B) The root lengths were recorded from 10 independent seedlings for each lines. (C) The number of females of *M. incognita* produced on wild type and different transgenic lines of *N. benthamiana* at 45 days post-infection. Data represent the mean and standard deviations from 5-8 plants and the independent experiments were repeated twice with similar results. Statistically significant differences using a Student's t-test ($P < 0.05$) are indicated with different letters.