1 Silencing *SmDI* in Solanaceae alters susceptibility to root-knot nematodes

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Summary

Root-knot nematodes (RKNs) are among the most damaging pests of agricultural crops. Indeed, *Meloidogyne* is an extremely polyphagous genus of nematodes that can infect thousands of plant species. A few genes for resistance (R-genes) to RKNs suitable for use in crop breeding have been identified, and new virulent strains and species of nematode emerge rendering these R-genes ineffective. Effective parasitism is dependent on the secretion, by the RKN, of effectors targeting plant functions, which mediate the reprogramming of root cells into specialised feeding cells. These cells, the giant cells, are essential for RKN development and reproduction. The EFFECTOR 18 protein (EFF18) from *M. incognita* interacts with the spliceosomal protein SmD1 in Arabidopsis, disrupting its function in alternative splicing regulation and modulating the giant cell transcriptome. We show here that EFF18 is a conserved RKN-specific effector. We also show here that EFF18 effectors also target SmD1 in *Nicotiana benthamiana* and *Solanum lycopersicum*. The alteration of *SmD1* expression by virus-induced gene silencing (VIGS) in Solanaceae affects giant cell formation and nematode development. Thus, *SmD1* is a susceptibility gene and a promising target for the development of broad resistance, especially in Solanaceae, for the control of *Meloidogyne* spp.

46 **Introduction**

47 Plant parasitic nematodes are major crop pests causing crop losses of several million dollars
 48 annually, through damage to almost all cultivated plants, and the transmission of plant viruses
 49 (Singh et al., 2013). Root-knot nematodes (RKNs) of the genus *Meloidogyne* are considered
 50 to be the most detrimental of these plant parasites, due to the magnitude of the economic
 51 losses they cause (Jones *et al.*, 2013). RKNs are widespread worldwide and can infect more
 52 than 5,500 different plant species, including many species of major agricultural interest.
 53 About 100 RKN species have been described, and those reproducing asexually by mitotic
 54 parthenogenesis (*M. incognita*, *M. javanica*, *M. arenaria* and *M. enterolobii*) are the most
 55 polyphagous and damaging pests. By contrast, those reproducing sexually or by meiotic
 56 parthenogenesis (*M. hapla*) have a smaller host range (Blok et al., 2008; Castagnone-Sereno,
 57 2006).

58 All RKNs are sedentary endoparasites that induce the formation of specialised feeding
 59 structures and typical root deformations, known as galls or root knots, that deprive the plant of
 60 nutrients (Escobar *et al.*, 2015; Favery *et al.*, 2016). After hatching from eggs, the stage 2
 61 juveniles (J2) of *M. incognita* penetrate the root apex and migrate between plant cells to reach
 62 the plant vascular system (Holbein *et al.*, 2019). Once there, the filiform J2 switch to a
 63 sedentary lifestyle, by selecting five to seven cells of the vascular parenchyma and inducing
 64 their reprogramming into specialised feeding cells, known as giant cells (Escobar *et al.*, 2015;
 65 Favery *et al.*, 2016; Olmo *et al.*, 2020). These hypertrophied and multinucleate cells act as
 66 metabolic sinks close to the xylem and phloem vessels that withdraw water and nutrients from
 67 the sap (Rodiuc *et al.*, 2014). The nematode uses these specific giant cells for feeding for the
 68 rest of its life. After successive moults, the sedentary swollen juveniles develop into an adult
 69 female that lays her egg masses on the root surface, thus completing the cycle. The giant cells
 70 are hypertrophied and multinucleate, harbouring hundreds of nuclei. They are produced by

71 successive nuclear divisions uncoupled from cytokinesis, followed by nuclear
72 endoreduplication (de Almeida Engler and Gheysen, 2013). RKN induce giant cells and gall
73 formation by recruiting the developmental pathways of post-embryonic organogenesis and
74 regeneration to promote transient pluripotency (Olmo *et al.*, 2020).

75 RKNs parasitise plants and induce the redifferentiation of vascular cells into giant cells by
76 secreting effectors, molecules that recruit/hijack plant functions (Mejias *et al.*, 2019; Toruño
77 *et al.*, 2016). RKN effectors, particularly those produced by the three oesophageal gland cells
78 and secreted into the host through a stylet, are involved in the four main functions underlying
79 parasitism: (i) the degradation and modification of plant cell walls during J2 migration within
80 the root; (ii) the suppression of host defences; (iii) the reprogramming of plant vascular cells
81 as giant cells and (vi) the maintenance of these feeding sites (Mitchum *et al.*, 2013; Truong *et al.*,
82 2015). The profound morphological and metabolic changes associated with giant cell
83 induction by RKNs and the transcriptional reprogramming occurring during the formation of
84 these cells require the secretion of effectors targeting key nuclear functions (Hewezi and
85 Baum, 2013; Quentin *et al.*, 2013). With the exception of plant cell wall-degrading enzymes
86 (Danchin *et al.*, 2010), very few effectors have been shown to be conserved and functional in
87 multiple RKN species. For example, 16D10 encodes a conserved secretory peptide conserved
88 in five RKN species (*M. incognita*, *M. arenaria*, *M. hapla*, *M. javanica*, *M. chitwoodi*) that
89 stimulates root growth and functions as a ligand for a putative plant transcription factor
90 (Huang *et al.*, 2006; Dinh, 2015). The silencing of *16D10* by RNA interference methods
91 confers broad resistance to RKNs (Huang *et al.*, 2006; Dinh, 2015). The chorismate mutases,
92 MiCM3 (Wang *et al.*, 2018) and MjCM1 (Doyle and Lambert, 2003), and the transthyretin-
93 like proteins, MjTTL5 (Lin *et al.*, 2016) and MhTTL2 (Gleason *et al.*, 2017), also appear to
94 be effectors conserved among RKNs. Interestingly, MhTTL2 is expressed in the amphids
95 (Gleason *et al.*, 2017), whereas MjTTL5 is expressed specifically in the subventral glands,

suggesting different roles for these two molecules in parasitism, encoded by the same gene family (Lin *et al.*, 2016).

We recently showed that MiEFF18, a nuclear effector from *M. incognita*, is secreted *in planta*, targets the giant cell nuclei and interacts with the SmD1 protein, a core component of the spliceosome (Mejias *et al.*, 2020). We show here that MiEFF18 is a specific and conserved RKN effector and that orthologous genes are specifically expressed in the salivary glands of RKNs. We also show that MiEFF18 and its orthologue in *M. enterolobii*, MeEFF18, interact with SmD1 proteins from different plant species. Moreover, virus-induced gene silencing (VIGS) approaches silencing the SmD1 genes of *N. benthamiana* and *S. lycopersicum* greatly impair RKN infection. These results are consistent with the targeting, by RKNs, of conserved spliceosomal functions, to drive the development of giant cells, facilitating parasitism on a large spectrum of host plants.

Results

EFF18 is a conserved RKN-specific effector targeting plant nucleus

MiEFF18 was first described in the *M. incognita* genome (Mejias *et al.*, 2020; Nguyen *et al.*, 2018; Rutter *et al.*, 2014). Database queries showed that MiEFF18 displayed no sequence homology or known domains, and that it was absent from nematodes of other genera, such as cyst nematodes and free-living nematodes. By contrast, EFF18 orthologues were identified in seven of the eight RKNs for which genome sequences were available: *M. incognita*, *M. javanica*, *M. arenaria* (Blanc-Mathieu *et al.*, 2017), *M. hapla* (Opperman *et al.*, 2008), *M. enterolobii* (syn. *M. mayaguensis*) (Koutsovoulos *et al.*, 2020), *M. floridensis* (Lunt *et al.*, 2014) and *M. luci* (Susič *et al.*, 2020) (Figure 1, Table S1, Figure S1). No EFF18 orthologue was identified in *M. graminicola* (Somvanshi *et al.*, 2018). Three paralogous copies were identified, in the *M. incognita*, *M. javanica* and in *M. luci* genomes. Four copies were detected

in *M. arenaria* and a single copy was detected in *M. hapla*, *M. floridensis* and *M. enterolobii*. A sequence alignment and analysis of the RKN EFF18 protein sequences showed that they were more than 60% identical, between 279 and 316 amino acids (aa) long and that they had an N-terminal secretion signal peptide (SSP), a low-complexity acidic D/E-rich region and a C-terminal lysine (K)-rich domain carrying direct repeats (Figure 1a, Figure S2). Only the C-terminal K-rich domain displayed marked differences between copies.

A phylogenetic tree based on an alignment of the 17 RKN EFF18 protein sequences showed divergences between copies among the same species (Figure 1a). EFF18 proteins more closely related to MiEFF18a/Minc18636 harboured one monopartite NLS and one bipartite NLS, whereas other copies are more divergent (*e.g.* MiEFF18b/Minc15401 and MiEFF18c) and contained only one monopartite NLS (Figure 1a). MiEFF18a fused at its C- or N-terminus to GFP (green fluorescent protein) and MeEFF18a fused at its N-terminus to GFP were transiently expressed in *Nicotiana benthamiana* leaf epidermis. For EFF18 constructs, GFP fluorescence was only detected in the nucleus, with a strong GFP signal accumulating in the nucleolus (Figure 1b). In contrast, GFP alone was detected in the cytoplasm and the nucleus, but not in the nucleolus (Figure 1b). The EFF18s with bipartite NLS were 98% to 100% identical to the MiEFF18a protein, whereas those with only monopartite NLS were only 79 to 89% identical to this protein (Figure 1c). *M. hapla* had the most divergent genome of the *Meloidogyne* species tested. It was found to have a single copy of the gene, 63-65% identical to the closest copies and the most divergent copies, which suggests that the ancestor of RKN species had an EFF18 gene, and providing support for the role of EFF18 as a conserved effector.

RKN EFF18s are specifically expressed in the subventral glands

MiEFF18 have been shown to be more strongly expressed at parasitic stages and to be expressed specifically in the subventral glands of *M. incognita* J2s (Rutter et al., 2014; Nguyen et al., 2018; Mejias et al., 2020). We studied the pattern of expression of genes encoding orthologous sequences of MiEFF18 in two other RKN species, by performing *in situ* hybridisation (ISH) for the *M. enterolobii* *MeEFF18a* and the *M. arenaria* *MaEFF18a* sequences. A specific signal was detected in the subventral oesophageal gland cells of pre-J2s after hybridisation with digoxigenin-labelled *MeEFF18a* and *MaEFF18a* antisense probes (Figure 2). No signal was detected in pre-J2s with sense negative controls. This finding suggests that *MaEFF18a* and *MeEFF18a*, may, like *MiEFF18a*, be secreted and play an important role in nematode parasitism.

MiEFF18a and MeEFF18a interact with the SmD1 proteins of *A. thaliana*, *N. benthamiana* and *S. lycopersicum*

We have demonstrated an interaction between MiEFF18 and the nuclear ribonucleoproteins SmD1s from *S. lycopersicum* and *A. thaliana*, modulating the pattern of alternative splicing and promoting the formation of giant cells (Mejias et al., 2020). Two genes, *AtSmD1a* (AT3G07590) and *AtSmD1b* (AT4G02840), encode SmD1 proteins in *Arabidopsis* (Koncz et al., 2012) and two genes encode 100% identical SmD1 proteins (SlSmD1) in *S. lycopersicum*: *SlSmD1a* (Soly06g084310) and *SlSmD1b* (Soly09g064660). In *N. benthamiana*, we identified three genes encoding SmD1s: *NbSmD1a* (*Niben101Scf01782g05006*), *NbSmD1b* (*Niben101Scf05290g01011*), and *NbSmD1c* (*Niben101Scf04283g03011*). A multiple sequence alignment showed that SmD1 was highly conserved in these species, with 93% identity between SlSmD1 and the sequence from which it diverged most strongly, *AtSmD1b* (Figure 3a). Like all Sm proteins, SmD1s carry two conserved Sm motifs mediating protein-protein interactions during small nuclear ribonucleoprotein (snRNP) biogenesis (Figure 3b). We

investigated the subcellular localisation of SmD1 in plant cells, by transiently expressing constructs encoding GFP-SmD1 fusion proteins in *N. benthamiana*. We confirmed a strong accumulation of SlSmD1a and AtSmD1b in the nucleolus and in Cajal bodies, and a weaker accumulation in the nucleoplasm (Figure 3c).

We then investigated whether MeEFF18a was also able to interact with SmD1 proteins from *S. lycopersicum* and *A. thaliana*, like MiEFF18 (Mejias et al., 2020). Using a pairwise yeast-two hybrid approach, we showed that MiEFF18a and MeEFF18a interact with SmD1 proteins from plants of different clades, such as *A. thaliana*, *S. lycopersicum* and *N. benthamiana* (Figure 3d). As a control, we tested SmD1 interactions with another *M. incognita* effector, MiEFF16, encoded by the *Minc16401* gene and expressed in the subventral glands, with the same nuclear location *in planta* as MiEFF18 (Mejias et al., 2020). No interaction was observed between MiEFF16 and SmD1 proteins in yeast (Figure 3d). These results demonstrate that EFF18 proteins are conserved among RKNs and that they interact with SmD1 proteins, which are conserved among plant species.

***SmD1* acts as a susceptibility gene for infection in plants of different clades**

We recently demonstrated an important role for the AtSmD1b protein in giant cell formation and successful nematode infection (Mejias et al., 2020). We investigated whether *SmD1* is a conserved susceptibility gene required to ensure infection, and essential for RKN parasitism in Solanaceae species, by using a virus-induced gene silencing (VIGS) approach to alter the expression of *SmD1* genes in *S. lycopersicum* and *N. benthamiana*.

We first performed a VIGS assay to silence *SmD1* genes in *S. lycopersicum* (Figure 4a). We evaluated silencing efficiency by RT-qPCR on emerging leaves. Treated tomatoes had much lower levels of *SmD1* transcripts (Figure 4b). Tomatoes in which SmD1 genes were silenced displayed developmental defects on emerging leaves and had a shorter root system (Figure S3). In tomato plants infected with *M. incognita*, in which *SmD1* genes were silenced, the

number of females producing egg masses was much smaller than that in control plants treated with the TRV-GFP virus (Figure 4c).

Because of adverse effect of *SmDI* silencing on development in tomato, we then silenced the *SmDI* genes in *N. benthamiana*, which allows performing a VIGS assay at a later developmental stage when roots have already developed substantially (Figure 5a and 5b). An evaluation of silencing efficiency by RT-qPCR showed that *N. benthamiana* roots subjected to VIGS had much lower levels of *SmDI* transcripts, particularly for the most strongly expressed gene, *NbSmDIb* (Figure 5c). We observed no significant decrease in the expression of the two mostly weakly expressed genes, *NbSmDIa* and *NbSmDIc* (Figure 5c; Figure S4). *N. benthamiana* plants in which *SmDI* was silenced produced a much smaller number of galls (up to 80% fewer) following infection with *M. incognita* (Figure 5d).

We studied the effect on nematode and giant cell development in detail, by investigating J2s *in planta* by the fuchsin acid staining method, to determine the proportions of migrating filiform and sedentary swollen parasitic juveniles and their ratio. The percentage of migrating filiform J2s was higher (90%) in plants in which *SmDI* was silenced, which had a lower percentage of swollen juveniles, indicating a defect in the RKN development (Figure 6a). We also investigated whether the giant cells formed on plants in which *SmDI* was silenced displayed developmental defects. We observed these cells directly, under a confocal microscope, after clearing in benzyl alcohol/benzyl benzoate (BABB ; Cabrera et al., 2018). A comparison of the mean surface areas of the largest giant cells in each gall showed that giant cells from plants in which *SmDI* was silenced were 36% smaller than those from control plants (Figure 6b and 6c). These results confirm the important role of *SmDI* in giant cell formation in Solanaceae species and the requirement of this protein for successful nematode development.

Discussion

The ability of plant pathogens to infect their hosts is generally dependent on the secretion of effectors. Most pathogens secrete effectors to overcome host physical defences, such as the plant cell wall, and to suppress plant immune responses (Toruño *et al.*, 2016). Other effectors are more specific to the parasitic strategy of the pathogen and may regulate host gene expression or trigger changes in host cell morphology and physiology to allow pathogen feeding and development. Most obligatory biotrophs form specific feeding structures, such as the haustoria of biotrophic filamentous pathogens, and produce sets of specific effectors (Chaudhari *et al.*, 2014; O’Connell and Panstruga, 2006). RKNs are root endoparasites that manipulate host cells to form specialised giant cells for feeding. These giant cells constitute the sole source of nutrients for the nematode, and are, therefore, essential for nematode survival. RKNs induce giant cells by manipulating root cell developmental programmes. Indeed, massive transcriptomic reprogramming occurs during giant cell formation (Favery *et al.*, 2016; Mitchum *et al.*, 2013). Genes associated with root meristem function, lateral root formation and the establishment of the vasculature, in particular, are tightly regulated upon giant cell induction (Cabrera *et al.*, 2014; Olmo *et al.*, 2020; Yamaguchi *et al.*, 2017). Alternative splicing has recently been shown to occur in *Arabidopsis* following infection with *M. incognita*, and this process contributes to transcriptome and proteome diversity (Mejias *et al.*, 2020).

EFF18 is a nuclear conserved RKN-specific effector

Nuclear effectors are thought to mediate the transcriptional reprogramming required for giant cell formation (Mejias *et al.*, 2019; Quentin *et al.*, 2013). They may interfere with the function of transcription factors, as described for Mi16D10, which interacts with SCARECROW-like transcription factors (Huang *et al.*, 2006), or may themselves act as transcription factors, as

reported for Mi7H08 (Zhang *et al.*, 2015). MiEFF18 is another RKN effector that has been shown to be secreted within host cells, in which it localises to the nucleus. MiEFF18 has been shown to interact with the SmD1 protein, a core component of the spliceosome conserved in all eukaryotes, thereby modulating alternative splicing and gene expression (Mejias *et al.*, 2020). MiEFF18 may corrupt the function of *Arabidopsis SmD1* function to modulate the expression of various genes encoding proteins involved in giant cell formation through processes such as DNA replication or cytokinesis (Mejias *et al.*, 2020). We show here that the manipulation of SmD1 function by MiEFF18 plays a key role in giant cell development in other plant species, such as *Nicotiana benthamiana* and the tomato *Solanum lycopersicum*.

Genes encoding the MiEFF18 effector were found in all available *Meloidogyne* spp. genomes other than the draft genome for the rice RKN *M. graminicola* (Somvanshi *et al.*, 2018). EFF18 is exclusive to RKN, being absent from all other parasitic nematodes and other plant pathogens with parasitic strategies not involving the induction of giant feeding cells. At least one orthologous copy of a MiEFF18 sequence was detected in each of the available *Meloidogyne* genomes, demonstrating that MiEFF18 is a conserved effector. The multiple copies identified in *M. incognita*, *M. javanica* and *M. arenaria* are consistent with the polyploidy of these mitotic parthenogenetic species (Koutsovoulos *et al.*, 2020). The absence of an EFF18 effector in *M. graminicola* may be explained by the particular host range and life cycle of this nematode. *M. graminicola* may have lost the EFF18 effector during specialisation on monocotyledonous hosts and adaptation to the infection of submerged roots (Mantelin *et al.*, 2017). These growing conditions may have resulted in different requirements for the modulation of gene expression for giant cell ontogenesis, dependent on effectors other than EFF18.

The distribution of EFF18 orthologues in two major groups, with copies (e.g. MiEFF18a) carrying two NLS, and those of the most divergent group (e.g. MiEFF18b) carrying only one

NLS, suggested a possible duplication of the ancestral MiEFF18 gene in the ancestor of RKN species, with one of the duplicated genes either gaining or losing a bipartite NLS. The proteins from the closest group to the MiEFF18a gene would be expected to function similarly to MiEFF18a, through the modulation of SmD1 functions, due to the very high level of sequence identity between these proteins (98% identity). *M. enterolobii* is an extremely polyphagous species that reproduces through mitotic parthenogenesis, like *M. incognita*. Therefore, we investigated the functionality of proteins MeEFF18 orthologue. We found that, like MiEFF18, MeEFF18a was able to interact with SmD1 proteins from *A. thaliana*, *N. benthamiana* and *S. lycopersicum*, suggesting that orthologous copies of MiEFF18a are functional and target the same functions in different host plants. MiEFF18a and MeEFF18a are the first examples of conserved RKN effectors able to target the same conserved plant process in different plant species.

Targeting conserved effectors to engineer plant resistance

The identification of conserved effectors could lead to new strategies for developing broad resistance (Huang *et al.*, 2006; Landry *et al.*, 2020; Peeters *et al.*, 2013; Roux *et al.*, 2015). Only a few RKN effectors have been described to be conserved. The MAP (*Meloidogyne* avirulence protein) effector family, which includes *M. incognita* Mi-MAP1.2, was shown to be conserved in 13 of the 21 RKN species tested, and absent from other genera of plant-parasitic nematodes (PPNs) (Tomalova *et al.*, 2012). The genes of the MAP effector family harbour one or multiple CLE-like motifs, which may be involved in feeding site formation, as demonstrated for cyst nematode CLE-like peptides, which promote syncytium formation (Rutter *et al.*, 2014; Mitchum *et al.*, 2012). MjNULG1a, from *M. javanica*, is a nuclear effector with a demonstrated role in parasitism. Southern blot experiments have revealed the presence of MjNULG1a orthologues in *M. incognita* and *M. enterolobii*, but not in other

PPNs (Lin *et al.*, 2013). Similarly, the 16D10 effector is exclusive to RKNs (Huang *et al.*, 2006; Dinh, 2015). The use of host-induced gene silencing (HIGS) approaches to engineer plant resistance to RKNs has excited considerable interest (Ali *et al.*, 2017; Banerjee *et al.*, 2017). The targeting of genes involved in nematode development or encoding effectors has been considered. Silencing conserved effectors may allow specific resistance to RKNs with no impact on non-targeted species. Studies of Mi16D10 have demonstrated the feasibility of conferring RKN resistance in *Arabidopsis*, potato or grape through the targeting of this effector (Huang *et al.*, 2006; Yang *et al.*, 2013; Dinh, 2015). However, this strategy is constrained both by limited HIGS effectiveness, by the redundancy of the effector's function and the difficulty in targeting the point in time when the effector plays a key role in the interaction.

Targeting essential conserved effector targets to induce a loss of susceptibility

The use of resistant cultivars or rootstocks is an efficient and non-polluting method for controlling RKNs. Very few natural resistance genes (R-genes) have been identified to date, in a limited number of plant species. Furthermore, some RKN species or populations are not controlled by these genes, e.g. *M. enterolobii* (Elling, 2013; Kiewnick *et al.*, 2009) or can overcome these resistances, e.g. populations of *M. incognita* virulent against tomato *Mi1.2* (Castagnone-Sereno, 2006). One alternative would be to target conserved plant genes encoding proteins involved in host processes that are hijacked by the biotrophic pathogens for settlement and feeding, and that are essential for disease development. These susceptibility genes (S-genes) represent an alternative to R-genes for the deployment of pathogen resistance, and they may be more durable in the field (Dong and Ronald, 2019; Engelhardt *et al.*, 2018; van Schie and Takken, 2014). Well-characterised examples of S-genes include the genes encoding eukaryotic translation initiation factors, the sugar transporter SWEET14 or PMR6,

which are required for viral, bacterial and oomycete infections, respectively (Langner *et al.*, 2018; van Schie and Takken, 2014; Schmitt-Keichinger, 2019).

In recent decades, transcriptomic approaches have been widely used to identify genes regulated upon RKN infection, and, thus, host functions manipulated by RKNs. However, as thousands of genes are differentially regulated during a compatible interaction, the identification of S-genes from these data is a very time-consuming process, probably explaining why only a few genes to date have been shown to be important for the establishment of giant cells (Favery *et al.*, 2016). Interactomics approaches have recently been used to identify the direct plant targets manipulated by PPN effectors. Only a few targets of RKN effectors have been identified, but most have been shown to be instrumental in promoting nematode parasitism (Mejias *et al.*, 2019). SmD1 is a host target of an effector required for host susceptibility to RKNs in several plant clades. It exerts a conserved plant function targeted by a core effector in *Arabidopsis* and Solanaceae, common to diverse *Meloidogyne* species that have adopted the same successful parasitic strategy based on the induction of giant feeding cells in the root in several host species. SmD1 is thus a good candidate S-gene for targeting in the engineering of crop resistance to RKN. As SmD1 functions is required for plant development knockout mutations of this gene have adverse effects, it will be necessary to identify mutant alleles that can evade recognition by MiEFF18 whilst remaining competent to perform the functions of SmD1 in the regulation of plant development in a continually changing environment. This strategy has proven to be effective for potyvirus susceptibility *eIF4E* genes (Bastet *et al.*, 2019).

Experimental procedures

Nematode and plant materials

M. incognita (Morelos strain), *M. arenaria* (Guadeloupe strain) and *M. enterolobii* (Godet strain) were multiplied on tomato (*Solanum lycopersicum* cv. “Saint Pierre”) growing in a growth chamber (25°C, 16 h photoperiod). Freshly hatched J2s were collected as previously described (Caillaud and Favery, 2016). For VIGS experiments, *Nicotiana benthamiana* and *Solanum lycopersicum* (cv M82) seeds were sown on soil and incubated at 4°C for two days. After germination, *N. benthamiana* and tomato plantlets were transplanted into pots containing soil and sand (1:1), and were grown at 24°C and 16°C, respectively (photoperiod, 16 h: 8 h, light: dark).

Sequence analysis, alignment and phylogenetic tree

The sequences of EFF18 paralogues and orthologues were obtained from *Meloidogyne* genomic resources http://www6.inra.fr/meloidogyne_incognita and Wormbase parasite. Protein sequences were aligned with the MAFFT tool on the EBI server (<https://www.ebi.ac.uk/Tools/msa/mafft/>). The alignment was then used as input for the IQTree Web server <http://iqtree.cibiv.univie.ac.at/> (Trifinopoulos et al., 2016) to generate the maximum likelihood phylogenetic tree. The model chosen by the inbuilt model test was HIVb+F+G4. Support for the nodes was calculated with 100 bootstrap replicates. *M. hapla* was used as the outgroup in the phylogenetic tree for MiEFF18 orthologues. The tree was visualised in iTOL <https://itol.embl.de/>. The sequence alignment were coloured with Boxshade (https://embnet.vital-it.ch/software/BOX_form.html). The pairwise sequence identity matrix of RKN EFF18 protein sequences was generated with Sequence Demarcation Tool version 1.2 software (Muhire et al., 2014) (<http://web.cbio.uct.ac.za/~brejnev/>).

***In situ* hybridisation (ISH)**

ISH was performed on freshly hatched *M. arenaria* and *M. enterolobii* pre-J2s, as previously described (Jaouannet et al., 2018). The MaEFF18, and MeEFF18 gene fragments were

amplified with specific primers (Table S2). A sense probe for MeEFF18 was used as a negative control. Images were obtained with a microscope (Zeiss Axioplan2, Germany).

Pairwise yeast two-hybrid assays

For pairwise yeast two-hybrid (Y2H) assays, the coding sequences (CDS) of the MiEFF16, MiEFF18 and MeEFF18 effectors without their secretion signals were amplified (Table S1) and inserted into pB27 as C-terminal fusions with LexA. Full-length SmD1 CDS sequences (*SlSmD1*, *NbSmD1* and *AtSmD1b*) were amplified (Table S1) and inserted into pP6 as C-terminal fusions with Gal4-AD. The pB27 and pP6 constructs were verified by sequencing and used to transform the L40ΔGal4 (MATa) and Y187 (MATα) yeast strains, respectively. Y187 and L40ΔGal4 were crossed and diploids were selected on medium lacking tryptophan and leucine. Interactions were investigated on medium lacking tryptophan, leucine and histidine and supplemented with 0.5 mM 3-aminotriazole (3-AT).

***N. benthamiana* agroinfiltration**

Transient expression was achieved by infiltrating *N. benthamiana* leaves with *A. tumefaciens* GV3101 strains harbouring GFP-fusions, as previously described (Caillaud *et al.*, 2008). Leaves were imaged 48 hours after agroinfiltration, with an inverted confocal microscope equipped with an argon ion and HeNe laser as the excitation source. For simultaneous GFP imaging, samples were excited at 488 nm and GFP emission was detected selectively with a 505-530 nm band-pass emission filter.

Virus-induced gene silencing in Solanaceae

VIGS assays were performed on *N. benthamiana* and *S. lycopersicum*. We used the Sol Genomics Network VIGS-Tool (<https://vigs.solgenomics.net/>) to design the best sequence for

silencing *SlSmD1a* transcripts, and selected the full-length *SlSmD1a* (without the ATG and STOP codons) for amplification by PCR with the TRV2-*SlSmD1*-F/TRV2-*SlSmD1*-R primer pairs (Table S2). The PCR products were digested with *EcoRI* and *XhoI* and ligated to the tobacco rattle virus RNA 2 vector (TRV2) for the transformation of *A. tumefaciens* strain GV3101. VIGS assays were performed, as previously described, by the co-infiltration of leaves of three-week-old *N. benthamiana* plants (Lange *et al.*, 2013; Velasquez *et al.*, 2009) or 10-days-old tomato plants (Cox *et al.*, 2019) with agrobacterial strains containing the RNA 1 vector (TRV1) and TRV2. Tomato plants were incubated at 16°C for four weeks. Three independent biological replicates were established for each set of conditions ($n = 15$ per replicate). Two *N. benthamiana* root systems per set of conditions and per replicate, or *S. lycopersicum* leaves were collected upon RKN infection and frozen in liquid nitrogen for subsequent RNA extraction and the assessment of silencing efficiency by RT-qPCR.

RKN infection assay, juveniles in the plant and giant cell area measurements

N. benthamiana plants subjected to VIGS were inoculated with 200 *M. incognita* J2s per plant, 10 days post inoculation (dpi) with TRV, and incubated at 24°C. *S. lycopersicum* plants subjected to VIGS were inoculated with 150 *M. incognita* J2s per plant, 30 dpi with TRV, and incubated at 16°C for two weeks before transfer to 24°C. *N. benthamiana* infected roots were collected two weeks after infection whereas *S. lycopersicum* infected roots were collected six weeks after infection. Galls, and egg masses stained with 4.5% eosin were counted under a binocular microscope, and root system was weighted ($n=12$ to 19 and $n=18$ to 21 plants per replicates for *N. benthamiana* and *S. lycopersicum*, respectively). Three and two independent biological replicates were established for each set of conditions in *N. benthamiana* and *S. lycopersicum*, respectively. The impact of the plant lines on the number of galls per mg of root in *N. benthamiana* and the number of egg masses per plant in *S. lycopersicum* were

analyzed using Kruskal Wallis test since the dependent variable did not follow a Normal distribution using a Shapiro-Wilk Test. The different replicates of the numbers of galls per mg of roots in *N. benthamiana* were pooled for the analyzes because there was no difference between the replications ($X^2_2 = 2.8$, $P = 0.248$). By contrast, the different replicates of the number of egg masses per plant in *S. lycopersicum* varied depending on the replication ($X^2_1 = 5.3$, $P = 0.022$), and they were analyzed separately. Thus, both the number of galls per mg of root in *N. benthamiana* and the number of egg masses per plant in *S. lycopersicum* varied significantly between the two plant lines tested ($X^2_1 = 57.2$, $P < 0.001$; $X^2_1 > 25.6$, $P < 0.001$, respectively). For determination of the ratio of filiform-to-swollen nematodes, infected roots were collected 14 dpi, parasitic nematodes were stained with fuchsine acid, as previously described (Karssen and Moens, 1983), and nematodes were examined under a binocular microscope (model LSM 880; Zeiss) ($n = 3$ plants per replicate for TRV-empty lines and $n = 5$ plants per replicate for TRV-SmD1 lines, with a mean of 75 nematodes observed per condition and per replicate). Three independent biological replicates were established for each set of conditions. Statistical analyses were carried out with R software (R Development Core Team, version 3.1.3). For giant cell area measurements, galls were cleared in BABB, as previously described (Cabrera *et al.*, 2018), and examined under an inverted confocal microscope (model LSM 880; Zeiss). The mean areas of the biggest giant cell in each gall from *N. benthamiana*, for each genotype, were measured with Zeiss ZEN software ($n = 32$ and 26 galls for control and SmD1-VIGSed plants, respectively). One biological experiment was performed for giant cells measurement. The data were analysed with a *t*-test since the data followed a normal distribution ($t = 3.5$, $P < 0.001$).

Reverse transcription-quantitative PCR

We assessed silencing efficiency in Solanaceae species, by extracting total RNA with TriZol (Invitrogen) and subjecting 1 µg of total RNA to reverse transcription with the Superscript IV reverse transcriptase (Invitrogen). qPCR analyses were performed as described by Nguyen et al., 2016. Primers were designed to amplify both SlSmD1 transcripts or to discriminate between the three copies of SmD1 present in *N. benthamiana* specifically according to their UTR, to prevent the amplification of TRV2-SlSmD1 constructs (Table S2). We performed RT-qPCR in triplicate on cDNA samples from three independent biological replicates. The *EF-1α* and Actin genes were used for the normalisation of RT-qPCR data (Liu *et al.*, 2012). Quantifications and statistical analyses were performed with SATqPCR (Rancurel *et al.*, 2019), and the results are expressed as normalised absolute quantities.

Graphs and statistical analysis

Graphs and plots were created with Microsoft® Office Excel® 2016 and statistical calculations were performed in R.

Accession numbers

The sequence data from this article can be found in the *Arabidopsis* Information Resource (<https://www.arabidopsis.org/>), Solgenomics (<https://solgenomics.net/>) and/or GenBank/EMBL databases. All RKN EFF18 protein sequences are presented in the Figure S1. The accession numbers are summarised in Table S1 including MiEFF18a (KX907770), MeEFF18a (MW272456), NbSmD1a (MT683762), NbSmD1b (MT683763) and NbSmD1c (MT683764).

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Conflict of interest

The authors have no conflict of interest to declare.

Author contributions

J.M. designed and performed experiments, and interpreted data; J.M., YC and N.M.T. generated constructs and performed yeast two-hybrid analysis; YC performed *in situ*

hybridisation and *in planta* subcellular localisation experiments; KM performed VIGS in tomato and nematode infection tests; J.M., P.A., S.J.P., B.F. and M.Q. wrote the manuscript; B.F. and M.Q. obtained funding, designed the work and supervised the experiments and data analyses; all the authors read and edited the manuscript.

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Figure legends

Figure 1 Effector 18 (EFF18) is a conserved effector in root-knot nematodes. (a) Phylogenetic tree and schematic diagram of root-knot nematode EFF18 protein sequences. The tree scale corresponds to the number of substitutions per site based on the amino-acid matrix (JTT). In the schematic diagram of EFF18 proteins, the predicted secretion signal

peptide (SP; grey boxes), the aspartic acid and glutamic acid (D-E)-rich region (red boxes), the lysine (K)-rich C-terminal region (blue boxes) and the nuclear mono- (purple boxes) or bi- (orange boxes) partite localisation signals (NLS) are shown. EFF18 proteins from the closest group to MiEFF18a carry one mono- and one bipartite NLS, whereas the most divergent copies have only a single monopartite NLS. (b) MiEFF18 localised to the nucleus and nucleolus of plant cells. The MiEFF18 sequence was fused to that encoding GFP in an N- or C-terminal position and expressed in *N. benthamiana* leaves by agroinfiltration. GFP was used as a control and gave fluorescence in the cytoplasm and the nucleus (n), but not the nucleolus (arrowhead). Bars = 10 μ m. (c) Pairwise sequence identity matrix for RKN EFF18 protein sequences.

Figure 2 RKN EFF18s are specifically expressed in the subventral glands. *In situ* hybridisation, showing EFF18 transcripts in the subventral glands of J2s of *M. incognita*, *M. enterolobii* and *M. arenaria*, respectively. A sense probe for the MeEFF18 transcript was used as a negative control. SvG, subventral glands. Bar = 50 μ m.

Figure 3 Conserved SmD1 proteins are targeted by EFF18. (a) MAFFT protein sequence alignment of the *S. lycopersicum* (Sl), *N. benthamiana* (Nb) and *A. thaliana* (At) SmD1 proteins. (b) Schematic representation of Sm1 and Sm2 motif in SmD1 proteins. (c) GFP-AtSmD1b and GFP-SlSmD1a accumulate in the nucleus and particularly in the nucleolus when transiently expressed in *N. benthamiana* epidermal leaf cells. GFP was used as a nucleocytoplasmic control. n= nucleoplasm; Black arrows show nucleolus. Bars = 5 μ m. (d) Pairwise yeast two-hybrid assays showed that the MiEFF18 and MeEFF18 proteins were able to interact with the SmD1 proteins of *A. thaliana*, *S. lycopersicum* and *N. benthamiana*. We used MiEFF18 and MiEFF16 as a positive and negative control, respectively. Diploid yeasts containing the bait and prey plasmids carrying controls, effectors or SmD1 were serially

diluted and spotted on plates. The 10⁻² dilution is shown. SD-WL corresponds to the non-selective medium without tryptophan (W) and leucine (L). Only yeasts carrying a protein-protein interaction can survive on the SD-WLH (H, histidine) + 0.5 mM 3-aminotriazole (3-AT) selective medium.

Figure 4 The silencing of *SmD1* genes by VIGS affects susceptibility to *M. incognita* in *S. lycopersicum*. (a) Timeline used for the VIGS experiments in *S. lycopersicum*. (b) RT-qPCR demonstrating the effective silencing of *SlSmD1* in TRV-*SlSmD1* line when compared to the control TRV-GFP. RPN7 was used for data normalisation. (c) Infection test on tomato plants in which *SmD1* was silenced (TRV-*SlSmD1*) and control tomato plants (TRV-GFP). Females producing egg masses were counted seven weeks after inoculation with 150 *M. incognita* J2s per plant. Statistical significance was determined on the basis of three independent biological replicates, by Kruskal-Wallis tests. Clearly significant differences were observed between TRV-GFP control and TRV-*SmD1* plants (* $p \leq 0.05$).

Figure 5 The silencing of *SmD1* genes by VIGS affects susceptibility to *M. incognita* in *N. benthamiana*. (a) Timeline used for VIGS experiment in *N. benthamiana*. (b) *N. benthamiana* plants with silenced *SmD1* genes (TRV-*SmD1*, right panel) and TRV2-empty control plants (TRV-empty, left panel), showing some developmental defects of the leaves (upper panel) and a shorter root system (lower panel). Red arrow point-out galls on these pictures. (c) RT-qPCR showing that the *NbSmD1b* gene, the most strongly expressed and closest orthologue to the *SlSmD1* gene, was effectively silenced. The data shown are the means of three independent biological replicates. EF1a and actin were used for data normalisation. (d) Infection test on *N. benthamiana* control plants (TRV-empty) and plants in which *SmD1* was silenced (TRV-*SlSmD1*). Galls were counted and root weight was measured two weeks after

inoculation with 200 *M. incognita* J2s per plant. Statistical significance was determined on the basis of three independent biological replicates, by Kruskal-Wallis tests. Clear significant differences were found between the TRV-GFP control and the TRV-SmD1 plants (* $p \leq 0.05$; ** $p \leq 0.01$).

Figure 6 *SmD1* plays an important role in the formation of giant cells. (a) The filiform J2s/swollen juveniles (Js) ratio obtained by fuchsine acid staining in the *N. benthamiana* root system with (TRV-SmD1) and without (TRV-empty) silencing with the TRV-SlSmD1 construct, following infection with *M. incognita*. (b) Galls of negative control plants and plants with SmD1 silencing collected two weeks post infection for measurement of the area of giant cells (dotted line) by the BABB clearing method (Cabrera et al., 2018). The biggest giant cell measured is shown by a surrounding dashed white line. Bar = 100 μm . (c) Box-and-whisker plot of giant cell size (μm^2) measurements (n = 32 and 26 galls).

Supporting information

Figure S1 Identified EFF18 sequences in RKN species.

Figure S2 EFF18 is a conserved RKN effector.

Figure S3 Tomato phenotypes associated with VIGS of *SlSmD1* genes.

Figure S4 Semi-quantitative RT-PCR expression analysis of *NbSmD1s* in *N. benthamiana* roots VIGS experiments.

Table S1 Sequences used in this study and accession numbers

Table S2 Primers used in this study

M. incognita

M. arenaria

M. enterolobii

(a) **(b)** **(c)**

size of the giant cells
(μm^2)