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Comprehensive transcriptome profiling of root-knot nematodes during plant infection and characterisation of species specific trait

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Chinh Nghia Nguyen. Comprehensive transcriptome profiling of root-knot nematodes during plant infection and characterisation of species specific trait. Agricultural sciences. COMUE Université Côte d'Azur (2015 - 2019), 2016. English. NNT: 2016AZUR4124 . tel-01673793

HAL Id: tel-01673793

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Ecole Doctorale de Sciences de la Vie et de la Santé
Unité de recherche : UMR ISA INRA 1355-UNS-CNRS 7254

Thèse de doctorat

Présentée en vue de l'obtention du
grade de docteur en Biologie Moléculaire et Cellulaire

de **L'UNIVERSITE COTE D'AZUR**

par

NGUYEN Chinh Nghia

**Etude de la régulation du transcriptome de nématodes
parasites de plante, les nématodes à galles du genre**

Meloidogyne

Dirigée par ***Dr. Bruno FAVERY***

Soutenance le **8 Décembre, 2016**

Devant le jury composé de :

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**Doctoral School of Life and Health Sciences
Research Unity: UMR ISA INRA 1355-UNS-CNRS 7254**

PhD thesis

Presented and defended to obtain

Doctor degree in Molecular and Cellular Biology

from **COTE D'AZUR UNIVERITY**

by

NGUYEN Chinh Nghia

**Comprehensive Transcriptome Profiling of Root-knot
Nematodes during Plant Infection and Characterisation of
Species Specific Trait**

PhD directed by ***Dr Bruno FAVERY***

Defense on **December 8th 2016**

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Résumé

Les nématodes à galles du genre *Meloidogyne* spp. sont des parasites obligatoires des plantes qui maintiennent une relation particulière avec leur hôte pendant plusieurs semaines. Les larves pré-parasitaires de second stade (J2) infectent les racines puis migrent entre les cellules pour atteindre le cylindre vasculaire. Afin de se développer en femelle qui libèrera des centaines d'œufs dans le sol, les J2 doivent établir et maintenir un site nourricier spécialisé composé de « cellules géantes ». Ces cellules géantes constituent l'unique source de nutriments du nématode et résulte du dialogue moléculaire entre la plante et le nématode. Mon projet de thèse a pour objectif d'identifier des gènes spécifiques des nématodes à galles qui sont impliqués dans le parasitisme en se focalisant sur des gènes codant de nouvelles protéines potentiellement sécrétées, appelées effecteurs.

En utilisant la technologie de séquençage Illumina, nous avons comparé les transcriptomes de *M. incognita* au cours de son cycle de vie et identifié des gènes surexprimés aux stades parasitaires précoces par comparaison aux stades pré-parasitaire J2, œufs, femelles et males. A partir de 307 gènes surexprimés dans -au moins- un stade du cycle de vie, nous avons sélectionné 14 candidats d'effecteurs. L'expression au stade parasitaire de huit gènes a pu être confirmée par RT-qPCR. Les expériences d'hybridation *in situ* ont permis de localiser l'expression de sept effecteurs dans les glandes salivaires du nématode, suggérant qu'ils sont sécrétés et pourraient jouer un rôle au cours du parasitisme. De plus, des expériences d'ARN interférence ont été utilisées afin de réprimer l'expression de gènes et étudier leur rôle dans la pathogénicité. La diminution d'expression du gène spécifiquement exprimé dans les glandes dorsales, Minc18876 et ses paralogues, a montré une diminution significative et reproductible du nombre de masses d'œufs produites, démontrant qu'il pourrait jouer un rôle important dans les stades précoces de formation des cellules géantes.

Parallèlement, nous avons réalisé l'assemblage *de novo* du transcriptome de *M. enterolobii*. Ce nématode représente une nouvelle menace pour l'agriculture mondiale du fait de sa capacité à se reproduire sur la majorité des plantes résistantes aux autres nématodes à galles. Six banques d'ADNc ont été construites à partir d'échantillons de larves pré-parasitaires J2 et parasitaires J3-J4, et ribodéplétées. Les lectures Illumina de haute qualité ont été assemblées *de novo* afin de produire 127,355 contigs. Afin de mieux comprendre le rôle des protéines de *M. enterolobii*, nous avons réalisé une annotation fonctionnelle. Sur les 24 696 protéines comportant une annotation fonctionnelle, 1 632 nouveaux effecteurs putatifs de *M. enterolobii* ont été identifiés. Les premières comparaisons avec d'autres nématodes à galles nous ont permis d'identifier, non seulement des effecteurs en commun, mais aussi ceux qui sont spécifiques à certaines espèces de nématodes à galles et qui pourraient expliquer des différences de gamme d'hôtes.

En conclusion, les analyses de transcriptome de nématodes à galles nous ont permis de caractériser des nouveaux effecteurs candidats impliqués dans la pathogénicité. Une meilleure connaissance du rôle de ces effecteurs sécrétés au cours de l'interaction, en particulier par l'identification de leurs cibles végétales, constitue la prochaine étape dans le développement de méthodes de lutte plus sûres et plus saines contre ces nématodes.

Abstract

Root-knot nematodes (RKN) are obligate endoparasites that maintain a biotrophic relationship with their hosts over a period of several weeks. They infect roots as microscopic vermiform second-stage juveniles (J2) and migrate between cells to reach the plant vascular cylinder. To further develop and molt into a pear-shaped female that will release hundreds of eggs on the root surface, J2s need to successfully establish and maintain specialized feeding structures called “giant-cells” from which they withdraw water and nutrients. They result from the molecular dialog between the plants and the nematode. My PhD project aims to identify RKN genes specifically involved in plant parasitism with an emphasis on genes encoding new secreted proteins, named effectors.

Using Illumina RNA-seq technologies, we compared transcriptomes of *Meloidogyne incognita* during its life cycle and identified genes over-expressed in early parasitic stages as compared to pre-parasitic J2s, eggs, females and males. From 307 genes over-expressed at -at least- one stage of the life cycle, we selected 14 effector candidates. Eight of these selected genes were confirmed to be over-expressed at parasitic stage by RT-qPCR. *In situ* hybridisations were carried out to localize the spatial expression of these candidates in the nematode. Eight genes were detected in the nematode salivary glands, suggesting their putative secretion and role as effector of parasitism. Furthermore, siRNA soaking was used to silence these genes and study their role in pathogenicity. The silencing of the dorsal gland specific-Minc18876 and its paralogues resulted in a significant, reproducible decrease in the number of egg masses, demonstrating a potentially important role for the small cysteine-rich effector MiSCR1 it encodes in early stages of giant cell formation.

In parallel, we perform a *de novo* assembly of *M. enterolobii* transcriptome. This RKN species represents a new threat for the agriculture worldwide because of its ability to reproduce on the majority of known RKN-resistant plants. Six ribodepleted cDNA libraries were constructed using pre-parasitic J2s and parasitic J3-J4 samples. All high-quality Illumina reads were assembled *de novo* to produce 127,355 contigs. To gain functional insight on the *M. enterolobii* proteins, we performed a functional annotation. Out of the 24,696 annotated proteins, 1,632 novel putative effectors of *M. enterolobii* were identified. First comparisons with others RKN allowed us to identify, not only the common set of effectors, but also those specific to some RKN species and possibly involved in host range differences.

In conclusion, the transcriptome profiling of root-knot nematodes allowed the characterisation of new candidate effectors involved in the plant pathogenicity. A better knowledge of the role of the secreted effectors in the interaction, in particular the identification of their host targets, will represent a next step in the development of safer and healthier methods to control these pests.

Acknowledgments

First and above all, I would like to express my sincere gratitude to my thesis director, **Bruno FAVERY** for the continuous support of my Ph.D study and related research, for his patience, motivation, and immense knowledge. His warm encouragement and guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study.

Besides my thesis director, I express my deep thank to **Pierre ABAD** for hosting me in his research group. My sincere thanks also go to **Laetitia PERFUS-BARBEOCH**, **Michaël QUENTIN** and **Stéphanie JAUBERT-POSSAMAI** for their excellent advices, for teaching me much about research, for guiding me to the right way of my study, and of course, for their patience and encouragement.

I warmly thank **Ministry of Education and Training of The Socialist Republic of Vietnam** and **University of Science and Technology of Hanoi** for their funding of my PhD fellowship through the 911 program.

I would like to thank my thesis committee members: **Sébastien DUPLESSIS** and **Sébastien CUNNAC** for their insightful comments and encouragement, but also for the hard question which incited me to widen my research from various perspectives.

My sincere thanks to all members of **Plant-Nematode Interaction Team** and **SPIBOC Team** for their daily support. Thank you **Martine DA ROCHA** for the bioinformatics support and analysis, **Marc MAGLIANO** for advices in *in situ* hybridisation and siRNA soaking experiments, **Nathalie MARTEU** and **Cathy IACHIA** for biological material preparation, **Chantal CASTAGNONE** for ordering necessary products and for her appreciation to Vietnamese food, **Etienne G.J DANCHIN** for helpful discussions, and also thank other members of the teams: **Corine, Daniel, Philippe, Olivier, Alizée, Ulysse, Karine...**

I thank my fellow lab mates, **TRUONG Nhat My, Loris PRATX, Clémence MEDINA, Cristina MARTIN-JIMINEZ, Chami KIM** and other “**young people**” in the institute for the stimulating discussions, for their help in my experiments, for the “pourries” jokes, for “paëlla with chorizo”, for Shangri-la and for all the fun we have had in the last three years.

Last but not the least, I would like to thank **my Vietnamese friends**, and particularly my family: **my parents, my brother, sister-in-law, my nephews, my cousins** for supporting me spiritually throughout writing this thesis and my life in general.

“Đạo khả đạo phi thường đạo

Danh khả danh phi thường danh

Vô danh thiên địa chi thủy

Hữu danh vạn vật chi mẫu”

Dao (The Way, or The Origin) that can be spoken of is not the Constant Dao; The name that can be named is not a Constant Name; Nameless, is the origin of Heaven and Earth; The named is the Mother of all things. – Daodejing – LaozTu -

Table of Contents

List of abbreviations	7
INTRODUCTION	9
1. Generalities about Nematodes.....	9
1.1. Nematode structure	9
1.2. Nematode lifestyles.....	11
1.3. Nematode systematics	12
2. Plant-parasitic nematodes.....	14
3. Root-knot nematodes.....	18
3.1. Reproduction mode.....	19
3.2. Root-knot nematode control.....	21
3.3. RKN life cycle	23
4. Giant cells: formation and main characteristics	25
4.1. Cell cycle and cytoskeleton reorganization during giant cell formation.	27
4.2. Importance of the metabolism in GC	30
5. RKN effectors	31
5.1. Identification of RKN effectors.	32
5.2. Functional analysis of effectors	42
6. Transcriptomic approach to identify nematode effectors	52
7. Objectives	54
CHAPTER 1: Identification of Parasitism Effectors Expressed During Plant Infection from the Transcriptome of <i>Meloidogyne incognita</i>	55
Introduction.....	56
Materials and Methods	58
Results	61
Discussion	72
Acknowledgements	76
References.....	81
Supplementary data chapter 1.....	84
CHAPTER 2: Transcriptome Profiling of the Root-Knot Nematode <i>Meloidogyne enterolobii</i> During Parasitism and Identification of Novel Effector Proteins	97
Introduction.....	99
Material and methods	101
Results	104
Discussion	111

Acknowledgements	114
References	121
GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES	125
1. Identification of parasitism effectors expressed during plant infection from the transcriptome of <i>Meloidogyne incognita</i>	126
2. Transcriptome profiling of the root-knot nematode <i>Meloidogyne enterolobii</i> during parasitism and identification of novel effector proteins	130
3. Toward new resistance strategies against RKN	133
References	137
ANNEXES	153
Annex 1: <i>In situ</i> Hybridisation protocol for localisation of gene expression in nematode	155
Annex 2: siRNA soaking protocol and resistance test	163
Annex 3 : Curriculum vitae	171

List of abbreviations

ABPs	actin-binding proteins
AFLP	amplification fragment length polymorphism
BUSCO	Benchmarking Universal Single-Copy Orthologs
CAZymes	carbohydrate-active enzymes
CBP-1	cellulose-binding protein
CC	coiled-coil domain
CDKs	cyclin-dependent kinases
CM	chorismate mutase
CN	cyst nematode
CRT	calreticulin
DAMPs	damage-associated molecular patterns
DG	dorsal gland
DIG	digoxigenin
DNC	dorsal nerve cord
dsRNA	double-strand RNA
EST	expressed sequence tag
ETI	effector-triggered immunity
FITC	fluorescein isothiocyanate
G6PDH	glucose-6-phosphate dehydrogenase
GC	giant cell
GSH	glutathione
(h)GSH	homoglutathione
HIGS	host induced gene silencing
hpRNA	hairpin RNA
HR	hypersensitive response
IAA	indole-2-acetic acid
ISH	<i>in situ</i> hybridisation
J2	second-stage juvenile
KRP6	Kip-related protein 6
MAbs	monoclonal antibodies
MAPs	microtubule-associated proteins
MERCI	motif—emerging and with classes—identification
MF	microfilament
MT	microtubule
NB/LRR	nucleotide binding leucine rich repeat
nanoLC ESI MS/MS	nano-electrospray ionisation and tandem mass spectrometry
NILs	near-isogenic lines
NGS	next generation sequencing
NLDs	nucleus-localisation domains
NLS	nuclear localisation signal
OPPP	oxidative pentose phosphate pathway

PAMP	Plant-associated molecular pattern
PPN	plant parasitic nematodes
PPP	pentose phosphate pathways
PTI	PAMP-triggered immunity
R-genes	resistance genes
RISC	RNA-induced silencing complex
RKN	root knot nematodes
RNAi	RNA interference
RNA-seq	RNA sequencing
RPE	ribulose-phosphate-epimerase
SCR	small cysteine-rich protein
siRNA	small interference RNA
SSH	suppression subtractive hybridisation
SSU rDNA	small subunit ribosomal DNA
SUMO	small ubiquitin-like modifier
SvG	subventral gland
TCTP	translationally controlled tumour protein
TDF	transcript-derived fragments
TIP2	tonoplast-intrinsic protein 2
TIR	Toll/interleukin-1 receptor-like domain
VAP	Venom allergen-like protein
VIGS	virus induced gene silencing
VNC	ventral nerve cord
Y2H	yeast-two hybrid

INTRODUCTION

1. Generalities about Nematodes

Nematodes are one of the most distributed groups of animal in the world, found widespread from the marine to different soils or fresh water, in almost all habitats in the planet. These invertebrate roundworms have variable size from 100 μm to 8 m, but most of them are in microscopic size: less than 1 mm in length and between 15 to 20 μm in diameter. It is estimated that around one million nematode species are present in the planet, but only 27,000 species has been described in the phylum Nematoda (Quist *et al.*, 2015). This number is continuously increasing with the discovery or re-description of new species.

1.1. Nematode structure

Nematode has a simple structure. Their body is cylindrical, elongated and smooth with no limbs protruding, and covered by an elastic cuticle (Figure 1, 2). The nematode cuticle is secreted from a sheet of cells called epidermis or hypodermis, and, form an exoskeleton (Bird & Bird, 1991a). Nematode cuticle is non-living, contained mainly of collagen, permeable to allow ions and water to pass through and therefore plays a key role in maintaining the hydrostatic pressure. Moreover, it also acts as an anchoring point during locomotion and exhibits great diversity which is useful for identification of different nematode species. Most nematodes suffer four moults throughout their development from the juvenile stage (stages J1 to J4) until reaching the adult stage. During the moulting process, the cuticle is either shed completely or partially absorbed in case of *Meloidogyne* (Perry & Moens, 2011).

Underneath the nematode epidermis, their long muscles are all aligned longitudinally along the inside of the body. The muscles are activated by two nerves that run the length of the nematode on both the dorsal (back) and ventral (belly) side. The ventral nerve has a series of nerve centers along its length, and both nerves connect to a nerve ring and additional nerve centers located near the head (Bird & Bird, 1991b). The nematode head has a few tiny sense organs, e.g. amphids, and a “mouth” opening into a muscular pharynx (throat), an efficient pump to pull food in the intestine. This leads into a long simple gut cavity lacking any muscles, and then to an anus near the tip of the body. Interestingly, there is no vascular system for food digested distribution, neither respiratory system for the uptake or distribution of oxygen.

Rather, nutrients and waste are distributed in the pseudo coelomic body cavity, whose contents are regulated by an excretory canal along each side of the body (Bird & Bird, 1991c).

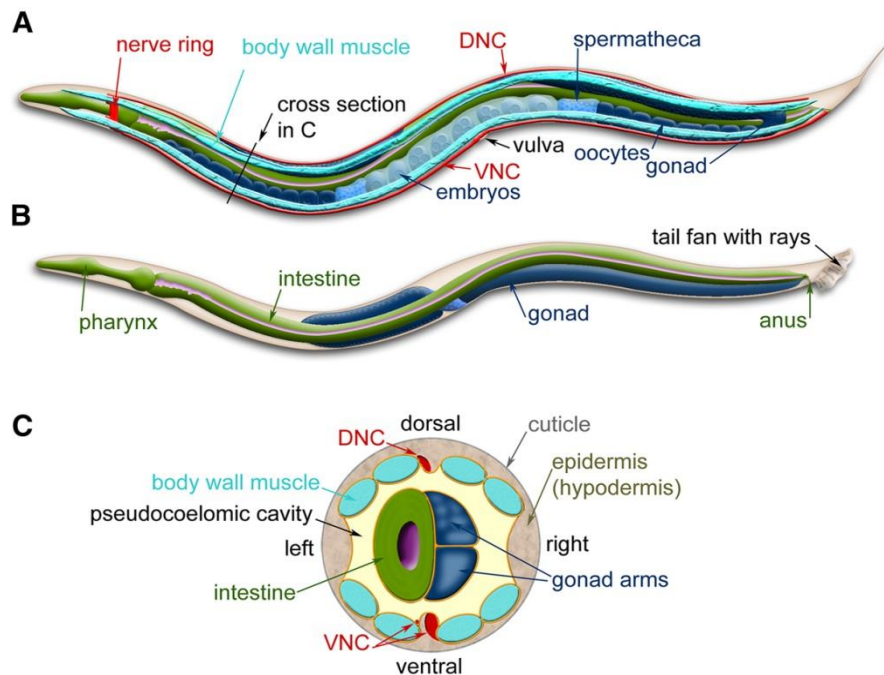


Figure 1: Anatomy of the model nematode *Caenorhabditis elegans* (Corsi et al., 2015)

Major anatomical features of a hermaphrodite (A) and male (B) *C. elegans* viewed laterally. (A) The dorsal nerve cord (DNC) and ventral nerve cord (VNC) run along the entire length of the animal from the nerve ring. Two of the four quadrants of body wall muscles are shown. (B) The nervous system and muscles are omitted in this view, more clearly revealing the pharynx and intestine. (C) Cross-section through the anterior region of the *C. elegans* hermaphrodite (location marked with a black line in A) showing the four muscle quadrants surrounded by the epidermis and cuticle with the intestine and gonad housed within the pseudo-coelomic cavity.

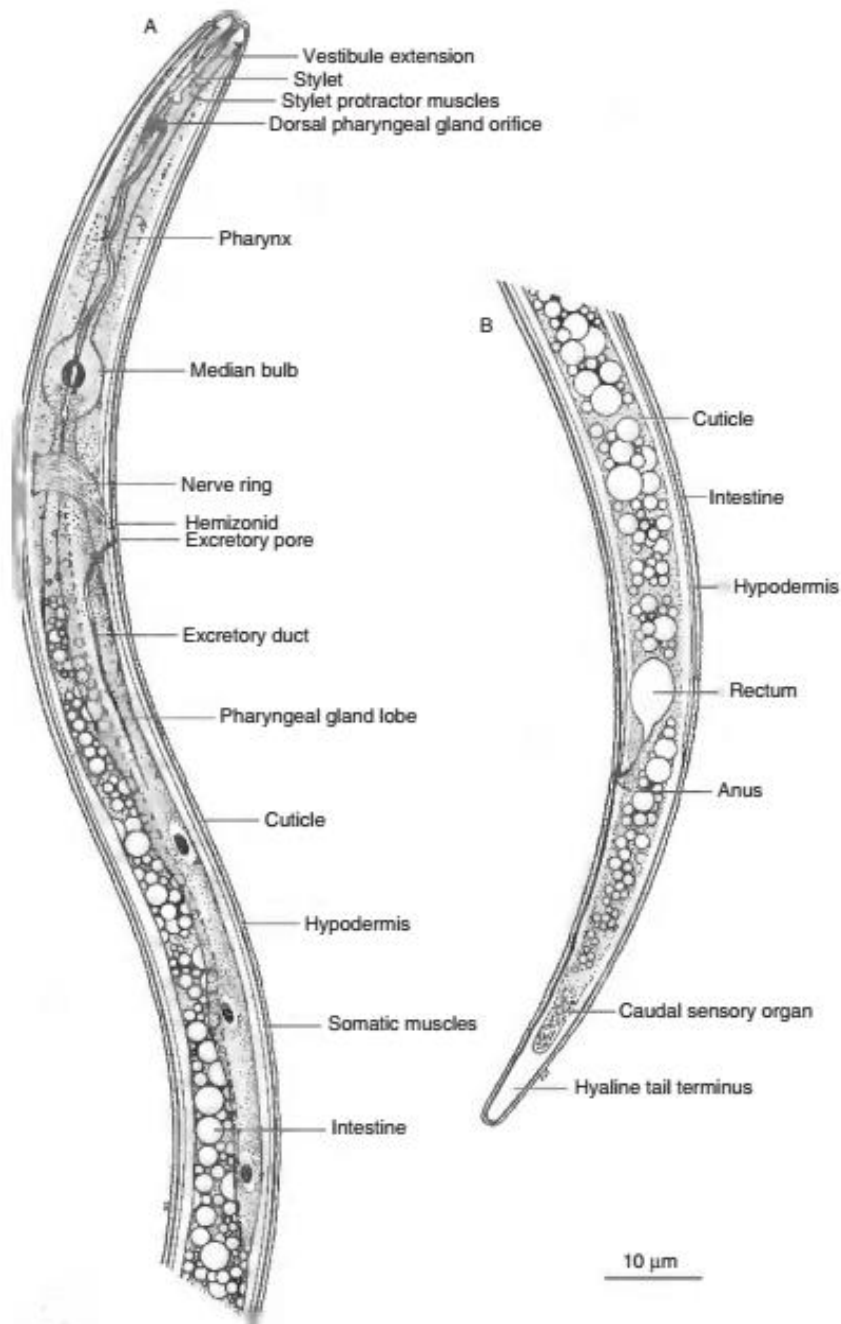


Figure 2: Drawings of second-stage juvenile root-knot nematode (Eisenback & Hunt, 2009)

A: anterior region; B: posterior region.

1.2. Nematode lifestyles

These ubiquitous organisms have varied lifestyles, including free-living and parasitic nematodes. Free-living nematodes live in soil or in water and feed on bacteria, fungi or nematodes whereas the parasitic ones have capacity to infect animals (from insects to human)

and crops plants as food resources (Perry & Moens, 2011). Among the known nematode species, around 10,600 are free-living, more than 4,000 are parasite of plants and about 12,000 are parasite of invertebrates or vertebrates (Hugot *et al.*, 2001). The vertebrate parasitic nematodes have a severe impact in public health and animal breeding production worldwide. In Africa, Onchocerciasis, caused by the filarial worm *Onchocerca volvulus*, is an important cause of blindness, skin disease and chronic disability. This disease currently infects around 17 million people (MH *et al.*, 2015). *Trichinella* spp., the causative agents for trichinellosis, not only affects human but also represents an economic problem in porcine animal production and food safety. Besides, the strongylid nematode *Haemonchus contortus* is one of the most important parasites of livestock that infects hundreds of millions of sheep and goat. It feeds on blood from capillaries in the stomach mucosa of affected animals that often leading to death in severely cases.

Although a lot of nematodes have devastating effects on plant production (see next chapter), animal and human health, there are also nematodes with “beneficial effects”. The free-living forms in the soil have been known as a good factor involving the soil nutrient turnover. The entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are commercialized to be used in crop protection as an efficient insect control agent (Lacey & Georgis, 2012). These two genera invade the target insects and release symbiotic bacteria into the host, where bacteria multiply and kill the insect by septicaemia (Gaugler, 2002). Moreover, the free-living nematode *C. elegans*, which genome was sequenced in 1998 (Consortium, 1998), is one of the most studied model in biological research. The simple structure, the facility to reproduce and the possibility of genetic manipulation make this nematode be an excellent organism addressing many molecular and cellular mechanism researches (Sulston *et al.*, 1983; Culetto & Sattelle, 2000; Kaletta & Hengartner, 2006; Shaye & Greenwald, 2011).

1.3. Nematode systematics

Blaxter *et al.* (1998) were among the first who exploited the potential of small subunit ribosomal DNA (SSU rDNA) data to reconstruct evolutionary pathways of the nematodes. Based on the combined use of multiple molecular data sets and a wide range of fossil records, the phylum Nematoda was devised into 12 major clades (Figure 3) (Holterman *et al.*, 2006; Quist *et al.*, 2015).

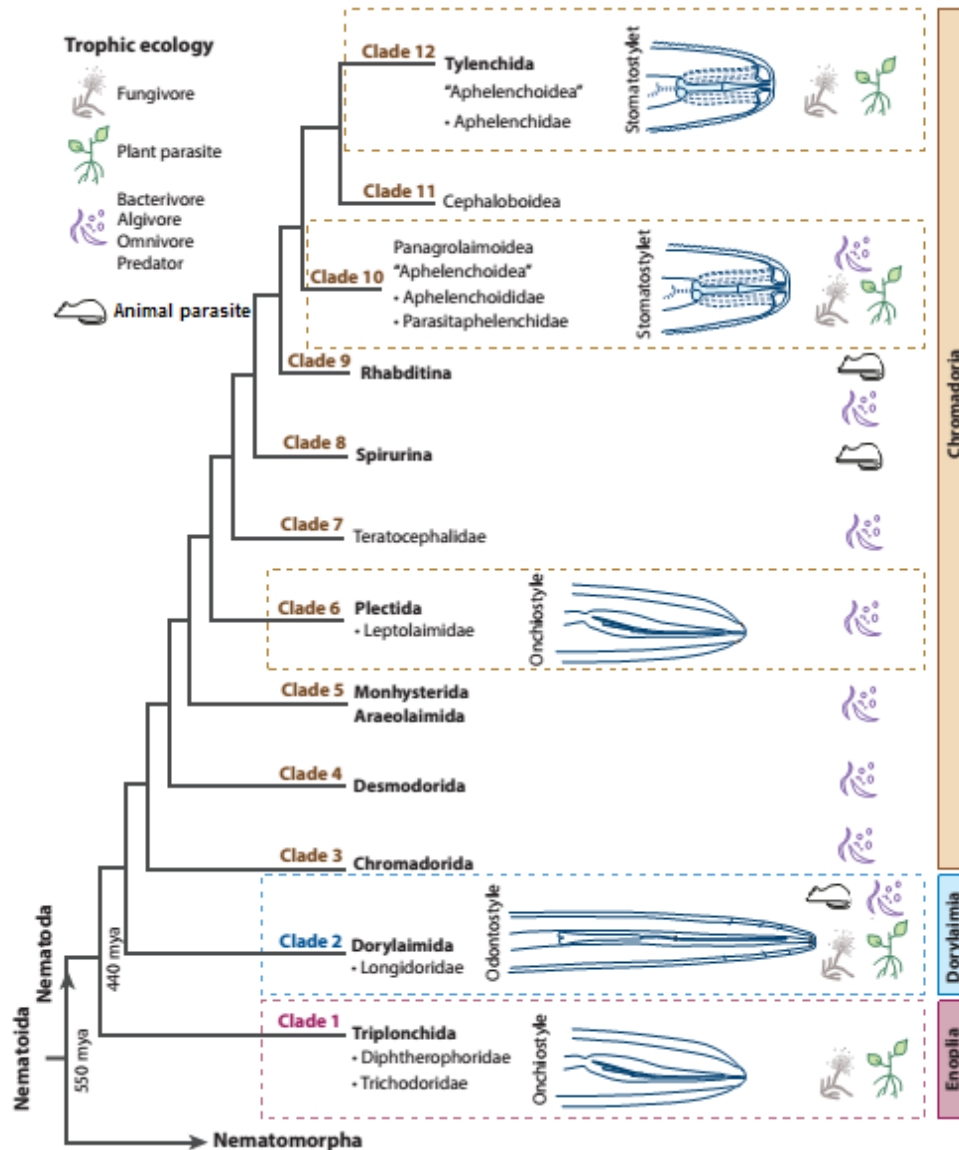


Figure 3: Schematic overview of the phylum Nematoda (adapted from Quist et al. 2015)

Division into 12 major clades is based on SSU rDNA sequence data (Holterman et al., 2006). Next to the individual branches only those (sub)order names (with the endings *-ina* and *-ida*, respectively) are given that are relevant in the context of this review. At the far right, subclass names (*-ia*) are given. Trophic ecology of free living and plant-parasitic nematodes are indicated.

In phylum Nematoda, we observe a multitude of times that (animal- or plant-) parasitic lifestyles have arisen. The newborn phylum Nematoda probably emerged at the early Silurian (440 million years ago), with the presence of the subclass Triplonchida on one branch and two subclasses Dorylaimida and Chromadoria on the other one. The fungivorous family Diphtherophoridae and Trichodoridae-Clade 1 or Longidoridae-Clade 2 are the most basal major groups of higher plant parasites within the phylum Nematoda; while the third plant parasite

lineage is found in Clade 10 – with two closely related families Aphelenchoididae and Parasitaphelenchidae. The fourth-the most diverse plant parasite group points in Clade 12; comprising of the order Tylenchida and the family Aphelenchidae. Interestingly, it seems likely that the nematodes in Clade 12 arose from (predominantly) fungivorous ancestors (Quist *et al.*, 2015). Nevertheless, the establishment of a stable phylum Nematoda has always been prevented by the convergent evolution of morphological, ecological or biological characters. This seems to be an important explanation for the persistent volatility of nematode systematics (Bert *et al.*, 2011).

2. Plant-parasitic nematodes

Unlike the free-living form in soil, plant-parasitic nematodes (PPNs) are scourge for agriculture worldwide. These small roundworms are able to infect thousands of plant species and cause a disastrous global crops yield losses (Blok *et al.*, 2008). Although PPNs have different lifestyles and feeding strategies, all species use a hollow, protrusible syringe-like stylet to penetrate the wall of plant cells, produce and inject secretions to facilitate infection, and then withdraw nutrients from the plant.

The principal source of nematode secretions are three enlarged oesophageal or “salivary” glands – two subventral glands (SvG) and one dorsal gland (DG) (Figure 4), adapted to enhance secretory activity for plant infection. Besides the oesophageal glands, the cuticle and the amphids, the principal chemosensory organs of nematodes made up of 12 sensory neurons, have been also demonstrated to secrete proteins during plant infection (Perry, 1996; Semblat *et al.*, 2001; Curtis, 2007).

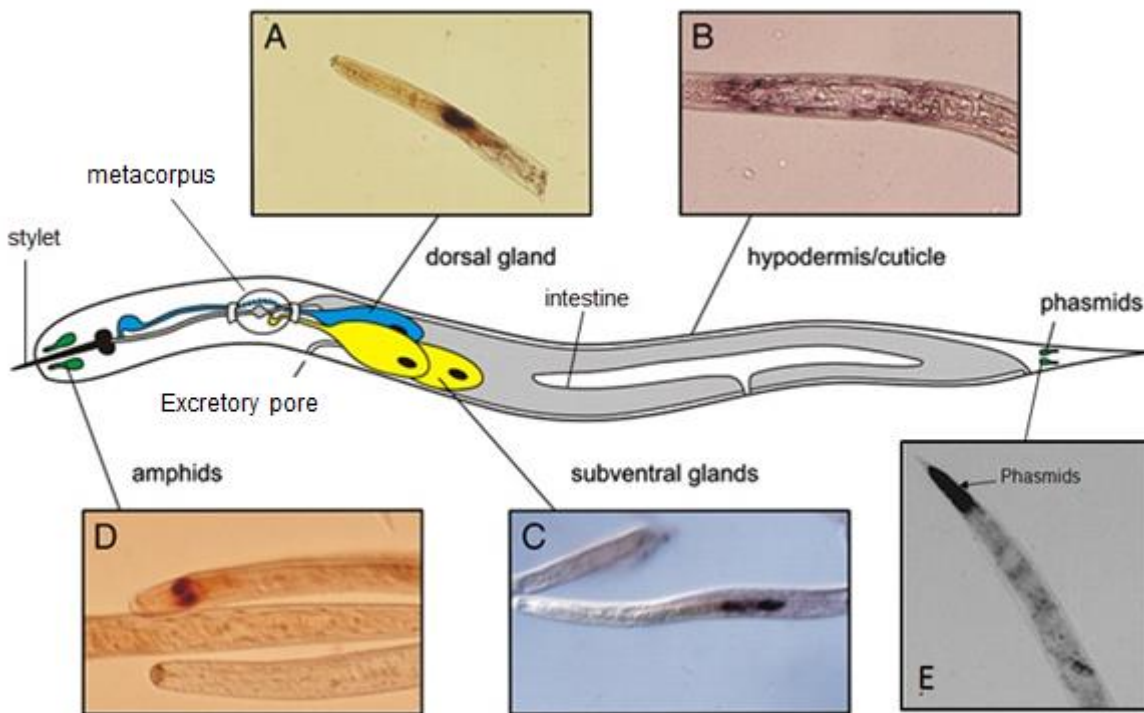


Figure 4: Secretory organs of a typical plant-parasitic nematode (adapted from Haegeman et al. 2012).

(A)-(E) *In situ* hybridisation images of nematode genes specifically expressed in secretory organs. (A) *SPRYSEC* in *G. pallida* (Jones et al., 2009) (B) *SXP-RAL2* protein in *G. rostochiensis* (Jones et al., 2000) (C) *SXP-RAL2* protein in *G. rostochiensis* (Jones et al., 2000) (D) *chorismate mutase* in *G. pallida* (Jones et al., 2003) (E) *CL1191Contig1_1* protein in *M. incognita* (Bellafiore et al., 2008)

Plant parasitic nematodes are classified according to their lifestyle and feeding habits. Those that feed externally on the root are called ectoparasites, whereas the nematodes that feed internally from different inner cell types are classified as endoparasites. They are further sub-classified into sedentary, fixed at a feeding site, or migratory, moving and feeding inside the root or the shoot (Figure 5) (Decraemer & Hunt, 2013).

Ectoparasite nematodes tend to gather in the soil rhizosphere (soil on and around root) to browse along root and then to feed. They can have a long stylet that helps them to penetrate the plant root, goes deeply inside for rich nutrients in plant cells. This feeding strategy makes them easier to switch hosts but also be harmed by environment or predators. The sedentary ectoparasite *Tylenchulus semipenetrans* are responsible for losses in citrus, olive and grapevine trees, whereas the migratory ectoparasite *Xiphinema* spp. (Figure 6) can transmit important plant viruses to grapes.

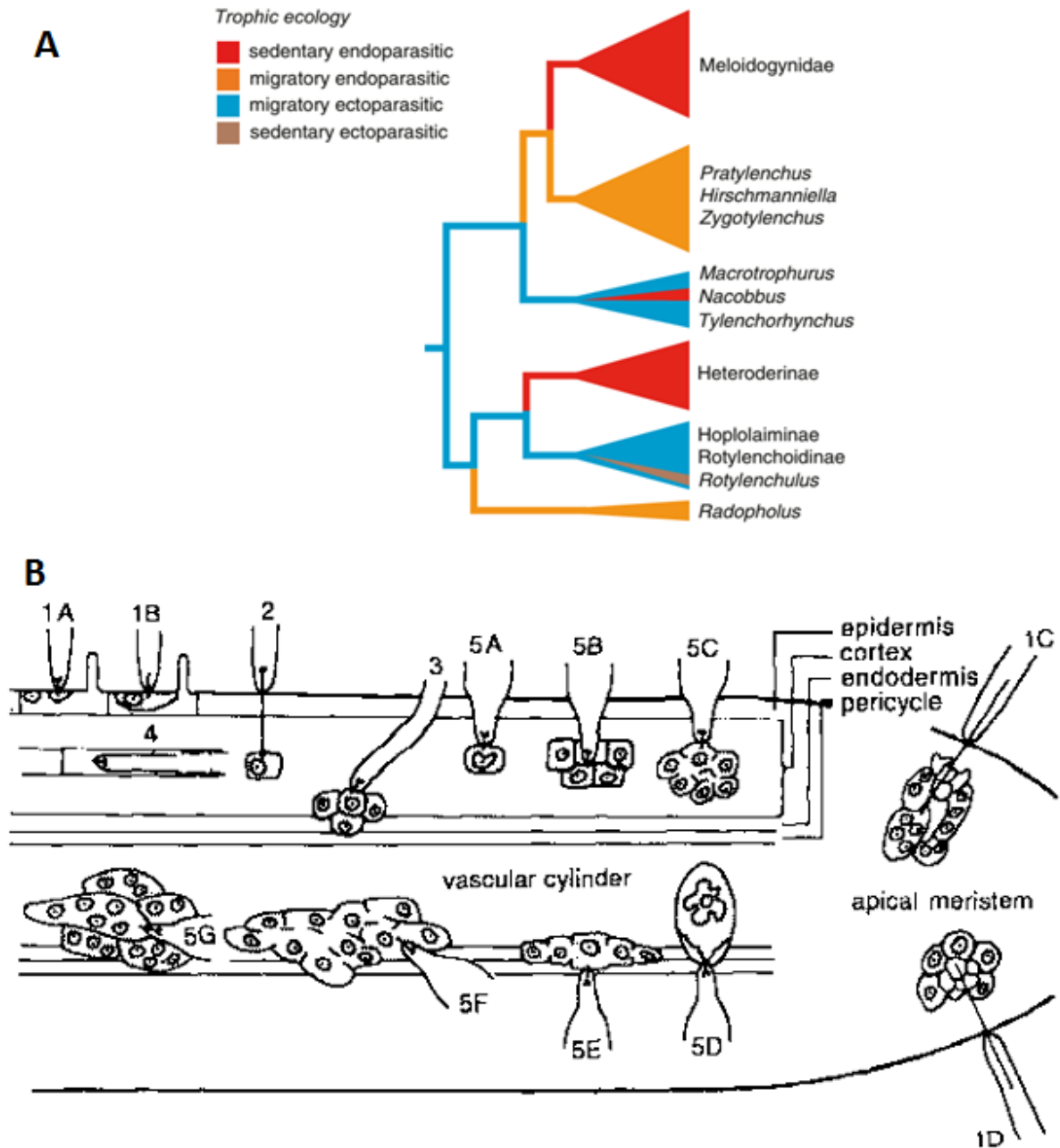


Figure 5: Phylogeny and lifestyle of Tylenchida
 (adapted from Bert et al. 2011; Sijmons et al. 1994)

(A) Phylogeny of Tylenchida. (B) Schematic representation of feeding sites and feeding structure of some selected root parasitic nematodes. 1= Migratory ectoparasites: 1A, *Tylenchorhynchus dubius*; 1B, *Trichodorus* spp.; 1C, *Xiphinema index*; 1D, *Longidorus elongates*; 2= Sedentary ectoparasites: *Cricone-malla xenoplax*; 3: Migratory ecto-endoparasites: *Helicotylenchus* spp.; 4: Migratory endoparasites: *Pratylenchus* spp.; 5: Sedentary endoparasites: 5A, *Trophotylenchulus obscurus*; 5B, *Tylenchulus semipenetrans*; 5C, *Verutus volvingentis*; 5D, *Cryphodera utahensis*; 5E, *Rotylenchulus reniformis*; 5F, *Heterodera* spp.; 5G, *Meloidogyne* spp.

Migratory endoparasitic nematodes cause massive plant tissue necrosis because of their migration and feeding. As they have no permanent feeding site, they simply withdraw the nutrients using their stylet, killing the plant cell and moving ahead of the lesion. Some examples of migratory endoparasites are *Pratylenchus* (lesion nematode-Figure 6), *Radopholus* (burrowing nematode), *Hirschmaniella* (rice root nematode). Furthermore, these nematodes could cause extensive wounds in plant roots, that leads to a potential secondary infection by bacteria and fungi (Zunke, 1990).

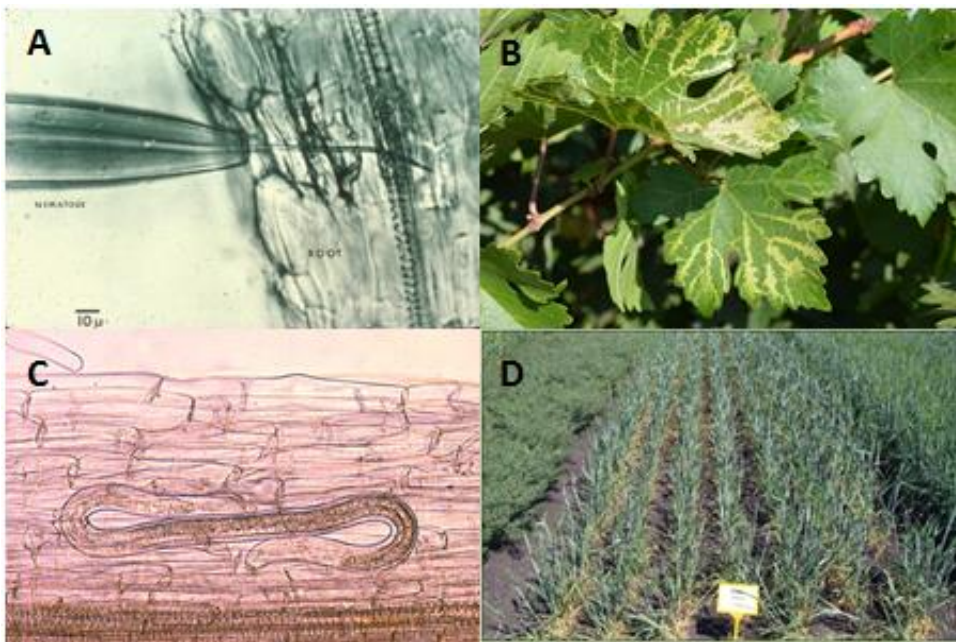


Figure 6: Migratory ectoparasitic and endoparasitic nematodes.

(A) Migratory ectoparasite *Xiphinema* spp. (B) Symptom of viruses transmitted by *Xiphinema* spp. on grapevine leaf (C) Migratory endoparasite *Pratylenchus* sp. (D) Symptom of *Pratylenchus* sp. on wheat include lower leaf yellowing, decreased tillers and wilting (Photo credit by: (A) NC State University (B) <http://plpnemweb.ucdavis.edu/> (C) Courtesy D. Wixted (D) Kirsty Owen, DAFF)

Sedentary endoparasites are, among PPNs, the most economical and dangerous ones. These pests enter host roots, establish a specialized feeding site within the root tissue and feed internally. They are represented by two major threats: root-knot nematodes (RKNs, *Meloidogyne* spp.) and cyst nematodes (CNs, *Globodera* spp. and *Heterodera* spp.). Both nematodes group preferentially infect plant root from the elongation zone and induced the formation of multinucleate and hypertrophied feeding cells (Figure 7). However, their ways to achieve the feeding site are different. The CN J2s migrate intracellular by cutting cortical cell walls and migrating through cells until they reach the differentiating vascular. By contrast, RKNs migrate intercellular. RKN J2s move towards the root tip until they reach the root apex, and then

migrate back up until they reach a site near the vascular cylinder (Figure 7) (Perry & Moens, 2011). While CNs are mainly found in a few plant species, RKNs show a capacity to infect almost cultivated plants throughout the world. In the next part of the introduction, I will focus on the description of RKNs.

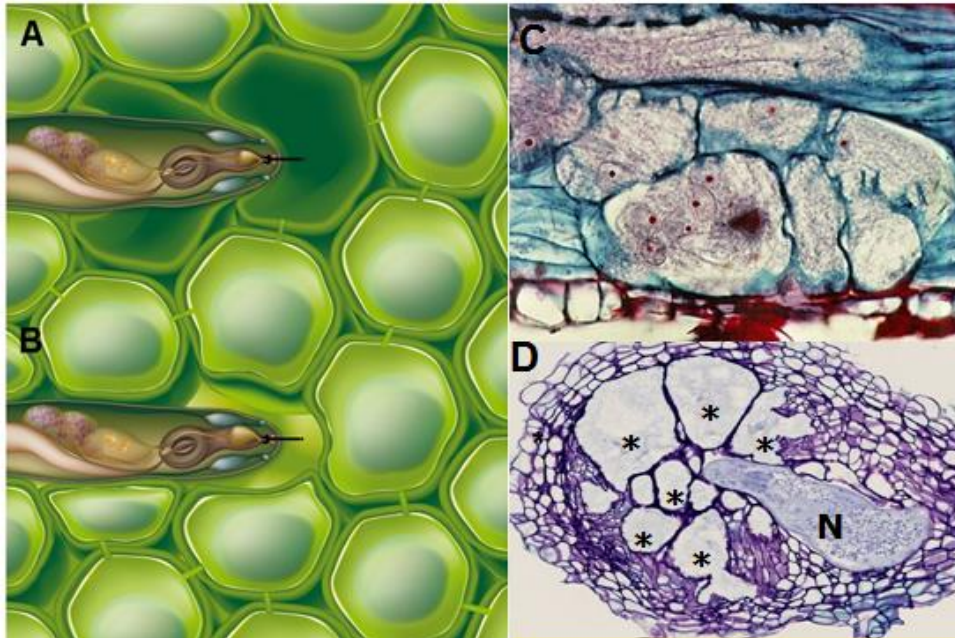


Figure 7: Parasitic strategies of cyst nematodes and root-knot nematodes during migration.

(A) CNs migrate intracellular and wound the plant cells while (B) RKNs migrate intercellular without impact on plant cells; (C) Feeding sites induced by CNs; (D) Feeding sites induced by RKNs. (Photo credit: (A), (B), (D) INRA Sophia-Antipolis; (C) www.apsnet.org)

3. Root-knot nematodes

Root-knot nematodes (RKNs, *Meloidogyne* species) are ones of the most economically devastating plant pathogens in the world (Trudgill & Blok, 2001), that causes a global crop losses of about 10 billion euros per year. RKNs could be found in the temperate and tropical regions all over the world (Blok *et al.*, 2008; Abad & Williamson, 2010) and are able to infect thousands of plant species (Figure 8). These microscopic worms induce typical root deformations, known as galls, which result a weak and poor-yielding plants. Until 2009, 97 RKN species have been described (Hunt & Handoo, 2009), in which those with asexual reproduction are the most damaging pests, e.g. *M. incognita*, *M. javanica*, *M. arenaria* and *M. enterolobii* (Figure 9). Climate change could promote nematodes to produce more generations per year, thus increase

the risk of nematode infections (Ghini *et al.*, 2008). Therefore, novel and efficacy strategies need to be developed to against these pests and secure global food production.

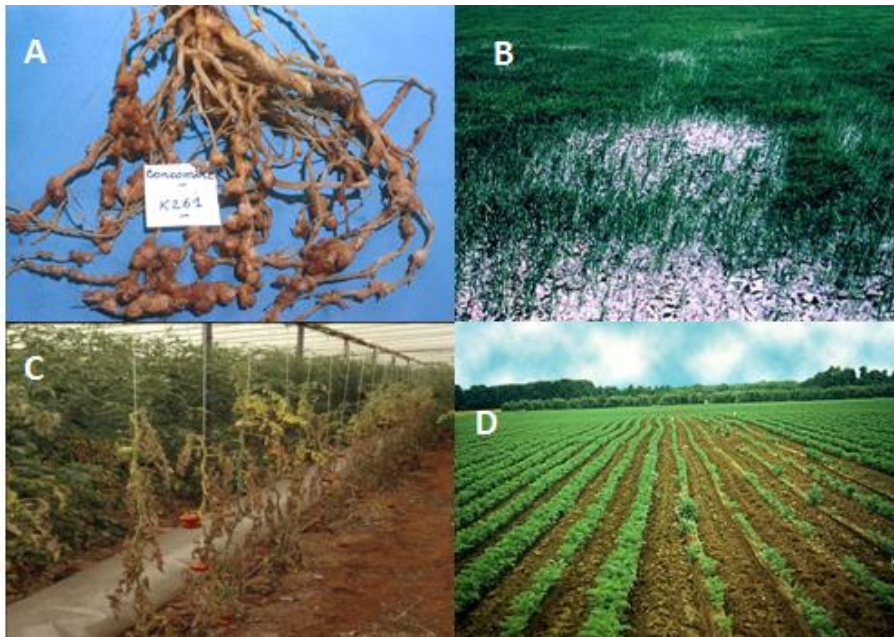


Figure 8: Example of wide host-range of RKNs.

(A) RKN symptom on cucumber root (B) *M. graminicola* infestation on rice field (C) *M. javanica* infestation on tomato (D) RKN damage in carrot field in New York state (Photo credit to: (A) INRA Sophia-Antipolis (B) Roger Lopez-Chaves, Universidad de Costa Rica (C) <http://www.nagref-her.gr/> (D) Courtesy G.S. Abawi).

3.1. Reproduction mode

There are three reproduction modes in RKNs: mitotic parthenogenesis, meiotic parthenogenesis and amphimixis (sexual reproduction) (Figure 9). There are a few RKNs that reproduce sexually (*M. carolinensis*, *M. megatyta*, *M. microtyla*, *M. pini*). They have a restricted distribution, a poor host-range and less impact in agriculture. Some species (*M. hapla*, *M. chitwoodi*, *M. fallax*) reproduce by cross-fertilization when males are present; or by meiotic (automixis) parthenogenesis when males are absent. Mitotic parthenogenesis, is the mode of reproduction of the most important RKN species in term of host-range and agronomic impact (*M. incognita*, *M. javanica*, *M. arenaria*, *M. enterolobii*) (Castagnone-Sereno *et al.*, 2013). This lack of sexual reproduction prevents the use of classical genetic approach to study these nematode species. Although information on nematode reproduction is still missing and lacking for several species, it is admitted that mitotic parthenogenesis species have wider host-range than the meiotic or amphimixis ones.

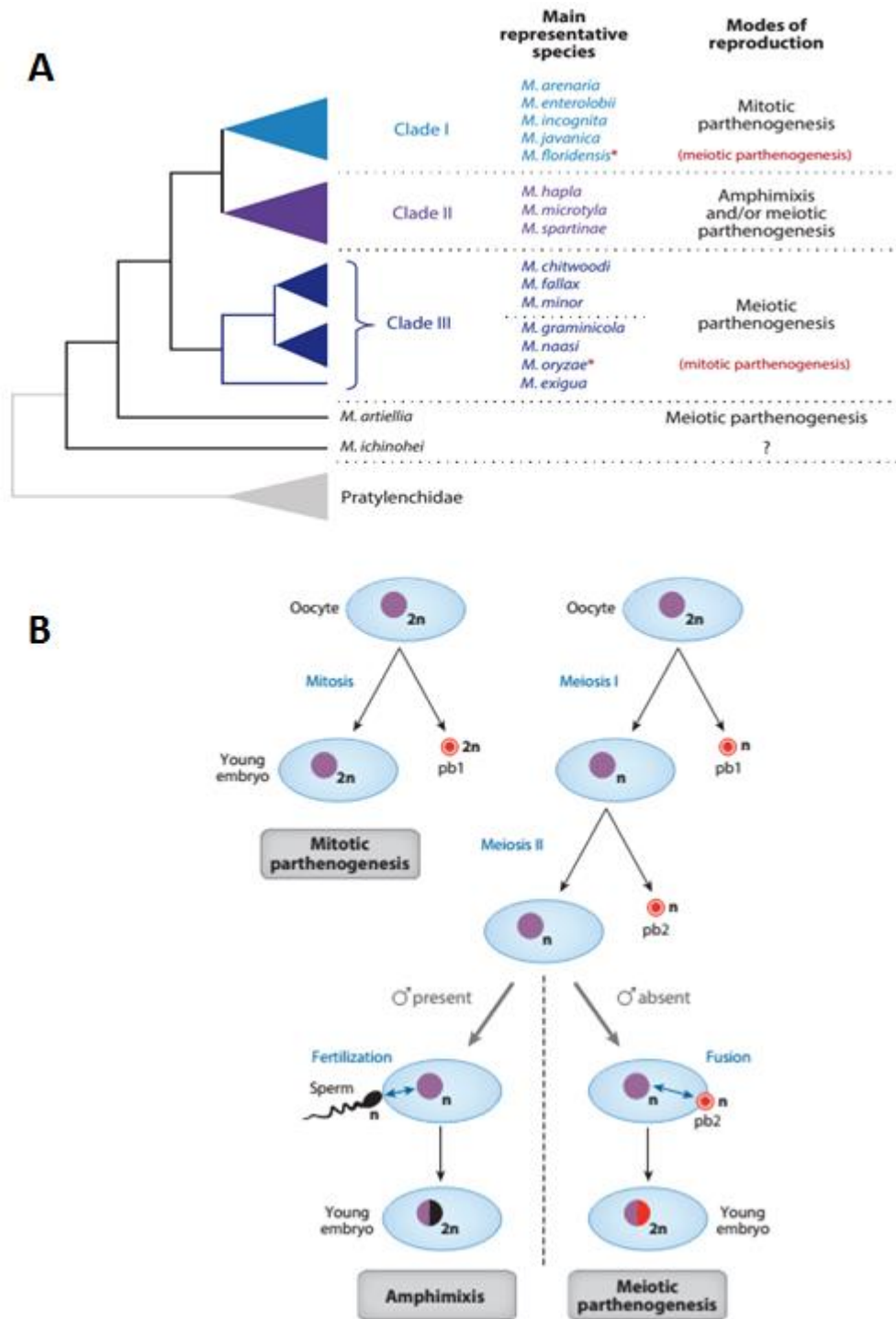


Figure 9: Mode of reproduction and reproductive pathway of root-knot nematodes (genus *Meloidogyne*) (Castagnone-Sereno *et al.*, 2013).

(A) Consensus tree of phylogenetic relationships in root-knot nematodes (genus *Meloidogyne*) and modes of reproduction (The tree is based on the analysis of SSU rDNA sequence); (B) Schematic representation of reproductive pathways in root-knot nematodes. $2n$ represents the somatic chromosome number independently of the ploidy level. Abbreviations: pb1, first polar body; pb2, second polar body.

3.2. Root-knot nematode control

There are several methods commonly used to control RKNs. These methods can be divided in to three main types: chemical control, biological control and resistant plants. The main goal of these methods is to limit the nematode population under an economically viable threshold. In reality, crop rotation is not efficient to control RKN due to large host range of these pests.

For long times, nematodes have been controlled using chemical nematicides. There are two types of nematicides, soil fumigants (gas) and non-fumigants (liquid or solid). Soil fumigants became popular because they limited practical methods; they drastically reduced nematode populations in the soil, and were cost effective for most crops. Non-fumigant nematicides such as fenamiphos (Nemacur) and aldicarb (Temik) were based upon the same kinds of active ingredients as many insecticides (i.e. nerve poisons) and could be applied in liquid or granular formulations (Lambert & Bekal, 2002). While non-fumigant nematicides reduce nematode populations, their effectiveness is not as consistent as that of fumigant nematicides. However, nematicides induced severe impacts to environment and human health. Therefore, in Europe, most of nematicides were banned by the application of Council Directive 91/414/EEC, except four active molecules: ethoprophos, fenamiphos (aka fenamiphos), fosthiazate and oxamyl. However, they are expensive, not so efficient, and the authorization to use these products will soon be expired in 2017.

An alternative nematode control method is to use natural predators or pathogens of nematodes. Most researches focused on isolating soil microorganisms, mainly fungi and bacteria as potential microbial control agents (Davies & Spiegel, 2011). The fungi *Arthrobotrys irregularis* was reported to be a predator for *Meloidogyne* spp. thanks to the formation of hyphae in lasso shaped (patent INRA n°7817624, (Cayrol, 1978)). The bacteria *Pasteuria penetrans* is also documented as control agent for *Meloidogyne* spp. The spore of this specie is able to parasite on the nematode and blocks its multiplication (Djian-Caporalino & Panchaud-Mattei, 1998). However, the efficiency and use of these biological agents were limited to some specific soil conditions and their commercialization was also limited by the difficulties and costs of the production of these agents.

Plant resistance constitutes an effective control method. Plant breeders cross natural nematode resistance genes (R-genes) into cultivated plant species to improve their resistance to nematodes. So far, there are about 30 R-genes to RKNs have been identified, mostly in the

Solanaceae family, such as *Mi-1.2* in tomato and *Me* genes in pepper, and *Ma* in Myrobalan plum (Castagnone-Sereno, 2006; Williamson & Kumar, 2006; Claverie *et al.*, 2011). Only two genes, *Mi-1.2* and *Ma*, were cloned (Figure 10). The *Mi-1.2* gene encodes a typical nucleotide binding leucine rich repeat (NB/LRR) type resistance gene, which confers resistance to *M. incognita*, *M. javanica*, *M. arenaria* (Rossi *et al.*, 1998). *Mi-1.2* is remarkable in that it also confers resistance to the potato aphid (*Macrosiphum euphorbiae*) and whitefly (*Bemisia tabaci*). The *Ma* gene is a Toll/Interleukin1 Receptor (TIR)-NBS/LRR (TNL) gene that confers a high and wide-spectrum RKN resistance comprising, besides the mitotic parthenogenetic RKNs controlled by *Mi-1.2* gene, the uncontrolled *M. enterolobii* (Claverie *et al.*, 2011). The wide-spectrum and resistance induced by *Ma* gene may be best explained by an indirect interaction - guard hypothesis between the resistance gene product and putative nematode avirulence factors (Claverie *et al.*, 2011). Whereas efficiency of several R-genes against RKNs is temperature-dependent, e.g. the tomato *Mi-1.2* gene or the pepper *N* gene, *Ma*, as pepper *Me3* and *Me1* are stable at high temperature (Djian-Caporalino *et al.*, 1999).

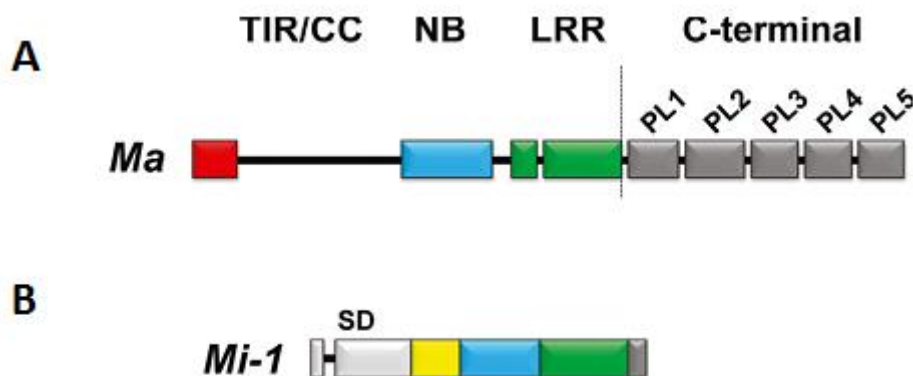


Figure 10: Structure of *Ma* and *Mi-1* resistance proteins (adapted from Rossi *et al.* 1998; Saucet *et al.* 2016).

Predicted protein structure of (A) the plum Ma and (B) the tomato Mi-1.2. red: TIR, Toll/interleukin-1 receptor-like domain; cyan: NB, a nucleotide binding site; green: LRR, leucine-rich repeat region; dark gray: C-terminal unknown domain; PL, huge-post LRR sequence; PL1-PL5, five repeated exon; light gray: N-terminal Solanaceae domain (SD); yellow: coiled-coil (CC) domain.

The histological characterisation of resistance to RKNs involved by *Mi-1.2*, *Me* and *Ma* genes showed that the complete absence of gall symptoms is associated with cell necrosis and corresponding hypersensitive (HR)-like reactions occurring either early at the penetration site (*Mi-1.2* and *Me3*) (Ho *et al.*, 1992; Milligan *et al.*, 1998), during migration in the cortex and the

stele (*Ma*) (Khallouk *et al.*, 2011) or later at the feeding site (*Me1*) (Pegard *et al.*, 2005). Nematode attacks often disorganized the meristematic apical tissues of *Ma*-R accessions, which induced the development of subterminal lateral roots replacing primary terminal apices and, thus, provided an active resistance reaction to HR damage.

However, the plant resistance is first limited by the low number of available resistance genes. R-genes are not available for some host species and some families, such as Cucurbitaceae, do not have characterized RKN R-genes. It takes years to screen for resistant plant varieties and more time to breed resistance traits into commercial varieties. Indeed, attempts to transfer cloned natural resistance genes to new hosts also have limited success. The transfer of tomato R-genes *Mi-1.2* to eggplant or the cyst nematode *Hero A* R gene to potato disappointingly did not function (Sobczak *et al.*, 2005; Goggin *et al.*, 2006). Further complications are that natural sources of nematode resistance do not exist for all cultivated species and some RKN species are not controlled by resistant plants. Thus, *M. enterolobii* is a new risk for global agriculture because of its worldwide distribution as well as its capacity of reproduction in all commercial plants rootstock resistant to *Meloidogyne* spp. (Kiewnick *et al.*, 2008). The second main limitation is the increasing nematode populations able to overcome R genes, such as the tomato *Mi1-2* gene (Jarquin-Barberena *et al.*, 1991; Castagnone-Sereno *et al.*, 1994; Castagnone-Sereno, 2006; Williamson & Kumar, 2006). The emergence of these “virulent” nematode populations has significantly decreased the efficacy of elite crop lines. Interestingly, virulent *M. incognita* populations were obtained for *Mi1-2* and *Me3*, both in natural (i.e., in the field) and artificial (i.e., in the laboratory) conditions, whereas, to date, no evidence showed the emergence of *Me1*-virulent populations (Castagnone-Sereno *et al.*, 1994; Barbary *et al.*, 2014), which suggests a possible relationship between the mode of action of these R-genes and their durability (Barbary *et al.*, 2014). The study on sustainable nematode control by R-gene is an important issue, in which research on the nematode evolution under stress of R-genes is essential.

3.3. RKN life cycle

The life cycle of RKNs spans 3-10 weeks depending on the nematode species and environmental conditions (Figure 11). RKNs vermiform second-stage juveniles (J2) hatch from the eggs to the soil in order to infect host roots. These pre-parasitic J2s penetrate the root in elongation zone, where they migrate between cells to reach the root apex, and then enter the

vascular cylinder of plant to establish the feeding sites, called giant cells (GCs). These GCs are hypertrophied and multinucleate. They are generated by repeated nuclear divisions and cell growth in the absence of cell division (Jones & Payne, 1978; Caillaud *et al.*, 2008b). The GCs constitute the sole source of nutrients for the nematode, which punctures the cells to gain access to their cytoplasmic content (Abad & Williamson, 2010). In addition, the division of the vascular cells surrounding the nematode and the feeding cells lead to the formation of a typical gall, observed as symptoms of the infection. Like all other plant parasitic nematodes, RKNs use a hollow, protrusible syringe-like stylet to penetrate the wall of plant cells, to inject secretions from their oesophageal glands into the cell, and to withdraw nutrients from the cytoplasm. Once the nematodes have established their feeding sites, they become sedentary and then grow up through three further moults (parasitic J3 and J4) to adult females or males. The females are always sedentary whereas the males return to vermiform and mobile again to leave the root to the soil. Sex is determined by environmental conditions and the number of males increases in poor nutrition conditions (Papadopoulou & Triantaphyllou, 1982). At the end, the female nematodes continue their development, become pear-shaped and then produce hundreds to thousands eggs to the outer surface of the root in a protective and gelatinous matrix, which will be released directly into the rhizosphere.

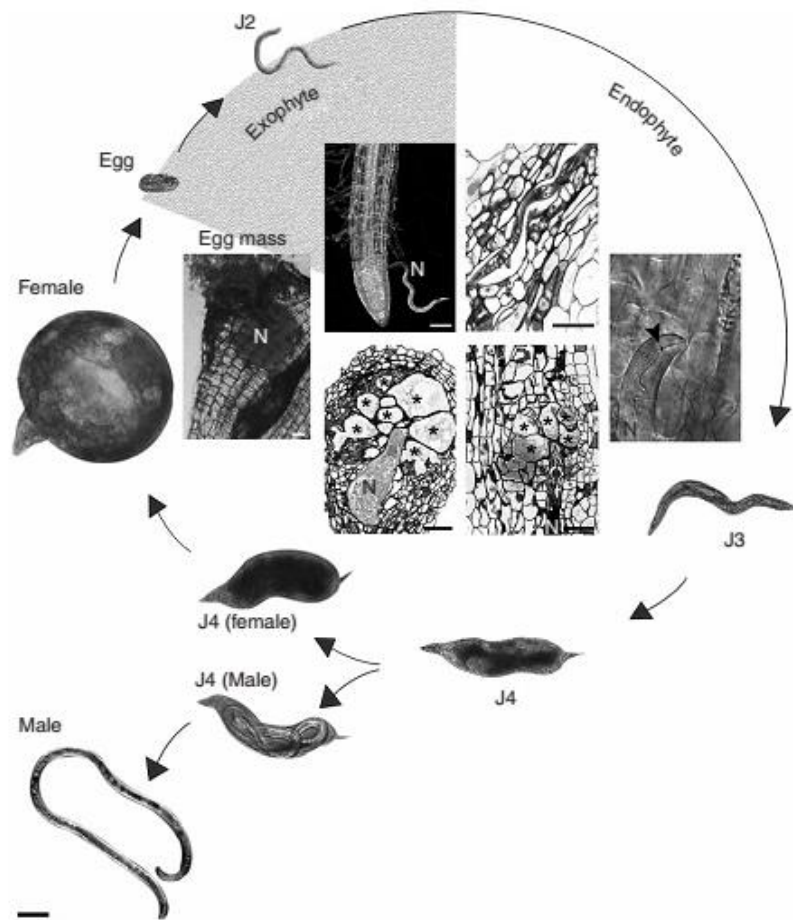


Figure 11: The parasitic life cycle of *Meloidogyne incognita* (Abad et al., 2008)

Infective second stage juveniles (J2) penetrate the root and migrate between cells to reach the plant vascular cylinder. The stylet (arrowhead) connected to the oesophagus is used to pierce plant cell walls, to release oesophageal secretions and to take up nutrients. Each J2 induces the dedifferentiation of five to seven root cells into multinucleate and hypertrophied feeding cells (). These GCs supply nutrients to the nematode (N). The nematode becomes sedentary and goes through three moults (J3, J4, adult). Occasionally, males develop and migrate out of the roots. However, it is believed that they play no role in reproduction. The pear-shaped female produces eggs that are released on the root surface. Embryogenesis within the egg is followed by the first moult, generating second-stage juveniles (J2). Scale bars, 50 μm .*

4. Giant cells: formation and main characteristics

The formation of the giant cells (GC) plays a special attention due to the fact that they are essential for nematode development and reproduction. Mature GCs (Figure 10) reach 100 times the size of a normal root vascular cell and can contain more than 100 enlarged nuclei. Mature GC nuclei are highly amoeboid and have dispersed chromatin, reflecting intensive gene transcription (Jones and Payne, 1978). GCs contain small vacuoles, and display proliferation of the endoplasmic reticulum, ribosomes, mitochondria and plastids. GCs have several features

typical of highly metabolic transfer cells, such as wall ingrowths developing in contact with the xylem elements and increasing the contact area for exchanges at the associated membrane. The complex changes in cell structure and physiology leading to feeding cell establishment result from profound changes to the profile of gene expression in the infected root cells (Gheysen & Fenoll, 2002; Caillaud *et al.*, 2008b; Barcala *et al.*, 2010; Damiani *et al.*, 2012). The genome-wide expression profiling of isolated GCs and galls has identified thousands of genes involved in diverse processes, including cell cycle activation, cytoskeleton reorganisation, cell wall and metabolism modification, and hormone and defence responses, as differentially expressed during feeding cell formation. Functional analyses of these differentially expressed genes have been carried out, to identify nematode susceptibility genes essential for the development of RKNs and their feeding sites. Only few mutations impairing nematode infection have been characterised to date (Caillaud *et al.* 2008b). Unique defects in GC ontogenesis have been described in the absence of regulators of microtubule (MT) or microfilament (MF) dynamics, highlighting the importance of changes to cytoskeleton architecture for correct giant cell development (Caillaud *et al.* 2008c; Clement *et al.* 2009). However, the molecular events underlying the formation and development of nematode feeding cells remain to be identified. Two key characteristics of the giant cells, i.e. the cell cycle and cytoskeleton reorganization, and the metabolism reprogramming, are now presented in more details.

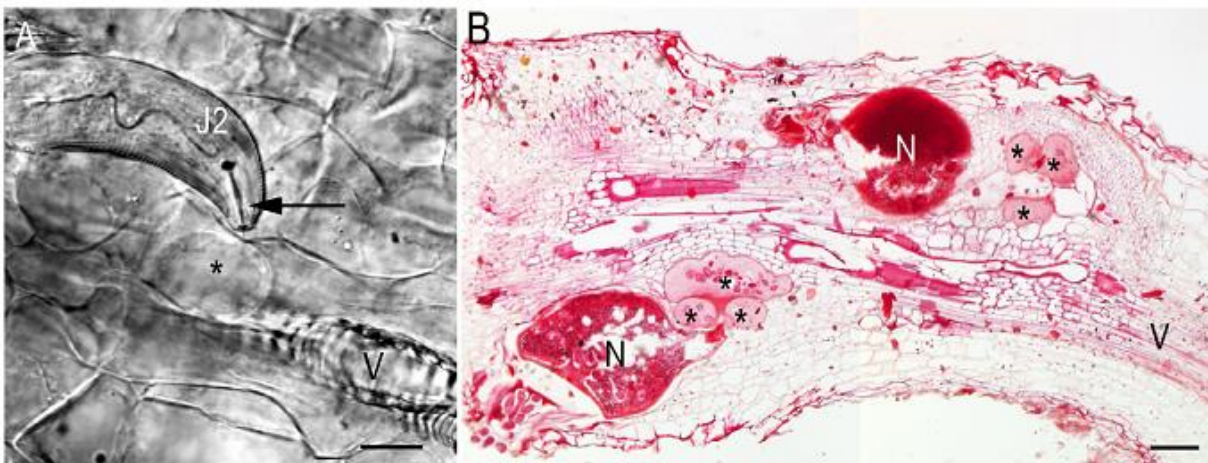


Figure 12: Galls and giant cell induced by root-knot nematodes (Favery *et al.*, 2016)

(A) *M. incognita* second-stage juvenile J2 into a vascular cell (*) that will become a GC. Section through a gall in *Arabidopsis*, 12 hours post infection. V: vessels. Scale bar = 10 μ m. (B) Section through a gall in tomato, 15 days post infection, containing two nematode feeding sites. Asterisks: GCs; N: female root-knot nematode; V: vessels. Scale bar = 40 μ m.

4.1. Cell cycle and cytoskeleton reorganization during giant cell formation.

The process of GC formation follows a typical developmental pathway, where RKNs are involved in the re-programming of plant cell differentiation in a specialized way. The first sign of GC induction is the formation of one or several binucleate cells, in which the vesicles align apparently after mitosis as in a normal cell plate, but subsequently fail to fuse and then disperse. This nuclear division cycle – nuclear mitosis without cytokinesis- repeats and generates more nuclei, could up to 100 enlarged nuclei, during the development of GCs (Jones & Payne, 1978; Caillaud *et al.*, 2008a). In addition, endoreduplication, i.e. , occurs during later stages (Jones & Goto, 2011; de Almeida Engler & Gheysen, 2013). The use of chemical inhibitors to block either DNA synthesis or the G1 to S transition of cell cycle, resulted in a failure of RKNs to induce their feeding sites (de Almeida Engler *et al.*, 1999; Wiggers *et al.*, 2002). Genes encoding cell cycle regulators, such as cyclins or cyclin-dependent kinases (CDKs), have been shown to be upregulated at early stage of the interaction (de Almeida Engler *et al.*, 1999; Jammes *et al.*, 2005; Barcala *et al.*, 2010; Ibrahim *et al.*, 2011; Ji *et al.*, 2013). A detail expression analysis of 61 core cell cycle genes showed that most of them were expressed in GC (de Almeida Engler & Gheysen, 2013). Indeed, the silencing of Arabidopsis cell cycle gene *AtCDKA;1* led to a reduced susceptibility to RKN (Van De Cappelle *et al.*, 2008). Analysis of Kip-related protein 6 (KRP6)-overexpressing Arabidopsis lines showed a role for this particular KRP as an activator of the mitotic cell cycle by delaying mitosis progression (Vieira *et al.*, 2014). KRP6 expression was found parallel with the induction of a mitotic state in plant and GCs prompting their multinucleate and acytokinetic state. Furthermore, expression of plant genes *CCS52s*, *DEL1* and *CPR5* in GC suggested their direct involvement in endoreduplication (Koltai *et al.*, 2001; Favery *et al.*, 2002; Jammes *et al.*, 2005; de Almeida Engler *et al.*, 2012). Down-regulation or over-expression of *CCS52* and *DEL1* in Arabidopsis drastically affected giant cell growth, resulting in restrained nematode development, illustrating the need for mitotic activity and endo-reduplication for feeding site maturation (de Almeida Engler *et al.*, 2012).

The plant cytoskeleton, composed mainly of microtubules and actin filaments, plays a central role in intracellular transport, cell division, cell differentiation and morphogenesis. Thus, the manipulation of plant cytoskeleton is an important step during the giant cell formation and the success of RKN parasitism. It has been reported that RKNs are able to induce important changes in the cytoskeleton organization of GCs (Wiggers *et al.*, 2002; de Almeida Engler *et al.*, 2004,

2010; Caillaud *et al.*, 2008a). The treatment of infected roots with cytoskeleton inhibitor taxol during the feeding site initiation led to the arrest of proper GC development and consequently nematode development (de Almeida Engler *et al.*, 2004). Moreover, histochemical analysis and the use of fluorescence markers demonstrated that, during the GC formation, the actin network is significantly de-organized (de Almeida Engler *et al.*, 2004, 2010; de Almeida-Engler & Favery, 2011) while the microtubule network is also rearranged for GC ontogenesis (Figure 12) (de Almeida Engler *et al.*, 2004; Caillaud *et al.*, 2008a; de Almeida-Engler & Favery, 2011). Indeed, these cytoskeleton changes may be triggered by actin-binding proteins (ABPs) and microtubule-associated proteins (MAPs).

Three genes coding for actin-nucleating formins of *Arabidopsis*, AtFH1, AtFH6, and AtFH10 that may participate in actin cytoskeleton remodeling, were observed to be upregulated in developing feeding sites (Favery *et al.*, 2004; Jammes *et al.*, 2005; Barcala *et al.*, 2010). Among them, AtFH6 was shown to be anchored and uniformly distributed throughout the giant cell plasma membrane. It is hypothesized that this protein could regulate the GC isotropic growth via controlling assembly of actin cables guiding the vesicle trafficking needed for membrane and cell wall extension of GC formation (Favery *et al.*, 2004). Furthermore, the *Arabidopsis* MAP65-3 and the actin depolymerizing factor ADF9 have been shown to be essential for the development of GC induced by *M. incognita* (Caillaud *et al.*, 2008c; Clement *et al.*, 2009). The subcellular localization of MAP65-3 demonstrated that this protein is linked to the microtubule networks in all dividing plant cells. In GCs, MAP65-3 was associated with mini cell plates formed between daughter nuclei during cytokinesis initiation in developing GC. The absence of this protein led to an incomplete formation of GCs, in which GC started to develop but accumulation of mitosis defects during nuclear division prevented the normal development of feeding cells (Caillaud *et al.*, 2008c).

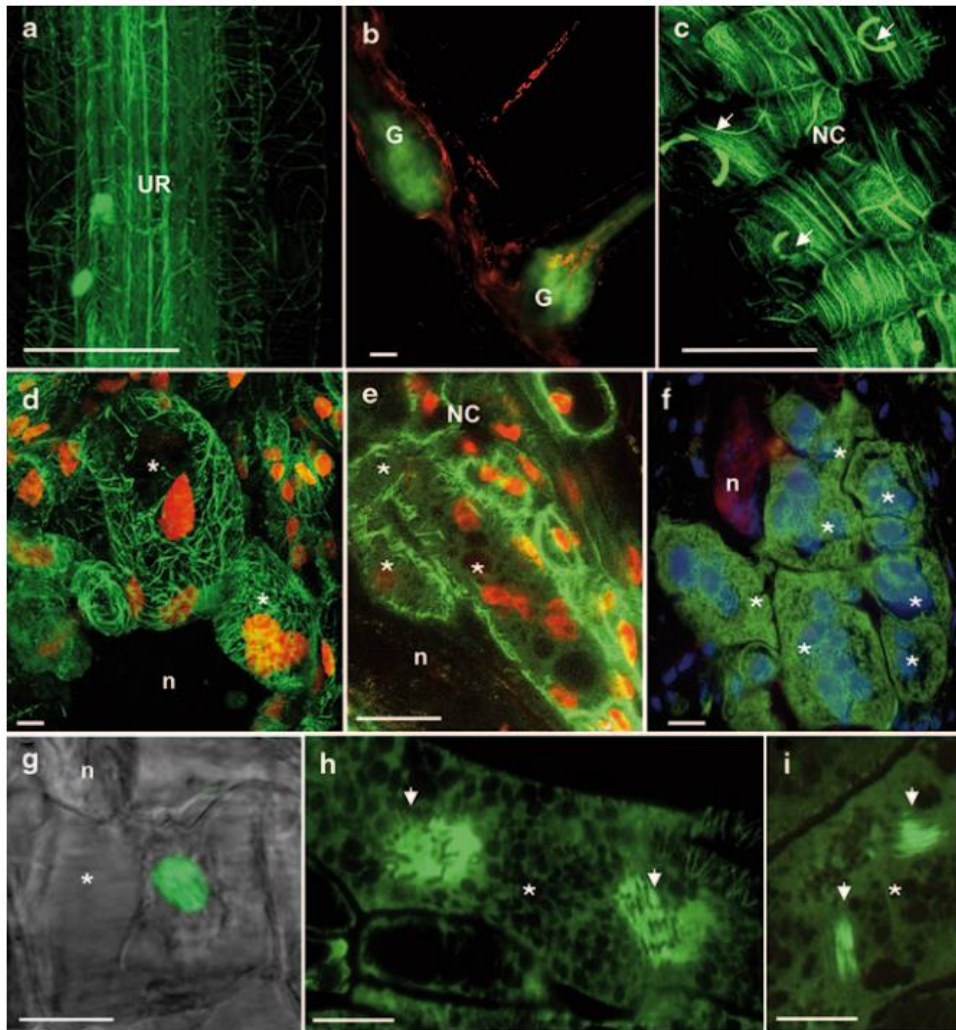


Figure 13: The microtubule cytoskeleton in galls (de Almeida-Engler & Favery, 2011)

(a) *In vivo* confocal microscopy of MTs (green) in an uninfected *Arabidopsis* root expressing MBD:GFP. (b) Galls induced by *M. incognita* in *Arabidopsis* showing GFP fluorescence of MTs (green and autofluorescence in red). (c) *In vivo* confocal microscopy of MTs of gall cells neighbouring the GC showing curved phragmoplasts (arrows). (d) *In vivo* confocal microscopy of a young gall co-expressing DNA (H2B:YFP in red) and MT markers (MBD-GFP in green). Interphasic young (7 dpi) GCs showing bundles of cortical MTs. (e) *In vivo* confocal microscopy of cortical MTs and a diffuse cytoplasmic fluorescence (green) in maturing (10 dpi) GCs containing several nuclei (red). (f) Immunocytochemical analysis of MTs in a section of a gall in *Pisum sativum* (pea) showing a diffuse fluorescence in the cytoplasm of GC (MTs in green, autofluorescence in red and nuclei in blue). (g) A mitotic spindle in a cell neighboring a giant cell expressing MBD:GFP. (h) Large malformed spindles (arrowheads) of mitotic nuclei of a GC. (i) Phragmoplasts (arrowheads) with misaligned microtubules (green) in a mitotic GC. UR, uninfected root; G, gall; NC, neighbouring cells; n, nematode; Asterisks, GCs. Bars = 50 μm (a, c–e), 100 μm (b, f), 10 μm (g) and 20 μm (h, i)

4.2. Importance of the metabolism in GC

The formation of the highly specialized feeding cells, i.e. GCs, and unique source of water and nutrients for RKN development, requires extensive changes in cellular structure and metabolism. Increased activities of malate, isocitrate, succinate, esterase, peroxidase, cytochrome oxidase and pentose phosphate pathways (PPP) have been observed in the infected sites (Veech & Endo, 1969). High levels of activity of G6PDH (glucose-6-phosphate dehydrogenase), the first enzyme in the PPP, has been first detected in histochemical preparations of galls. Indeed, ribulose-phosphate-epimerase (RPE), a key enzyme in the reductive Calvin cycle and the oxidative pentose phosphate pathway (OPPP), has been reported to be essential for the early steps of GC formation (Favery *et al.*, 1998). These results illustrated the importance of PPP in the formation of GCs. Moreover, large amounts of solutes and water are transported from the xylem through the cell wall ingrowths of the GC, probably via transmembrane transporter proteins that facilitate the passage across biological membranes (Gheysen & Fenoll, 2002; Bartlem *et al.*, 2014).

Microarray analysis of *Arabidopsis* roots infected with *M. incognita* and *M. javanica* of hand-excised galls as compared to non-infected roots was performed in *Arabidopsis* and tomato along different stages of development (Jammes *et al.*, 2005; Portillo *et al.*, 2013). The functional categories with the highest number of genes were those related to metabolism, which is in accordance with the hypothesis that GCs act as strong sinks. Transcript abundance for most of the genes involved in cell cycle, energy metabolism, protein synthesis and DNA processing increased in galls as compared to control roots. Although transcriptome analysis of galls provided a detailed view of gene expression, they include GCs and the surrounding tissues, which might lead to a dilution of the specific mRNA population within GCs. Therefore, Barcala *et al.* (2010) used laser capture microdissection for microarray analysis of very young GCs at 3 dpi in *Arabidopsis* roots induced by *M. javanica* and Portillo *et al.* (2013), in tomato GCs at 3 and 7 dpi. Again, the functional categories with the highest number of upregulated genes included metabolism, RNA and protein. Similarly, isolation of GCs induced by *M. graminicola* on rice roots and subsequent transcriptome analysis revealed a general induction of primary metabolism (Ji *et al.*, 2013).

The importance of glutathione (GSH), a major antioxidant molecule involved in plant development, in plant microbe interaction and in abiotic stress response, was analyzed in galls

induced in *Medicago truncatula* by *M. incognita*. Starch contents were also measured using an enzymatic assay (Baldacci-Cresp *et al.*, 2012) and differences were observed between galls and uninfected roots. A metabolomics analysis revealed that, out of 37 identified metabolites, six amino acids (glucose, sucrose, trehalose, malate and fumarate) accumulated at high levels in galls compared with uninfected roots. The amount of starch increased threefold in galls, suggesting that starch acts as a carbohydrate buffer during nematode development (Baldacci-Cresp *et al.*, 2012). Furthermore, depletion of homoglutathione ((h)GSH) content impaired nematode egg mass formation and modified the sex ratio of offspring. These results suggest that (h)GSH have a key role in the regulation of GC metabolism.

Massive water transport is supported by upregulation of genes encoding water channel proteins, such as aquaporins. These proteins are also involved in osmoregulation and growth control (Maurel & Chrispeels, 2001). Microarray analysis of Arabidopsis aquaporin families showed the downregulated of TIPs and PIP families, including AtTIP1.1 and AtPIP1.5 genes (Jammes *et al.*, 2005). Indeed, another Arabidopsis PIP family, AtPIP2.5 was reported to be specially upregulated in galls by qRT-PCR and promoter GUS fusion (Hammes *et al.*, 2005). Moreover, the tomato aquaporin TIP2 was recently shown to be a direct target of the Mi8D05 RKN secreted protein (Xue *et al.*, 2013). This interaction illustrated that solute and water transport within GC could be regulated by RKN effectors to promote the parasitism.

5. RKN effectors

Our knowledge of the dialogue between plants and RKNs remains fragmentary, but nematode secretions, named effectors, are thought to be instrumental in manipulating developmental and defence signalling pathways in host cells. The nematode effectors are all pathogen proteins and small molecules that are secreted by nematode into the host plant in order to alter host-cell structure and function (Hogenhout *et al.*, 2009).

A large part of this section was published in a review paper published in Advances in Botanical Research: Plant Nematode Interaction: A view of compatible interrelationships, 2015 (Truong, Nguyen et al. 2015).

5.1. Identification of RKN effectors.

As other PPNs, RKN effectors produced by three oesophageal gland cells, from which they are secreted into the host through the stylet, may play an important role in the induction and maintenance of the giant cells (Davis et al. 2004; Hewezi and Baum, 2013; Mitchum et al. 2013). Secreted proteins are also known to play an important role in other aspects of the host-parasite interaction, including invasion, migration, and protection against host defence responses (Abad and Williamson, 2010; Mitchum et al. 2013). The three oesophageal gland cells are large and complex secretory cells that have undergone adaptation to increase their secretory activity. Two are in a subventral location and the third is in a dorsal location. Interestingly in the early stages of parasitism, the subventral glands are highly active. Following the onset of parasitism and throughout the rest of the parasitic cycle, the dorsal gland cell becomes the leading source of effector proteins. Most studies have focused on secretions originating from these glands, but putative effector proteins may also be produced by other secretory organs, such as the cuticle, the chemosensory amphids, the excretory/secretory system and the rectal glands. Like the oesophageal glands, these organs probably undergo changes in function on adoption of the parasitic stage of the life cycle (Jones *et al.*, 1993).

The biotrophic life cycle and lack of sexual reproduction of the main *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. arenaria*) preclude the development of forward genetic screens for identifying nematode parasitism genes. Research has thus focused on the cloning and characterisation of nematode secreted proteins with functions likely to promote the parasitism of plants by nematodes (candidate approach), the presence of these proteins in secretions (proteomics), and gene expression patterns in secretory organs or for genes containing predicted specific secretory signals in their sequences (transcriptomics/genomics). The

sequencing of the full genomes of two RKN species has opened up new opportunities for studying plant-nematode interactions (Bird et al. 2009).

From secretions...

Most of effectors are secreted into host cells and tissues through the stylet. Direct qualitative analysis of the proteins secreted via the stylet by J2s was therefore considered an evident way to identify effectors. However, this approach has not been successful, due to the small size of the nematodes, the minute amounts of secretory material recovered from RKNs and their obligate biotrophy. The analysis of stylet secreted proteins was, for a long time, limited to one-dimensional electrophoresis (Veech *et al.*, 1987). Studies of the proteins secreted via the stylet began to advance much more rapidly with the advent of a strategy based on monoclonal antibodies (MAbs). MAbs were developed against secretory granules formed in the oesophageal glands of *M. incognita*, as a first step towards the identification of biologically important secretions (Hussey, 1989). MAbs have been used to isolate two high-molecular weight secretory glycoproteins from the oesophageal glands of RKNs (Hussey *et al.*, 1990). The amino-acid sequence of the *M. incognita* 6D4 protein has yet to be determined (Vieira *et al.*, 2011). MAbs binding specific structures in RKNs have also been developed by an immunisation procedure involving the injection of homogenates of the anterior regions or stylet secretions from *M. incognita* adult females (Davis *et al.*, 1992). Nine MAbs have been shown to bind to secretory granules formed in the dorsal oesophageal gland and two have been shown to bind to such granules in the subventral glands. One MAb, MGR48, developed from J2s of the potato cyst nematode, *Globodera rostochiensis*, binds specifically to the subventral oesophageal glands and was used for the immunopurification of the first parasitism protein from a plant-parasitic nematode ever identified, a β -1,4 endoglucanase (Smant *et al.*, 1998). Cyst nematode cellulases were the first endogenous cellulases to be identified in animals. All previously identified cellulases from the digestive systems of animals originate from symbiotic microorganisms. A first cellulase gene, *Mi-ENG-1*, was cloned by PCR from *M. incognita* J2s and shown, by mRNA *in situ* hybridisation (ISH), to be expressed in the subventral glands (Figure 14) (Rosso *et al.*, 1999). The nematode cellulases identified were thought to facilitate migration through plant roots by mediating the partial degradation of the plant cell wall. These enzymes were the first of a long list of cell wall degrading or modifying effectors to be identified in RKNs and other plant-parasitic nematodes (Table 1).

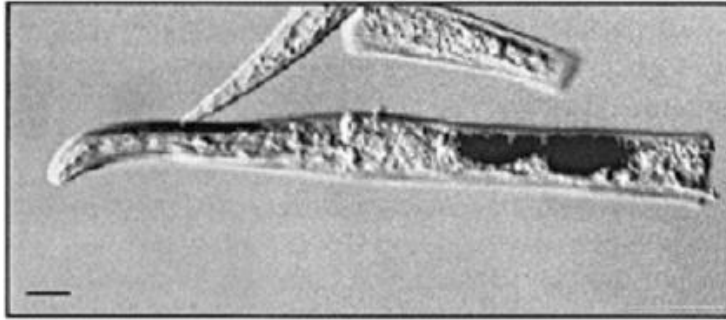


Figure 14: Localization of β -1,4-endoglucanase transcripts in the subventral glands of *Meloidogyne incognita* second-stage juveniles (Rosso *et al.*, 1999)

Jaubert *et al.* (2002b) established a new procedure for the direct qualitative analysis of stylet-secreted proteins from *M. incognita* infective juveniles. A large-scale procedure was established, for the production of stylet secretions in semi-sterile conditions by the incubation of J2s in a solution of resorcinol. Resorcinol is a neurostimulant that stimulates stylet thrusting and the accumulation of secretions in the lip region of J2s without impairing the ability of the nematode to infect plants (Jaubert *et al.*, 2002b; Rosso *et al.*, 1999). The purified proteins were separated by two-dimensional electrophoresis and the seven most abundant proteins were identified by microsequencing. Genes encoding a calreticulin (CRT) and 14-3-3-like proteins were identified and shown to be expressed in the oesophageal glands of infective juveniles (Jaubert *et al.* 2002a, 2004) (Table 1). The *M. incognita* secretome was then explored in greater detail, by nano-electrospray ionisation and tandem mass spectrometry (nanoLC ESI MS/MS; Bellafiore *et al.*, 2008). These sensitive methods for high-throughput proteomics-based liquid chromatography led to the identification of 486 proteins secreted by *M. incognita*. These secreted proteins were then annotated to indicate their functions and classified according to their potential roles in disease development. ISH showed that most of the analysed secreted proteins were produced by the subventral glands, but phasmids also secreted proteins (Table 1). A new bacterial contamination-resistant method for collecting soluble proteins directly from the oesophageal gland cells of female *M. incognita* nematodes has recently been developed (Wang *et al.*, 2012). This approach has proved successful for *M. incognita* female and opens up new possibilities for identifying RKN effectors at different stages of life cycle. Indeed, the combination of proteomics and bioinformatics approaches could provide an experimentally verified essential tool for the biologically meaningful discovery-based proteomic analysis of nematode parasitism (Mbeunkui *et al.*, 2010), which would enrich our knowledge of RKN effector repertoires.

Table 1: *M. incognita* proteins produced in secretory organs and predicted to be involved in parasitism

Effector	Predicted function	Organs	ISH	IL	References
Mi-PEL-1	pectate lyase	SvG	+		(1, 2)
Mi-PEL-2	pectate lyase	SvG	+		(3, 2)
Mi-PEL-3	pectate lyase	SvG		+	(4, 5)
Mi-ENG-1	beta-1,4-endoglucanase	SvG	+		(3, 6, 7)
5A12B	beta-1,4-endoglucanase	SvG	+		(3)
8E08B	beta-1,4-endoglucanase	SvG	+		(3)
Mi-PG-1	polygalacturonase	SvG	+		(8)
Mi-XYL-1	beta-1,4-endoxylanase	SvG	+		(9)
Mi-CBP-1	cellulose-binding protein	SvG	+		(1, 10)
Mi-CM-1	chorismate mutase	SvG	+		(3, 11)
Mi-CM-2	chorismate mutase	SvG	+		(3, 11)
Mi-ASP2	aspartyl protease-like	SvG		+	(12)
Mi-GST-1	glutathione-S-transferase	SvG	+	+	(13)
16D10	CLE-like peptide	SvG	+	+	(1)
Mi-SXP-1	SXP/Ral-2 protein	SvG	+		(14)
Mi-VAP-2	venom allergen-like protein	SvG	+		(15)
Mi-MSP-1	venom allergen-like protein	SvG	+		(16)
5G05	zinc metallopeptidase	SvG	+		(1)
30G11	acid phosphatase	SvG	+		(1)
10A07	sodium/calcium/potassium exchanger	SvG	+		(1)
CL5Contig2_1	Sec-2 protein	SvG	+		(7)
CL2552Contig1_1	transthyretin-like protein	SvG	+		(7)
CL321Contig1_1	translationally controlled tumour protein	SvG	+		(7)
CL480Contig2_1	triosephosphate isomerase	SvG	+		(7)
Minc01696	protein kinase	SvG	+		(17)
Minc03866	C-type lectin	SvG	+		(18)
CL312Contig1_1	unknown	SvG	+		(7)
Minc00344	unknown	SvG	+		(17)

Minc04584	unknown	SvG	+		(17)
Minc18033	unknown	SvG	+		(17)
Minc13292	unknown	SvG	+		(17)
Minc08073	unknown	SvG	+		(17)
Minc00469	unknown	SvG	+		(17)
Minc15401	unknown	SvG	+		(17)
Minc10418	unknown	SvG	+		(17)
Minc03328	unknown	SvG	+		(17)
Minc03325	unknown	SvG	+		(17)
Minc18636	unknown	SvG	+		(17)
Minc08146	unknown	SvG	+		(19)
2G02	unknown	SvG	+		(1)
4D01	unknown	SvG	+		(1)
8D05	unknown	SvG	+	+	(1,25)
8H11	unknown	SvG	+		(1)
8E10B	unknown	SvG	+		(1)
30H07	unknown	SvG	+		(1)
31H06	unknown	SvG	+		(1)
35A02	unknown	SvG	+		(1)
HM1	unknown	SvG	+		(12)
6D4	unknown	SvG&DG		+	(5, 20)
Mi-CRT	calreticulin	SvG&DG		+	(21)
Mi-1.24-3-3-b	14-3-3	DG	+	+	(5, 22)
10G02	thioredoxin	DG	+		(1)
Minc00108	metallopeptidase	DG	+		(18)
Minc02097	unknown	DG	+		(17)
Mi-EFF1/Minc17998	unknown	DG	+	+	(18)
Minc18861	unknown	DG	+		(17)
Minc12639	unknown	DG	+		(17)
Minc11817	unknown	DG	+		(17)
Minc01595	unknown	DG	+		(17)
1C05B	unknown	DG	+		(3)
1D08B	unknown	DG	+		(3)
2E07	unknown	DG	+		(1)

2G10	unknown	DG	+		(1)
4D03	unknown	DG	+		(1)
4F05B	unknown	DG	+		(3)
5C03B	unknown	DG	+		(3)
6F07	unknown	DG	+		(1)
6G07	unknown	DG	+		(1)
7A01	unknown	DG	+		(1)
7E12	unknown	DG	+		(1, 23)
7H08	unknown	DG	+		(1)
9H10	unknown	DG	+		(1)
11A01	unknown	DG	+		(1)
12H03	unknown	DG	+		(1)
13A12	unknown	DG	+		(1)
17H02	unknown	DG	+		(1)
25B10	unknown	DG	+		(1)
14E06	unknown	DG	+		(1)
16E05	unknown	DG	+		(1)
21E02	unknown	DG	+		(1)
34D01	unknown	DG	+		(1)
34F06	unknown	DG	+		(1)
35F03	unknown	DG	+		(1)
35E04	unknown	DG	+		(1)
28B04	unknown	DG	+		(1)
HM7	unknown	DG	+		(12)
HM12	unknown	DG	+		(12)
Mi-MAP1	unknown	amphids	+	+	(5, 24)
CL1191Contig1_1	CDC48-like	phasmids	+		(7)
Minc00801	unknown	RG	+		(17)

ISH, *in situ* hybridisation; IL, immunolocalisation; SvG, subventral glands; DG, dorsal gland; RG, rectal gland. RPKM.

(1) Huang *et al.* 2003; (2) Huang *et al.*, 2005b (3) Huang *et al.* 2004; (4) Vieira *et al.* 2011; (5) Vieira *et al.* 2012; (6) Rosso *et al.* 1999; (7) Bellafiore *et al.* 2008; (8) Jaubert *et al.* 2002a; (9) Dautova *et al.* 2001; (10) Ding *et al.* 1998; (11) Huang *et al.*, 2005a; (12) Neveu *et al.* 2003; (13) Dubreuil *et al.* 2007; (14) Tytgat *et al.* 2005; (15) Wang *et al.* 2007; (16) Ding *et al.*, 2000; (17) Rutter *et al.* 2014; (18) Danchin *et al.* 2013; (19) Jaouannet *et al.* 2012; (20) Davis *et al.* 1992; (21) Jaubert *et al.* 2005; (22) Jaubert *et al.* 2004; (23) Souza *et al.* 2011; (24) Semblat *et al.* 2001; (25) Xue *et al.* 2013

...To secretory organs

Many studies have focused on the oesophageal cells as a target for the identification of expressed genes encoding secretory proteins (Davis *et al.*, 2004). Micro-aspiration of the cytoplasm of oesophageal gland cells from parasitic-stage nematodes has been used to generate cDNA libraries for gland cell-expressed genes. This approach has been shown to be useful for identifying candidate parasitism genes (Huang *et al.*, 2003; Davis *et al.*, 2004, 2008). Extensive expressed sequence tag (EST) analyses of gland cell libraries from *M. incognita*, together with secretion signal peptide prediction and high-throughput ISH on 185 cDNAs led to the identification of 37 unique clones encoding parasitism effectors and specifically hybridising to transcripts accumulating within the subventral (13 clones) or dorsal (24 clones) oesophageal gland cells of *M. incognita* (Huang *et al.*, 2003). Interestingly, 73% of the predicted proteins were previously unknown. Those with similarities to known proteins included a pectate lyase, acid phosphatase, and hypothetical proteins similar to proteins from other organisms (Table 1). In addition, a cDNA library constructed from the oesophageal gland region of *M. javanica* was used to identify genes differentially expressed in the oesophageal glands, for the characterisation of a potentially secreted chorismate mutase (CM) and pectate lyase (Lambert *et al.*, 1999; Doyle & Lambert, 2002) (Table 1). More recently mRNA extracted from microaspirated gland cells has been analysed by a next-generation sequencing (NGS) approach using Roche 454 technology (Rutter *et al.* 2014). By combining *in silico* analysis and ISH on 91 cDNA, Rutter *et al.* (2014) identified 17 *M. incognita* genes encoding putative effector proteins that are expressed specifically in the oesophageal gland cells. Most of these candidate effectors are pioneers with no significant sequence similarity to any proteins in the databases (Table 1). Finally, a new technique has been developed for the separation and isolation of individual oesophageal gland cells from multiple species of plant-parasitic nematodes (Maier *et al.*, 2013). The isolated gland cells can then be used for transcriptomic analyses by NGS. This technique has been successfully used in several nematode species and will facilitate the identification of effectors not only from RKNs, but also from nematodes with different modes of parasitism.

Differential gene expression

Genes differentially expressed between specific stages in the nematode life cycle, and particularly those differentially expressed between the preparasitic exophytic stage J2 and the endoparasitic stages (J3, J4, female), have been characterised by various transcriptomic strategies, including the global analysis of gene expression based on the ESTs generated from nematodes, RNA fingerprinting, cDNA-AFLP (amplification fragment length polymorphism), suppression subtractive hybridisation (SSH)-based strategies or real time Q-PCR analysis (Ding *et al.*, 1998; Dautova *et al.*, 2001; Neveu *et al.*, 2002; Tytgat *et al.*, 2005; Jaouannet *et al.*, 2012). A cDNA encoding a secretory cellulose-binding protein (CBP-1) was cloned from *M. incognita* by RNA fingerprinting (Ding *et al.*, 1998). The cDNA-AFLP method was used to identify genes differentially expressed between two pairs of near-isogenic *M. incognita* lines (NILs) or between two *M. javanica* strains, one avirulent and the other virulent against the tomato *Mi-1.2* resistance gene (Gleason & Williamson, 1999; Neveu *et al.*, 2002; Gleason *et al.*, 2008). Gene expression profiles were compared for the infective *M. incognita* J2s, and 22 of the 24,025 transcript-derived fragments (TDF) generated were found to display differential expression (i.e., present in both avirulent NILs and absent from both virulent NILs or vice versa). Fourteen of the TDF sequences displayed no significant similarity to known proteins, whereas eight matched reported sequences from nematodes and other invertebrates. ISH on five of the sequences showed that two were specifically expressed in the intestinal cells (HM10), one in the subventral oesophageal glands (HM1), and two in the dorsal oesophageal gland of J2s (HM7 and HM12) (Neveu *et al.*, 2002). The *Cg-1* cDNA fragment, which was present in the avirulent *M. javanica* strain but not in the virulent strain, was found to encode a small nematode protein required for *Mi-1.2*-mediated resistance (Gleason *et al.*, 2008). Genes upregulated during the endophytic stage were isolated by SSH (Huang *et al.*, 2004; Dubreuil *et al.*, 2007). Upregulation was demonstrated for genes involved in detoxification (e.g. glutathione S-transferase *GST-1*) and protein degradation, for a gene encoding a putative secreted protein and for genes of unknown function. Secreted GSTs may protect the parasite against reactive oxygen species or modulate the plant responses triggered by pathogen attack (Dubreuil *et al.*, 2007). Using RT-Q-PCR, Rutter *et al.* (2014) showed transcripts of newly discovered putative effectors to be specifically up-regulated during different stages of the nematode's life cycle, indicating that they function at specific stages during *M. incognita* parasitism.

Genome and secretome mining

The genomes of RKNs were first compared by the AFLP fingerprinting of three pairs of *M. incognita* NILs, for the identification of genetic markers displaying differential expression between nematode genotypes avirulent or virulent against the tomato *Mi-1.2* resistance gene. For the avirulent genotypes, a cDNA encoding a secretory protein with a sequence characterised by internal repeat motifs, named MAP-1, was cloned (Semblat et al., 2001). In 2008, the sequencing of the complete genomes of two RKN species, *M. incognita* and *M. hapla*, provided new opportunities for studying plant-nematode interactions and initiating comparative genomics studies (Abad et al., 2008; Opperman et al., 2008; Bird et al., 2009; Danchin et al., 2013).

The genome size of *M. hapla*, a RKN species with sexual reproduction, is only 54 Mb, the smallest nematode genome to have been sequenced (Opperman et al., 2008). The assembled genome sequence of *M. incognita*, with a size of 86 Mb, revealed a more complex structure with homologous but divergent segment pairs potentially derived from former alleles in this species (Abad et al., 2008). In total, 19,212 and 14,420 protein-coding genes were identified in *M. incognita* and *M. hapla*, respectively. InterPro protein domains were identified in 55% of *M. incognita* proteins and 22% of these proteins were predicted to be secreted. Interestingly, 2,578 secreted proteins without known domains were predicted to be specific to *M. incognita*. *M. incognita* has an unprecedented set of 61 plant cell wall-degrading, carbohydrate-active enzymes (CAZymes), including 21 cellulases, six xylanases, two polygalacturonases and 30 pectate lyases (Abad et al., 2008). Striking similarities between RKN and bacterial proteins led to the discovery that these cell wall-degrading or -modifying enzymes, which are generally absent from other metazoans, were acquired by multiple independent lateral gene transfers from different bacterial sources (Danchin et al. 2010). In addition, four chorismate mutases, 20 cysteine proteases of the C48 SUMO (small ubiquitin-like modifier) deconjugating enzyme family were shown to be specifically present or more abundant in *M. incognita* genomes than in those of free-living nematodes. Twenty-seven previously described *M. incognita*-restricted pioneer genes expressed in oesophageal glands were retrieved from the genome and a further 11 copies were identified; all of these genes are specific to *Meloidogyne* spp. (Abad et al., 2008).

The increasing availability of full-genome sequences for nematodes and of NGS transcriptomes and functional information databases, such as that of the Uniprot project, has

made it possible to use genome mining as an approach for the identification of effectors. This bioinformatic analysis makes use of several filter steps, in which genes that do not correspond to predefined criteria, such as the presence of orthologues in non-pathogenic species or the presence of transmembrane domains, are eliminated (or vice versa). A method for the identification of discriminant motifs in biological sequences has been used to define a set of motifs specifically present in known secreted effectors but absent from evolutionarily conserved housekeeping proteins (Vens *et al.*, 2011). The proteome of *M. incognita* has been shown to include 2579 proteins containing specific MERCI (motif—emerging and with classes—identification) motifs, which can be considered to be new putative effectors. Comparative genomics studies led to the identification of a set of genes in RKN genomes that were conserved during the evolution of plant-parasitic nematodes and are only found in organisms parasitic on plants (Danchin *et al.*, 2013). A combination of genome mining and protein function analysis identified large panels of putative effector-like proteins, which generally present an N-terminal signal peptide and no transmembrane domain (Roze *et al.*, 2008; Haegeman *et al.*, 2013; Danchin *et al.*, 2013). In addition, bioinformatic analyses of predicted effectors revealed the presence of nuclear localisation signals (NLS) in several RKN secreted proteins (Huang *et al.*, 2003; Roze *et al.*, 2008; Quentin *et al.*, 2013), suggesting that these molecules may target the host cell nucleus.

None of these approaches, including genome/secretome mining, can identify the complete set of effectors when used alone. Effectors not satisfying the predefined criteria may be missed (Mitchum *et al.*, 2013). For example, several effector candidates without a detectable signal peptide have been reported to be secreted but are not picked up by current genome mining strategies due to the lack of this peptide. Indeed, although most studies focus on protein effectors, non-protein molecules, such as plant hormones and carbohydrate effectors, have also been reported to play an important role in parasitism (McCarter *et al.*, 2003). Nevertheless, the characterisation of genes specifically expressed in secretory organs is a first step towards understanding their function in plant-RKN interactions. If a gene is identified as “effector-like” by various approaches, extensive analyses are required, together with a detailed study of its pattern of expression in cells, to confirm its role as a key parasitism effector and for the accurate dissection of gene function during disease development.

5.2. Functional analysis of effectors

The repertoire of putative RKN effectors appears to be large: about a hundred RKN proteins can be localised within secretory organs by ISH and/or immunolocalisation (Table 1) and Bellaifiore et al. (2008) have suggested that the *M. incognita* secretome contains 468 proteins. The expression of parasitism genes appears to be regulated developmentally during the parasitic cycle. However, a precise function in parasitism has been attributed to only a very small number of secreted proteins. The contribution to parasitism of some of the effectors identified is easy to deduce. Indeed, cell wall-degrading enzymes have been shown to play a role in nematode penetration and intercellular migration and in the cell wall expansion and thickening associated with giant cell formation (Davis *et al.*, 2011). It has also been suggested that nematode chorismate mutases, which act on the plant shikimate pathway, thereby decreasing the synthesis of salicylic acid and phytoalexins through competition with chorismate, may prevent the triggering of host defences (Doyle and Lambert, 2003). However, most candidate effectors have no clearly identified function or display no sequence similarity to genes in databases. Detailed functional analyses are therefore required to elucidate their role in parasitism. We will review here the approaches that have been (and are) used to elucidate the function of RKN effectors, particularly “pioneers”.

Effector localisation

Identification of the plant cell compartments for which nematode secreted proteins are destined will provide compelling evidence about the molecular functions of these proteins in parasitism. Only a few RKN effectors have been shown to be secreted into host tissues and, for most, it remains unclear in which compartment of the plant cell they act during parasitism.

The expression *in planta* of RKN fusion proteins tagged with fluorescent proteins e.g. GFP has made it possible to localise effectors without signal peptides within the cytoplasmic and/or nuclear compartments of plant cells (Figure 15), leading to speculations about the functions of RKN effectors (Jaubert *et al.*, 2004; Jaouannet *et al.*, 2012; Lin *et al.*, 2013; Jaouannet & Rosso, 2013). The transient expression using infiltration of *Agrobacterium tumefaciens* into leaves represents a fast and convenient strategy but do not allow the localisation of RKN effectors during infection. However, plants stably transformed with translational fusions have not yet been used to localise effectors within GCs during plant-RKN interactions.

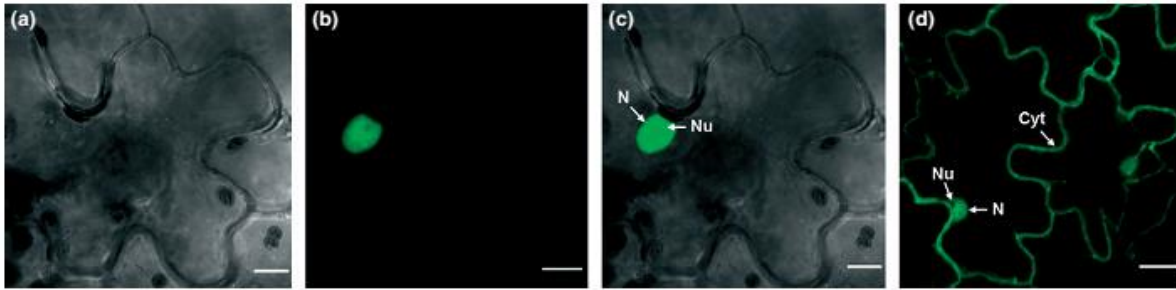


Figure 15: Targeting of Mi-EFF1 to the nucleus of agroinfiltrated tobacco cell (Jaouannet *et al.*, 2012)

Single-plane confocal images of tobacco epidermal leaf cells infiltrated with Agrobacterium tumefaciens and expressing Mi-EFF1 fused to a green fluorescent protein (GFP) reporter gene. (a–c) The fusion protein Mi-EFF1–GFP accumulated in the nucleus. The merged image (c) shows the overlay of bright field projection (a) and GFP signal (b). The fusion protein Mi-EFF1–GFP with a mutated version of the nuclear localization signal (PLAAGAE) was localized in the cytoplasm and the nucleus of agroinfiltrated cells, probably as a result of a passive diffusion. N, nucleus; Nu, nucleolus; Cyt, cytoplasm. Bars, 10 μm (a–c); 20 μm (d).

Immunolocalisation techniques have provided the most reliable data concerning the distribution of effectors within host cells. Mi-CRT was the first molecule shown to be secreted into the feeding site via the nematode stylet and to accumulate in large amounts at the cell wall of GCs (Figure 16) (Jaubert *et al.*, 2005). The apoplasm was subsequently confirmed as an important destination compartment for nematode secreted proteins during migration and feeding cell formation in the host plant (Rosso *et al.*, 2011; Vieira *et al.*, 2011). Three putative effectors secreted by the subventral glands — a pectate lyase (Mi-PEL3), an aspartyl protease (Mi-ASP2) and the 6D4 protein of unknown function — and the amphidial protein Mi-MAP1 were shown to be secreted into the apoplasm during the intercellular migration of infective juveniles. Mi-6D4 and Mi-MAP1 have also been detected in the apoplasm at the feeding site of sedentary parasitic juveniles, indicating a possible role for these secreted proteins in giant cell formation and/or maintenance (Figure 16) (Vieira *et al.*, 2011).

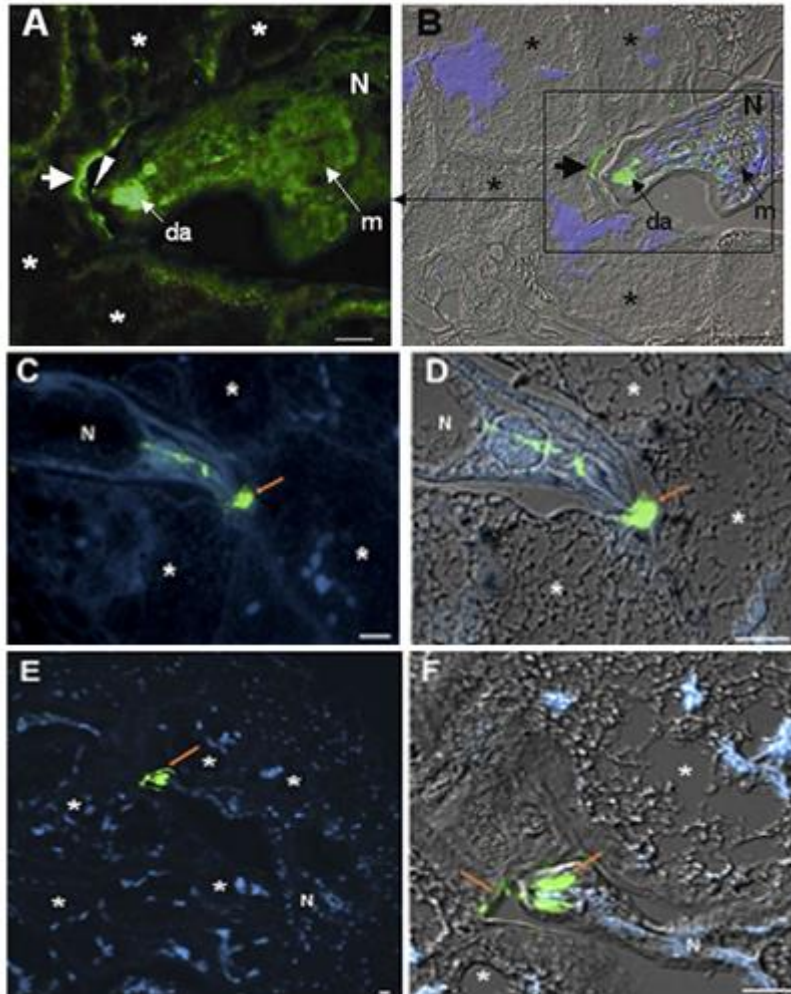


Figure 16: Localization of *M. incognita* effector at the nematode feeding site in Arabidopsis galls (Jaubert et al. 2005; Vieira et al., 2011)

(A) and (B), Mi-CRT localization in parasitic *M. incognita* juvenile (J4) and at the cell wall of the giant cells in sectioned Arabidopsis gall (5 μm) 21 days after inoculation (DAI). The Mi-CRT protein is observed in the DG ampulla (da), at the tip of the stylet (arrowhead), and along the cell wall of adjacent giant cells (arrow). A Observations with an fluorescein isothiocyanate (FITC) filter; B, overlays of FITC, differential interference contrast, and di-aminido phenyl indol-stained nuclei images; m, metacarpus; N, nematode. Giant cells are indicated by asterisks. Scale bars = 10 μm . (C) and (D), gall containing a sedentary nematode displaying 6D04 secreted and accumulated at the head of the nematodes and along the GC wall (arrow) within the apoplasm (21 dai). (E) and (F), gall containing a sedentary nematode, showing the significant accumulation of MAP-1 in the amphids of the nematode and along the GC wall (arrow) within the apoplasm (21 dai).

It has recently been shown that three RKN effectors are injected into the cytoplasm for the GCs, and that these proteins then target the nuclei. Jaouannet et al. (2012) carried out immunolocalisation experiments on infected tomato roots and confirmed the secretion *in planta* of Mi-EFF1, a 122-amino acid protein with a signal peptide for secretion and an NLS. Despite the detection of Mi-EFF1 in the DG of migratory juveniles, no signal was observed along the

migratory path of the nematode, suggesting that this protein is not secreted during migration. By contrast, Mi-EFF1 was secreted via the stylet, by sedentary nematodes settled at the feeding site. The secreted Mi-EFF1 was located in the nuclei of GCs (Figure 17). MiEFF1 seems to be specific to early steps in the plant-nematode interaction, but its function during parasitism remains to be determined. Similarly, the *M. javanica* Mj-NULG1a protein is a 274-amino acid pioneer protein with a signal peptide for secretion and two NLS motifs. This effector is produced in the DG of the nematode and is injected into the cytoplasm of the GCs (Figure 17C). It subsequently accumulates within the nuclei, as demonstrated by immunocytochemical studies of infected tomato roots (Lin *et al.*, 2013). Recently, *M. incognita* effector 7H08 was reported to localize in the nuclei of plant cells and contained two independent nucleus-localisation domains (NLDs). This protein was found to activate expression of the reporter genes in both yeast and plant system. So far, 7H08 is the first reported PPN effector with transcriptional activation activity (Zhang *et al.*, 2015). Giant-cell ontogenesis and maintenance requires the manipulation of host nuclear processes, such as the cell cycle and transcriptional regulation, and undoubtedly involves the targeting of the host nucleus by secreted effectors. It is also clear that RKN effectors must target various nuclear processes to corrupt plant cell fate and immunity in ways similar to those observed for other plant pathogens e.g. bacteria, oomycetes, fungi and virus (Rivas & Genin, 2011; Canonne & Rivas, 2012; Deslandes & Rivas, 2012).

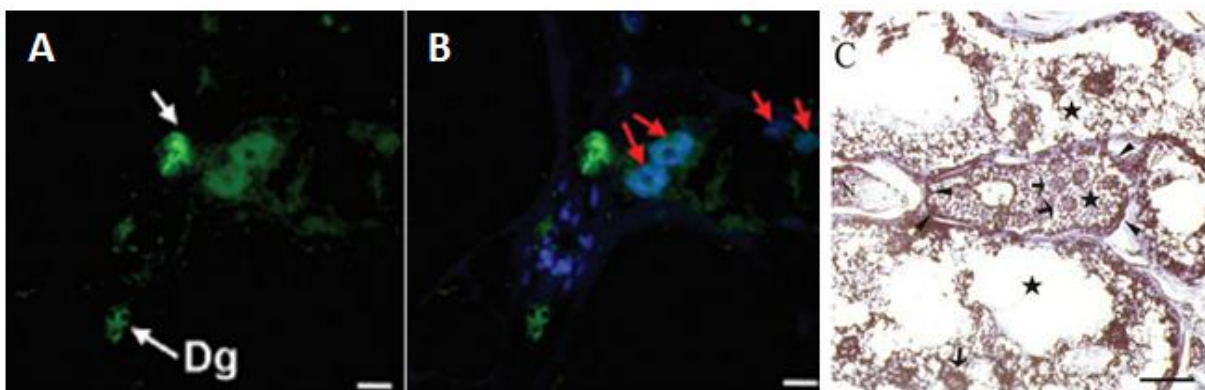


Figure 17: Two RKN effectors are injected into the GCs and target the nuclei (Jaouannet *et al.*, 2012; Lin *et al.*, 2013)

(A) and (B), Localization of the secreted Mi-EFF1 at the tip of the stylet of the sedentary parasitic juveniles (white arrow) in a young gall (14 dai) and in the nuclei of GCs (red arrow). A signal was also observed in the DG. Micrograph (A) is observation of Fluorescein Isothiocyanate (FITC)-conjugated secondary antibody. Micrograph (B) is superposition of FITC-conjugated secondary antibody and DAPI-stained nuclei; (C), Gall containing a fourth-stage juvenile (J4) at 18 dpi, showing the MjNULG1a protein at the cell wall of adjacent GCs (arrowhead) and GC nuclei (arrow). Dg, dorsal gland. Asterisks, GCs. Scale bars: 20 μm (A-B); 10 μm (C).

RNA interference-mediated gene silencing

It is not currently possible to transform RKNs, but RNA interference (RNAi) approaches have been developed, for studies of the role of candidate effectors (Rosso *et al.*, 2009). In RNAi approaches, the small interfering RNA (siRNA) is generated from a double-stranded RNA (dsRNA) by Dicer-mediated cleavage and is processed to yield an RNA-induced silencing complex (RISC) that drives the degradation of the targeted transcript (Fire *et al.*, 1998; Tabara *et al.*, 2002). The dsRNA or siRNA inactivating the effector genes can be delivered to nematodes by the *in vitro* “soaking” of pre-parasitic J2s in exogenous dsRNA or siRNA molecules or *in planta*, by transgenic methods involving the expression of hairpin RNA (hpRNA) or through host/virus induced gene silencing (HIGS/VIGS) (Figure 18) (Rosso *et al.* 2009). Correlations between candidate effector gene silencing and the reduced parasitic success of the nematode suggest that secreted effectors do indeed play a key role in parasitism.

The silencing of a glutathione-S-transferase, Mi-GST-1, and of a cellulose-binding protein, Mj-CBP-1, has been achieved by soaking parasites in a solution of long dsRNA molecules homologous to the targeted transcripts of the genes to be silenced (400 and 255 nt, respectively), and was shown to lead to lower levels of parasitic success. The targeting of Mi-GST-1 with dsRNA led to a 90% decrease in *gst* transcript abundance in the treated nematodes and decreased the ability of the nematode to develop and reproduce on tomato plants infected with the treated parasitic juveniles (Dubreuil *et al.*, 2007), whereas the silencing of Mj-CBP-1 decreased the penetration success of *M. javanica* (Adam *et al.*, 2008). Mj-CBP-1 silencing, however appeared rather heterogeneous between treated nematode lines (Adam *et al.*, 2008), and such drawback, that may depend on the targeted gene, could limit the use of RNAi through soaking. However, impressive results were obtained with a synthetic small interfering RNA (siRNA) only 21 nucleotides in length. With this siRNA, Arguel *et al.* (2012) validated this strategy by silencing Mi-CRT in nematode oesophageal glands, thereby decreasing nematode virulence. *In vitro* RKN gene silencing by siRNA soaking has since been successfully used to demonstrate the function in parasitism of new RKN target genes including an effector expressed in *M. incognita* oesophageal glands encoding a C-type lectin (Danchin *et al.*, 2013). C-type lectins appear to be secreted by various plant parasitic nematodes, including cyst nematodes and RKNs, and may be involved in modulating plant immune responses (Urwin *et al.*, 2002; Roze *et al.*, 2008; Ji *et al.*, 2013; Danchin *et al.*, 2013; Ganji *et al.*, 2014).

Alternatively, siRNA can be delivered to nematodes by feeding on transgenic plants expressing a dsRNA (usually a hpRNA). This technology was developed in the model plant *Arabidopsis* and used to demonstrate the function of five candidate effectors (16D10, 8D05, Mi-CRT, fatty acid- and retinol-binding Mj-FAR-1 and Mj-NULG1a) in parasitism (Huang *et al.*, 2006a; Xue *et al.*, 2013; Iberkleid *et al.*, 2013; Lin *et al.*, 2013; Jaouannet & Rosso, 2013). *M. incognita* 16D10 is a small peptide displaying sequence similarity to the proteins of the plant CLE protein family. The *in vivo* expression of 16D10 dsRNA in *Arabidopsis* resulted in resistance effective against the four major RKN species, *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla*, suggesting that this effector is conserved among RKNs (Huang *et al.*, 2006b). Indeed, *M. chitwoodi* has also been shown to secrete a 16D10-like protein (Dinh *et al.*, 2014). The *in planta* silencing of 16D10 could also be applied to crop plants such as grapes and potato, and this approach may provide interesting opportunities to provide crops with broad resistance to RKNs (Yang *et al.*, 2013; Dinh *et al.*, 2014).

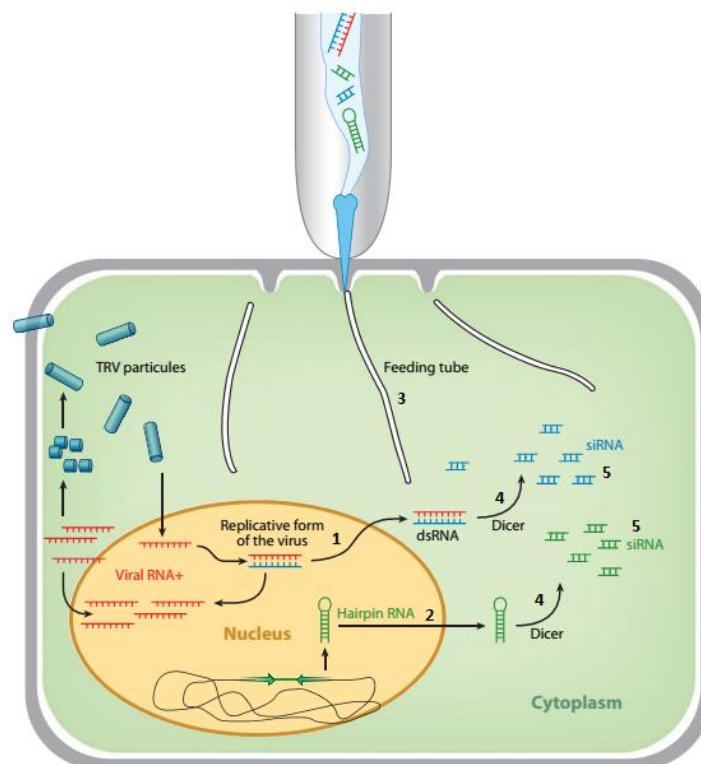


Figure 18: Schematic diagram of plant mediated RNAi in plant parasitic nematodes

Double-stranded RNA (dsRNA) molecules are produced in the cytoplasm of plant cells as the replicative form of a positive strand RNA virus (1) or as hairpin RNA (2) produced by transgenic plants. The dsRNA may be ingested by the feeding nematodes through the feeding tubes (3). Alternatively, dsRNA molecules are processed by the plant RNAi machinery (4) and siRNA are ingested (5). Abbreviation: TRV, Tobacco Rattle Virus (adapted from Rosso *et al.* 2009)

Interestingly, Souza Junior et al. (2013) showed that this technology could be used to silence three *M. incognita* proteases simultaneously, with the use of transgenic RNAi tobacco lines. This ability to silence several parasitic genes simultaneously renders this strategy promising for the study of effector functions and the development of new tools for nematode control. Again, efficiency of *in planta* RNAi may vary considerably, depending on level and pattern of target gene expression, size and sequence composition of the dsRNA segment and its position in the target gene. Silencing of a targeted RKN gene may not be achieved similarly in every generated transgenic line, and questions remain regarding the stability and inheritance of this resistance (Gheysen & Vanholme, 2007; Rosso et al., 2009). Viruses have also been used to deliver dsRNA fragments within the host plant, and appeared effective for delivery of RNAi triggers to feeding nematodes. Tobacco rattle virus-mediated silencing of Mi-CRT was correlated with the decreased ability of the nematode to induce disease (Dubreuil et al., 2009). Nevertheless, a high heterogeneity in virus propagation in root tissues has been observed which is limiting for the use of VIGS for functional screens (Dubreuil et al., 2009; Rosso et al., 2009). Finally, tools developed for the expression of artificial microRNA *in planta* (Schwab et al., 2006; Carbonell et al., 2014) will undoubtedly prove useful for the development of new types of resistance to nematodes based on effector silencing in the near future.

***In planta* effector overexpression**

In addition to RNAi analyses, *in planta* gene overexpression approaches have also been used to attribute actions in both plant cell physiology and pathogenicity to effectors, according to the phenotypes observed on the transgenic plants. Feeding cell ontogenesis involves impressive effects of parasitism proteins on root cell physiology, and the *in planta* overexpression of RKN effectors can result in major changes to plant phenotype. The inhibition of vascularisation and lateral root initiation induced by Mj-CM-1 in soybean hairy roots provides the first clearest example of an oesophageal gland-specific protein altering plant development (Doyle & Lambert, 2003). The phenotype of Mj-CM-1-overexpressing plants can be rescued by adding indole-2-acetic acid (IAA). Doyle and Lambert (2003) hypothesised that RKN chorismate mutase is active in the cytoplasm, where it alters the shikimate pathway to prevent auxin formation. Mj-CM-1 thus has the potential to act as a multifunctional enzyme in the promotion of nematode pathogenicity. Mj-CM-1 can assist in the developmental reprogramming required to generate GCs, by interfering with the auxin signalling pathway and/or inhibiting the production of plant

defence compounds, as discussed above. The overexpression of 16D10 in both tomato and *Arabidopsis* results in greater root growth and the extensive production of lateral roots; it also induces the formation of callus on tomato roots (Huang *et al.*, 2006b), demonstrating a strong ability of this effector to alter plant development. The constitutive expression of 8D05 in *Arabidopsis* plants greatly increases shoot growth and early flowering, but has no effect on root growth. Susceptibility to *M. incognita* infection was found to be greater in all lines overexpressing Mi8D05 than in wild-type *Arabidopsis*, leading to the conclusion that this protein plays a key role in parasitism (Xue *et al.*, 2013). Lin *et al.* (2013) demonstrated the key role of Mj-NULG1a in parasitism in a similar manner. They showed a clear effect of transgene expression on susceptibility to nematode attack, with larger numbers of nematodes in the infected *Arabidopsis* roots and larger numbers of galls formed when Mj-NULG1a was overexpressed. By contrast, Mj-NULG1a had no apparent effect on the growth of the transgenic plants (Lin *et al.*, 2013). Tomato roots constitutively expressing Mj-FAR-1 were more susceptible to RKN infection and displayed faster gall induction and expansion, with a higher percentage of the nematodes developing into mature females than observed in control roots. Further histological analysis of the infected MJ-FAR-1-overexpressing plants indicated that the galls contained larger feeding cells potentially able to support faster nematode development and maturation. Nevertheless, a phenotypic analysis of MJ-FAR-1-overexpressing root lines revealed no significant change in root development and growth (Iberkleid *et al.*, 2013). An analysis of defence-related target gene expression in the Mj-FAR-1-overexpressing plants led the authors to conclude that this effector might facilitate infection by manipulating host lipid-based defences (Iberkleid *et al.* 2013). The putative effector 7E12, expressed in the *M. incognita* DG, has also been shown to disrupt host root physiology to favour parasitism (Souza *et al.*, 2011). Gall formation and egg hatching occur more rapidly in 7E12-overexpressing tobacco plants than in wild-type tobacco plants. The morphology of the GCs was shown to be affected, with larger numbers of vacuoles and wall ingrowths, and the apparent proliferation of neighbouring cells. Souza *et al.* (2011) suggested that 7E12 may act by regulating host cell division. Finally, Jaouannet *et al.* (2013) recently showed that Mi-CRT overexpression increases susceptibility to *M. incognita* in *Arabidopsis*, probably by interfering with the triggering of defence responses.

Defence suppression assays

In general, the first line of plant defence against the invading nematode is triggered by damage-associated molecular patterns (DAMPs) or conserved pathogen-associated molecular patterns (PAMPs). These patterns induce various defence mechanisms referred to as “basal resistance” or “PAMP-triggered immunity” (PTI). Plants can also recognise specific pathogen-derived effectors and have acquired a highly specific defence response known as effector-triggered immunity (ETI). Together, PTI and ETI limit the entry of microbes, restrict pathogen propagation or kill pathogens within the host plant. These immune signalling pathways are common targets of many plant pathogen effectors (Jones & Dangl, 2006; Dou & Zhou, 2012). Several assays have been developed and used to demonstrate the functions of bacterial, fungal and oomycete effectors in defence suppression. However, such approaches were not applied to RKNs until very recently. Using *Arabidopsis* lines overexpressing of Mi-CRT and MiMsp40, Jaouannet et al. (2013) and Niu et al. 2016 demonstrated that these effectors were able to suppress the defences induced by the PAMP elf18 (N-terminal 18 amino acids of elongation factor Tu), which usually triggers callose deposition and the expression of defence marker genes of salicylic acid, jasmonate and ethylene pathways. Interestingly, co-agroinfiltration assay indicated that MiMsp40 also suppressed macroscopic cell death triggered by MAPK cascades or by the ETI cognate elicitors R3a/Avr3a, suggested the role of MiMsp40 as novel nematode-secreted ETI suppressor (Niu *et al.*, 2016). Venom allergen-like protein (VAP) effectors have been identified in many PPNs. The modulation of basal immunity by ectopic VAPs in *A. thaliana* involved extracellular protease-based host defense and non-photochemical quenching in chloroplast. Non-photochemical quenching regulates the initiation of the defense-related programmed cell death, that was suppressed by VAP-1 in *G. rostochiensis*, *H. schachtii* and *M. incognita* (Lozano-Torres *et al.*, 2014). Moreover, translationally controlled tumour protein (TCTP) in *M. enterolobii*, MeTCTP, was reported to be able to suppress programmed-cell death triggered by the pro-apoptotic protein BAX. Silencing of MeTCTP by *in planta* RNAi resulted in an attenuation of parasitism, suggested the role of MeTCTP as effector by suppressing programmed cell death in host plants (Zhuo *et al.*, 2016). Furthermore, VIGS approach showed the potential role of Misp12 in the manipulation of SA signalling pathways in the root cell to support nematode parasitism at the later stages, and this gene was also able to suppress the defense genes from the JA pathways (Xie *et al.*, 2016). Recently, yeast two-hybrid (Y2H) was used to

identify the specially interaction between *M. javanica* transthyretin-like protein (MjTTL5) and Arabidopsis ferredoxin: thioredoxin reductase catalytic subunit (AtFTRc), that could drastically increase host reactive oxygen species-scavenging activity, and result in suppression of plant basal defenses and attenuation of host resistance (Lin *et al.*, 2016). This result revealed a novel mechanism of PPNs to subjugate plant immunity for parasitism.

Search for the host targets of effectors

RKN effectors must manipulate host cellular processes through specific interactions with certain host proteins, to favour parasitism. We currently know almost nothing about the host targets of RKN effectors. Only few RKN effectors have been identified to date, but Y2H screens have successfully been used for the identification of potential host targets (Huang *et al.* 2006b; Xue *et al.* 2013; Lin *et al.* 2015; Zhang *et al.* 2015; Quentin *et al.*, unpublished results). Studies with a tomato root cDNA library have shown that two SCARECROW-like (SCL) plant transcription factors are targeted by the *M. incognita* 16D10 effector (Huang *et al.*, 2006b). The 16D10 effector can also interact with *Arabidopsis SCL6 and SCL21* in yeast. These interactions have been confirmed in co-immunoprecipitation experiments. In plants, SCARECROW-like transcription factors regulate root meristem identity and root development. The identification of interactions with these proteins thus provided the first evidence that plant-parasitic nematode-secreted peptides may function as signalling molecules, inducing root proliferation by specifically targeting host proteins regulating transcription. However, the function of these transcriptions factors in the giant cell ontogenesis was not yet demonstrated. Recently, a Y2H screen in which *M. incognita* 8D05 was used as bait revealed the occurrence of multiple interactions with the plant aquaporin TIP2 (tonoplast-intrinsic protein 2) in tomato (Xue *et al.*, 2013). This interaction suggests a potential role for the 8D05 effector protein in regulating water and solute transport within GCs, promoting their enlargement and nematode feeding. A similar Y2H was applied and found the interaction between MjTTL5 and Arabidopsis ferredoxin: thioredoxin reductase catalytic subunit (described above in “defense suppression assays” part) (Lin *et al.*, 2016). These results demonstrate the efficiency of Y2H approach to identity nematode effector’s host targets.

6. Transcriptomic approach to identify nematode effectors

From the appearance of next generation sequencing (NGS) over ten years ago, whole transcriptome RNA-sequencing (RNA-seq) is becoming increasingly attractive as a new approach for quantitative studies of differential gene expression in all species. This method provides unprecedented access to sequence and expression variation in the transcriptome and allows for additional insights into alternative splicing, cis vs. trans gene regulation or small non-coding RNA dynamics of interested species, changing the focus from individual genes to gene networks. RNA-seq studies are more challenging in non-model species than in model organisms. These challenges are at least in part associated with the lack of quality genome assemblies for some non-model species and the absence of genome assemblies for others (Hekman *et al.*, 2015). Nevertheless, there are more and more studies focused on PPN using RNA-seq approach (Haegeman *et al.*, 2013; Danchin *et al.*, 2013; Eves-van den Akker *et al.*, 2014; Bauters *et al.*, 2014; Rutter *et al.*, 2014; Zheng *et al.*, 2015; Fosu-Nyarko *et al.*, 2016; Petitot *et al.*, 2016; Pogorelko *et al.*, 2016).

Using Illumina sequencing, Zheng *et al.* 2015 performed the analysis on transcriptomes of early parasitic second-stage juveniles (30 hours, 3 days and 9 days post infection) of the cereal cyst nematode *Heterodera avenae* in the host *Aegilops variabilis*. Among all assembled unigenes, 681 putative genes of parasitic nematode were found, in which 56 putative effectors were identified, including novel pioneer genes and genes corresponding to previously reported effectors. Moreover, by comparing the differentially expressed gene between the pre-parasitic and the early parasitic larvae, the hydrolase activity was reported to be over-expressed in the pre J2s whereas binding activity was upregulated in infective J2s, suggesting the possible secretion of proteins and their putative role in infection (Zheng *et al.*, 2015). The 454 sequencing was carried out for the transcriptome of the rice root nematode *Hirschmanniella oryzae* on mix stages of population. By screening the data for the putative plant cell wall-modifying proteins, which facilitate nematode migration through host root, and for putative effector proteins that may alter the host defence mechanism, a β -mannanase, not previously reported, and two enzymes, chorismate mutase and isochorismatase, thought to be involved in the salicylic acid pathway, were identified (Bauters *et al.*, 2014). So far, transcriptomic approach represent also interesting data set to study the evolution of nematodes. Eves-van den Akker *et al.* 2014 presented the first large-scale genetic resource of the “false RKN” *Nacobbus aberrans*.

Comparing parasitism genes of typical RKNs and CNs to those of *N. aberrans* has revealed interesting similarities. Importantly, genes that were believed to be either CN- or RKN- “specific” have both been identified in *N. aberrans*. This result has revealed insights into the evolution, phylogenetic history, and biology of biotrophic plant-nematode interactions (Eves-van den Akker *et al.*, 2014).

Recently, an important number of new *Meloidogyne* spp. effectors has been described (Rutter *et al.*, 2014; Petitot *et al.*, 2016). *De novo* transcriptome of *M. graminicola* samples enabled the identification of 15 putative effector genes, including two homologues of well-characterized effectors from CN (CLE-like and VAP1) and a metallothionein (Petitot *et al.*, 2016). Otherwise, from 17,741 isotigs and 72,397 singletons of isolated gland-cell-specific mRNA of *M. incognita*, Rutter *et al.* 2014 identified 91 *M. incognita* secreted candidate effector proteins. Among them, 18 were detected to be expressed in the salivary glands of the nematode. Moreover, 11 genes produced a cytoplasmic YFP signal when expressed into onion epidermal cells (Rutter *et al.*, 2014). Interestingly, most of these proteins are unique for *M. incognita*, showing no homology to proteins in the non-redundant database and containing no detectable functional domains. This unique nature of effector proteins often makes it difficult to predict their functions or their targets in the host.

So far, Illumina sequencing has been carried out for the transcriptome of *M. incognita* in all stages of its life cycle (Danchin *et al.*, 2013; Perfus-Barbeoch and Danchin, unpublished data). RNA-seq reads were detected for almost all known putative effectors of *M. incognita* (Table 1). These results opened a new method to identify nematode effectors based on RNA-seq transcriptomes, in which we could focus on the putative candidates specific for each stage of the parasitism.

7. Objectives

My PhD project is dedicated to the analysis of the transcriptomic regulation in RKNs and is composed of two parts:

- 1) Analyse the transcriptome of *M. incognita* during the plant infection, focusing on early parasitic stages.** In this part, based on the available RNAseq dataset in our laboratory, I will identify the genes that are over-expressed in parasitic J3-J4 stage, and then used a pipeline filtering to obtain a list of candidate effector proteins. Once the over-expression of those genes in parasitic stage will be confirmed by RT-qPCR, *in situ* hybridisation test will be carried out to localize the candidates in the nematode secretion organs. Furthermore, siRNA soaking will be used to study the infection capacity of nematode when the effectors were altered and demonstrate the key role of these effectors in the pathogeny.
- 2) Compare the transcriptomes between *M. incognita* and *M. enterolobii*.** This part aims to answer why *M. enterolobii* is not controlled by the classical plant resistance genes, e.g. *Mi* and *Me*, and therefore to find an efficiency method to control this pest. Pre-parasitic J2s and J3-J4 parasitic *M. enterolobii* will be collected for sequencing by Illumina method. The transcriptome datasets of *M. enterolobii* will be compared with the ones of *M. incognita* in order to identify, not only the common feature, but also the specificity of each nematode.

Besides the effectors, other candidate genes that are over-expressed at parasitic stage could also play an important physiological role in the parasitism or the development of this nematode. The functional analysis of all these factors could increase our knowledge about the parasitism of these pests and represent a first step in the development of new control methods.

CHAPTER 1: Identification of Parasitism Effectors Expressed During Plant Infection from the Transcriptome of *Meloidogyne incognita*

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This chapter was submitted to New Phytologist on October 6th, 2016

Summary

- Root-knot nematodes, *Meloidogyne* spp., are obligate endoparasites that maintain a biotrophic relationship with their hosts. They infect roots as microscopic vermiform second-stage juveniles (J2), and migrate to reach the vascular cylinder. The J2s must then successfully establish and maintain specialised feeding structures called “giant-cells”, from which they withdraw water and nutrients, to enable them to develop and reproduce. The effector proteins secreted *in planta* are key elements in the molecular dialogue of parasitism.
- Here, we compared Illumina RNA-seq transcriptomes for *M. incognita* obtained at various points in the lifecycle, and identified 31 genes more strongly expressed in early parasitic stages than in preparasitic juveniles. We then selected candidate effectors for functional characterisation.
- RT-qPCR and *in situ* hybridisations showed that the validated differentially expressed genes are predominantly specifically expressed in oesophageal glands of the nematode. We also soaked the nematodes in siRNA to silence these genes and to determine their role in pathogenicity.
- The silencing of the dorsal gland specific-*Minc18876* and its paralogues resulted in a significant, reproducible decrease in the number of egg masses, demonstrating a potentially important role for the small cysteine-rich effector MiSCR1 it encodes in early stages of giant cell formation.

Key words: effector, giant cell, pathogenicity, RNA-seq, root-knot nematode, transcriptome.

Introduction

Root-knot nematodes (RKNs, *Meloidogyne* species) are among the most economically devastating plant pathogens in the world (Trudgill & Blok, 2001). Those with asexual modes of reproduction, such as *M. incognita*, *M. javanica*, and *M. arenaria*, are particularly damaging pests. These obligate sedentary endoparasitic nematodes complete their lifecycle in three to 10 weeks, depending on the RKN species and environmental conditions. Preparasitic RKNs infect roots as microscopic vermiform second-stage juveniles (J2s) that hatch from eggs in the soil. J2s penetrate the root apex and migrate between cells to reach the vascular cylinder of the plant. There, they establish and maintain specialised multinucleate feeding structures called giant cells, from which they withdraw water and nutrients to sustain their sedentary biotrophic lifestyle (Caillaud *et al.*, 2008b). After establishing feeding sites, the J2s become sedentary, developing through successive moults into 3rd- and 4th-stage juveniles (parasitic J3 and J4) and then into adults, to complete their life cycle. J2 males leave the roots, whereas the pear-shaped females generate offspring through parthenogenetic reproduction, and release hundreds of eggs onto the root surface in a protective gelatinous matrix, the egg mass.

Despite significant advances in recent years, the molecular mechanisms driving the interaction between plants and RKNs remain poorly understood. Like other plant pathogens, RKNs secrete effector proteins into the host plant, to modify host-cell structure and function for their own purposes (Hogenhout *et al.*, 2009; Mitchum *et al.*, 2013). Plant parasitic nematodes are characterised by hypertrophied salivary/oesophageal glands, one dorsal gland (DG) and two subventral glands (SvGs), connected to a hollow protrusive stylet (Hussey, 1989). RKN effectors are produced primarily in the DG and SvGs, and are released into the plant tissues via the stylet; they play an important role in RKN parasitism, from J2 migration to the formation and maintenance of giant cells (Davis *et al.*, 2004, 2008; Rosso *et al.*, 2011; Haegeman *et al.*, 2012; Hwezi & Baum, 2013; Truong *et al.*, 2015). Various strategies have been developed for identifying RKN effector candidates directly from the secretions of *M. incognita*. These approaches include gland microaspiration/dissection coupled with expressed sequence tag (EST) sequencing (Huang *et al.* 2003) and secretome analysis (Jaubert *et al.*, 2002b; Bellafiore *et al.*, 2008). Rutter *et al.* (2014) identified 91 candidate effector proteins secreted by *M. incognita*, by 454 sequencing of isolated gland cell-specific mRNA from *M. incognita*. Eighteen of the candidate genes were expressed in the salivary glands. Genes differentially expressed

between stages in the nematode life cycle or between virulent and avirulent strains have also been characterized by various strategies, including quantitative RT-PCR, cDNA-AFLP and suppression subtractive hybridisation (SSH) (Neveu *et al.*, 2003; Williamson & Gleason, 2003; Dubreuil *et al.*, 2007; Jaouannet *et al.*, 2012; Rutter *et al.*, 2014). Moreover, a combination of genomic data mining for two RKN species, *M. incognita* and *M. hapla* (Abad *et al.*, 2008; Opperman *et al.*, 2008), and protein function analysis identified a new panel of putative effector-like proteins in *M. incognita* (Danchin *et al.*, 2013). These approaches led to the identification of about 80 candidate genes involved in parasitism in *M. incognita*, for which expression in the glands was validated by *in situ* hybridisation (Davis *et al.*, 2008; Quentin *et al.*, 2013; Truong *et al.*, 2015).

The lack of transformation and genetic analysis, due to the asexual mode of reproduction, together with the obligate biotrophy of this species, make it difficult to validate the function of these putative effectors. However, a few effectors have been validated as essential for *M. incognita* parasitism, by RNA interference (RNAi), achieved by soaking the nematodes in siRNA solution or *in planta*: the glutathione-S-transferase Mi-GST1 (Dubreuil *et al.*, 2007), the CLE-like peptide 16D10 (Huang *et al.*, 2006b), the C-type lectin Minc03866 (Danchin *et al.*, 2013), the unknown proteins 8D05, MSP40 and MiSP12 (Xue *et al.*, 2013; Niu *et al.*, 2016; Xie *et al.*, 2016), and the calreticulin MiCRT (Jaouannet *et al.*, 2013). Several factors have been shown to be present in the apoplast *in planta* (Vieira *et al.*, 2011), but three RKN effectors have recently been shown to be injected into the giant cells. These three proteins, Mi-EFF1, MjNULG1 and 7H08, target the giant cell nuclei and constitute interesting candidates for giant cell reprogramming (Jaouannet *et al.*, 2012; Lin *et al.*, 2013; Zhang *et al.*, 2015).

Whole-transcriptome RNA-sequencing (RNA-seq) is an attractive approach for quantitative studies of differential gene expression in plant-pathogen interactions (Hekman *et al.*, 2015). The transcriptome of *M. incognita*, at all stages of its life cycle, has been sequenced with Illumina technology, to support the functional annotation of *M. incognita* genes (Danchin *et al.*, 2013). In this study, we used this RNAseq dataset to develop a pipeline for identifying new effector candidate genes overexpressed during the parasitic stages of the *M. incognita* lifecycle. The pattern of gene expression was validated by quantitative real-time PCR (RT-qPCR), and a functional analysis was then performed, coupling *in situ* hybridisation and gene silencing with siRNA, to determine the role of these effectors in plant infection. Our findings demonstrate a

key role for a new family of *M. incognita* effectors — including Minc18876, a 102-amino acid (aa) protein, and its paralogues — in RKN pathogenicity.

Materials and Methods

Sample preparation

Freshly hatched J2s and eggs of *M. incognita* were collected as previously described (Rosso *et al.*, 1999). Nematodes at parasitic stages J3-J4 were collected from tomato roots (*Solanum esculentum* cv. St Pierre) 14 days after inoculation, by incubation in 15% (v/v) Pectinex Ultra SP-L (Novozymes, Bagsvaerd, Denmark) and 7.5% cellulase from *Trichoderma reesei* ATCC 26921 (Sigma-Aldrich, USA) overnight. The samples were purified from root debris by filtering through sieves with 40 µm pores followed by manual collection under a binocular microscope.

Gene expression analysis

RNA-seq libraries were generated from seven stages of the lifecycle of *M. incognita* (Danchin *et al.*, 2013). Illumina RNA-seq reads were aligned with the *M. incognita* v1 genome with TopHat (version 2.0.7) software (Kim *et al.*, 2013). All transcripts from individual stages were merged with Cuffmerge, to obtain a final transcriptome with no redundancy. Gene expression patterns were deduced from the aligned reads with Cufflinks and are presented as FPKM values. Cuffdiff was then used to re-estimate transcript abundance. The analysis focused on genes with at least one statistically significant difference in expression levels between stages. *P*-values were estimated and subjected to Benjamini-Hochberg correction (*q*-value) (Benjamini & Hochberg, 1995; Trapnell *et al.*, 2010). The difference in expression was considered significant for *q*-values below 0.05. A graphic representation of differential expression was generated with CummeRbund (version 1.0.0) in R software.

Searching for effector-like proteins

Using 307 genes overexpressed at different stages of the parasite lifecycle, we searched for genes more strongly expressed at parasitic than at pre-parasitic stages and meeting the following criteria: FPKM > 50 in J3-J4 and in J2-J3-J4, and FPKM J2-J3-J4/J2 and J3-J4/J2 ratios > 10. The protein sequences encoded by the genes were then analysed with PHOBIUS (<http://phobius.sbc.su.se/>) and PSORT II (<http://psort.hgc.jp/form2.html>) software, for the

prediction of signal peptides, non-transmembrane domains, DNA-binding domains and NLS. BLASTp analyses were carried out with an e-value threshold of 0.01 and without low complexity against *M. hapla* or NCBI non-redundant protein database for homologue identification. BLASTp hits were considered as significant when identity > 50% and bit score > 100. Interproscan was performed on the candidate proteins to identify protein signature referenced in the InterPro database (Mitchell *et al.*, 2015).

Reverse transcription-quantitative PCR

Total RNA was extracted with TriZol (Invitrogen, Carlsbad, CA, USA) according to the Invitrogen protocol, and resuspended in 11 μ l nuclease-free water. Total RNA concentration was measured with a NanoDrop 2000 spectrophotometer (NanoDrop Products, Wilmington, DE). We subjected 1 μ g of total RNA to reverse transcription with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Marnes la Coquette, France). The primers for qPCR (Table S1) were designed with primer3 software (Rozen & Skaletsky, 2000) and synthesized by Eurogentec (Seraing, Belgium). The cDNA was diluted 1:50 and 5 μ l of the dilution was used for each qPCR reaction. We added 7.5 μ l SYBR Green MasterMix Plus (Eurogentec, Liege, Belgium), 0.2 μ l each of forward and reverse primer (from a 10 μ M solution), and 2.1 μ l water to the cDNA, to obtain a total volume of 15 μ l/reaction. Amplification and detection were performed in an Opticon 2 system (MJ Research, Bio-Rad). Thermocycling was performed as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s. The dissociation curve of the final products was examined to check that there was only one amplification product. We performed qPCR on duplicate samples of each cDNA from three independent biological replicates. *GAPDH* (*Minc12412*) and *HK14* (*Minc18753*) were used for the normalisation of RT-qPCR data. Quantifications were performed by the modified Δ Ct method (Livak & Schmittgen, 2001) in qBase1.3.5 software, and as the results are expressed as normalised relative quantities.

Localisation of the transcript by *in situ* hybridisation

Amplicons of 81 to 229 bp in length were amplified using forward and reverse primers (Table S1) from a cDNA pool generated from *M. incognita*. These amplicons were used as the template in a unidirectional PCR, to produce sense and antisense DIG-labelled probes. *In situ* hybridisation was performed as previously described (Jaubert *et al.*, 2002b). We used 10,000

J2s for each candidate effector. These nematodes were fixed by overnight incubation in 10% deionised formaldehyde before permeabilisation by chopping with a razor blade on a glass slide. The chopped nematodes were then digested in 0.1% (w/v) proteinase K solution (AM2546, ThermoFisher, USA). The tissues were hybridised with a DIG-labelled specific probe overnight at 42°C. Probes hybridising to the nematode were detected with an anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics, Mannheim, Germany). Samples were then observed under a Zeiss Axioplan 2 microscope.

siRNA design and treatment for infection assays

The siRNA primers were prepared as described in the instruction manual of the Ambion Silencer siRNA Construction Kit (AM1620, Ambion, Austin, TX), with two online software suites: siDirect2 (<http://sidirect2.rnai.jp/>) and Vienna RNA Web Services (<http://rna.tbi.univie.ac.at/>). A target sequence for the siRNA was identified within the qPCR amplicon, to ensure specificity for the tested gene and to make it easier to check for a decrease in gene expression after siRNA treatment. The J2s were treated by immersion in siRNA solution (2 µg siRNA/10,000 J2s) for 1 h, followed by incubation for 24 h in mineral water (Danchin *et al.*, 2013). The soaked J2s were then used to infect 24 tomato plants, with 120 larvae/plant. After soaking for 24 h, larvae were also retained for a qPCR experiment to check that the predicted reduction in expression of the targeted gene had actually occurred. Six weeks after infection, tomato roots were recovered with care, to compare infection success between nematodes treated with a specific siRNA and control nematodes treated with a random siRNA not targeting a nematode transcript (Danchin *et al.*, 2013). Galls and egg masses were counted and the results were normalised as the percentage difference in the number of galls or egg masses/g root relative to the control. Statistical analysis was performed with Mann-Whitney tests.

Accession numbers

Minc02654 (KX907741), Minc00328 (KX907742), Minc00331 (KX907743), Minc00672 (KX907744), Minc01625 (KX907745), Minc01681 (KX907746), Minc03314 (KX907747), Minc03471 (KX907748), Minc03750 (KX907749), Minc04572 (KX907750), Minc04712 (KX907751), Minc04729 (KX907752), Minc07254 (KX907753), Minc07307 (KX907754), Minc08014 (KX907755), Minc08146 (KX907756), Minc08148 (KX907757), Minc10604 (KX907758), Minc10606 (KX907759), Minc11260 (KX907760), Minc11888 (KX907761), Minc12024 (KX907762), Minc12754 (KX907763), Minc12921 (KX907764), Minc13038 (KX907765), Minc14652 (KX907766), Minc14707 (KX907767), Minc15508 (KX907768), Minc18288 (KX907769), Minc18636 (KX907770), Minc18876 (KX907771), Minc17611 (KX907772), Minc04822 (KX907774).

Results

The *Meloidogyne incognita* transcriptome reveals putative effector genes overexpressed during parasitic stages

RNA-seq libraries were generated from seven stages of the lifecycle of *M. incognita*: preparasitic J2s; parasitic J2s in the early stages of parasitism; parasitic J2-J3-J4 including late J2 parasites and young J3-J4 parasites; J3-J4 parasites; females; males and eggs (Danchin *et al.*, 2013). In total, 104,847,779 reads were obtained, including 7,333,207 for the J2-J3-J4 mixed parasitic stages, 3,112,384 for the J3-J4 parasitic stage and 27,818,226 for the preparasitic J2 stage. We aligned 42,586,639 (40.6%) reads against the *M. incognita* v1 genome with TopHat 2. Interestingly, 18,219 of the 19,212 predicted genes (94%) were supported by the RNA-seq data. A statistical analysis was carried out to investigate transcription dynamics over the lifecycle of *M. incognita*. Only 343 transcripts were identified as displaying significant differences in expression between lifecycle stages, and 307 of these genes were annotated in the *M. incognita* v1 genome (Abad *et al.*, 2008; Fig. S1).

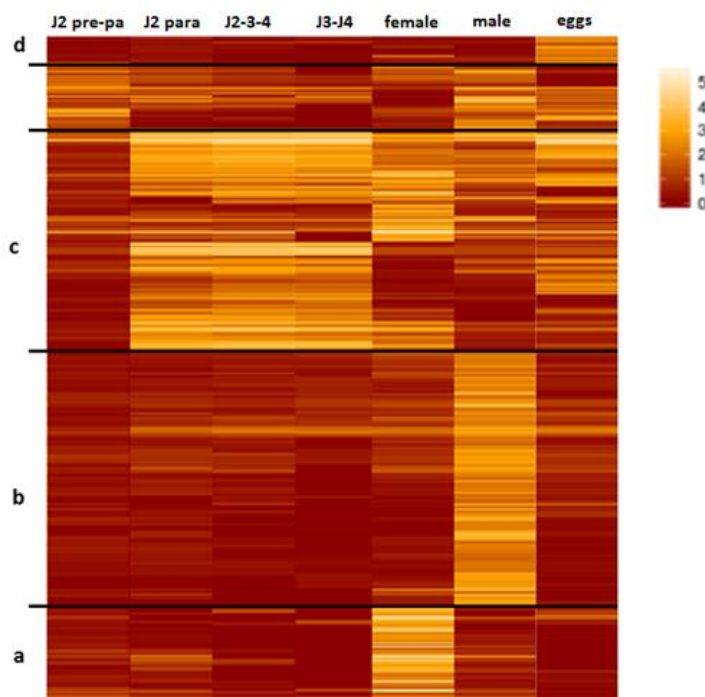


Fig. S1 Clustering of the 343 *M. incognita* transcripts differentially expressed at different life stages. Each line corresponds to a transcript, and each column represents a stage in the life cycle. Low Log_{10} FPKM value appears brown, and then tends to lighten to white when it rises. Representative groupings of genes appear in certain stages: a) representative gene of the female stage; b) of the male stage; c) of the parasitic stages; d) of the egg stage.

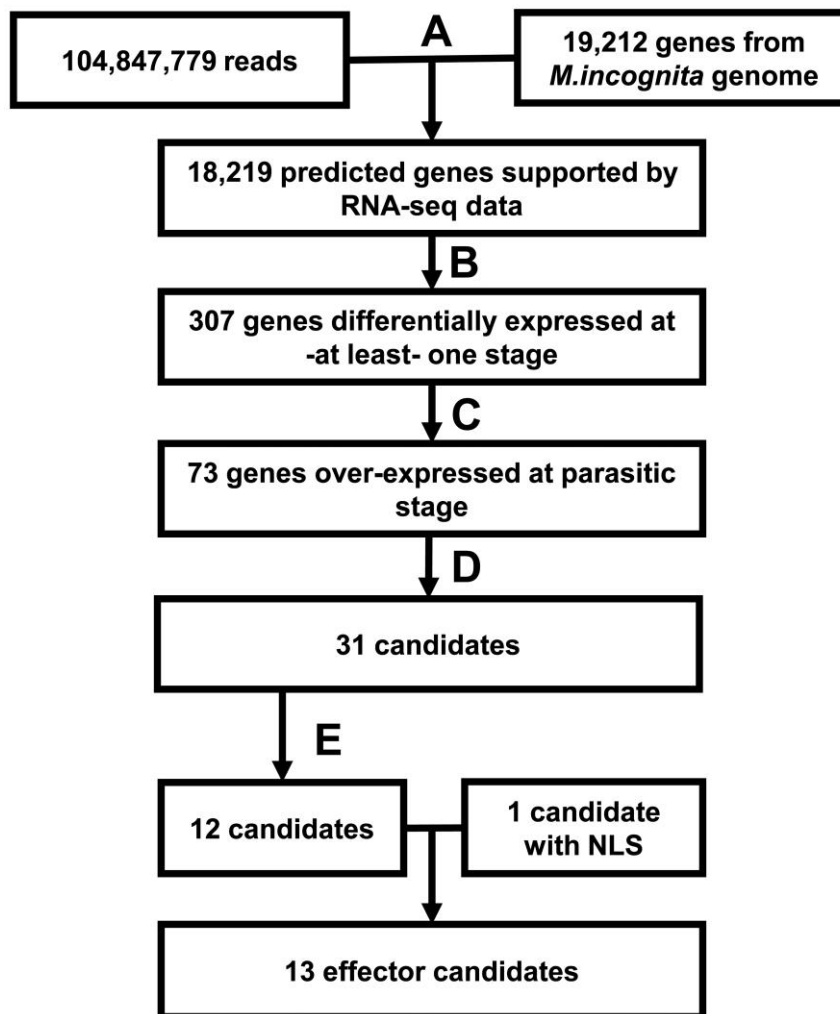


Fig. 1 Diagram of the filtering pipeline method for the identification of novel effectors from the *Meloidogyne incognita* transcriptome. **A**, Reads from seven libraries were aligned with the *M. incognita* genome, with TopHat software. **B**, Cufflinks and Cuffdiff were used to identify genes differentially expressed between lifecycle stages. **C**, Identification of genes more strongly expressed at the parasitic stages than at the pre-parasitic stage, with a FPKM > 50 in J3-J4 and in J2-J3-J4, and FPKM J2-J3-J4/J2 and J3-J4/J2 ratios > 10. **D**, The candidate effectors from *M. incognita* were analysed for the presence of an N-terminal secretion signal and the absence of transmembrane domains, with Phobius. **E**, Combination of 12 candidate effectors chosen from the filtering pipeline and one candidate with nuclear localisation signals (NLS) predicted by PsortII.

We hypothesized that effectors involved in the formation of giant cells *in planta* would be produced in larger amounts during parasitic stages than in preparasitic J2s, and would accumulate during infection. We selected 73 genes from the 307 differentially expressed genes

on the basis of their expression levels being 10 times higher in J2-J3-J4 and J3-J4 parasitic stages than in pre-parasitic J2s (i.e. with a ratio of FPKM (fragments per kilobase of exon per million reads mapped) J2-J3-J4/J2 and J3-J4/J2 > 10) (Fig. 1). These genes included 31 (42%) genes encoding proteins with an N-terminal secretion signal peptide but no transmembrane domains (Table 1). Twenty-seven of the proteins were less than 300 aa long and 17 contained more than four cysteine residues (from 4 to 49) and could be considered to be small cysteine-rich proteins, as defined in a previous study (Hacquard *et al.*, 2012) (Table S2). Almost all these proteins (27/31) were specific to *Meloidogyne* spp., and were found only in *M. incognita* and/or *M. hapla*, whereas four proteins (Minc01681, Minc00672, Minc12921 and Minc15508) had homologues in other free-living or parasitic nematodes. Twelve genes were unique to the *M. incognita* genome, whereas 19 others had one to three paralogues displaying 84% to 100% identity in the *M. incognita* v1 genome (Table 1). Only six proteins from among the 31 candidates and their 16 paralogs, Minc18636/Minc15401, Minc14652, Minc08014, Minc15508 and Minc04573, have been identified as putative effectors in previous studies (Danchin *et al.*, 2013; Rutter *et al.*, 2014) (Table 1). Interproscan analysis identified five proteins with predicted functional domains: nematode cuticle collagen (Minc01681), calcium-binding EF hand (Minc00672), colipase (Minc04712), saposin (Minc15508) and thioredoxin (Minc12921) domains. Three proteins (Minc07307, Minc14652, and Minc18636) had predicted nuclear localisation signals (NLS) and another protein (Minc12754) had a putative DNA-binding domain (leucine zipper pattern, PS00029), suggesting that they might be nuclear proteins. Finally, 22 of the 31 proteins (-71%) displayed no similarity to proteins present in databases and had no predicted functional domains (pioneers).

Twelve of the 31 candidate proteins (Minc03314, Minc04712, Minc08148, Minc12024, Minc12754, Minc13038, Minc14652, Minc18288, Minc18636 and Minc18876/Minc10604/Minc10606) were selected for further study (Table 1) because (i) they were pioneers specific to *Meloidogyne* species and (ii) they were 22 (Minc12754) to 7192 (Minc03314) times more strongly expressed in J3/J4 than in J2, constituting a large range of overexpression. All members of the Minc18876 family were 100% identical at the nucleotide level and were analysed together (Fig. S2). We also included one additional candidate in this study: Minc17611, which had a J3-J4/J2 FPKM ratio > 10 and contained NLS. Thus, we selected 13 effector candidates for functional characterisation.

Table 1 List of the identified candidate effectors over-expressed in parasitic J3-J4 stages

Gene (aa) ^a	<i>M. incognita</i> paralogs (%ID, bit score) ^b	Blastp		FPKM value ^d			Predicted domain ^e
		<i>M. h</i> ^c	NCBI ^c	J2	J2-J3-J4	J3-J4	
Minc02654 (229)	N/A	+	N/A	2	441	315	N/A
Minc00328 (221)	Minc04572 (100%, 427) Minc00331 (84%, 336) Minc04573 ^f (85%, 335)	+	N/A	27	39 849	38,342	N/A
Minc00331 (352)	Minc04573 ^f (97%, 668) Minc00328 (84%, 336) Minc04572 (84%, 336)	+	N/A	27	39 849	38,342	N/A
Minc00672 (149)	Minc02346 (93%, 276) Minc12334 (100%, 140)	+	N/A	5	224	190	EF-Hand type; Calcium-binding EF-hand
Minc01625 (79)	Minc14707 (100%, 169) Minc03471 (97%, 167)	N/A	N/A	0	14 216	8,060	N/A
Minc01681 (294)	Minc04518 (100%, 646)	+	+	0	3 070	3,230	Nematode cuticle collagen, N- terminal
Minc03314 (87)	N/A	+	N/A	4	40 645	28,767	N/A
Minc03471 (79)	Minc01625 (97%, 167) Minc14707 (97%, 167)	N/A	N/A	2	17 326	6,152	N/A
Minc03750 (127)	Minc09283 (98%, 224) Minc00296 (97%, 212)	+	N/A	2	1 003	525	N/A
Minc04572 (221)	Minc00328 (100%, 427) Minc00331 (84%, 336) Minc04573 ^f (85%, 335)	+	N/A	6	14 571	10,984	N/A
Minc04712 (406)	Minc11889 (93%, 646)	+	N/A	2	5 729	1,750	Colipase
Minc04729 (101)	Minc04730 (80%, 154)	+	N/A	1	773	635	N/A
Minc07254 (210)	N/A	N/A	N/A	0	168	141	N/A
Minc07307 (274)	N/A	N/A	N/A	2	212	75	NLS
Minc08014 ^f (59)	N/A	N/A	N/A	5	487	219	N/A
Minc08146 (74)	N/A	N/A	N/A	0	756	210	N/A

Minc08148 (72)	N/A	N/A	N/A	2	1 106	3,967	N/A
Minc10604 (102)	Minc10606 (100%, 235) Minc18876 (100%, 235) Minc04822 (100%, 209)	N/A	N/A	10	11 102	17,298	N/A
Minc10606 (102)	Minc10604 (100%, 235) Minc18876 (100%, 235) Minc04822 (100%, 209)	N/A	N/A	10	11 073	17,226	N/A
Minc11260 (109)	Minc08754 (91%, 243) Minc02843 (88%, 189)	N/A	N/A	2	1 920	1,922	N/A
Minc11888 (46)	N/A	+	N/A	1	1 784	405	N/A
Minc12024 (77)	Minc06089 (96%, 130)	N/A	N/A	1	294	137	N/A
Minc12754 (96)	Minc13608 (93%, 186) Minc01345 (92%, 184)	+	N/A	11	128	243	DNA-binding domain
Minc12921 (240)	Minc14988 (98%, 484)	+	+	4	824	435	Thioredoxin fold
Minc13038 (93)	N/A	N/A	N/A	6	935	562	N/A
Minc14652^f (387)	N/A	+	N/A	2	401	495	NLS
Minc14707 (79)	Minc01625 (100%, 169) Minc03471 (97%, 167)	N/A	N/A	1	9 298	4,920	N/A
Minc15508 ^g (158)	N/A	N/A	N/A	1	471	302	Sapoin-like
Minc18288 (104)	N/A	N/A	N/A	4	812	278	N/A
Minc18636 (312)^{f,g}	Minc15401 ^{f,g} (89%, 556)	+	N/A	4	279	148	NLS
Minc18876 (102)	Minc10604 (100%, 235) Minc10606 (100%, 235) Minc04822 (100%, 209)	N/A	N/A	9	10 998	17,052	N/A
Minc17611 (183)	N/A	+	-	8	346	318	NLS

^a Name of the identified effector candidates and their protein length in amino acids. ^b Amino acid ^a Name of the identified effector candidates and their protein length in amino acids. ^b Amino acid similarity to paralogous gene in the *M. incognita* V1 genome (Blastp % identity and bit score) and N/A= not applicable. ^c (+) indicates that a homologous sequence has been found in *Meloidogyne hapla* (Mh) or in NCBI non-redundant protein database (Blastp identity > 50% and bit score > 100). ^d FPKM value at pre-parasitic stage J2 and parasitic stages J2-J3-J4 and J3-J4 of effector candidates. ^e Protein domains predicted using Interproscan and nucleus localization signal (NLS) predicted using PSORTII. ^f effectors also identified in Danchin et al., 2013. ^g effectors also identified in Rutter et al., 2014. The effector candidates selected for functional analysis are indicated in bold.

Validated differentially expressed genes are predominantly expressed in oesophageal glands.

The overexpression of these 13 candidates at J3-J4 parasitic stages was confirmed by collecting J2s and J3-J4s from infected tomato plants and performing RT-qPCR as an independent validation method (Fig. 2a-c). We designed specific primers for each candidate. However, the RT-qPCR primers used for *Minc12024* and *Minc18876* actually targeted the whole family, due to the high degree of similarity between paralogues. The gene encoding Mi-EFF1, which was known to be overexpressed at J3-J4 parasitic stages (Jaouannet *et al.*, 2012), was used as a positive control for this experiment. Eight candidate effector genes (73%) (*Minc03314*, *Minc08148*, *Minc12024/Minc06089*, *Minc12754*, *Minc14652*, *Minc17611*, *Minc18636*, *Minc18876* family) were found to be significantly more strongly expressed at the J3-J4 parasitic stages than at the J2 stage (2.7- to 46.2-fold overexpression), whereas three other genes were not overexpressed at the J3-J4 stages (*Minc04712*, *Minc18288* and *Minc13038*).

Most of these genes encode novel candidate effectors. We therefore investigated the sites at which they were expressed in the nematode. *In situ* hybridisation (ISH) assays were performed on the eight genes for which an overexpression at the parasitic stage was validated. Full-length coding sequences (*Minc12754*, *Minc14652* and *Minc17611*) or qPCR amplicons (*Minc03314*, *Minc08148*, *Minc12024/Minc06089*, *Minc18636* and *Minc18876* family) were used as *in situ* probes (Suppl. Table 2). A visible signal was obtained for all eight antisense probes with preparasitic J2s (Fig. 3a-h), whereas no signal was obtained with the sense control probe (Fig. 3i). One gene (*Minc08148*) was found to be expressed in the intestine and tail of the nematode. Seven genes (*Minc03314*, *Minc12024/Minc06089*, *Minc12754*, *Minc14652*, *Minc17611*, *Minc18636*, *Minc18876* family) were expressed specifically in the oesophageal glands. Four of these genes (*Minc12754*, *Minc14652*, *Minc17611* and *Minc18876* family) were identified in the DG (Fig. 3d,e,f,h). We searched for the DG promoter element motif (the DOG Box, ATGCCA) identified in the promoter of *Globodera rostochiensis* genes encoding DG effectors (Eves-van Den Akker *et al.*, 2016) in the 500 bp upstream from the start codons of these genes, but this motif was not detected. Two genes (*Minc03314* and *Minc12024/Minc06089*) were specifically expressed in the SvGs (Fig. 3a,c). *Minc18636* expression was also observed in the SvGs (Fig. 3g), consistent with previous reports (Rutter *et al.*, 2014). Thus, seven of the eight upregulated candidate effector genes selected were specifically expressed in the nematode secretory organs.

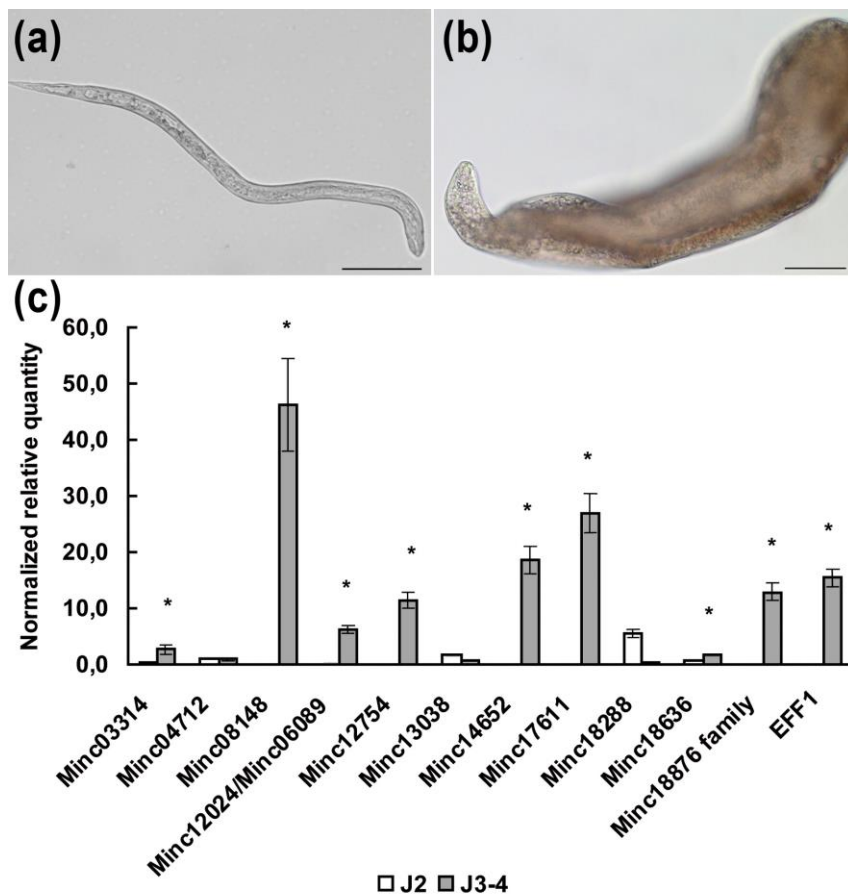


Fig. 2 Expression of the 12 *M. incognita* candidate effector genes at the at preparasitic J2 and parasitic J3/J4 stages. (a) Free-living *M. incognita* J2. (b) Adult parasitic *M. incognita* J3-J4. (c) Normalized relative quantities of the candidate effector transcripts in J2 and J3/J4 samples were determined by RT-qPCR. Eight putative effectors (*Minc03314*, *Minc08148*, *Minc12024/Minc06089*, *Minc12754*, *Minc14652*, *Minc17611*, *Minc18636* and *Minc18876* family) were validated as significantly overexpressed during parasitic stages. *EFF1* (Jaouannet et al., 2012) was used as a positive control. The data shown are means \pm SD from three independent biological replicates.

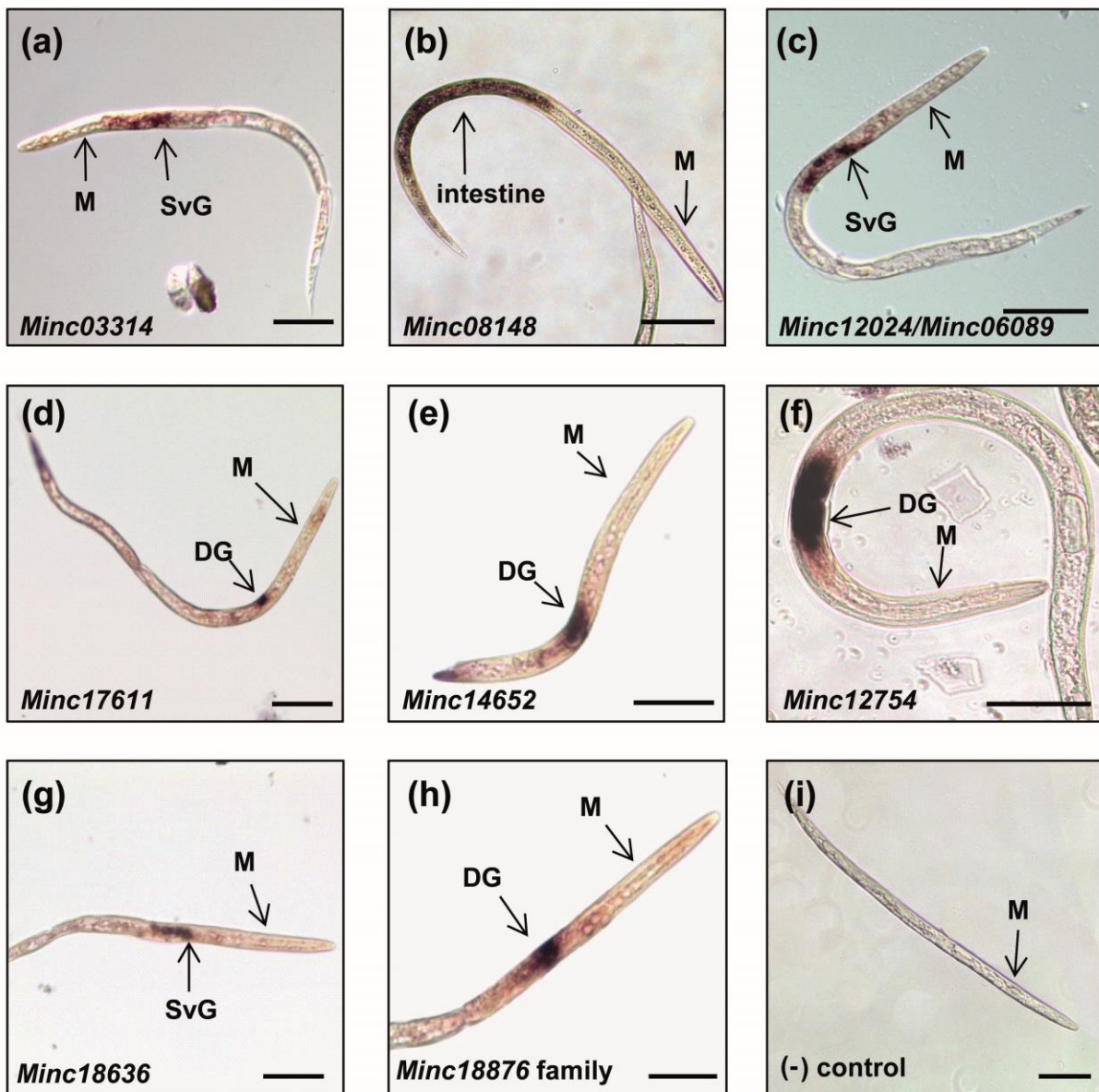


Fig. 3 Tissue expression of candidate secreted effectors in *M. incognita* pre-parasitic juveniles. (a-h) Transcripts were localised by *in situ* hybridisation with gene-specific digoxigenin-labelled probes. Transcripts from *Minc03314* (a), *Minc08148* (b), *Minc12024/Minc06089* (c), *Minc17611* (d), *Minc14652* (e), *Minc12754* (f), *Minc18636* (g) and the *Minc18876* family (h). (i) The sense control probe (-) of *Minc12754* yielded no labelling of nematode tissues. Scale bars represent 50 μ m. M, metacarpus.

Gene silencing experiments reveal that the *Minc18876* family is required for *M. incognita* parasitism

We used gene silencing techniques to investigate the roles of the seven candidates localised in the oesophageal glands by ISH as effectors in plant-nematode interactions. We used small interfering RNA (siRNA) molecules to target the selected genes in infectious *M. incognita* J2

larvae, and we infected host tomato plants with treated larvae 24 h after soaking in the siRNA solution. The siRNA target sequence was designed from the sequence of the qPCR amplicon, and we checked for specificity for the tested genes, and the efficiency of gene silencing after siRNA treatment (Fig. S3).

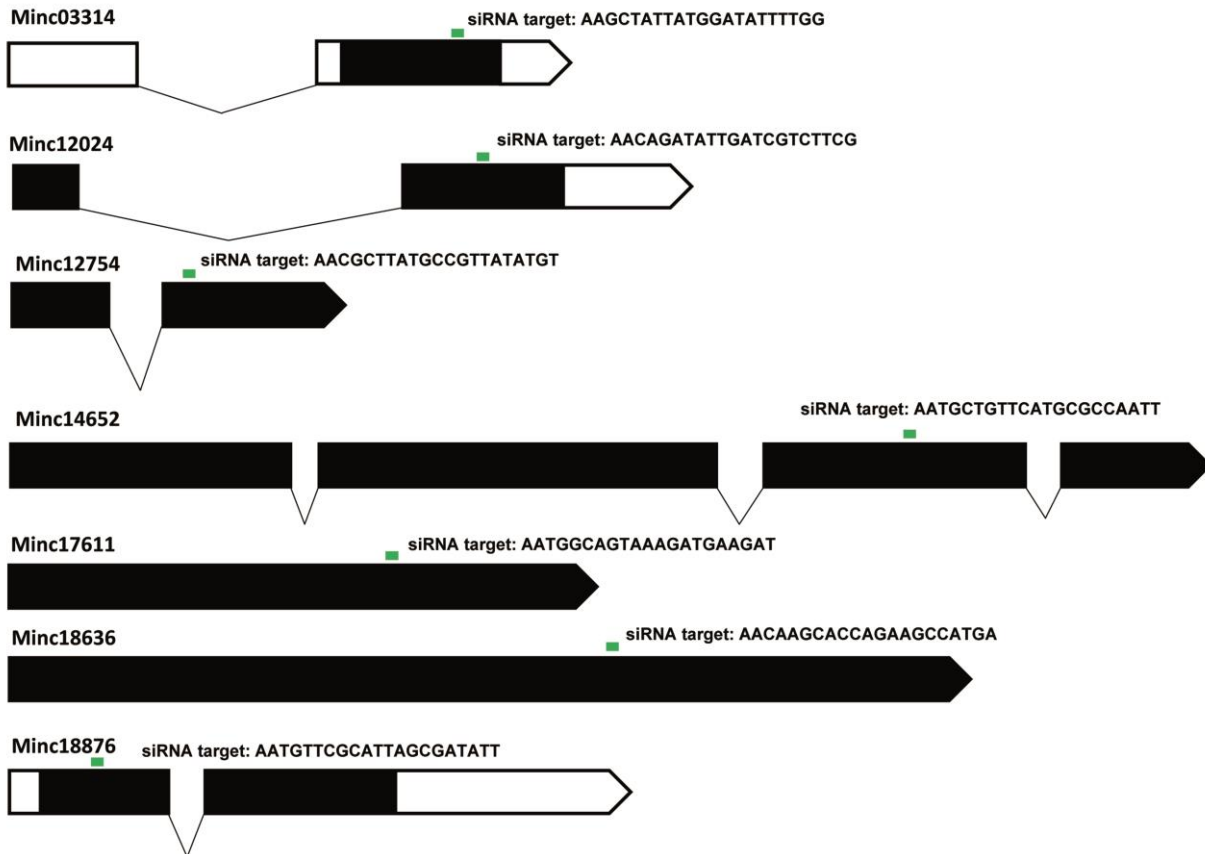


Fig. S3 Gene organization of the seven *M. incognita* effector candidates and position and sequences of the siRNA target sequences. The 5' and 3' UTR are presented in white boxes, the exons in black boxes, the introns in broken line and the siRNA target sequence in green boxes.

Our RT-qPCR results indicated that five siRNAs significantly decreased the amounts of the targeted transcripts (*Minc03314*, *Minc12754*, *Minc17611*, *Minc18636* and *Minc18876* family) present 24 h after soaking, relative to the amounts of these transcripts present in control J2 samples treated with a siRNA with no target in the *M. incognita* genome (Fig. 4). The largest effect was observed for *Minc17611*, which decreased transcript levels by 80%, whereas a 40-60% decrease was observed for the other four genes. Finally, targeting *Minc12024/Minc06089* or *Minc14652* with siRNA resulted in no significant decrease in transcript abundance.

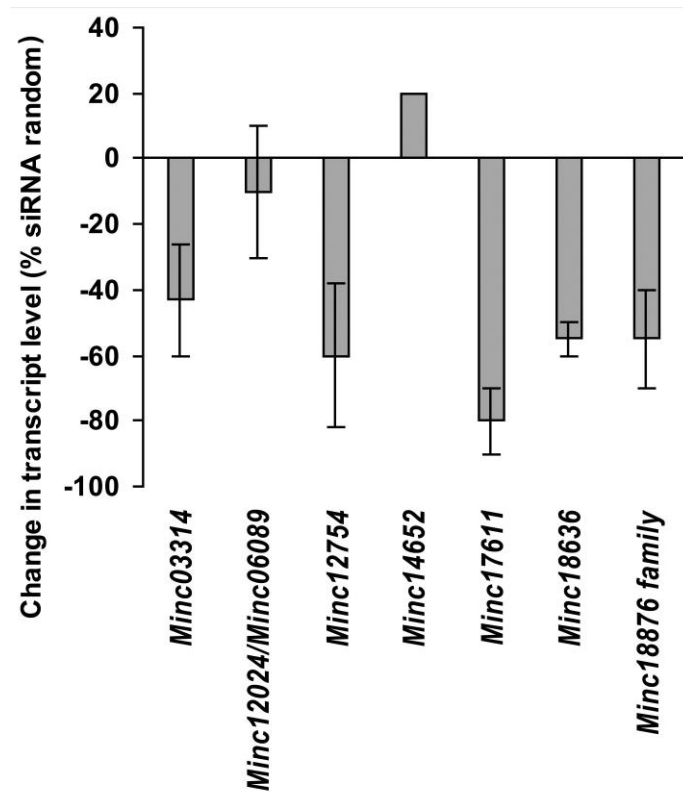


Fig. 4 Percent difference in transcript abundance between J2s soaked in specific siRNA and control J2s. Soaking in a specific siRNA solution induced a significant change in the level of the targeted transcript. For each targeted gene, transcript level was measured by qPCR 24 h after the soaking treatment, and compared with that in control J2 samples treated with a siRNA with no target in the *M. incognita* genome. The figure shows the results for two biological replicates, except for Minc14652, for which there was only one biological replicate.

Galls and egg masses were counted only for samples displaying significantly lower levels of gene expression after soaking in siRNA solution. Four siRNA-treated samples (Minc03314, Minc12754, Minc17611 and Minc18636) displayed no significant or reproducible decrease in the numbers of galls or egg masses (Fig. 5a). By contrast, treatment of the nematodes with a siRNA targeting the Minc18876 family had no significant effect on gall number, but did significantly decrease (60% and 32% for the first and second replicates, respectively) the number of egg masses on infested plants, relative to the control (Fig. 5b). This family has four members encoded by ORFs with 100% identical nucleotide sequences. Only three mismatches were detected in the 3'UTRs of these transcripts (Fig. S2). These genes, specifically overexpressed in parasitic juveniles (Fig 6a), encode a small (102 aa) *Meloidogyne*-specific protein with a putative 30 aa signal peptide and no known functional domains (Table 1, Fig.

6b,c). The mature protein is 72 aa long (6.0 kDa), glycine-rich (57%, by frequency) and contains 10 cysteine residues. This small cysteine-rich effector, encoded by the *Minc18876* family genes and named MiSCR1, may play an important role in the early stages of giant cell formation.

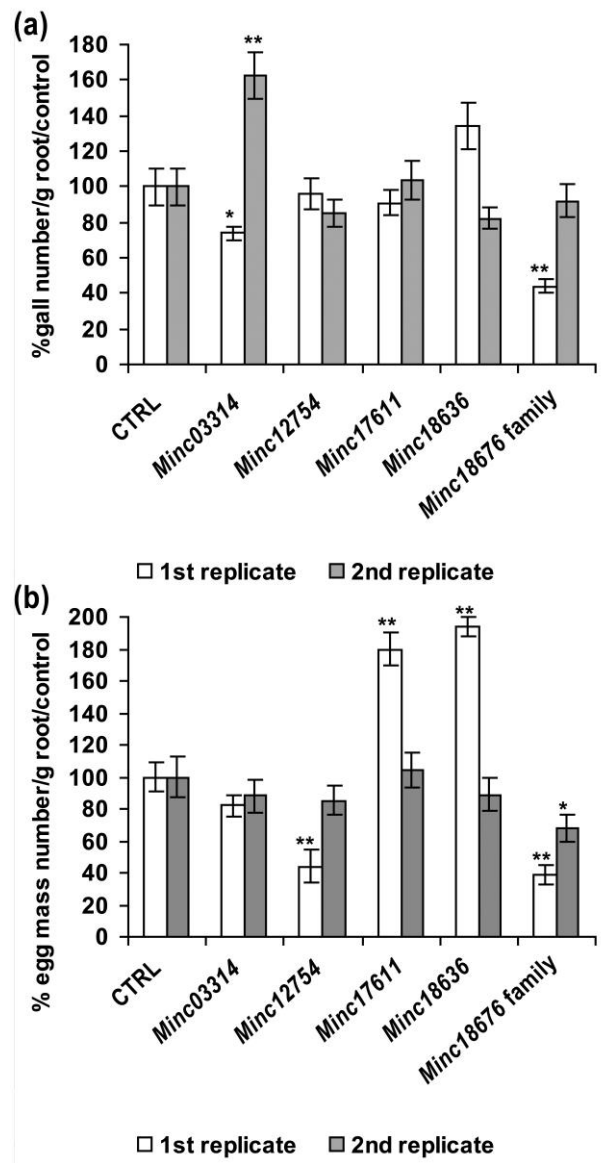


Fig. 5 Effect of candidate gene silencing on nematode infection and reproduction. *J2s* were soaked in a solution of siRNA specifically targeting *Minc* genes or in a solution of siRNA with no target in the *M. incognita* genome (Control, CTRL). They were then used to inoculate tomato plants. (a) Percent difference in gall number/g root relative to the control. (b) Percent difference in the number of egg masses/g root relative to the control. The white and grey columns represent the results for the first and second independent biological replicates, respectively. Error bars correspond to the standard error of the mean (SEM). Statistical analysis was performed with Mann-Whitney tests. P-values: <0.001 ‘***’, <0.005 ‘**’, <0.05 ‘*’.

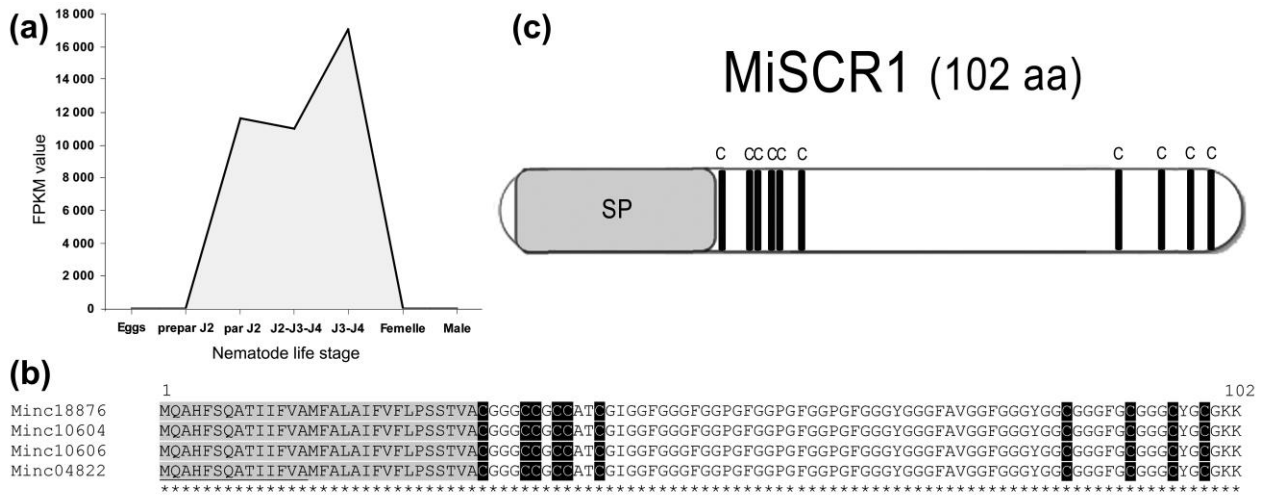


Fig. 6 The genes of the *Minc18876* family encode a small glycine- and cysteine-rich effector, MiSCR1. (a) Expression pattern of *Minc18876* during *M. incognita* life cycle. FPKM values obtained in the transcriptome of eggs, preparasitic J2, parasitic J2, parasitic J2-J3-J4, parasitic J3-J4, female and male. (b) CLUSTAL alignment of the protein sequences of the four members of this family. The first 14 aa of *Minc04822* (underlined) were missing from the *M. incognita* V1 proteome and were corrected by manual annotation. * indicates identical amino acids. (c) Schematic representation of the MiSCR1 protein. The predicted signal peptide (SP) is indicated in grey, the cysteine residue (C) is shown in black.

Discussion

The development of high-throughput sequencing technologies for genomics and transcriptomics has provided new opportunities for studying plant-nematode interactions. New candidate RKN effectors were recently identified by a transcriptomic approach based on RNA-seq (Haegeman *et al.*, 2013; Rutter *et al.*, 2014; Petitot *et al.*, 2016). Rutter *et al.* (2014) sequenced transcripts isolated from *M. incognita* gland cells. In the absence of a reference genome, a *de novo* transcriptome was obtained for the rice RKN *M. graminicola*, from samples of preparasitic J2s and parasitic stages in rice root tips. This made it possible to identify 15 candidate effector genes displaying upregulation in pre parasitic J2s or two to four days post infection (Petitot *et al.*, 2016).

***Meloidogyne incognita* parasitic stage gene profiling reveals new putative effectors expressed in oesophageal glands**

In this study, we used whole RNA-seq transcriptomes from various points in the lifecycle of *M. incognita* to identify new candidate secreted effector proteins. Using the available whole-

genome sequence for *M. incognita* (Abad *et al.*, 2008), we obtained the RNA-seq profiles of almost all *M. incognita* genes for the various stages of its lifecycle. Combining RNA-seq data with statistical approaches and functional annotation, we focused on RKN genes overexpressed in J3-J4 parasitic stages isolated by dissection from plants, and selected candidates that might play a role in giant cell formation, for functional characterisation. As observed in previous studies, most of the proteins had never been described before, were specific to *Meloidogyne* spp., and had no detectable functional domain. Only six proteins (13%) had already been described as putative effectors in previous studies (Danchin *et al.*, 2013; Rutter *et al.*, 2014). Searches for known protein domains revealed five proteins with nematode cuticle collagen, calcium-binding EF hand, colipase, saposin or thioredoxin domains. Another calcium-binding protein, the *M. incognita* calreticulin MiCRT, is produced in the SvGs and DG and secreted at the feeding site (Jaubert *et al.*, 2005). A dorsal gland-specific thioredoxin (10G02 / Minc14653) has also been described in a previous study (Huang *et al.*, 2003). Ten genes displayed no similarity to proteins in databases, suggesting that they might be involved in specific adaptations to parasitism in RKNs. Overall, 2,578 secreted proteins with no known functional domains were predicted to be produced from the genome of *M. incognita* (Abad *et al.*, 2008). This specificity makes it difficult to predict and analyse their function.

RT-qPCR confirmed that eight of the 11 candidates tested (67%) were more strongly expressed at the parasitic stage than at the preparasitic stage. A similar proportion of candidate genes was validated in a transcriptomic analysis of *M. graminicola* (Petitot *et al.*, 2016). For a protein to be considered an effector, it must be secreted from one of the nematode's secretory organs: the hypoderm, amphids, rectal glands or, as for most nematode-secreted effectors, the oesophageal glands (Huang *et al.*, 2003; Davis *et al.*, 2004; Rutter *et al.*, 2014). Proteomics and molecular approaches led to the identification of 89 *M. incognita* candidate effectors, the expression of which was specifically detected in the glands by ISH (Truong *et al.*, 2015). Two thirds of these candidate genes encoded proteins of unknown function (Davis *et al.*, 2008; Truong *et al.*, 2015). Almost half were expressed in the two SvGs: the genes encoding plant cell wall-modifying enzymes (Rosso *et al.*, 2011), the 16D10 CLE-like peptide (Huang *et al.*, 2003) and MSP40 (Niu *et al.*, 2016), which have been observed to be more active in the early stage of parasitism (Davis *et al.*, 2000). The other half of these genes (Mi-EFF1 (Jaouannet *et al.*, 2012) and numerous pioneers (Huang *et al.*, 2003; Rutter *et al.*, 2014; Xie *et al.*, 2016) were expressed in the DG, and were highly active *in planta* after the migration phase (Davis *et al.*, 2000). Thirty-

seven of the 185 candidates obtained by the microaspiration of cytoplasm from the salivary glands (20%) were found to be expressed in the glands (Huang *et al.*, 2003). A similar proportion was obtained in other studies on *M. incognita*: 19% (17/91 genes) (Rutter *et al.*, 2014) or 27% (3/11 genes) (Jaouannet *et al.*, 2012). A higher proportion of genes with expression specifically localised to the gland (50%; 3 of 6 genes) was obtained, by a transcriptomic approach, with *M. graminicola* (3/6 genes) (Petitot *et al.*, 2016). In this study, 87% (7/8) of the selected candidate effectors were localised in the glands, three in the SvGs and four in the DG, validating our strategy coupling RNA-seq expression and functional annotation for the identification of new RKN effectors. Two candidate nuclear effectors, *Minc12754* and *Minc17611*, were specifically expressed in the DG, as observed for the two RKN effectors shown to be targeted to feeding cell nuclei (Jaouannet *et al.*, 2012; Lin *et al.*, 2013).

Small cysteine-rich effector MiSCR1 is involved in early stages of giant cell formation

Finally, the validation of the candidates identified as secreted effectors requires an analysis of their function during plant infection, particularly after gene silencing. As previously reported, only a few *M. incognita* effectors were found to be required for RKN parasitism. The asexual reproduction of this biotrophic, genetically untransformable organism by mitotic parthenogenesis greatly limits functional analyses. RNAi is currently the only tool available for knocking down the expression of RKN genes (Gheysen & Vanholme, 2007; Rosso *et al.*, 2009). Five of the seven candidate effector genes tested here displayed a significant decrease (from 40% to 80%) in expression after soaking in siRNA solution. However, knocking down the expression of these genes did not decrease gall number, indicating a lack of involvement of these genes in the initial stages of infection: penetration, migration and the sedentary phase. The number of egg masses was significantly smaller in siRNA-treated J2s than in controls for only one candidate, *Minc18876*, and its paralogues. A similar effect was observed for one SvG-specific candidate effector, *Minc03866*, encoding a C-type lectin, in a previous siRNA study (Danchin *et al.*, 2013). A decrease in the number of egg masses but not in the number of galls indicates that the gene targeted by the siRNA has functional consequences in processes occurring between giant cell formation and egg production. In addition, the effect of the siRNA generally only lasts a few days, depending on the gene considered. RNAi approaches based on siRNA are, therefore, most suitable for functional analyses of genes involved in the early stages of infection (Arguel *et al.*, 2012).

Our findings suggest that the small cysteine-rich (SCR) protein produced by the Minc18876 family may play an important role in giant cell formation, which occurs early and specifically in infection. SCR proteins are typically highly represented in the list of predicted candidate effectors secreted by filamentous pathogens (Saunders *et al.*, 2012; Zhang *et al.*, 2016) and they were also abundant among the list of candidate secreted effectors overexpressed at parasitic stages obtained in this study. SCRs dominate the secretome of the causal agent of poplar rust, *Melampsora larici-populina*, with 63% of small secreted proteins containing more than four cysteine residues (Duplessis *et al.*, 2011; Hacquard *et al.*, 2012). SCR effectors may function extracellularly, like the *Cladosporium fulvum* Avr2 protein (Rooney *et al.*, 2005), or within the plant cell, like the avirulence protein AvrLm4-7 from *Leptosphaeria maculans* (Blondeau *et al.*, 2015). The cysteine residues of SCR effectors are often involved in the formation of intramolecular disulfide bonds that stabilise the protein and help it to function (Saunders *et al.*, 2012).

For other selected candidate effectors from RKNs, the lack of an observed phenotype following gene silencing may reflect functional redundancy, particularly for the Minc18636 paralogue, or a role at later stages of parasitism. An alternative way of analysing the function of these genes would be to use transgenic plants producing siRNA or dsRNA in giant cells. This technology has been successfully used in *Arabidopsis*, to demonstrate the role of several RKN candidate effectors in parasitism (Quentin *et al.*, 2013; Jaouannet & Rosso, 2013; Truong *et al.*, 2015). However *in planta* silencing require the feeding of the RKN on the giant cells and is thus not efficient to study early acting effectors.

In conclusion, this transcriptomic study of nematode genes overexpressed during parasitic stages of the lifecycle made it possible to search for new candidate *M. incognita* effectors and to identify a dorsal gland-specific small cysteine-rich protein, MiSCR1, involved in giant cell formation, which occurs early in infection. The novel candidate effectors add to the repertoire of known effectors in this fascinating plant pathogen. The next step will be to characterise the plant targets of these effectors. The host plant targets of only four RKN effectors (16D10, Mi8D05, Mc1194, MjTTL5) have been identified to date (Huang *et al.*, 2006b; Xue *et al.*, 2013; Davies *et al.*, 2015; Lin *et al.*, 2016).

Acknowledgements

We thank Etienne G.J. Danchin, Sébastien Duplessis and Alexandre Jamet for helpful discussions. This work was funded by INRA and the French Government (National Research Agency, ANR) through the ANR/Genoplante program NEMATARGETS and 'Investments for the Future' LabEx SIGNALIFE: program reference #ANR-11-LABX-0028-01. C.-N. Nguyen was supported by USTH fellowships from the Ministry of Education and Training of The Socialist Republic of Vietnam.

Author contributions

C.-N.N. performed the research, data analysis and interpretation. M.M. cloned effectors and set up ISH experiments; M.Q. cloned the effectors *Minc17611*, *Minc12754* and *Minc14652*. L.P.-B., M.Q. and B.F. supervised the experiments and data analyses; M.D.R., N.N. performed the transcriptome data analysis; P.A. and B.F. supervised the research; C.-N.N. and B.F. wrote the paper.

Table S1 Protein sequence of identified putative effectors over-expressed in parasitic J3-J4 stages and number of cysteine residues in the mature protein

Gene	Protein sequence	Cystein number
Minc12024	MMKLNILFLFFIILNSLAFGSPSLNSVRVKRQGWGGWGWNPQVQTDIDRLRIDKDKLRDMDRLRLDQDSSSWG GK	0
Minc00672	MEATPLIINLIIVLVLILVNAKESKVEETISNNTGAKKKANLFTAADENNDGKIDMEELKRFVNGPKFWKAVVVVENS EEAINKYRSFFTEGLKLLDIDGNGKVDDAELRGYSLQYIKEWMDIDHDGVISKWFEFEMNMNMLMGDGS	0
Minc17611	MRILTKILLIQFSIGFALLISPKEETGSKIDGLVKEKTEEKPEVTREKRESEKDELIPQEKPEGKDESETKNKENPQDK AKRGEDTIASIREKGERVMMRKRTRIFTAVPESKNGSKDEKEDKKEGKKEEKEEENEKISQTK EGLTKKEEKIEVK PKEKKETKEEKIETPKNKAQ	0
Minc00328	MRAFLIISLTLIISFVIANTADKTQASDSATGNIEHGNKKEETAAEKRATIGEKKIAADEASLPAAGISKENTGKLENK REESKIEGQQQHQANAEEETNEKTEKRDEQRAKNAEEKQEGIERRSDEKRGDAELEKGNKENIETSKTEK HESKRRTEERKNVGTTEEKREKREEAIAPEEKKEEIEKEKNVETPRKQQRWAIKAVRALF	0
Minc04572	MRAFLIISLTLIISFVIANTADKTQASDSATGNIEHGNKKEETAAEKRATIGEKKIAADEASLPAAGISKENTGKLENK REESKIEGQQQHQANAEEETNEKTEKRDEQRAKNAEEKQEGIERRSDEKRGDAELEKGNKENIETSKTEK HESKRRTEERKNVGTTEEKREKREEAIAPEEKKEEIEKEKNVETPRKQQRWAIKAVRALF	0
Minc04729	MSSSTFCFLIVAALLAQIVMSAPVADKTVQKRHAVYAPAYYYYPPYAPAAPASSSFVSGGGGGGGIQQSVIGGGYGL PGSSFVAGGGGGYGGGGIQQSVIGGR	0
Minc01625	MNKSLIFILLNILAISLNFVECGYNRGGGGFDSSSGGSEWSQSSGGGSSFDNGGGGGGGGFGSPGYNGGGSGYNR GKK	0
Minc03471	MNKSLIFVLLNILAISLNFVECGYNRGGGGFDSSSGGSEWSQSSGGGSSFDNGGGGGGGGFGSPGYNGGGSGYN RGKK	0
Minc14707	MNKSLIFILLNILAISLNFVECGYNRGGGGFDSSSGGSEWSQSSGGGSSFDNGGGGGGGGFGSPGYNGGGSGYNR GKK	0
Minc18636	MYFKAFILLISPFAAFVNNARTISNMDEVDVSATMAEIKNNVGEKSNQVLPQGEEMDDLTEYDSDSDSDDED EDYDDEEDDEDNDANNYDYLNSRIDHMDIDSFETYEELPHMDNYGKMNEAKQMMPVMHMHNAVENEHYKQ LPSIATQPIIEANPIMPPMPVVPQKMEQAPEAMKKEATPQASQPSSPKAATPKAKTSPKTELKPKSAKKVQAKKG AAKVAKDKTKPKDVKKSKDAKAKTAKGKVPKPKGAAKGPAPKAKGKPMKGPAPAKATKAGPKQPAKKGKSA SKKVTTKGGKKH	0
Minc00331	MRAFLIISLTLIISFVIANTADKTQASDSATGNIEHGNKKEETAAEKRATIGEKKIAADEASLPAAGISKENTGKLENK REESKIEGQQQHQANAEEETNEKTEKRDEQRAKNAEEKQEGIERRSDEKRGDAELEKGNKENIETSKTEK HESKRRTEEVQKNEGKTEGKREETNIHGAATNTEESKTVAEEQKQHKNREEIAGAEINKDEAAAKTGLAAATNN NNEKREEKREETGKATEQRKTAEEGKEKREELTGNNEEKITGHEEITKTEKRDEAAAAGTTEERKNVGTTEEKREK REEAIAPEEKKEEIEKEKNVETPRKQQRWAIKAVRALF	0
Minc11888	MLSYKLALFVIFLFSVAIADLTADNEGLVQQMDRRSQGCTNPLS	1
Minc12921	MKASTRLFLEIALIAASGILINAHENEKVDLTEMYKNPLSNGFGDDIDWIPWENAVETALERNKPVFLIHKTWCHA CKSLKVMQQSNARKAFKLSYFVMVNTADDEPEYEEYRPDGYVPRILFDKNGDLLPDFKNKAEYKNYAYYY PSPADILNSMKEVIAHYGIELSSEKGDKLPVPPPKPEAPEKTKDKKVSNETKKAKEVKEIKEGKKTKESS KTSEL	2
Minc18288	MTKLIKISILVLTLLVIYTNLNSAETNSDNNEGRLKRFGGWGGGTYRYGPGYGGCNGYGGGGWMMNGAGYG MRPVGWRPVRTGGGGRHSHESHESKESYDVN	2
Minc12754	MFGSPMFVGSLLRLTFVIVFGFVEGNEEPTCSKTLMPLYVVLGLLNVCLIGVIFLSYKLFVSKGNKEGKKEEDKGDK KKEGEGDKKEGEEPCK	2
Minc08148	MILLFISSVTIAEDEPSILTKRDAFKMVLRAKRCFCGCGYGGFGGFGGGLGIGLIGAGIGLFR	4

Minc03314	MKSYLCLFLILLFVLCFTVSMQVSTDNSESKLLRSKRYGGFGYGGGGGGCDTCGGGYGGYAPSNFYGGGCDMCG GGYRSYYGYFG	4
Minc15508	MQNKIIITVLFVTVFGFLGSEGLSKESNLEINNENLTKNWFDIGTCFVCKEAIIQMRFFLSNPEFEELRKYIRVNLCPK FGIFRQMCEDFVDNELADFFEDLEFYMNPNLACMEMNFCTTNLNNNTNTPKVNNNLLHKHIEEEKNTQENVVTE K	6
Minc07254	MFKFLILTCLIIKTHSWTWKDYPSRPGPDYSECQVSRPTYVCDPDGMLTDQEREIVHMVEDFKEKTKRPNNSNVPC MREGLRLVVALAKDKIGREDGWNGTTKLCFNDRKWTSLYTTDCDSVVQGIELNSDGFRCVYSLRWLMTLNYDEYK QLGYADEVHLENKNYFDALKNYIENMRMLYIHRFSIFDNPDVSNERASLQSSNKLEEL	6
Minc08146	MKFTSFFILFVCAIFVLSAYAEINEKLAVEEMGENAQFEKMAVERTSRAKRCCCGCCCGFYGGFGFYFGK	6
Minc02654	MSSKNKIIILLVFLIFLQIQSLTLNNESSCNPNRNLIFSTCKAGLPWPREDFTSISVLITHGIHCIDILQEDNPNFEKHL QFHKEHCELEKIIHKFFKENNNYVPELCKDFRQAAPGGRVRRGVWGCWGRHRCCRKRDIKEFKGLEELDDN DFKEMATKNFEKLLLEKNEKLEIEKYYVEGSRNPLAIRMAALGIYCKFESYGVVVDVIDGRIIFN	8
Minc08014	MALQKLIFVLIASLILINLSADAQNTPAFYDCNCAQLCSNPCKSGKSACDNCKSIDIPKQCSIQCKIN	8
Minc01681	MQETKVVIIGIACFSSLLAIMATLVVMPQLYSQINDLNRVRDGVQAFRVNTDSAWNDLMELQVAVTPQSKPRSNP FQSLYRQKRSRDPDYCICQPLEINCAPGPPGPPGPPGQPHGPGQPHVGPQSPGQPPAPPCPLPQACQRCRCPAGAP GTPGKQGPAGQPGQGRPGAPGKSSGAGPPGAPGQPPGAPGKHGGPGQPGQPKNGVSHPTIPGPKGPSGS PGQPGKPGPAGVPGKPGPEGPPGVPVGPAGPSGKPGAPGQPGPHGPPGQDQAYCPCPPRSSVLKAKKRA	8
Minc10604	MQAHFSQATIIIFVAMFALAIFFVLPSSTVACGGGCCGCCATCGIGGFGGGFGGPGFGGPGFGGPGFGGGYGGGFA VGGFGGGYGGCGGGFGCGGGCYGCGKK	10
Minc10606	MQAHFSQATIIIFVAMFALAIFFVLPSSTVACGGGCCGCCATCGIGGFGGGFGGPGFGGPGFGGPGFGGGYGGGFA VGGFGGGYGGCGGGFGCGGGCYGCGKK	10
Minc18876	MQAHFSQATIIIFVAMFALAIFFVLPSSTVACGGGCCGCCATCGIGGFGGGFGGPGFGGPGFGGPGFGGGYGGGFA VGGFGGGYGGCGGGFGCGGGCYGCGKK	10
Minc13038	MAIIILFIISAFIAESNLAAIKNGDDCVILKNGGCMKSGGKDCSLGHKKCCSGSIGIPAHKPECIGKGRTPTKGQKC CKGKTDSKGKCI	10
Minc14652	MISITSFFLLFLLFLDFDSTSNICIEYCFVPPQQYSGCNICGGYGYGSGYGTGGYGGYVQQTQQLYVPPQQQYVP QQVVVQVQAGGGVGGSGYPSGQGYIPQSGGVIPQTTGGQSYVGTGNGGGNVGGGTSSCGSGGCSAGGTGGTTG GSNYGGGESSGGTVSGSGLGGSTGGTGGGETGGTGGEEGTGGTSGGSSGGSEGGYGGSTGGTGTDTASGLGT GGSPGSSYSGSQSGTQTSYQPAATQVATGENKQCCSANSVGCYQTSPPQTAAGGSVAVQPVAVQPTGTA QASSPQKGYGDVRRKKFSSRHRQRQIFGRGTMVNHMAMLSQLQPIPPVKREKNNGPPKPFRLFNKSPVDAMKS KGQSFENRAATFKKI	10
Minc03750	MLVQSLNYLLFIFLIFETCSTINVEIIQKRFRQLTGCSEQDHRSCDDVCKGDSYWYGFCSAWDGRDLKCSGSRYP LDDGNVCGPSRQQKCVEECRGKGQESGGYCVLPSSENRRGVKPCSCFGKPR	12
Minc04712	MLSYNLTLFVIFLFSVAIADLTADSEGLVKQMDRSVRSKRYGYYECCCYGGYGGYGDLSSEVLTDFISFLPR KKLIKNVEPVNKYFELSKENVKSAHLITKNENFIQNLANNLEEAKQYFNLSSECSLAEHFVKNVFNVISSDEKTN NPPQYKRRCRYQMCVLRHSIDIFKNCFIKIDTDFGKNFTDSDLQEILAYLNILDSVSPSRITLHLSHSIHQTPVHLRYQI CQELLNNQAILNCQLEIFNDKIFNEPGVDSLFNWLHYKKNIFDNACRNLCLMKYMANELIEKCKATKNLAKDLNL LESVSTNDCRSYLLIFHSNKIMNEFSLENTKSGEILMKNIKDLNVEVEVKCYSLTRCQLNMEEGVIRNYFSKLFDRFES LPLIRIH	16
Minc11260	MSSVKFSTLFAFLSIILVELPLSQSFLFGLGGGGDSKGGCCCPQCAPPPPTPCGSPCCCNPCGGAPPPVPVA PSCGGGGCGGCGSSVPIIVNCGCGCGK	20
Minc07307	MAKNMYSLCFTLLSILIANEINAGLFGNGDILLPSLVRGLRMKKQCCGGGGGGGGGGCGGGPPPPPPITCECP QQPRCECQQQRCECPQQQRCECPQQQRCECPQQQRCECPQQQRCECQQQKCECQQQRCECPQQQRCEC QQQKCECQQQRCECPQQQRCECPQQQRCECQQQKCECQQQRCECPQQPRCECPQRPPPTIT LKISSSGGGGCCGPPPPPCGCGGGGGGGGGCGGGRKRLLALLQRTAAA	48

Table S2 Primers used for qRT-PCR analysis and ISH

Gene	Primers used for qRT-PCR	Primers used for <i>in situ</i> hybridisation
Minc03314	F: TGGAGGAGGCTATTTTGGAGGTTATGC R: AGCTTCGGTAACCACCTCCACACA	qRT-PCR primers
Minc04712	F: AAGCGTCGTTGTCGCTACCAAATGT R: AGGTAATTCTAGAGGGGCAAACAGAATCC	N/A
Minc08148	F: TACGTGCAAAACGTTGCTGTTTCGG R: AGTCCACCAAATCCTGGAAATCCGC	qRT-PCR primers
Minc12024/ Minc06089	F: AAACGTCAAGGCTGGGGAGGATG R: CGGTCCATATCTAATCGCAGTTTGTC	qRT-PCR primers
Minc12754	F: TGGAGGGGAATGAGGAACCGACTTG R: GAGAGAAAAGATAACGCCACCAATAAGGC	Sense: AAAAAGCAGGCTTCACCATGTTTGT GGAGGGGAATGAGGAACC Anti-sense: AGAAAGCTGGGTGTTACTTTTTTA GGCTCTCCCCCTCTTTC
Minc13038	F: TGGAGGAAAAGACTGCTGTTGTTAGG R: CCAATACATTCAGTTTTGGATGTGCAGG	N/A
Minc14652	F: TATCAACCAGCAGCCACTCA R: CGTTGTCTGTGCCTTGAAGA	Sense: AAAAAGCAGGCTTCACCATGAATTG TATTGAATATTGTTTTG Anti-sense: AGAAAGCTGGGTGTTAAATTTTC TTGAAGTTGCTGCTC
Minc17611	F: GCATCAATTCGTGAAAAAGGGGAGAGAG R: CTGCCATTTTTCGATTCAGGTACCGC	Sense: AAAAAGCAGGCTTCACCATGAAGGA AGAAACAGGGTCTGA Anti-sense: AGAAAGCTGGGTGTTATTGAGC TTATTTTTAGGAGTTTC
Minc18288	F: AGATATGGCCCTTGTGGTTACGGCG R: TTCATGACTATGACGGCCTCCTCCG	N/A
Minc18636	F: ATGCCTCCGATGCCAGTTGTCCAACCA R: AGCCTTTGGCGTTGCAGCCTTTGGTGA	qRT-PCR primers
Minc18876 family	F: TTACCAAGCTCCACAGTTGCTTGCG R: CCAAAGCCTGGACCACCAAACCTG	qRT-PCR primers
GAPDH (Minc12412)	F: CGTGCAGCGGTTGAGAAGGA R: GCGTCCGTGGGTGGAATCAT	N/A
HK14 (Minc18753)	F: GCGAGACTTGCTTTTCACGA R: GAACCTGATGCAGGTGTTC	N/A

F: Forward primer, R: Reverse primer, N/A = not applicable

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Minc18876      ttttaagtttcgcctttcctaaaaaattaaaatgcaagctcattttctcacaagcaacaatt
Minc04822     ----gtttcgcctttcctaaaaaattaaaatgcaagctcattttctcacaagcaacaatt
Minc10604     ttttaagtttcgcctttcctaaaaaattaaaatgcaagctcattttctcacaagcaacaatt
Minc10606     ttttaagtttcgcctttcctaaaaaattaaaatgcaagctcattttctcacaagcaacaatt
                *****

Minc18876     atttttgtcgcaatggttcgcattagcgatattcgttttcttaccagctccacagttgct
Minc04822     atttttgtcgcaatggttcgcattagcgatattcgttttcttaccagctccacagttgct
Minc10604     atttttgtcgcaatggttcgcattagcgatattcgttttcttaccagctccacagttgct
Minc10606     atttttgtcgcaatggttcgcattagcgatattcgttttcttaccagctccacagttgct
                *****

Minc18876     tgcggaggtggatgctgtggttgttgtgcaacatggttaagagttcttttaacattgacat
Minc04822     tgcggaggtggatgctgtggttgttgtgcaacatggttaagagttcttttaacattgacat
Minc10604     tgcggaggtggatgctgtggttgttgtgcaacatggttaagagttcttttaacattgacat
Minc10606     tgcggaggtggatgctgtggttgttgtgcaacatggttaagagttcttttaacattgacat
                *****

Minc18876     tctaataaaaaatccttcagcggaatcggtggcctttggtggcggttttgggtggtccagg
Minc04822     tctaataaaaaatccttcagcggaatcggtggcctttggtggcggttttgggtggtccagg
Minc10604     tctaataaaaaatccttcagcggaatcggtggcctttggtggcggttttgggtggtccagg
Minc10606     tctaataaaaaatccttcagcggaatcggtggcctttggtggcggttttgggtggtccagg
                *****

Minc18876     ttttgggtggtccaggctttggtggtccaggcttcggcggtggttacggtggcggttttcgc
Minc04822     ttttgggtggtccaggctttggtggtccaggcttcggcggtggttacggtggcggttttcgc
Minc10604     ttttgggtggtccaggctttggtggtccaggcttcggcggtggttacggtggcggttttcgc
Minc10606     ttttgggtggtccaggctttggtggtccaggcttcggcggtggttacggtggcggttttcgc
                *****

Minc18876     tgttggagggtttggcggtggttatggaggatgcgaggagggttttgggtgcggtggtgg
Minc04822     tgttggagggtttggcggtggttatggaggatgcgaggagggttttgggtgcggtggtgg
Minc10604     tgttggagggtttggcggtggttatggaggatgcgaggagggttttgggtgcggtggtgg
Minc10606     tgttggagggtttggcggtggttatggaggatgcgaggagggttttgggtgcggtggtgg
                *****

Minc18876     atgctacggttgcggaagaaataagacctttacgaaatttttcgcaatcaaaaatggt
Minc04822     atgctacggttgcggaagaaataagacctttacgaaatttttcgcaatcaaaaatggt
Minc10604     atgctacggttgcggaagaaataagacctttacgaaatttttcgcaatcaaaaatggt
Minc10606     atgctacggttgcggaagaaataagacctttacgaaatttttcgcaatcaaaaatggt
                *****

Minc18876     atatgatacaatttatcaggaatattttgtgcttttgttcaattatacttagtcatttta
Minc04822     atatgatacaatttatcaggaatattttgtgcttttgttcaattatacttagtcatttta
Minc10604     atatgatacaatttatcaggaatattttgtgcttttgttcaattatacttagtcatttta
Minc10606     atatgatacaatttatcaggaatattttgtgcttttgttcaattatacttagtcatttta
                *****

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Fig. S2 CLUSTAL alignment of the transcript sequences of four members of the *Minc18876* gene family. The ORF is indicated in grey and the three mismatches in the 3'UTR in black. * means identical bases.

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Supplementary data chapter 1

These supplementary data were not included in the submitted paper.

1. *M. incognita* proteins produced in secretory organs and predicted to be involved in parasitism

We summarize in the table 1 the information concerning the *M. incognita* candidate effectors, the expression of which was specifically detected in the glands by ISH and/or immunolocalisation. Blast analysis against the *M. incognita* v1 genome (Abad et al., 2008) revealed that 33 % of the candidate effectors were detected as a single copy, 36 % as two copies and 32% as three or more copies (Fig. 19A). As indicated in the submitted paper, almost half (45) candidate effectors were expressed in the two SvGs including the genes encoding plant cell wall-modifying enzymes (Rosso *et al.*, 2011). The other half (35) of these genes were expressed in the DG (Table 2). Interestingly, RNA seq data highlighted that 53% of the SvG-specific genes are overexpressed in J2s compared to J3/J4, whereas 80% of DG-specific genes are overexpressed in J3/J4 compared to J2s (Fig. 19B). These results are in agreement with the observations of SvGs and DG being active in the early stage of parasitism and *in planta* after the migration phase, respectively (Davis *et al.*, 2000).

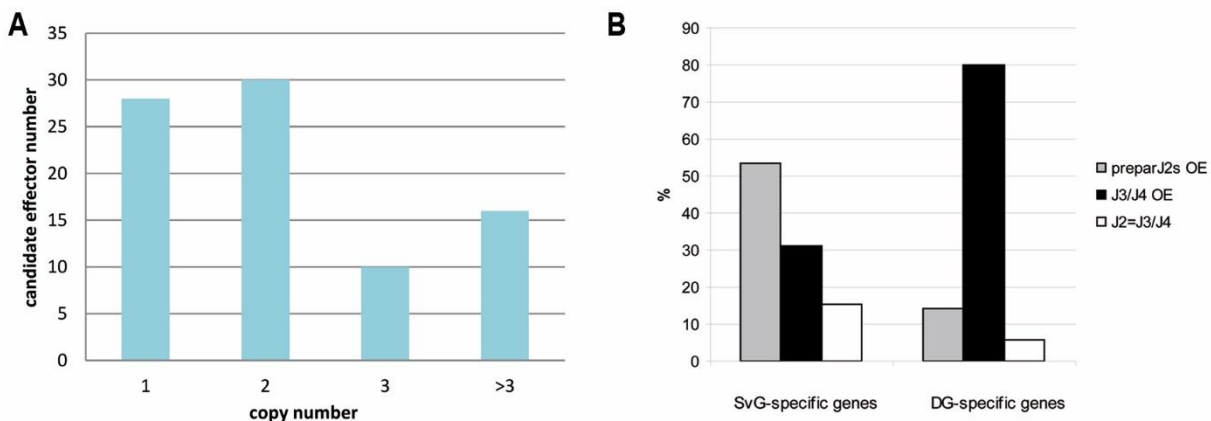


Figure 19: Copy number and expression of *M. incognita* candidate effector genes expressed in secretory organs.

(A) Copy number detected in *M. incognita* genome (version 1). (B) Expression profile in preparasitic J2s and parasitic J3/J4. OE, overexpression.

Table 2: *M. incognita* proteins produced in secretory organs and predicted to be involved in parasitism

Effector	NCBI accession	Length (bp)	Predicted function	Organs	SP	ISH	IL	References	Minc V1 ⁽³⁶⁾	Identities (Blastip)	RNAseq*		
											preJ2	J3-J4	J3-J4/preJ2
Mi-PEL-1	AAS88579	813	pectate lyase	SVG	+	+		(1, 2)	Minc04661	240/272	21	7	0.3
									Minc03619	231/238	27	0	0.0
									Minc08823	199/199	15	0	0.0
Mi-PEL-2	AAS88580	840	pectate lyase	SVG	-	+		(3, 2)	Minc11772	279/280	51	0	0.0
									Minc13942	240/255	13	0	0.0
Mi-PEL-3	AAW56829	837	pectate lyase	SVG	+	+	+	(4, 5)	Minc11928a	279/279	189	2	0.0
									Minc01521	223/275	0	0	nd
									Minc01517	223/275	0	0	nd
									Minc12947	214/277	0	0	nd
									Minc01515	186/224	0	0	nd
									Minc01514	184/265	1	0	0.0
Minc01810	157/159	115	0	0.0									
Mi-ENG-1	AF100549	1668	beta-1,4-endoglucanase	SVG	+	+	(3, 6, 7)	Minc13221a-b	506/506	242	1	0.0	
								Minc09298a-c	499/506	302	0	0.0	
								Minc09446a-c	482/505	198	0	0.0	
5A12B	AY422836	1706	beta-1,4-endoglucanase	SVG	+	+	(3)	Minc09298a-c	500/506	302	0	0.0	
								Minc13221a-b	493/506	242	1	0.0	
								Minc09446a-b	485/505	198	0	0.0	
								Minc09447b	431/455	1	0	0.0	
								Minc09447b	308/350	1	0	0.0	
8E08B	AY422837	1242	beta-1,4-endoglucanase	SVG	+	+	(3)	Minc09446a-b	308/350	198	0	0.0	
								Minc13221a-b	302/355	242	1	0.0	
								Minc09298a-c	300/355	302	0	0.0	
Mi-FG-1	AAM28240	1899	polygalacturonase	SVG	+	+	(8)	Minc18543b	627/633	30	0	0.0	
								Minc18543a	567/572	30	0	0.0	
Mi-XYL-1	AMF37276	987	beta-1,4-endoxylanase	SVG	+	+	(9)	Minc09178	307/328	3	0	0.0	
								Minc03215	275/293	11	0	0.0	
Mi-CBP-1	AF049139	995	cellulose-binding protein	SVG	+	+	(1, 10)	Minc14048	71/192	5	0	0.0	
								Minc00168	71/192	5	3	0.6	
Mi-CM-1	AAS82580	573	chorismate mutase	SVG	+	+	(3, 11)	Minc10536	47/155	17	12	0.7	
								Minc10536	48/163	17	12	0.7	
								nd	nd	nd	nd	nd	
Mi-ASP2	AAS82581	1209	aspartyl protease-like	SVG	+	+	(4)	Minc1599	105/202	655	518	0.8	
								Minc12868	105/202	205	72	0.4	
Mi-GSTS-1	ABN64198	609	glutathione-S-transferase	SVG	-	+	(13)	Minc11765	103/203	58	102	1.8	
								Minc08770	102/210	48	84	1.8	
16D10	AAZ77751 (Q6YKB)	129	CLE-like peptide	SVG	-	+	+	(1)	nd	nd	nd	nd	nd
Mi-SXP-1	576	SXP/Ral-2 protein	SVG	+	+	+	(14)	Minc16477	50/127	176	9	0.1	
								Minc11847	46/124	3	10	3.3	
								Minc10888	45/124	17	112	6.6	

Table 2, continue

Effector	NCBI accession	Length (bp)	Predicted function	Organs	SP	ISH	IL	References	Minc V1 ⁽³⁴⁾	Identities (Blastp)	RNAseq*		
											preJ2	J3-J4	J3-J4/preJ2
M-VAP-2	ABO38110	882	venom allergen-like protein	SvG	+	+		(15)	Mnci17115	269/294	153	0	0.0
									Mnci17155	245/294	50	0	0.0
									Mnci17117a-b	187/206	93	0	0.0
									Mnc099228a-b	180/201	6	0	0.0
									Mnci17154	176/191	40	0	0.0
									Mnci12763	171/207	1	0	0.0
									Mnci17158	166/168	173	0	0.0
									Mnci12508	155/160	18	0	0.0
									Mnci18036	155/160	20	0	0.0
									Mnc09032	154/160	16	0	0.0
Mnci18037	154/160	1	0	0.0									
Mi-MSP-1	AF013289	902	venom allergen-like protein	SvG	+	+		(16)	Mnci19171	112/117	33	0	0.0
5G05	AY135362	977	zinc metalloproteinase	SvG	+	+		(1)	Mnc07366	242/243	5	119	23.8
30G11	AY134440	1511	acid phosphatase	SvG	+	+		(1)	Mnci13373	178/426	26	71	2.7
									Mnc03442	146/430	4	11	2.8
									Mnc05138	137/382	2	5	2.5
10A07	AY142117	656	sodium/calcium/potassium exchanger	SvG	+	+		(1)	Mnc01732	105/326	3	17	5.7
									Mnc03667	60/149	5	2	0.4
									Mnc12295	55/148	15	3	0.2
CL5Contig2_1		675	Sec-2 protein	SvG	-	+		(7)	Mnc01079	190/190	342	1364	4.0
									Mnc08986	190/190	204	948	4.6
									Mnc03266	179/181	302	1101	3.6
CL2552Contig1_1		375	transhyrin-like protein	SvG	-	+		(7)	Mnc03267b	162/162	1	0	0.0
									nd	nd	nd	nd	nd
									Mnc02150	178/179	63	1429	22.7
CL321Contig1_1		537	translationally controlled tumour protein	SvG	-	+		(7)	Mnc06933	203/203	26	269	10.3
									Mnc09728	176/179	40	499	12.5
									Mnc07894b	429/429	11	83	7.5
CL312Contig1_1		1287	unknown	SvG	-	+		(7)	Mnc07893a-c	429/429	91	7	0.1
									Mnc18706a-c	421/429	85	7	0.1
									Mnc03829a-c	357/364	162	51	0.3
Minc01696		1728	protein kinase	SvG	+	+		(17)	Mnc01696	576/576	34	37	1.1
									Mnc04534	558/576	15	14	0.9
									Mnc03866	484/484	59	1	0.0
Minc00344		1155	unknown	SvG	+	+		(17)	Mnc00344	385/385	32	5	0.2
									Mnc04584	174/202	15	7	0.5
									Mnc04584	209/209	15	7	0.5
Minc04584		627	unknown	SvG	+	+		(17)	Mnc00344	174/202	32	5	0.2
									Mnc00344	467/467	2	1	0.5
									Mnc18033	467/467	2	1	0.5
Minc13292		1602	unknown	SvG	+	+		(17)	Mnc13292	534/534	145	6	0.0
									Mnc03325	433/539	1069	155	0.1
									Mnc08073	710/710	1	12	12.0
Minc08073		2130	unknown	SvG	+	+		(17)	Mnc10418	697/709	0	13	nd
									Mnc10418	697/709	0	13	nd

Effector	NCBI accession	Length (bp)	Predicted function	Organs	SP	ISH	IL	References	Minc V1 ⁽⁶⁴⁾	Identities (Blastp)	RNAseq*		
											preJ2	J3-J4	J3-J4/preJ2
Minc18033		1311	unknown	SvG	+	+		(17)	Minc18033	467/467	2	1	0.5
Minc13292		1602	unknown	SvG	+	+		(17)	Minc13292 Minc03325	534/534 433/539	145 1069	6 155	0.0 0.1
Minc08073		2130	unknown	SvG	+	+		(17)	Minc08073 Minc10418	7107/10 697/709	1 0	12 13	12.0 nd
Minc00469		195	unknown	SvG	+	+		(17)	Minc00469	65/65	1	0	0.0
Minc15401		948	unknown	SvG	+	+		(17)	Minc15401 Minc18636	3163/16 2823/16	5 4	186 148	37.2 37.0
Minc10418		2127	unknown	SvG	+	+		(17)	Minc10418 Minc08073	709/709 697/709	0 1	13 12	nd 12.0
Minc03328		1446	unknown	SvG	+	+		(17)	Minc03328 Minc11908	378/378 345/378	1 0	3 0	3.0 nd
Minc03325		1590	unknown	SvG	+	+		(17)	Minc03325	539/539	1069	155	0.1
Minc18636		936	unknown	SvG	+	+		(17)	Minc18636 Minc15401	3163/16 2823/16	4 5	148 186	37.0 37.2
Minc08146		222	unknown	SvG	+	+		(19)	Minc08146	74/74	608	4	0.0
2G02	AF531161	776	unknown	SvG	+	+		(1)	Minc09288	194/211	152	48	0.3
4D01	AF531162	785	unknown	SvG	+	+		(1)	nd	nd	nd	nd	nd
8D05	AF531169	1282	unknown	SvG	+	+		(1)	nd	nd	nd	nd	nd
8H11	AF531170	1228	unknown	SvG	+	+		(1)	nd	nd	nd	nd	nd
8E10B	AY422833	1217	unknown	SvG	+	+		(3)	nd	nd	nd	nd	nd
30H07	AY134439	994	unknown	SvG	+	+		(1)	Minc15159	227/261	20	15	0.8
31H06	AY134441	362	unknown	SvG	+	+		(1)	Minc04178	25/26	3	0	0.0
Minc16401		210	unknown	SvG	+	+		lab candidate	Minc16401 Minc13790 Minc18308	67/69 65/69	48 47 164	0 0 0	0.0 0.0 0.0
35A02	AY134444	2210	unknown	SvG	+	+		(1)	Minc02097 Minc01595	517/531 454/522	11 3	13 11	1.2 3.7
Minc03314		180	unknown	SvG	+	+		lab candidate	Minc03314		4	28767	7191.8
Minc12024		234	unknown	SvG	+	+		lab candidate	Minc12024 Minc06089	77/77 58/60	1 0	137 310	137.0 nd
Minc09973		1386	unknown	SvG	+	+		lab candidate	Minc09973		4222	2890	0.7
Minc09716		1386	unknown	SvG+intestine	+	+		lab candidate	Minc09716	462/462	1.9	45.2	23.8
6D4			unknown	SvG&DG	+	+		(5,20)	nd	nd	nd	nd	nd
Mi-CRT		1245	calreticulin	SvG&DG	+	+		(21)	Minc0693a	415/415	63	701	11.1
Mi-14-3-3-b	AAL40719	783	14-3-3	DG	-	+		(5,22)	Minc10258 Minc16550 Minc18869	260/261 257/261 247/261	11 32 3	19 19 16	7.2 0.6 5.3
10G02	AY135365	1499	thioredoxin	DG	+	+		(1)	Minc14653	400/424	1	2	2.0
Minc00108		612	metallopeptidase	DG	+	+		(19)	Minc00108 Minc11496 Minc00121	204/204 158/204 110/155	1 1 100	46 1 52	46.0 1.0 0.5

Table 2, continue

Effector	NCBI accession	Length (bp)	Predicted function	Organs	SP	ISH	IL	References	Minc V1 ^(M)	Identities (Blastp)	RNAseq*		
											preJ2	J3-J4	J3-J4/preJ2
Minc00469		195	unknown	SvG	+	+		(17)	Minc00469	65/65	1	0	0.0
Minc15401		948	unknown	SvG	+	+		(17)	Minc15401 Minc18636	316/316 282/316	5 4	186 148	37.2 37.0
Minc10418		2127	unknown	SvG	+	+		(17)	Minc10418 Minc08073	709/709 697/709	0 1	13 12	nd 12.0
Minc03328		1446	unknown	SvG	+	+		(17)	Minc03328 Minc11908	378/378 345/378	1 0	3 0	3.0 nd
Minc03325		1590	unknown	SvG	+	+		(17)	Minc03325 Minc13292	539/539 433/539	1069 145	155 6	0.1 0.0
Minc18636		936	unknown	SvG	+	+		(17)	Minc18636 Minc15401	316/316 282/316	4 5	148 186	37.0 37.2
Minc08146		222	unknown	SvG	+	+		(19)	Minc08146	7474	608	4	0.0
2G02	AF531161	776	unknown	SvG	+	+		(1)	Minc09288	194/211	152	48	0.3
4D01	AF531162	785	unknown	SvG	+	+		(1)	nd	nd	nd	nd	nd
8D05	AF531169	1282	unknown	SvG	+	+	+	(1)	nd	nd	nd	nd	nd
8H11	AF531170	1228	unknown	SvG	+	+		(1)	nd	nd	nd	nd	nd
8E10B	AY422833	1217	unknown	SvG	+	+	+	(3)	nd	nd	nd	nd	nd
30H07	AY134439	994	unknown	SvG	+	+		(1)	Minc15159	227/261	20	15	0.8
31H06	AY134441	362	unknown	SvG	+	+		(1)	Minc04178	25/26	3	0	0.0
Minc16401		210	unknown	SvG	+	+		lab candidate	Minc16401 Minc13790	67/69 65/69	48 164	0 0	0.0 0.0
35A02	AY134444	2210	unknown	SvG	+	+		(1)	Minc02097 Minc01595	517/531 454/522	11 3	13 11	1.2 3.7
Minc03314		180	unknown	SvG	+	+		lab candidate	Minc03314		4	28767	7191.8
Minc12024		234	unknown	SvG	+	+		lab candidate	Minc12024	71777	1	137	137.0
Minc09973		1386	unknown	SvG	+	+		lab candidate	Minc06089 Minc09973	58/60	0	310	nd
Minc09716		1386	unknown	SvG+intestine	+	+		lab candidate	Minc09716	462/462	19	45.2	23.8
6D4		1245	unknown	SvG&DG	+	+		(5, 20)	nd	nd	nd	nd	nd
M-CRT		1245	calreticulin	SvG&DG	+	+		(21)	Minc06693a	415/415	63	701	11.1
Mi-14-3-3-b	AAL40719	783	14-3-3	DG	-	+	+	(5, 22)	Minc10258 Minc16550 Minc18869	260/261 257/261 247/261	11 32 3	79 19 16	7.2 0.6 5.3
10G02	AY135365	1499	thioredoxin	DG	+	+		(1)	Minc14653	400/424	1	2	2.0
Minc00108		612	metallopeptidase	DG	+	+		(19)	Minc00108 Minc11496 Minc00121	204/204 158/204 110/155	1 1 100	46 1 52	46.0 1.0 0.5
Minc02097		1596	unknown	DG	+	+		(17)	Minc02097	532/532	11	13	1.2
Vf-EFF1/Minc17998		366	unknown	DG	+	+	+	(19)	Minc01595	458/520	2	8	4.0
Minc18861		450	unknown	DG	+	+		(17)	Minc17998 Minc18861 Minc11817	122/122 150/150 150/150	3 4 4	509 105 104	169.7 26.3 26.0

Effector	NCBI accession	Length (bp)	Predicted function	Organs	SP	ISH	IL	References	Minc V1 ⁽⁹⁴⁾	Identities (Blastp)	RNAseq*		
											preJ2	J3-J4	J3-J4/preJ2
Minc02097		1596	unknown	DG	+	+		(17)	Minc02097	532/532	11	13	1.2
									Minc01595	458/520	2	8	4.0
Mi-EFF1/Minc17998		366	unknown	DG	+	+	+	(19)	Minc17998	122/122	3	509	169.7
Minc18861		450	unknown	DG	+	+		(17)	Minc18861	150/150	4	105	26.3
									Minc11817	150/150	4	104	26.0
Minc12639		300	unknown	DG	+	+		(17)	Minc12639	100/100	25	5	0.2
Minc11817		450	unknown	DG	+	+		(17)	Minc11817	150/150	4	104	26.0
									Minc18861	150/150	4	105	26.3
Minc01595		1650	unknown	DG	+	+		(17)	Minc01595	520/520	2	8	4.0
									Minc02097	458/520	11.1	13.2	1.2
1C05B	AY422829	617	unknown	DG	+	+		(3)	nd	nd	nd	nd	nd
1D08B	AY422830	547	unknown	DG	+	+		(3)	Minc05757	170/171	0	0	nd
									Minc02313	97/109	1	169	169.0
									Minc02312	58/60	1	169	169.0
2E07	AF531160	694	unknown	DG	+	+		(1)	Minc06491	157/158	5	103	20.6
									Minc12379	93/103	2	43	21.5
2G10	AY135363	999	unknown	DG	-	+		(1)	Minc11979	102/109	4	2	0.5
									Minc01856	64/64	3	3	1.0
4D03	AY135364	864	unknown	DG	+	+		(1)	Minc03699	174/186	0	0	nd
									Minc08210	179/186	0	14	nd
4F05B	AY422831	763	unknown	DG	-	+		(3)	nd	nd	nd	nd	nd
5C03B	AY422832	589	unknown	DG	+	+		(3)	nd	nd	nd	nd	nd
6F07	AF531163	1339	unknown	DG	+	+		(1)	Minc03667	57/168	37	980	26.5
6G07	AF531164	762	unknown	DG	+	+		(1)	nd	nd	nd	nd	nd
									Minc06489	180/180	0	13	nd
									Minc18502	158/161	0	21	nd
7A01	AF531165	778	unknown	DG	+	+		(1)	Minc06490	156/160	0	20	nd
									Minc18501	158/163	0	23	nd
									Minc18500	153/160	0	7	nd
7E12	AF531166	757	unknown	DG	+	+		(1, 23)	Minc06482	170/174	0	5	nd
									Minc14900	167/176	4	18	4.5
									Minc12523	166/176	4	15	3.8
7H08	AF531168	1091	unknown	DG	+	+		(1)	Minc12377	92/102	1	5	5.0
9H10	AF531167	737	unknown	DG	+	+		(1)	Minc10160	97/166	0	0	nd
									Minc12617	178/181	3	11	3.7
11A01	AY134431	1266	unknown	DG	+	+		(1)	Minc12379	103/103	2	43	21.5
									Minc06491	94/104	5	103	20.6
									Minc06489	175/180	0	13	nd
									Minc06490	163/164	0	20	nd
12H03	AY134432	723	unknown	DG	+	+		(1)	Minc18502	157/161	0	21	nd
									Minc18500	154/160	0	7	nd
									Minc18501	157/163	1	23	23.0

Table 2, continue

Effector	NCBI accession	Length (bp)	Predicted function	Organs	SP	ISH	IL	References	Minc V1 ⁽⁹⁴⁾	Identities (Blastp)	RNAseq*		
											preJ2	J3-J4	J3-J4/preJ2
16E05	AY134436	1687	unknown	DG	+	+		(1)	Mnc18033	436/437	2	1	0.5
21E02	AY134438	704	unknown	DG	+	+		(1)	Mnc14900	174/176	4	18	4.5
									Mnc12523	173/176	4	15	3.8
									Mnc06482	164/174	0	5	nd
									Mnc12377	100/102	1	5	5.0
34D01	AY134442	782	unknown	DG	+	+	(1)	Mnc06489	172/180	0	13	nd	
								Mnc18500	156/160	0	7	nd	
								Mnc18502	154/161	0	21	nd	
								Mnc06490	151/160	0	20	nd	
34F06	AY134443	801	unknown	DG	+	+		(1)	nd	nd	nd	nd	nd
	AY142120	398	unknown	DG	-	+		(1)	Mnc01678	234/237 (blasin)	4	2	0.5
35E04	AY142121	836	unknown	DG	+	+		(1)	Mnc15702	124/141	0	2	nd
									Mnc07023	124/124	1	9	9.0
28B04	AY142119	748	unknown	DG	+	+		(1)	Mnc14900	168/174	4	18	4.5
									Mnc12523	167/174	4	15	3.8
									Mnc06482	162/175	0	5	nd
									Mnc12377	101/102	1	5	5.0
Mnc12754		291	unknown	DG	+	+		lab candidate	Mnc12754	97/97	11	243	22.1
Mnc17611	522	unknown	DG	+	+	+		lab candidate	Mnc13608	90/96	15	571	38.1
									Mnc01345	91/96	8	184	23.0
Mnc17612	396	unknown	DG	+	+	+		lab candidate	Mnc17611	174/174	8	304	38.0
									Mnc13062	112/129	18	1838	102.1
Mnc18876	309	unknown	DG	+	+	+		lab candidate	Mnc18876	102/102	9	17052	1894.7
									Mnc10606	102/102	10	17226	1722.6
									Mnc10604	102/102	10	17298	1729.8
									Mnc04822	88/88	11	17821	1620.1
Mnc18016	714	unknown	DG	+	+	+		lab candidate	Mnc18016	235/235	7	2	0.3
									Mnc18873	230/235	4	9	2.3
Mnc14652	1164	unknown	DG+intestin	+	+	+		(18)	Mnc18347	218/235	5	8	1.6
									Mnc14652	388/388	2	495	247.5
Mnc09978	759	unknown	glands+intestin	+	+	+		lab candidate	Mnc09978	237/237	1837	1223	0.7
									Mnc11857a,d	201/237	2538	1978	0.8
Mnc08335	555	unknown	amphids	+	+	+		lab candidate	Mnc08335	185/185	5	0	0.0
									Mnc15577	573/575	11	145	13.2
CL1191Contig1_1	1740	CDC48-like	phasmsids	-	+	+		(7)	Mnc13545	572/580	14	202	14.4
									Mnc0801	361/361	19	384	20.2

ISH in situ hybridisation; IL, immunolocalisation; SvG, subventral glands; DG, dorsal gland; RG, rectal gland. * FPKM (fragments per kilobase of exon per million reads mapped)

(1) Huang et al. 2003; (2) Huang et al. 2005b; (3) Huang et al. 2004; (4) Vieira et al. 2011; (5) Vieira et al. 2012; (6) Rosso et al. 1999; (7) Bellafiore et al. 2008; (8) Jaubert et al. 2002a; (9) Dautova et al. 2001; (10) Ding et al. 1998; (11) Huang et al. 2005a; (12) Neveu et al. ; 2003; (13) Dubreuil et al. 2007; (14) Tytgat et al. 2005; (15) Wang et al. 2007; (16) Ding et al. 2000; (17) Rutter et al. 2014; (18) Danchin et al. 2013; (19) Jaouannet et al. 2012; (20) Davis et al. 1992; (21) Jaubert et al. 2005; (22) Jaubert et al. 2004; (23) Souza et al. 2011; (24) Sembliat et al. 2001; (25) Doyle and Lambert 2002; (26) Hu et al. 2013; (27) Doyle and Lambert 2003; (28) Lin et al. 2013; (29) Iberkleid et al. 2013; (30) Haegeman et al. 2008; (31) Roze et al. 2008; (32) Dinh et al. 2014; (33) Gleason et al. 2008; (34) Abad et al. ; 2008.

RKN candidate avirulence proteins

Twenty-four candidate avirulence proteins have been identified in *M. incognita* and *M. javanica* as present in avirulent near-isogenic lines and absent in Mi-1 virulent lines (Semblat *et al.*, 2001; Neveu *et al.*, 2003; Gleason *et al.*, 2008). Eighteen were shown to correspond to one to four genes in the *M. incognita* genome (Table 3). These genes appeared preferentially overexpressed in the J3/J4 stages compare to the J2 stage except *MAP1* genes that are overexpressed (even with small FPKM values) in the preparasitic J2s.

Table 3. *M. incognita* and *M. javanica* candidate avirulence proteins

Effector	NCBI accession	Length (bp)	Predicted function	Organs	SP	ISH	IL	References	Minc V1	Identities (Blastn)	RNAseq*		
											preJ2	J3-J4	J3-J4/preJ2
ET1	AJ544640	321	unknown	nd	-			(3)	Minc07013 Minc00126	305/305 291/305	10 5	69 22	6.9 4.4
ET2	AJ544641	319	ubiquitin carboxyl-terminal hydrolase 2 peptidase C19 spectrin repeats	nd	-			(3)	Minc06101 Minc06004	318/319 257/267	18 3	164 65	9.1 21.7
ET3	AJ544642	167	SPK1 component	nd	-			(3)	Minc03310 Minc08411 Minc16634	120/120 92/117 77/96	6 11 0	116 4 5	19.3 0.4 nd
ET4	AJ544643	223	MIF4G-like, type 3; initiation factor eIF-4 gamma, IM3	nd	-			(3)	Minc02744	171/173	8	49	6.1
ET5	AJ544644	167	HECT domain (ubiquitin transferase)	nd	-			(3)	nd	nd	nd	nd	nd
HM1	AJ544623	525	membrane bound O-acyl transferase, MBOAT	SvG	+	+		(3)	Minc00115	263/267	7	57	8.1
HM2	AJ544624	97	ribosomal protein	nd	-			(3)	Minc05791	83/85	56	525	9.4
HM3	AJ544625	559	nematode cuticle collagen	nd	-			(3)	Minc01400a,b,c Minc04389a,b,c Minc01401a,b Minc04390	559/559 552/559 527/527 530/537	1 0 0 0	163 246 246 59	163.0 nd nd nd
HM4	AJ544626	169	unknown	nd	-			(3)	Minc08488 Minc07195	157/167 114/121	5 6	631 1	126.2 0.2
HM5	AJ544627	80	unknown	nd	-			(3)	Minc03315 Minc13287	75/76 73/76	13 0	6 3	0.5 nd
HM6	AJ544628	274	laminin B; immunoglobulin	nd	-			(3)	Minc00113	250/259	20	576	28.8
HM7	AJ544629	205	unknown	DG	-	+		(3)	nd	nd	nd	nd	nd
HM8	AJ544630	221	linnexin	nd	-			(3)	Minc03309	216/219	7	95	13.6
HM9	AJ544631	650	peptidase A1	nd	-			(3)	-	nd	0	0	nd
HM10	AJ557572	1152	peptidase C1A, papain	intestine	+			(3)	Minc13498 Minc13497	453/459 470/474	73 73	2745 2745	37.6 37.6
HM11	AJ544633	300	unknown	nd	-			(3)	nd	nd	nd	nd	nd
HM12	AJ544634	936	unknown	DG	-	+		(3)	Minc11007	837/870	13	30	2.3
HM13	AJ544635	204	unknown	nd	-			(3)	Minc15525 Minc15960	145/149 143/149	0 0	0 0	nd nd
PM1	AJ544636	672	WW/Rep5/WWP	intestine	-			(3)	Minc12830	370/370	7	38	5.4
PM2	AJ544637	183	SKP1 component	nd	-			(3)	Minc03310	138/151	6	116	19.3
PM3	AJ544638	201	Gamma-tubulin complex component 2	nd	-			(3)	Minc07324 Minc01067	200/201 193/201	9 2	6 8	0.7 4.0
PM4	AJ544639	169	pyrokinin, histidine kynase	nd	-			(3)	nd	nd	nd	nd	nd
Mi-MAP1	CAC27774	1374	unknown	amphids	+	+		(1,2)	Minc00158 Minc10366 Minc00365 Minc10365	438/495 (blastp) 225/234 231/338 221/259	9 3 25 5	1 0 2 0	0.1 0.0 0.1 0.0
MiCG1	EU214531	3154	unknown	nd	-			(4)	Minc15966	167/168	12	42	3.5

ISH, *in situ* hybridisation; IL, immunolocalisation; SvG, subventral glands; DG, dorsal gland; RG, rectal gland. * FPKM (fragments per kilobase of exon per million reads mapped)

(1) Sembilat et al. 2001; (2) Vieira et al. 2012; (3) Neveu et al. ; 2003; (4) Gleason et al. 2008

2. *In situ* hybridization and functional annotation of laboratory putative effectors.

In another approach to identify *M. incognita* effectors in our laboratory, Nhat-My Truong and Michaël Quentin focused on candidate secreted nuclear proteins. Based on previous functional annotation analysis and transcriptomic data (EST, RNA-seq) (Danchin et al. 2013, Jaouannet et al. 2012, Nghia et al., submitted, unpublished data), 167 proteins were identified as potential secreted proteins and containing NLS or DNA-binding domain. All these candidates are small proteins with a size less than 500 amino acids. I was in charge to initiate the functional analysis of seven of these candidates performing *in situ* hybridisations (ISH) and silencing experiments.

ISH was carried out two times for two candidates (*Minc16401* and *Minc17612*) and one time for four candidates (*Minc03948*, *Minc09716*, *Minc15167* and *Minc09973*) to investigate their localization in the nematode. Full length CDS of these seven proteins were used to synthesize *in situ* probes. A visible staining signal was observed for all seven antisense probes in pre-parasitic J2s whereas no signal was observed in the sense control probe (Figure 19). Three genes were observed to be specifically expressed in the esophageal glands, two in the SvG (*Minc16401* and *Minc09973*) and one in the DG (*Minc17612*), whereas three genes were found in the intestine (*Minc03948* and *Minc15167*). One gene (*Minc09716*) was detected in both intestine and SvG.

Furthermore, two candidate detected in the SvG (*Minc16401* and *Minc17612*) and the dorsal gland-specific EFF1 (*Minc17998*) (Jaouannet et al. 2012) were chosen for functional analysis using small interfering RNA (siRNA) to validate their potential role as effectors in plant-nematode interaction. *M. incognita* treated J2 larvae were treated 1 h with specific-siRNA or a control siRNA with no target in the *M. incognita* genome. 24 h after soaking, treated J2s were used for qRT-PCR and for tomato plant infection.

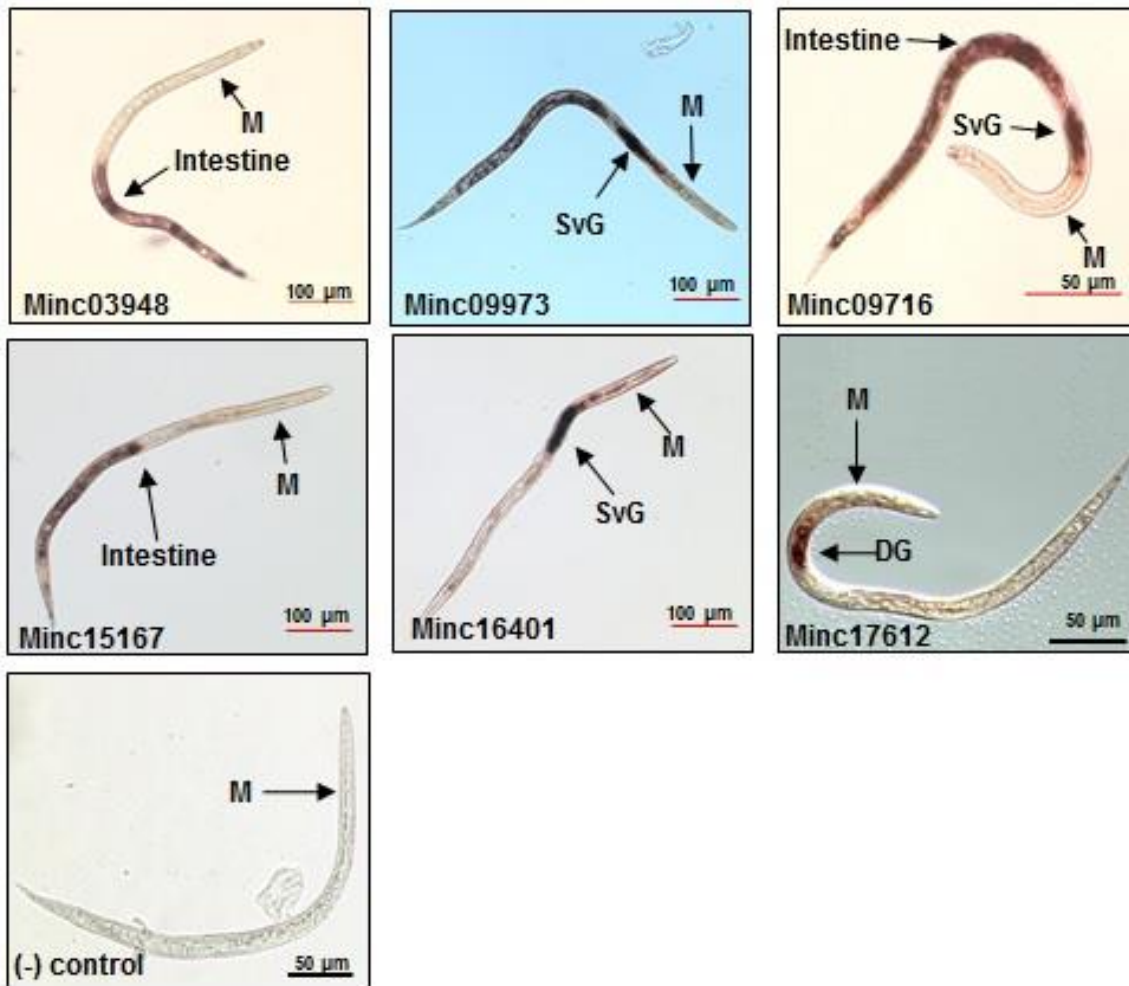


Figure 19: Tissue expression of candidate secreted effector in *M. incognita* pre-parasitic juveniles.

Transcripts were localized by *in situ* hybridisation using gene-specific digoxigenin-labelled probes. Transcripts from Minc16401 and Minc09973 were localized in the subventral gland (SvG); transcript from Minc 17612 was localised in the dorsal gland (DG); transcripts from Minc09716 were localized in the SvG and also in the intestine; transcript from Minc03948, Minc15167 was localized in the intestine. The sense control probe (-) of Minc16401 showed no labelling of nematode tissues. M, metacarpus.

So far, qRT-PCR demonstrated that all three genes showed a reduction of the targeted transcript expression 24h after soaking compared to their expression in the control sample (Figure 20). Only 20% reduction was observed for *EFF1/Minc17998* since a strong effect was observed for *Minc17612* with 85% depletion of transcript expression.

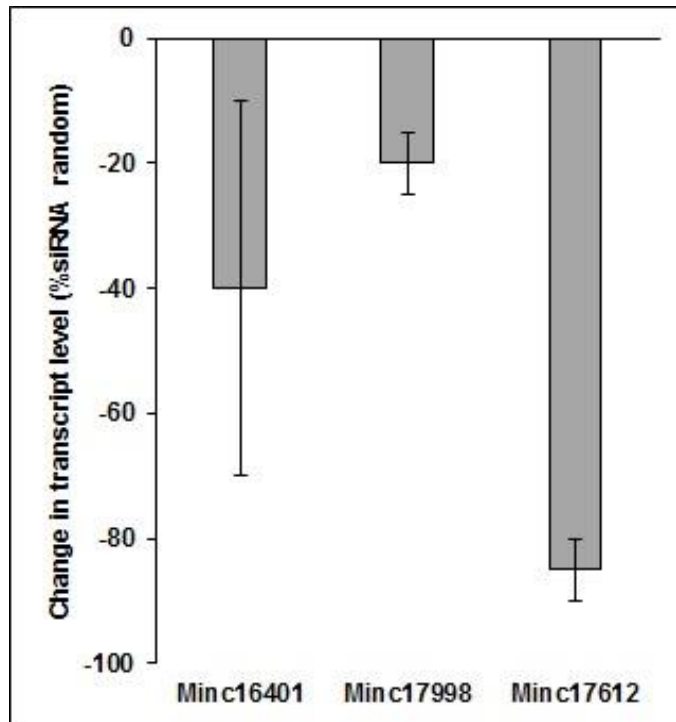


Figure 20: Transcript abundance percentage change in siRNA soaked J2s relative to control.

siRNA induced significant change in the targeted transcript expression level. Transcript level for each of the targeted gene was measured by qPCR 24h after soaking treatment and compared to their transcript level in control J2 samples treated with siRNA with no target in the M. incognita genome. The figure showed results of two biological replicates.

Six weeks after infection, galls and egg masses were counted for the three candidates and the control. All these siRNA-treated samples showed neither significant nor reproducible reduction in galls or egg masses numbers (Figure 21).

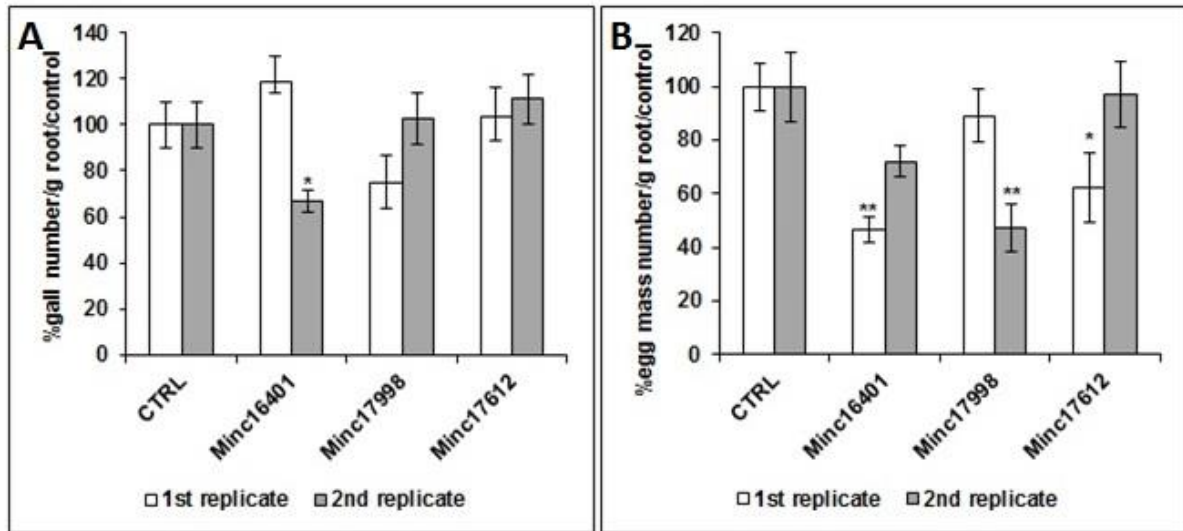


Figure 21: Effect of candidate gene silencing on nematode infection and reproduction.

J2s larvae were soaked with siRNA targeting specifically *Minc* genes or with siRNA with no target in *M. incognita* genome (Control, CTRL) and used to inoculate tomato plants. **A**, Variation of percentage of galls number/g root compared to control. **B**, Variation of percentage of egg masses number/g root compared to control. The white and grey columns represent results of the first and second independent biological replicates, respectively. Error bars were calculated by standard error of the mean (SEM). Statistical analysis was performed by Mann-Whitney test. *P*-value signification codes are as follows: 0.001 ‘***’, 0.005 ‘*’.

CHAPTER 2: Transcriptome Profiling of the Root-Knot Nematode *Meloidogyne enterolobii* During Parasitism and Identification of Novel Effector Proteins

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In this manuscript, we reported a preliminary analysis of the *M. enterolobii* transcriptome. Actually, this study is performed in collaboration with Dr. Sebastian Kiewnick (Agroscope, Switzerland) and will be completed before submission.

ABSTRACT

The root-knot nematode species *Meloidogyne enterolobii* (syn. *M. mayaguensis*) represent a new threat for global agriculture due to its wide host range and its capacity to reproduce on commercial plant rootstocks resistant to other *Meloidogyne* species. *M. enterolobii* induces severe root gall and plant stunted growth in many plant species worldwide. New strategies to control this pest are urgently needed. However, data on the pathogenicity of this obligate endoparasite are scarce at the molecular level. To investigate the genes and pathways that might control plant infection and host range specificity, we used Illumina sequencing to perform a *de novo* assembly of *M. enterolobii* transcriptome. In the present study, the transcriptomic profiles of the preparasitic J2s and isolated parasitic J3-J4s were investigated by next-generation sequencing. The *de novo* assembly of 174 million high-quality read pairs yielded 127,355 contigs with an average length of 495 bp. A first analysis of the *M. enterolobii* proteome consisting of 103,075 proteins indicated that 24,696 possessed a functional annotation. In total 11,399 and 9,613 proteins have been assigned a Pfam domain and a Gene Ontology term, respectively. Among them, 1,227 were found to participate in 103 KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. In addition, 1,632 putative secreted proteins without transmembrane domain were identified, in which 280 showed at least one GO term. The study of known RKN candidate secreted effectors showed that these proteins were well conserved across RKNs, except five proteins absent from *M. enterolobii* transcriptome. This transcriptomic analysis provides new insights into the development and host-parasite interactions of this plant-pathogen and highlight differences and similarities with other RKN species.

Key words: effector, *Meloidogyne enterolobii*, *Meloidogyne mayaguensis*, pathogenicity, RNA-seq, root-knot nematode, transcriptome.

Introduction

Root-knot nematodes (RKNs), *Meloidogyne* spp., are ones of the most damaging plant pathogens in the world with a broad host range encompassing all crop species (Trudgill & Blok, 2001; Favery *et al.*, 2016). These microscopic worms induce typical root deformations, known as galls, which result in weak and poor-yielding plants. About one hundred RKN species have been described so far (Hunt & Handoo, 2009), and those with asexual reproduction are the most polyphagous and damaging pests, e.g. *M. incognita*, *M. javanica*, *M. arenaria* and *M. enterolobii*. *M. enterolobii* was described from a population isolated from a tree species (*Enterolobium contortisiliquum*) in China (Yang & Eisenback, 1983) and it was shown later that *M. mayaguensis* was a junior synonym of *M. enterolobii* (Karssen *et al.*, 2012). This species has received a great attention as a new risk for global agriculture because of its worldwide distribution and progressive invasion of new territories (Tigano *et al.*, 2010; Castagnone-Sereno, 2012). In addition, this aggressive species is not controlled by, and reproduce on, commercial plant rootstocks resistant to *Meloidogyne* spp., e.g. tomato and pepper genotypes carrying the *Mi*, *Me* and *N* resistance genes, respectively (Brito *et al.*, 2007; Cetintas *et al.*, 2007; Kiewnick *et al.*, 2009). Recently, this pest was added to the EPPO A2 quarantine list in Europe (OEPP/EPPO, 2014).

The life cycle of this microscopic sedentary endoparasitic nematode takes three to ten weeks to complete, depending on the environmental conditions. Free-living second-stage juveniles (J2s) invade the root in the elongation zone. After the migratory phase, the nematode turns into sedentary life style, establishes and maintains a specialized feeding site, composed of few giant cells, that are hypertrophied and multinucleate as described for *M. incognita* (Caillaud *et al.*, 2008b). The division of the vascular cells surrounding the nematode and the giant cells lead to the formation of a typical gall, observed as symptoms of the infection. After giant cell initiation, the parasitic J2s grow up through three further moults (parasitic J3 and J4) to adult females or males. Finally, pear-shaped females reproduce by mitotic parthenogenesis and release eggs onto the root surface in a protective gelatinous matrix to complete the nematode life cycle. Considering its pathogenic peculiarities, our knowledge about the molecular determinants of interactions between plants and *M. enterolobii* is poorly understood (Castagnone-Sereno, 2012).

RKNs have developed various strategies for their successful parasitism in host plants, including the secretion of numerous proteins, named effectors, into their host to suppress the plant defense and promote the formation of giant cells (Mitchum *et al.*, 2013). Since the sequencing of the genome of the free-living model nematode *Caenorhabditis elegans* (Consortium, 1998), the development of high through-put sequencing technologies over ten years ago has opened new doors to identify the parasitic nematode features, including effectors, necessary for disease development. So far, genome sequences have been successfully reported for five plant-parasitic nematodes, including two RKN species, *M. incognita* and *M. hapla* (Abad *et al.*, 2008; Opperman *et al.*, 2008; Blanc-Mathieu *et al.*, 2016), the pine-wood nematode *Bursaphelenchus xylophilus* (Kikuchi *et al.*, 2011) and recently, two cyst nematode species *Globodera pallida* and *G. rostochiensis* (Cotton *et al.*, 2014; Eves-van Den Akker *et al.*, 2016). Comparison of the two RKN genomes pointed to a series of features common to the two species but also revealed differences that may be associated with the different modes of reproduction (Bird *et al.*, 2009; Castagnone-Sereno *et al.*, 2013). Genome mining combined with protein function analysis led to the identification of novel effector-like proteins (Danchin *et al.*, 2013). In addition, transcriptomic analyses, allowing additional insights for quantitative studies of differential gene expression, have been developed at the level of organisms or specific tissues, and made possible the characterisation of numerous plant-parasitic nematode effectors (Jaouannet *et al.*, 2012; Haegeman *et al.*, 2013; Danchin *et al.*, 2013; Eves-van den Akker *et al.*, 2014; Bauters *et al.*, 2014; Rutter *et al.*, 2014; Zheng *et al.*, 2015; Fosu-Nyarko *et al.*, 2016; Petitot *et al.*, 2016; Pogorelko *et al.*, 2016). Thus, Rutter *et al.* 2014 identified 91 putative effectors from the transcriptome of *M. incognita* isolated gland-cells. *De novo* analysis of the rice RKN *M. graminicola* allowed the identification of 15 effector candidates (Petitot *et al.*, 2016). Moreover, combining genomic and transcriptomic information have been used to improve genomic annotation and identified 117 potential novel effectors in the cyst nematode *G. pallida* (Cotton *et al.* 2014).

To date, little is known about *M. enterolobii* genes involved in plant parasitism. Only one candidate effector named MeTCTP, showing similarities with translationally-controlled tumour proteins was functionally characterized (Zhuo *et al.*, 2016). The silencing of MeTCTP by *in planta* RNAi resulted in an attenuation of parasitism. Moreover, MeTCTP was shown to be able to suppress programmed-cell death triggered by the pro-apoptotic protein BAX. In the present study, we performed Illumina RNA-sequencing of *M. enterolobii* pre-parasitic J2 stage and J3-J4

parasitic stages dissected from the root tissue, respectively. We combined the transcriptomic data with protein function analysis to describe the *de novo* transcriptome of the nematode, developed a pipeline to identify a set of new *M. enterolobii* effector candidates and initiate the comparison with other *Meloidogyne* species.

Material and methods

Establishment, maintenance and sample preparation of *M. enterolobii*

A population of *M. enterolobii* (Godet, Guadeloupe) isolated from tomatoes in French West Indies was multiplied on resistant tomato cv. Piersol in growth chambers at 25°C with a 12 h photoperiod. To initiate the culture, infected tomato root stored at -80°C were cut in small pieces and use to inoculate a tomato plant. After seven weeks, the infected root was cut, mixed in the infected soil and use to inoculate 12 tomato plants in separate pots. Nine plants were used to collect J3-J4, female and eggs. The remaining three infected roots and soil were used to reinfect 12 tomato plants and produce the next *M. enterolobii* generation. Eggs and freshly hatched J2s were collected as described previously (Rosso *et al.*, 1999). Parasitic stages J3-J4 and females were collected from tomato roots 14 and 21 days after inoculation, respectively, by incubation in 15% (v/v) Pectinex Ultra SP-L (Novozymes, Bagsvaerd, Denmark) and 7.5% Cellulase from *Trichoderma reesei* ATCC 26921 (Sigma-Aldrich, USA) overnight. The samples were purified from root debris by filtering through 40 µm sieves and then collected manually under binocular microscope. The population was verified based on isoenzyme phenotype from *Meloidogyne* spp. females as described previously (Esbenshade & Triantaphyllou, 1985). The specific identification was then confirmed by PCR on genomic DNAs using sequence characterised amplified region (SCAR) species-specific markers for *M. enterolobii*, *Meloidogyne* spp. and *M. incognita* as previously described (Zijlstra *et al.* 2000; Tigano *et al.* 2010). Nematode genomic DNA was purified from aliquots of 200–300 µl eggs by a phenol-chloroform method (Sambrook *et al.*, 1989). The primers used are given in the Table S1. Previously characterized *M. incognita* and *M. enterolobii* genomic DNA were used as controls.

Experimental determination of nuclear DNA content

Flow cytometry was used to perform accurate measurement of cells DNA contents in *M. enterolobii* compared to internal standards with known genome sizes: *Caenorhabditis elegans*

strain Bristol N2 (approximately 200 Mb at diploid state) and *Drosophila melanogaster* strain Cantonese S. (approximately 350 Mb at diploid state) as previously described (Blanc-Mathieu *et al.*, 2016). Briefly, nuclei were extracted from two hundred thousand J2s as previously described (Perfus-Barbeoch *et al.*, 2014) and stained with 75 µg/mL propidium iodide and 50 µg/mL DNase-free RNase. Flow cytometry analyses were carried out using a LSRII / Fortessa (BD Biosciences) flow cytometer operated with FACSDiva v6.1.3 (BD Biosciences) software. The DNA contents of the *M. enterolobii* samples were calculated by averaging the values obtained from three biological replicates.

Construction of cDNA libraries and Illumina sequencing

Total RNAs were extracted from preparasitic J2s and parasitic J3-J4 (three independent biological replicates) using TriZol Reagents (Invitrogen, Carlsbad, CA, USA) according to protocol available from Invitrogen. Total RNAs were re-suspended in 11 µl of Nuclease free water and RNA purity and concentration were detected by 2100 Bioanalyser (Agilent technologies, Santa Clara, CA, USA). Six cDNA libraries were constructed using the Ovation Universal RNA-seq system (Part No. 0343) (Nugen technologies, Inc, San Carlos, CA, USA) with an input of 100 ng total RNA. Ribodepletion was performed using 105 primers designed from the 18S and 28S rRNA consensus sequences in respecting the requirement of Nugen system: 15-25 nucleotides in length spaced every 70-100 bp of the target region, melting temperature from 60-65°C and with minimal secondary structure (Table S2 and Figure S1, at the end of the manuscript). Each library was sequenced using 75 base-length read chemistry in a single flow cell on the Illumina NextSeq500 (IPMC, France Genomic platform, Sophia Antipolis, France).

***de novo* assembly of the *M. enterolobii* transcriptome**

Ribosomal RNA sequences were identified and removed from the raw sequences using SortmeRNA (Kopylova *et al.*, 2012). The quality of each library was then controlled with FastQC, followed by the cleaning of contaminating sequences (adapters, sequence copies, short or long sequences, low-quality sequences) by Cutadapt and PrinSeq. Using Trinity, high quality reads were aligned for *de novo* assembly of *M. enterolobii* transcriptomes. Redundancy was eliminated with a home-made program with a minimum identity of 93 and minimum of overlap of 100. BUSCO (Benchmarking Universal Single-Copy Orthologs) was then used to blast against the core

genome of Metazoan or Eukaryota to evaluate the quality of the transcriptome (Simao *et al.*, 2015).

Prediction and annotation of the proteome

Transcripts were first translated using the Transeq program (Li *et al.*, 2015). InterProScan was performed on the candidate proteins to identify protein signatures referenced in the InterProScan database, transmembrane domains (TM) and signal peptides (Jones *et al.*, 2014). Blast2GO (Conesa *et al.*, 2005) was used for the functional annotation including Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis. GO-term enrichment of putative *M. enterolobii* effectors was estimated by using the Bioconductor package GStat (Falcon & Gentleman, 2007) with a p-value cutoff of 0.01. Briefly, GO-terms carried by proteins with signal peptide and no TM domain were extracted and were checked for enrichment in Molecular Functions and Biological Pathways against whole *M. enterolobii* GO-terms containing the proteome. For known RKN effectors and putative avirulence proteins, BLASTp analyses were carried out with an e-value threshold of 0.01 and without low complexity against *M. enterolobii* protein database for homologue identification. BLASTp hits were considered as significant when identity > 50% and bit score > 100.

Results

M. enterolobii characterization and transcriptome sequencing output

To ensure the purity of the *M. enterolobii* isolate used in this study, an isoenzyme analysis was performed from females collected from tomato roots. The analysis of enzymatic profile of all tested females showed a *M. enterolobii* esterase phenotype with two major bands (Fig. S2A, arrows) as previously described (Esbenshade & Triantaphyllou, 1985). *M. javanica* females, used as a standard control, showed a typical profile with three major bands (Fig. S2A). The species identification was then confirmed by the analyses of SCAR species-specific molecular markers. A visible band was obtained for all positive control samples whereas no band was obtained with the negative ones. Genomic DNA (gDNA) from produced *M. enterolobii* eggs was successfully amplified at 900 bp and 520 bp with *Meloidogyne*-specific and *M. enterolobii*-specific primers, respectively. No signal was observed using *M. incognita*-specific primers (Fig. S2B).

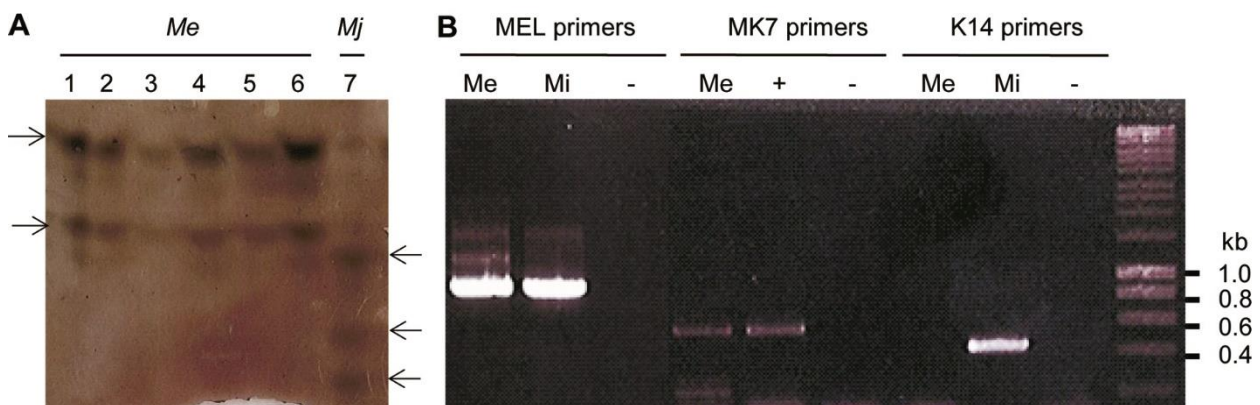


Fig. S2 *Meloidogyne enterolobii* population identification.

A, Isoenzyme esterase phenotype of *M. enterolobii* female extracted from tomato root. 1-6, *M. enterolobii* female. 7, *M. javanica* female used as standard control. **B**, Specific amplification with nematode genomic DNA extracted from eggs and the SCAR species-specific primers for *Meloidogyne* spp., *M. incognita* and *M. enterolobii* populations. Me, *M. enterolobii*. Mj, *M. javanica*. Mi, *M. incognita*. +, positive control. -, negative control (water instead of genomic DNA).

In addition, in the absence of *M. enterolobii* genome data, we measured DNA content in J2 nuclei with a flow cytometer. Flow cytometry experiments gave a DNA content estimated value of 274.69 ± 18.52 Mb (Fig. S3). Based on these measures and previous RNA-seq experiments on *M. incognita* (Danchin et al., 2013), we estimated that the depth for optimal transcriptome sequencing will be achieved with 3 replicates, for statistical analysis, with a minimum of 40 M paired-end reads per replicate.

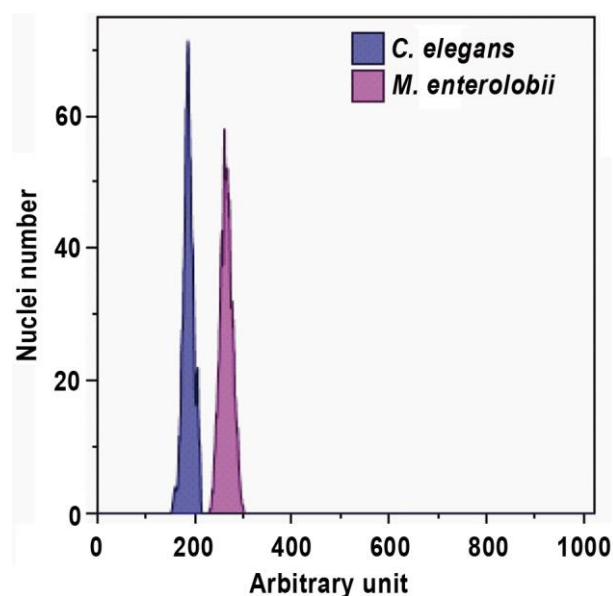


Fig. S3 Nuclei DNA content of *M. enterolobii* determined by flow cytometry.

Cytogram example obtained after gating on G0/G1 nuclei (arbitrary units) from M. enterolobii when processed mixed together with C. elegans as an internal standard (diploid genome size is 200 Mb).

To comprehensively cover the *M. enterolobii* transcriptome, total RNAs were extracted from preparasitic J2s and dissected parasitic J3-J4s. The six ribodepleted cDNA libraries were subjected to Illumina NextSeq500 sequencing and a total of 520,733,592 reads were obtained (Fig. 1). Ribosomal RNA (rRNA) was still highly represented in the libraries from 34.42% of the reads in the J2.3 library to 88.1% in the J3-4.1 library (Table S3).

Table S3. Number of reads and presence of ribosomal RNA in the libraries after sequencing

	J2-1	J2-2	J2-3	J3-4-1	J3-4-2	J3-4-3
Total reads	100,974,060	85,227,192	86,571,576	79,910,338	85,195,294	82,855,132
Mean read length (bp)	72	73	72	72	72	73
28S rRNA (%)	25.84	48.84	20.44	63.03	55.74	52.63
18S rRNA (%)	11.05	20.11	9.98	18.85	22.98	18.23
Total rRNA (%)	41.19	74.65	34.42	88.09	86.01	78.19
Reads after cleaning	58,198,212	21,071,954	56,003,348	8,977,252	12,236,338	17,531,838

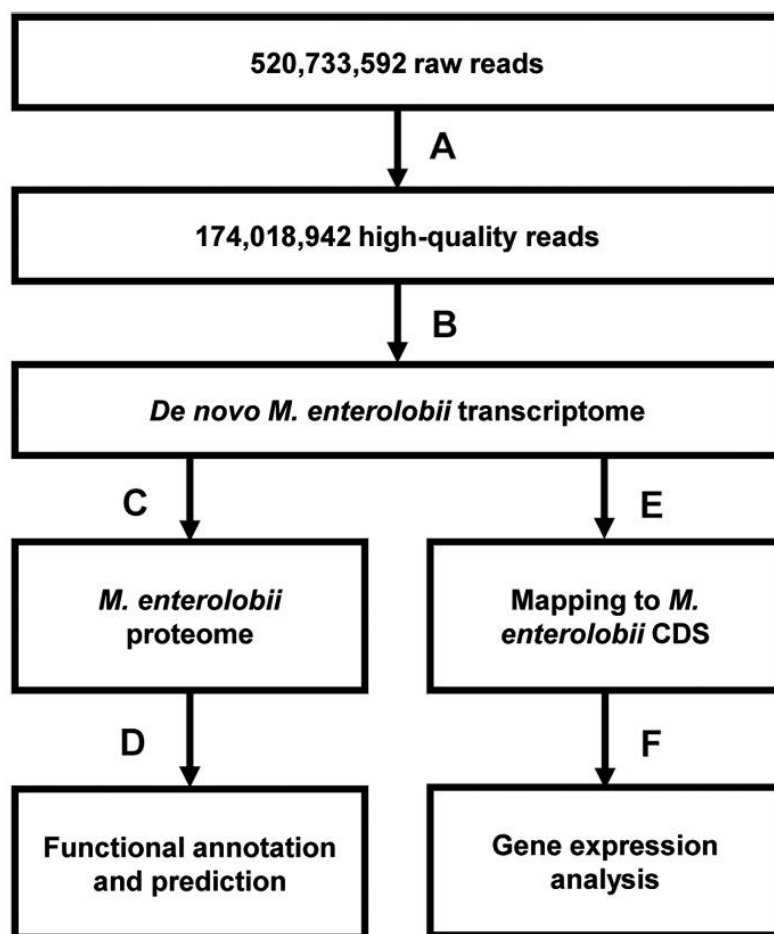


Fig. 1 Workflow summary of the bioinformatics strategy used for transcriptome sequencing analysis.

A, Removal of ribosomal RNA, contaminating sequences (adaptors, sequence copies) and low-quality sequences by SortmeRNA, Cutadapt and PrinSeq. **B**, Alignment of high-quality reads using Trinity for de novo assembly of *M. enterolobii* transcriptome. **C**, Translation of *M. enterolobii* transcripts to proteins using Transeq. **D** Interproscan was performed on the proteins to identify protein signature reference in the Interproscan database, for the presence of a N-terminal signal peptide and the absence of transmembrane domain. **E**, *M. enterolobii* transcripts were mapped to *M. enterolobii* coding sequences (CDS) with STAR. **F**, Gene expression patterns will be deduced from the aligned reads.

After removal of rRNA sequences, adaptor sequences, ambiguous reads and low-quality reads, we obtained a total of 174,018,942 high-quality clean read pairs of 72-bp long (Table 1). All high-quality reads were assembled *de novo* using the Trinity program in Galaxy environment, which produced 127,355 contigs, with an N50 of 631 bp (i.e. 50% of the assembled bases were incorporated into contigs of 631 bp or longer) and the total contig length was 62,994,138 bp (Table 1).

Table 1. Summary of the sequence assembly after removing the redundancy.

Total high-quality reads	174,018,942
Contig numbers	127,355
N50 length (bp)	631
Minimum contig length (bp)	185
Maximum contig length (bp)	11,359
Average contig length (bp)	495
Total length (bp)	62,994,138

Moreover, BUSCO was performed to assert quality of *de novo* assembly. The program determined the percentage of mis-assembled transcripts by trying to align all transcripts to highly conserved proteins within the BUSCO dataset of Eukaryota. Among the 429 BUSCO groups searched in our Trinity assembly, 188 genes (44%) were complete (106 single-copy and 82 duplicated genes) and 129 genes (30%) were fragmented. Only 112 (26%) genes were missing (Fig. 2).

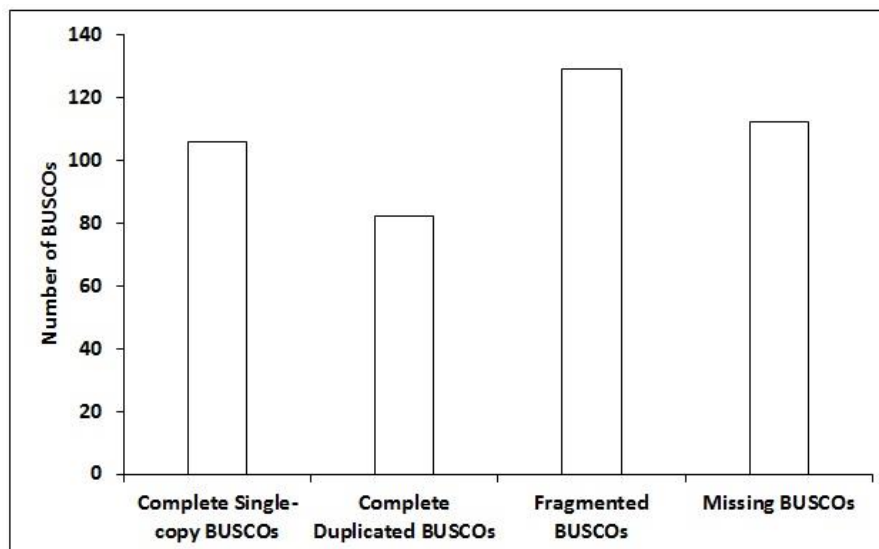


Fig 2. BUSCO analysis in the Trinity assembly after removing the redundancy

Functional Annotation of the *M. enterolobii* transcriptome and predicted secreted proteins

To gain functional insight on the *M. enterolobii* proteins, we searched and retrieved a series of functional annotations using interpro scan. This included a search for signal peptides for secretion, a search for transmembrane regions, a search for known protein domains and

associated functional annotations. Out of the 103,075 *M. enterolobii* proteins, only 24,696 (24%) have an Interpro annotation and 11,399 (11%) have been assigned a Pfam domain. BLAST2GO assigned 9,613 of the proteins to a Gene Ontology (GO) term. Among them, 1,227 were found to participate in 103 KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. The distribution of the main occurrences of the GO annotations within the three main categories were presented in Figure 3.

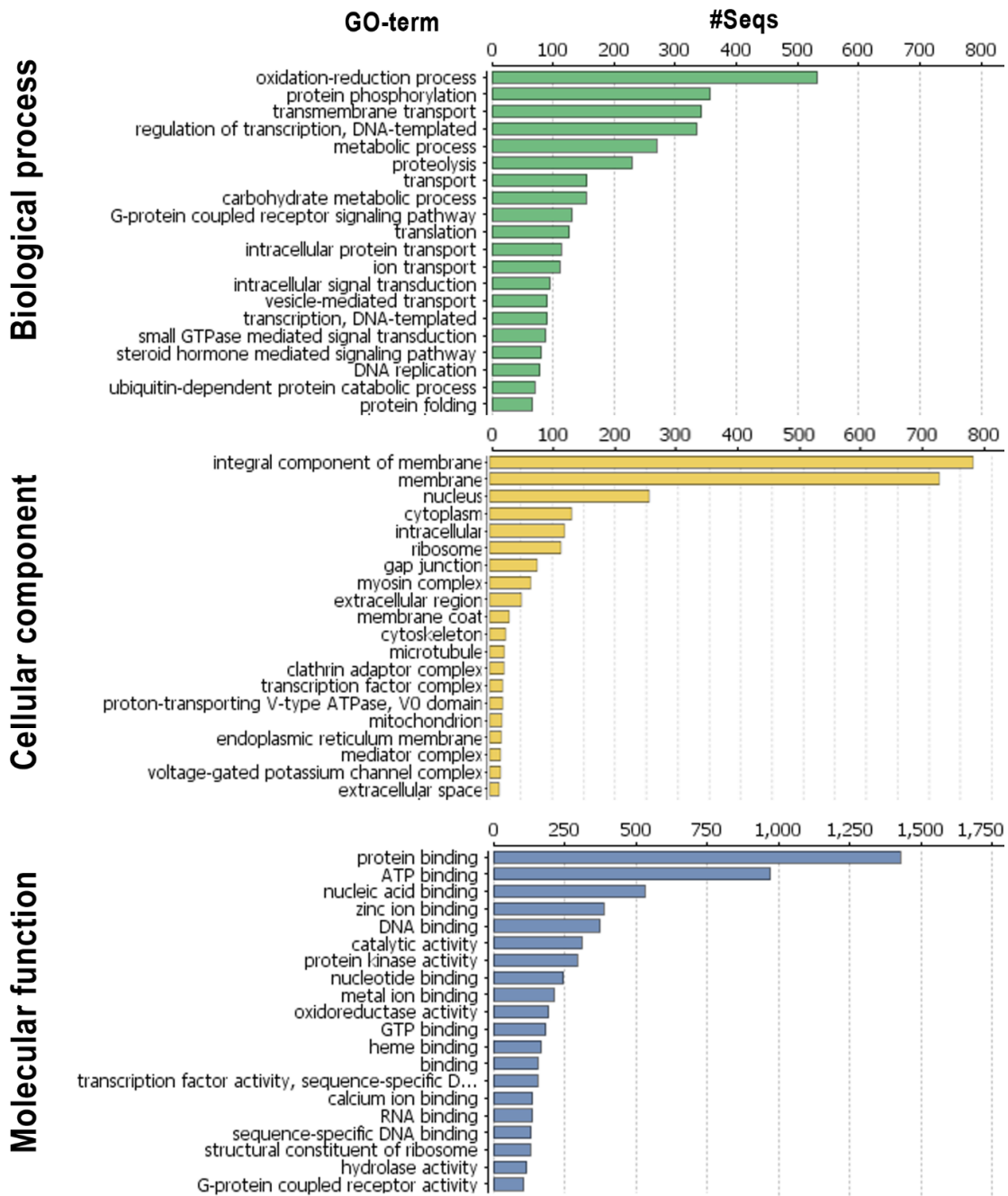


Fig 3. Distribution of the main occurrences of the GO annotations within the three main categories, biological processes, molecular functions and cellular components.

The most highly represented activities under each category were oxidation-reduction process, protein binding and integral component of membrane for biological processes, molecular functions and cellular components, respectively. In our annotated transcriptome datasets, we identified 1,632 proteins with putative signal peptide and without transmembrane domains. Among these secreted candidate effectors, 280 showed at least one GO term. Interestingly, twelve secreted proteins were predicted to bind DNA and/or to localise in the nucleus. We searched GO-term enrichment in molecular functions and biological pathways present in this set of proteins. Twenty-two molecular functions and nine biological pathways GO-terms were enriched in *M. enterolobii* effector set (p value < 0.01) (Table 2). The two more significant enrichments, i.e. the smallest p value scores, were associated with pectate lyase (18 proteins) and peptidase activity regulator (45-48 proteins). We searched the presence or absence of known RKN candidate effectors and avirulence proteins in the *M. enterolobii* proteome. Interestingly, among 106 known described RKN effectors, 101 proteins had homologues in *M. enterolobii* transcriptome expressed at J2 and/or J3-J4 stages (Table S4), including the cell-wall degrading enzyme proteins *Mi-PEL-1*, *Mi-PEL-2*, *Mi-ENG-1* (Huang et al. 2003, 2004, 2005a), the venom allergen-like protein *Mi-VAP-2* (Wang et al., 2007), the C-type lectin protein Minc03866 (Danchin et al., 2013), the nuclear protein *Mi-EFF1* (Jaouannet et al., 2012) and the putative avirulence proteins MAP1 (Semblat et al., 2001), ET1-4, HM1-6, HM8, HM10-13 (Neveu et al., 2003) and MjCG1 (Gleason et al., 2008). Only five proteins (Minc00469, 4D01/MSP3, 5C03B/MSP39, 9H10/MSP11 and Minc18876/SCR1) showed no homologues in our *M. enterolobii* transcriptome data, indicating the lack of expression at these developmental stages and/or the lack of these genes in the *M. enterolobii* genome. Among them, three proteins (Minc00469, 5C03B/MSP39, SCR1/Minc18876) were also not detected in the *M. hapla* genome, nor in *M. javanica* transcriptome but were detected, in addition to *M. incognita*, in *M. arenaria* transcriptome (Table 3). In contrast, *MSP3* and *MSP11* were specifically absent from *M. enterolobii* transcriptome.

Table 2 GO-term enrichment of putative *M. enterolobii* effectors

GO-ID	GO-term	P value	Odds Ratio	Exp Count	Count	Size
Molecular functions						
GO:0030570	pectate lyase activity	6.1E-06	17.9	0.5	6	18
GO:0016837	carbon-oxygen lyase activity, acting on polysaccharides	6.1E-06	17.9	0.5	6	18
GO:0061135	endopeptidase regulator activity	2.8E-05	7.8	1.3	8	45
GO:0004866	endopeptidase inhibitor activity	2.8E-05	7.8	1.3	8	45
GO:0061134	peptidase regulator activity	4.6E-05	7.2	1.3	8	48
GO:0030414	peptidase inhibitor activity	4.6E-05	7.2	1.3	8	48
GO:0004857	enzyme inhibitor activity	0.0001	6.3	1.5	8	54
GO:0030246	carbohydrate binding	0.0001	6.3	1.5	8	54
GO:0008146	sulfotransferase activity	0.0002	53.1	0.1	3	5
GO:0003993	acid phosphatase activity	0.0005	14.2	0.4	4	14
GO:0004675	transmembrane receptor protein serine/threonine kinase activity	0.0008	Inf	0.1	2	2
GO:0010576	metalloenzyme regulator activity	0.0011	21.2	0.2	3	8
GO:0048551	metalloenzyme inhibitor activity	0.0011	21.2	0.2	3	8
GO:0008191	metalloendopeptidase inhibitor activity	0.0011	21.2	0.2	3	8
GO:0042578	phosphoric ester hydrolase activity	0.0013	3.2	3.8	11	135
GO:0005509	calcium ion binding	0.0016	3.1	3.8	11	138
GO:0008081	phosphoric diester hydrolase activity	0.0018	5.2	1.3	6	47
GO:0016835	carbon-oxygen lyase activity	0.0018	5.2	1.3	6	47
GO:0019199	transmembrane receptor protein kinase activity	0.0023	70.5	0.1	2	3
GO:0030234	enzyme regulator activity	0.0041	3.1	3.1	9	113
GO:0015232	heme transporter activity	0.0045	35.2	0.1	2	4
GO:0035299	inositol pentakisphosphate 2-kinase activity	0.0045	35.2	0.1	2	4
Biological processes						
GO:0045454	cell redox homeostasis	0.0004	9.5	0.7	5	25
GO:0019725	cellular homeostasis	0.0005	7.0	1.0	6	39
GO:0042592	homeostatic process	0.0018	4.5	1.8	7	67
GO:0051181	cofactor transport	0.0040	37.4	0.1	2	4
GO:0015886	heme transport	0.0040	37.4	0.1	2	4
GO:1901678	iron coordination entity transport	0.0040	37.4	0.1	2	4
GO:0065008	regulation of biological quality	0.0056	3.6	2.2	7	82
GO:0018193	peptidyl-amino acid modification	0.0066	6.0	0.8	4	29
GO:0006457	protein folding	0.0081	3.7	1.8	6	67

1740 GO-terms carried by 281 proteins with signal peptide and without TM domain were extracted and were checked for enrichment in (A) molecular functions and (B) biological pathways) against whole GO-annotated proteome (9613 genes with at least one GO-term, 55088 GO-terms) with a p-value cutoff of 0.01. GO-ID (identity), GO-term, P-value, odds ratio, expected protein count (Exp Count), and actual protein count (Count) are indicated. Inf, infinity.

Table 3. *M. incognita* effector proteins produced in secretory organs without orthologs in *M. enterolobii* J2 and J3-J4 transcriptome

Effector	Length (aa)	SP (aa)	Organs	Predicted function	C residues	Ref	<i>M.e</i>	<i>M.h</i>	<i>M.j</i>	<i>M.i</i>	<i>M.a</i>
4D01/MSP3	174	24	SvG	ground-like domain containing protein	13	(1)	-	+	+	+	+
9H10/MSP11/Minc12617	180	23	DG	unknown	14	(1)	-	+	+	+	+
Minc00469	65	25	SvG	unknown	8	(2)	-	-	-	+	+
5C03B / MSP39	92	29	DG	unknown	1	(3)	-	-	-	+	+
SCR1/Minc18876	102	30	DG	Small cysteine-rich protein	10	Unpub.	-	-	-	+	+

aa: amino acid, *SvG*: subventral glands, *DG*: dorsal gland, *M.e*, *M. enterolobii*; *M.h*, *M. hapla*; *M.j*, *M. javanica*; *M.a*, *M. arenaria*; *M.i*, *M. incognita*. (+): indicated that homologue sequences have been found in this species (Blastp against *M.e*, *M.j* or *M.a* transcriptomes or *M. hapla* genome with identity > 50% and bit score > 100), (-): indicated that no homologue sequence was found in this species; C, cysteine in the mature protein; Ref, reference, (1) Huang et al. 2003; (2) Rutter et al. 2014, (3) Huang et al. 2004; unpub, unpublished results.

Discussion

M. enterolobii has the capacity to reproduce in commercial plant rootstock resistant to the three *Meloidogyne* species, *M. incognita*, *M. javanica* and *M. arenaria*, e.g. tomato or pepper carrying the *Mi-1*, *Me* or *N* resistant genes, respectively (Kiewnick et al. 2008, 2009). The few characterized sources of *M. enterolobii* resistance were found in Guava (*Psidium* spp.) (Carneiro et al., 2007) and in the Myrobalan plum (*Prunus cerasifera*) carrying the *Ma* gene. This *Ma* gene confers complete-spectrum resistance to *Meloidogyne* species and encodes a TNL with a huge repeated C-terminal post-LRR (leucine-rich repeat) region (Claverie et al., 2011; Saucet et al., 2016). New strategies to control this pest are urgently needed because of nematicide bans and lack of resistance genes. Efficient alternative *control* strategies could be based on a better *knowledge* of the parasitism of this pest and of the disease development.

Here, we used whole RNA-seq transcriptomes from preparasitic J2 and parasitic J3-J4 of *M. enterolobii* to identify the specificities of this species and to identify new candidate secreted effector proteins. High-throughput cDNA sequencing technology is especially suitable for gene expression profiling in non-model organisms that lack genomic sequence data. Recent transcriptomic studies on plant-parasitic nematodes revealed insight into the identification of plant-parasitic nematode parasitism features. Zheng et al. 2015 performed the analysis on transcriptomes of early parasitic J2s (30 hours, 3 days and 9 days post infection) of the cereal

cyst nematode *Heterodera avenae* in the host *Aegilops variabilis*. By comparing the differentially expressed gene between the pre-parasitic and the early parasitic larvae, the hydrolase activity was reported to be over-expressed in the preparasitic J2s whereas binding activity was upregulated in infective J2s, suggesting the possible secretion of proteins and their putative role in infection (Zheng *et al.*, 2015). The 454 sequencing of the rice root nematode *Hirschmanniella oryzae* on mix stages of population led to the identification of putative effector proteins that may alter the host defence mechanism, a β -mannanase, not previously reported, and two enzymes, chorismate mutase and isochorismatase, thought to be involved in the salicylic acid pathway (Bauters *et al.*, 2014). So far, transcriptomic approach represented also interesting data set to study the evolution of nematodes. Comparing parasitism genes of typical RKNs and CNs to those of “false RKN” *Nacobbus aberrans* has revealed interesting similarities, in which, genes that were believed to be either CN- or RKN- “specific” have both been identified in *N. aberrans*. This result has revealed insights into the evolution, phylogenetic history, and biology of biotrophic plant-nematode interactions (Eves-van den Akker *et al.*, 2014). However, to date, little is known about *M. enterolobii* genes involved in the parasitism. Only four putative effectors (MeTCTP, No. 5, No. 8 and No. 10 proteins) have been identified to be able to induce the suppression of programmed cell death in host plant (Li *et al.*, 2016; Zhuo *et al.*, 2016). In this study, we provided a transcriptomic approach combining with gene differently expression analysis to figure out its putative effectors. The polyploid genome size of *M. enterolobii*, measure *via* flow cytometry experiments, was estimated at ~275 Mb. This genome size is in the range of those recently described for *M. incognita* (189 Mb), *M. javanica* (297 Mb) and *M. arenaria* (304 Mb) and reflects the complexity of apomictic RKN genomes (Blanc-Mathieu *et al.* 2016).

Among the 103,075 *M. enterolobii* proteins obtained, 24,696 have an Interpro annotation. This is in contrast with the two whole RKN proteomes. Indeed, a total of 20,359 *M. incognita* proteins (Danchin *et al.*, 2013) and 14,421 *M. hapla* proteins (Opperman *et al.*, 2008) have been identified. However, 43,718 *M. incognita* proteins have been detected in the third version of the genome (Blanc-Mathieu *et al.*, 2016). The high number of the predicted *M. enterolobii* proteins could be due to fractioning of the proteins that could be improved by increasing read number. Only 9,613 proteins (9%) have been assigned a GO term. By comparison, GO terms were assigned to 6,881 (33.8%) and 4,673 (32.4%) of *M. incognita* and *M. hapla* whole proteomes, respectively (Danchin *et al.*, 2013).

Among 106 known described in RKN, including putative avirulence proteins, only five *M. incognita* proteins showed no homologues in J2 and J3-J4 *M. enterolobii* transcriptome. They encoded proteins of unknown functions except MSP3 which presents a ground-like domain. In *C. elegans*, it has been proposed that the ground-like domain containing proteins may bind and modulate the activity of Patched-like membrane molecules, reminiscent of the modulating activities of neuropeptides (Aspöck *et al.*, 1999). In *M. incognita*, *Minc00469* and *MSP3* genes have been shown, by *in situ* hybridization, to be expressed specifically in the subventral glands (Huang *et al.*, 2003; Rutter *et al.*, 2014), whereas *MSP39*, *MSP11*, and *Minc18876* that encode a small cysteine-rich protein SCR1, have been shown to be specifically expressed in the dorsal glands (Huang *et al.* 2003, 2004, Nguyen, unpublished results). Three of these proteins (*Minc00469*, *MSP39* and *SCR1*) were not detected in the two species that are not controlled by the *Mi-1*, *Me* and *N* resistance genes, *M. enterolobii* and *M. hapla*, and were detected in two of the three species, *M. arenaria* and *M. incognita*, controlled by these resistance genes. These three effectors could constitute putative avirulence proteins with respect to the *Mi1*, *Me* and *N* resistance genes or to the peach resistance gene R-Mia which controls *M. incognita* and *M. arenaria* but not *M. javanica* and *M. enterolobii* (Saucet *et al.*, 2016). Their lack may explain why *M. enterolobii* are not controlled by these resistance genes. However, this hypothesis should be tested to confirm the presence/absence of expression in the *Meloidogyne* species, and also in virulent *M. incognita* populations or selected lines (Neveu *et al.*, 2003). Finally, this transcriptomic analysis provides new insights into the development and host-parasite interactions of this plant-pathogen and highlight differences and similarities with other RKN species. The availability of nematode genomes and transcriptomes provides new opportunities for studying plant-nematode interactions and facilitates the applying of post-genomic technologies for the development of new strategies to control these pests.

RKN effector	<i>Mi-1, Me and N genes</i>					<i>R-Mia gene</i>				
	M.e	M.h	M.j	M.i	M.a	M.e	M.h	M.j	M.i	M.a
Minc00469	-	-	-	+	+	-	-	-	+	+
5C03B / MSP39	-	-	-	+	+	-	-	-	+	+
SCR1/Minc18876	-	-	-	+	+	-	-	-	+	+

S	NI	R
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Fig. S4. Correlation between presence/absence of RKN effectors and spectra of action of RKN resistance genes.

Mi-1, Me and N genes control *M.j, M.i* and *M.a* (R, resistance in red) but not *M.e* and *M.h* (S, susceptible in green). *R-Mia* control *M.i* and *M.a* (R, red) but not *M.e* and *M.j* (S, green). *M.h* do not infect peach (no interaction, NI, grey). *M.e, M. enterolobii; M.h, M. hapla; M.j, M. javanica; M.a, M. arenaria; M.i, M. incognita.* (+): indicated that homologue sequences have been found in this species

Acknowledgements

We thank Etienne G. J. Danchin and Philippe Castagnone for helpful discussions. This work was funded by SYNGENTA and INRA, and supported by the French Government (National Research Agency, ANR) through the “Investissements d’Avenir” LabEx SIGNALIFE (ANR-11-LABX-0028-01) and by the France Génomique National infrastructure (ANR-10-INBS-09). The authors are grateful to the GenoToul bioinformatics platform Toulouse Midi-Pyrenees for providing computing resources. C.-N. Nguyen was supported by USTH fellowships from the Ministry of Education and Training of The Socialist Republic of Vietnam.

Author contributions

C.-N.N. produced nematodes, constructed libraries, and performed data analysis and interpretation. L.P.-B. and C.M.J. performed the flow cytometry analysis; L.P.B designed RNA-seq experiment; L.P.-B. and B.F. supervised the experiments and data analyses; M.D.R. and LP performed the transcriptome data analysis; C.-N.N., L.P. and B.F. performed the functional annotation; P.A. and B.F. supervised the research; C.-N.N. and B.F. wrote the paper.

Table S1. Primers used in *M. enterolobii* identification.

SCAR Primers	Sequence
<i>Meloidogyne</i> spp.-specific (MEL)	Forward: TACGGACTGAGATAATGGT Reverse: GGTTC AAGCCACTGCGA
<i>M. enterolobii</i> -specific (MK7)	Forward: GATCAGAGGCGGGCGCATTGCGA Reverse: CGAACTCGCTCGAACTCGAC
<i>M. incognita</i> -specific (K14)	Forward: GGGATGTGTAAATGCTCCTG Reverse: CCCGCTACACCCTCAACTTC

Table S2 Ribodepleted primers used for library construction

Primers	Sequence	Length	%GC
18S_rb1	TTTATCGAGAAAACCGCGAAC	20	45
18S_rb1he	TGATTGTCTAAATGGATAACTGTGG	25	59
18S_rb2Me	TGCACCAAAGCTTTGTCCTCTCG	23	52
18S_Ribo1	TCCTCTCGGAAAAGCGCATTTATT	24	41.6
18S_rb3	CGGCTGCTTCTTGTGACTC	20	55
18S_rb28	CGTGTCTTTCAAGCGTCCAC	20	55
18S_rb4	ACTTGACGGGAGCATAATCG	20	50
18S_rb29	ACGTCTAAGGATGGCAGCAG	20	55
18S_rb6	GCTCGAGGAGGTAGTGACGA	20	60
18S_Ribo5.1	ACGAGATCGTTCTCTTTGAGGCCG	24	54
18S_rb30	AGCAGAGGGCAAGTCTGGT	19	57.9
18S_rb31	TTGCTGCGGTTAAAAAGCTC	20	45
18S_rb8	GCGGTAATTCCAGCTCTGC	19	57.9
18S_rb9Me	CCCTTCGGGTGTTTCTGGGT	20	60
18S_rb10	TCGGTTTTGAGTCCCTAACAGG	22	45.45
18S_Ribo8	TGCTTCAAACAGGCGTTTTCGCT	23	47.8
18S_Ribo9	TGGTTAACAGAGACAAACGGGGG	23	52
18S_rb13	ACCGTGGCCAGACAACTAC	20	55
18S_rb32	TTCGAAGGCGATCAGATACC	20	50
18S_rb14	GACCGTAAACGATGCCAACT	20	50
18S_rb33	GATCCGCCGATGGAAATTAT	20	45
18S_rb15	AACGAAAGTCTTCCGGTTCC	20	50
18S_rb16	AAGGGCACCACCAGGAGT	18	61.1
18S_rb17	CTCAACACGGGGAAACTCAC	20	55
18S_RN7	GTCTGGTTTATTCCGATAACGAGCG	25	48
18S_R12.1	GGGATTTGCGGTGTTCAGCC	20	60
18S_rb19	TCAGCCGAAAGAAATTGAGC	20	45
18S_rb20	ACTGGCAAATCAACGTGCT	20	45
18S_Ribo14	TTGCCGTGATTGGGATCGGA	20	55
18S_RN8	TGCGAGTCATCAGCTCGCGTT	21	57
18S_rb22	TTACGTCCCTGCCCTTTGTA	20	50
18S_rb36	AATTTGGGGACCGTTGATTT	20	40
18S_rb23	TAATCGCAGTGGCTTGAACC	20	50
18S_rb24	TGTAGGTGAACCTGCTGCTG	20	55
18S_RN10	AACGGCTGTCGCTGGTGTCT	20	60
18S_rb26	CGTCCGTGGCTGTATATGTG	20	55
18S_rb37	GGGCAAAAGTCCCAACG	17	58.82
18S_rb37_KJ	GGGCAAAAGTCGTAACA	17	47
18S_rb38	ATGGGCATAGCTGTTTCTCTG	20	50
18S_rb39	CGCTCACAATTCCACACAAC	20	50
18S_rb40	AGTGAGGCCGCCAGCAACCTTT	22	59
18S_rb41	ATTAATTGCGTTGCGCTCAC	20	45
28S_rd1	ATCACTAGGCTCGTGGATCG	20	55
28S_rd2	CCGCATTGAGGTCAAACCTCT	20	50
28S_rd46	CCGCATTGGTCAAACCTTTT	20	45
28S_23	TCTGGTTCAGGGTCATTTTCTCTT	24	60
28S_25	TGAACTCAGTCGAGAGCACCC	21	57
28S_rd4	GCCTCAGGCATTATGAGGTG	20	55
28S_rd5	GGTTCCACAGAAGGTGCAAG	20	55
28S_rd6	CGTGCTTTAGAGTCGGGTTG	20	55
28S_rd7	CCACGAGACCGATAGCAAAC	20	55

28S_rd8.1	TGAAACCGGTGAGGTGGAAA	20	45
28S_rd9	GAGCTCCAGATTGGGACAGA	20	55
28S_6	GGTGCTTGGGGATGTTTGAGGCA	23	56
28S_rd11	CAGCTTGCTGGTACCCAAAC	20	55
28S_rd12	GCGACACGTGCTTTTCAATTA	21	42.85
28S_rd13	CCATGTAAAAGCCGGTCATC	20	50
28S_rd14	AGCATGGCCCCATTCTAACT	20	50
28S_10	TGGTGAAGTCCGAAGCGGT	20	60
28S_11	GTCTGACTGGGTATAGGGGCGA	23	56
28S_rd17	AGTTTCCCCCAGGATAGCTG	20	55
28S_rd18	CTCGGTAAAGCGAATGATTAGG	22	45
28S_rd49	GAGGACTTGGGAACGAAATGT	21	47.6
28S_30	GGCGTTGAATACGAGCTCCA	20	55
28S_rd50	TAAGCAGAAGTGGCGATGTG	20	50
28S_31.2	TGCCAAAGTGCCCGCTCAT	19	57
28S_rd20	GTCGGAATCCGCTAAGGAGT	20	55
28S_12	ACCTGCCGAATCAACTAGCCCT	22	54
28S_rd51	AGCCCAGCGTTGCTTAACT	19	52.6
28S_14	AGAGGGTCGTAGTGGTTGCGT	21	57
28S_rd52	CAACGCGGTATGGTCGTAAT	20	50
28S_rd23	CCACTAGTGCAATCTTGGTGGT	22	50
28S_rd53	GGATTTGATGTTTCGCTGTT	20	45
28S_rd24	GAATGTGGGTGAGTCGATCA	20	55
28S_rd25	GTCTAGACACTGCGGGGAGA	20	60
28S_rd26	CAGGCATGGGAGATGGTGAT	20	50
28S_17	CGCGGTGACGCAAACGAACT	20	60
28S_rd54	TTTATTGACTCTCGTGCAAAA	22	30.4
28S_rd55	AATTGTCAGGAATTTAGCCGATT	23	34
28S_rd28	TCAGCCTGAGATAGGGATGC	20	55
28S_rd29	TCCACATGAGCCGTGAAAAT	20	45
28S_rd56	TTTCGACTTTTATTTGCGGATTT	23	30
28S_32	ATCCGCAGCAGGTCTCCAAG	20	60
28S_rd57	CCAGCAGTCTCGGTAATTCAA	21	47
28S_rd58	ATCTGGTTGATCCTGCCTGA	20	50
1_c5068_g1_i1_0	TGGCTCTAAAGTTGGGTCA	20	50
28S_rd32	GGCTGCTTGCCTTCTTTTC	20	55
28S_rd59	TTTATCGAGAAACCGGAAC	20	45
28S_rd33	GCTTCAGCTGCGTGCTATTT	20	50
3_c5068_g1_i1_140	AGTACTTCGATTGGCGCTGA	20	50
4_c5068_g1_i1_210	AGAGGAGACGGATGGTCCTT	20	55
5_c5068_g1_i1_280	GGTAAACGGCGGGAGTAACTA	21	52
6_c5068_g1_i1_350	AAGGTAGCCAAATGCCTCGT	20	50
28S_rd37	CGAGATTCCCACTGTCCCTA	20	55
7_c5068_g1_i1_420	CTAGCGAAACCACAGCCAAG	20	55
28S_34	GGGAAAGAAGACCCTGTTGAGC	22	54.5
8_c5068_g1_i1_490	GGTGTAGCATAAGTGGGAGTCG	22	54.5
28S_rd40	TCGGCTTCTCCTATCATTGC	20	50
28S_36	AGGGAACGTGAGCTGGGTTT	20	55
28S_rd42	GAACCGCAGTTTACAGCATT	20	50
28S_37	GATAGGCCAATGGCGGAAG	20	60
28S_38	GTCAGAATCCCGCCAGTCA	20	60
28S_rd43	TATATCGCTCTCCGGTGTCG	20	55
28S_rd44	AGCCCCAGTATCTGGCATT	20	50
28S_rd45	CCTCGTGCAGGTGTAACGTC	20	55

Table S4 *M. incognita* effector proteins produced in secretory organs with orthologs in *M. enterolobii* J2 and J3-J4 transcriptome

Effector	Length (bp)	Organs ^a	SP	Predicted function	<i>M. enterolobii</i> score,%, Identities (Blastp)	References
Mi-PEL-1	813	SvG	+	pectate lyase	496, 84%, 228/271	(1, 2)
Mi-PEL-2	840	SvG	-	pectate lyase	557, 95%, 268/280	(3, 2)
Mi-PEL-3	837	SvG ^b	+	pectate lyase	415, 97%, 198/203	(4, 5)
Mi-ENG-1	1668	SvG	+	beta-1,4-endoglucanase	959, 92%, 468/506	(3, 6, 7)
5A12B	1706	SvG	+	beta-1,4-endoglucanase	959, 92%, 468/506	(3)
8E08B	1242	SvG	+	beta-1,4-endoglucanase	635, 86%, 308/355	(3)
Mi-PG-1	1899	SvG	+	polygalacturonase	766, 92%, 378/409	(8)
Mi-XYL-1	987	SvG	+	beta-1,4-endoxylanase	138, 94%, 67/71	(9)
Mi-CBP-1	995	SvG	+	cellulose-binding protein	358, 88%, 182/205	(1, 10)
Mi-CM-1	573	SvG	+	chorismate mutase	135, 76%, 66/86	(3, 11)
Mi-CM-2	573	SvG	+	chorismate mutase	128, 73%, 63/86	(3, 11)
Mi-GSTS-1	609	SvG	-	glutathione-S-transferase	269, 92%, 127/137	(13)
Mi-SXP-1	576	SvG	+	SXP/Ral-2 protein	217, 92%, 106/114	(14)
Mi-VAP-2	882	SvG	+	venom allergen-like protein	504, 91%, 236/257	(15)
Mi-MSP-1	902	SvG	+	venom allergen-like protein	415, 89%, 184/205	(16)
5G05	977	SvG	+	zinc metallopeptidase	528, 97%, 250/257	(1)
30G11	1511	SvG	+	acid phosphatase	639, 97%, 301/310	(1)
10A07	656	SvG	+	sodium/calcium/potassium exchanger	224, 66%, 125/189	(1)
CL5Contig2_1	675	SvG	-	Sec-2 protein	354, 96%, 184/190	(7)
CL2552Contig1_1	375	SvG	-	transthyretin-like protein	260, 100%, 125/125	(7)
CL321Contig1_1	537	SvG	-	translationally controlled tumour protein	357, 100%, 179/179	(7)
CL480Contig2_1	609	SvG	-	triosephosphate isomerase	407, 99%, 201/203	(7)
CL312Contig1_1	1287	SvG	-	unknown	827, 98%, 422/429	(7)
Minc01696	1728	SvG	+	protein kinase	1092, 94%, 574/579	(17)
Minc03866	1452	SvG	+	C-type lectin	417, 76%, 206/269	(18)
Minc00344	1155	SvG	+	unknown	184, 86%, 87/101	(17)
Minc04584	627	SvG	+	unknown	140, 70%, 65/92	(17)
Minc18033	1311	SvG	+	unknown	289, 85%, 139/163	(17)
Minc13292	1602	SvG	+	unknown	578, 63%, 346/546	(17)
Minc08073	2130	SvG	+	unknown	270, 95%, 129/135	(17)
Minc15401	948	SvG	+	unknown	538, 86%, 273/317	(17)
Minc10418	2127	SvG	+	unknown	267, 96%, 127/132	(17)
Minc03328	1446	SvG	+	unknown	125, 85%, 60/70	(17)
Minc03325	1590	SvG	+	unknown	622, 67%, 365/538	(17)
Minc18636	936	SvG	+	unknown	553, 89%, 280/314	(17)
Minc08146	222	SvG	+	unknown	158, 97%, 72/74	(19)
2G02	776	SvG	+	unknown	398, 90%, 191/211	(1)
8D05	1282	SvG	+	unknown	306, 82%, 167/202	(1)
8H11	1228	SvG	+	unknown	436, 71%, 237/330	(1)
8E10B	1217	SvG	+	unknown	453, 72%, 236/327	(3)
30H07	994	SvG	+	unknown	454, 86%, 223/259	(1)
31H06	362	SvG	+	unknown	blastx: 62, 91%, 31/34	(1)
35A02	2210	SvG	+	unknown	195, 65%, 102/155	(1)
Mi-CRT	1245	SvG&DG ^b	+	calreticulin	858, 97%, 405/415	(20)
Mi-14-3-3-b	783	DG	-	14-3-3	508, 98%, 256/261	(5, 21)
10G02	1499	DG	+	thioredoxin	242, 88%, 123/139	(1)
Minc00108	612	DG	+	metallopeptidase	286, 70%, 145/205	(19)
Minc02097	1596	DG	+	unknown	195, 65%, 102/155	(17)
Mi-EFF1	366	DG	+	unknown	238, 94%, 116/123	(19)

Table S4 (continue)

Effector	Length (bp)	Organs ^a	SP	Predicted function	<i>M. enterolobii</i> score, % Identities (Blastp)	References
Minc18861	450	DG	+	unknown	296, 94%, 141/150	(17)
Minc12639	300	DG	+	unknown	170, 94%, 86/91	(17)
Minc11817	450	DG	+	unknown	296, 94%, 141/150	(17)
Minc01595	1650	DG	+	unknown	209, 69%, 108/155	(17)
1C05B	617	DG	+	unknown	135, 90%, 70/77	(3)
1D08B	547	DG	+	unknown	139, 94%, 64/68	(3)
2E07	694	DG	+	unknown	297, 85%, 149/174	(1)
2G10	999	DG	-	unknown	120, 96%, 57/59	(1)
4D03	864	DG	+	unknown	373, 91%, 170/186	(1)
4F05B	763	DG	-	unknown	116, 84%, 53/63	(3)
6F07	1339	DG	+	unknown	542, 84%, 280/332	(1)
7A01	778	DG	+	unknown	300, 85%, 155/181	(1)
7E12	757	DG	+	unknown	264, 78%, 139/177	(1, 22)
7H08	1091	DG	+	unknown	124, 86%, 64/74	(1)
11A01	1266	DG	+	unknown	197, 83%, 100/120	(1)
12H03	723	DG	+	unknown	302, 86%, 156/181	(1)
13A12	824	DG	+	unknown	290, 84%, 147/174	(1)
17H02	783	DG	+	unknown	240, 70%, 122/172	(1)
25B10	414	DG	+	unknown	190, 86%, 96/111	(1)
14E06	696	DG	+	unknown	264, 78%, 139/178	(1)
16E05	1687	DG	+	unknown	287, 84%, 138/163	(1)
21E02	704	DG	+	unknown	276, 81%, 144/177	(1)
34D01	782	DG	+	unknown	292, 83%, 151/181	(1)
35F03	398	DG	-	unknown	36, 53%, 23/43	(1)
35E04	836	DG	+	unknown	131, 85%, 60/70	(1)
28B04	748	DG	+	unknown	266, 79%, 141/178	(1)
Mi-MAP1	1374	amphids	+	unknown	251, 74%, 125/167	(5, 23)
CL1191Contig1_1	1740	phasmid	-	CDC48-like	1155, 100%, 580/580	(7)
Minc00801	1083	RG	+	unknown	504, 96%, 263/273	(17)
Minc03314	180	SvG	-	unknown	69, 92%, 37/40	unpub
Minc12024	234	SvG	+	unknown	162, 97%, 75/77	unpub
Minc12754	291	DG	+	DNA-binding domain	182, 96%, 88/91	unpub
Minc14652	1164	DG	+	nuclear localisation signal	773, 96%, 372/387	(18)
Minc17611	522	DG	+	nuclear localisation signal	291, 81%, 148/182	unpub
ET1	321	nd	-	unknown	215, 94%, 101/107	(12)
ET2	319	nd	-	peptidase C19	217, 97%, 102/105	(12)
ET3	167	nd	-	SPK1 component	116, 98%, 54/55	(12)
ET4	223	nd	-	initiation factor eIF-4 gamma	124, 91%, 61/67	(12)
HM1	525	SvG	+	membrane bound O-acyl transferase	174, 82%, 81/98	(12)
HM2	97	nd	-	ribosomal protein	53, 100%, 25/25	(12)
HM3	559	nd	-	nematode cuticle collagen	410, 98%, 183/186	(12)
HM4	169	nd	-	unknown	111, 96%, 53/55	(12)
HM5	80	nd	-	unknown	47, 92%, 23/25	(12)
HM6	274	nd	-	lamininB; immunoglobulin	177, 98%, 85/86	(12)
HM8	221	nd	-	innexin	107, 69%, 50/72	(12)
HM10	1152	nd	+	peptidase C1A, papain	659, 86%, 318/366	(12)
HM12	936	DG	+	unknown	281, 95%, 134/141	(12)
HM13	204	nd	-	unknown	104, 95%, 41/43	(12)
PM1	672	nd	-	WW/Rsp5/WWP	449, 95%, 214/224	(12)
PM2	183	nd	-	SKP1 component	122, 91%, 55/60	(12)
PM3	201	nd	-	gamma-tubulin complex component 2	270, 88%, 128/145	(12)
MjCG1	3154	nd	-	unknown	125, 100%, 58/58	(24)

a) secretion organ-specific expression observed by *in situ* hybridization except b) immunolocalisation; SvG: subventral glands, DG: dorsal gland, RG: rectal gland, SP, predicted signal peptide: yes (+) or no (-). References: (1) Huang et al. 2003; (2) Huang et al. 2005b; (3) Huang et al. 2004; (4) Vieira et al. 2011; (5) Vieira et al. 2012; (6) Rosso et al. 1999; (7) Bellafiore et al. 2008; (8) Jaubert et al. 2002a; (9) Dautova et al. 2001; (10) Ding et al. 1998; (11) Huang et al. 2005a; (12) Neveu et al. ; 2003; (13) Dubreuil et al. 2007; (14) Tytgat et al. 2005; (15) Wang et al. 2007; (16) Ding et al. 2000; (17) Rutter et al. 2014; (18) Danchin et al. 2013; (19) Jaouannet et al. 2012; (20) Jaubert et al. 2005; (21) Jaubert et al. 2004; (22) Souza et al. 2011; (23) Semblat et al. 2001; (24) Gleason et al. 2008; unpub, unpublished results.

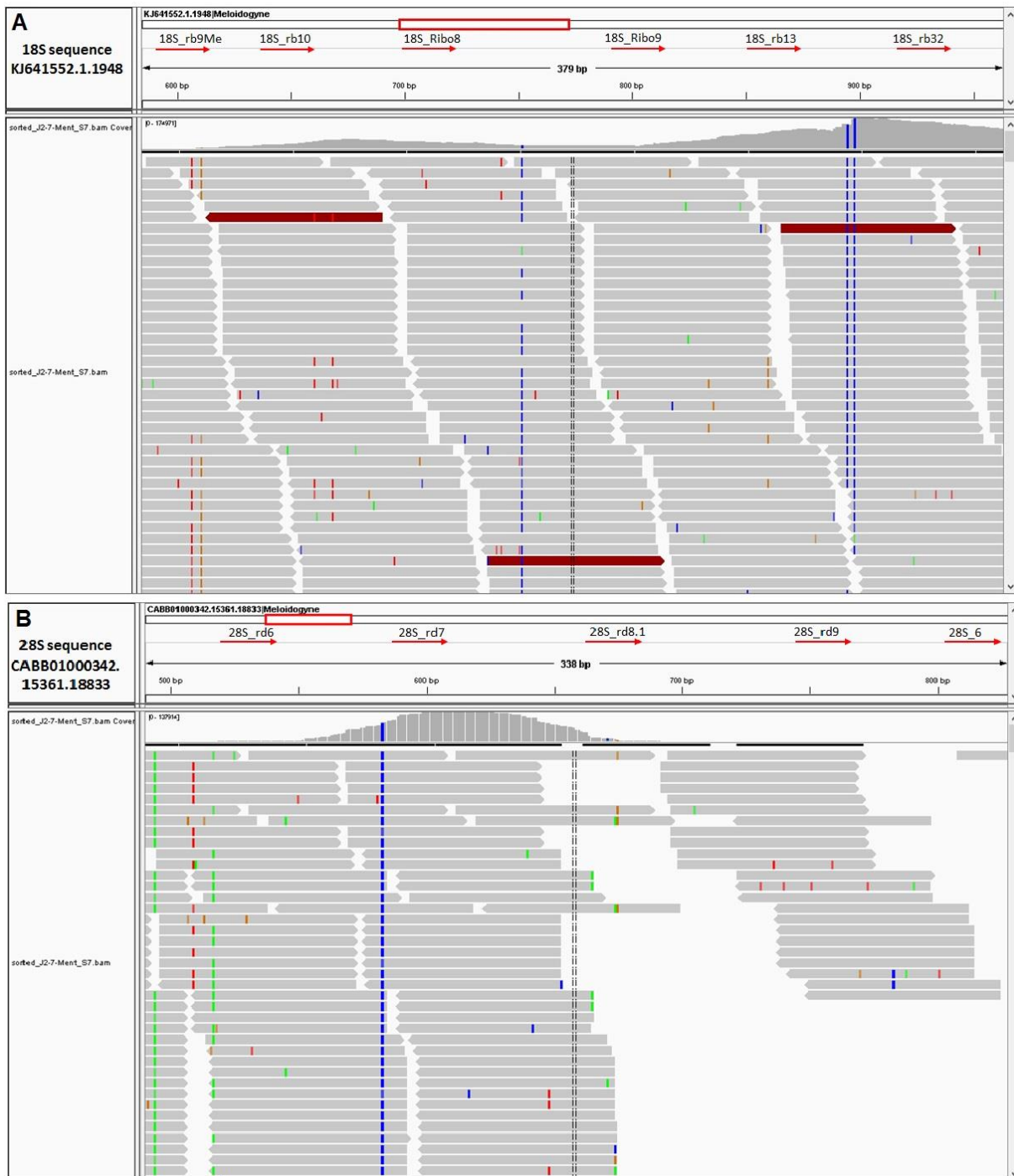


Fig. S1 Distribution map of *M. enterolobii* J2 rRNA reads to 18S and 28S *M. incognita* longest rRNA sequences.

Due to the lack of full length sequences of *M. enterolobii* rRNAs, we used 18S and 28S rRNA of *M. incognita* to design the ribodepleted primers. 97 rRNA sequences of *M. incognita* were extracted from Silva Database (Quast et al., 2013), including 57 sequences for 18S and 40 sequences for 28S. *M. enterolobii* J2 rRNA reads (in grey) were mapped to the 18S (**A**) (KJ641552.1.1948) and 28S (**B**) (CABB01000342.15361.18833) *M. incognita* longest rRNA sequences using IGV (Integrative Genomics Viewer) (Thorvaldsdóttir et al., 2013). Ribodepleted primers were designed according to the distribution map with polymorphism (red arrows in the upper part of the figures).

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GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

RKNs have been reported as one of the most damaging plant pathogens in the world (Jones *et al.*, 2013). Nowadays, the methods to fight against these pests appear limited. Widely used chemical nematicides were recently banned due to their toxicity for human health and environment. An efficient method is to use plant carrying resistance genes, but it is limited by the low number of available resistance genes and increasing “virulent” RKN populations or species (like *M. enterolobii*) able to reproduce on several crops carrying RKN resistance genes, such as the tomato *Mi* gene, significantly impeding the efficacy of these elite lines (Castagnone-Sereno, 2006, 2012; Williamson & Kumar, 2006). As an alternative, new control strategies may emerge from the knowledge of mechanisms underlying the plant-pathogen interactions.

Different strategies have been developed for better understanding the dialogues between plants and nematodes, particularly focusing on the parasitism features, i.e. the secreted proteins, named effectors, produced in the salivary glands. Gland micro-aspiration/dissection coupled with expressed sequence tag (EST) sequencing (Huang *et al.*, 2003, 2004), secretome analysis (Jaubert *et al.*, 2002b; Bellaifiore *et al.*, 2008), gene differentially expressed between stages in the nematode life cycle (Neveu *et al.*, 2003; Dubreuil *et al.*, 2007) approaches led to the identification of numerous putative secreted proteins and nematode virulence/avirulence genes. In the recent years, thanks to the development of high-throughput sequencing technologies, genomic and transcriptomic approaches provided new perspectives for researches on the interaction between plants and RKNs, that figured out new sets of nematode putative effectors (Haegeman *et al.*, 2013; Danchin *et al.*, 2013; Rutter *et al.*, 2014; Petitot *et al.*, 2016).

During my PhD project, a genome-wide transcriptomic approaches combined with functional annotation analysis was performed on two main *Meloidogyne* species, *M. incognita* and *M. enterolobii*. First, we compared Illumina RNA-seq transcriptomes for *M. incognita* obtained at various points in the lifecycle, and identified genes more strongly expressed *in planta* in early parasitic stages compared to preparasitic juveniles. We then selected some candidate parasitism effectors for functional characterization. Among them, the small cysteine-rich effector MiSCR1 was demonstrated to potentially play an important role in the early stages of parasitism. In parallel, we performed a preliminary analysis of the transcriptome of *M. enterolobii*, a species that could reproduce on plants resistant to the major *Meloidogyne* spp. in order to highlight

differences and similarities with other RKN species. We focused our study on genes encoding putative effectors that may account for the particular host range of this species.

1. Identification of parasitism effectors expressed during plant infection from the transcriptome of *Meloidogyne incognita*.

In a previous study in our laboratory, Danchin et al. (2013) generated RNA-seq transcriptome for various points of *M. incognita* life cycle (preparasitic J2s, parasitic J2s in the early stages of parasitism, parasitic J2-J3-J4 including late J2 parasites and young J3-J4 parasites, J3-J4 parasites, females males and eggs). This transcriptomic dataset supported 94% of the 20,359 protein-encoding genes predicted in the *M. incognita* genome (version 1, 86 Mb; Abad et al., 2008). During this PhD work, the statistical analysis of this dataset detected 307 genes displaying significant differential expression between lifecycle stages. Among them, 74 genes were identified as over-expressed at the parasitic stages J2-J3-J4 and J3-J4 compared to the pre-parasitic stage J2. RT-qPCR analysis confirmed RNA-seq data for 8 of the 11 selected candidates. However, the number of genes displaying significant differences in expression in parasitic stages appeared limited compared to other studies. *De novo* assembly of *M. graminicola* transcriptome with two biological replicates figured out 343 genes up-regulated at 8 dpi and 140 genes down-regulated at 12 dpi (Petitot *et al.*, 2016). Moreover, the analyses on transcriptomes of early parasitic J2s (30 hours, 3 days and 9 days post infection) of the cereal cyst nematode *H. avenae* identified 271 unigenes highly expressed at early steps of parasitism (Zheng *et al.*, 2015).

In our study, the statistical analysis was stringent due to the lack of real biological replicates for Illumina sequencing. The difficulty of collecting the parasitic stages led us to prepare only one biological sample at that time for a pilot study dedicated to the functional annotation of the version 1 of *M. incognita* genome (Danchin et al., 2013). Thereby, we compared the RNA-seq values of two parasitic samples: mix J2-J3-J4 and J3-J4 to that of the pre-parasitic J2. While those were quasi-replicates, they were not true biological replicates, and could not handle the whole problem. For example, *Minc17611*, a candidate with a ratio FPKM J2-J3-J4/J2 and J3-J4/J2 higher than 10 and containing NLS, was not detected in the list of the 307 differently expressed genes. After RT-qPCR validation, this gene was confirmed to have a higher expression at J3-J4 stages when compared to J2 stage. In order to improve this transcriptomic approach, new

transcriptomes of *M. incognita* at several key points of life cycle (eggs, J2, J3-J4 and female) were carried out by Illumina sequencing with three biological replicates (Laetitia Perfus-Barbeoch and Etienne G.J Danchin, personal communication). In the next future, the high-quality reads generated will be aligned to the last version of the *M. incognita* genome (v3, 189 Mb; Blanc-Mathieu et al., 2016). These analyses will improve the support of RNA-seq data on *M. incognita* predicted genes, therefore allowing the identification of missed effector.

Focusing on secreted proteins, we identified a set of 31 *M. incognita* putative effectors more strongly expressed in early parasitic stages than in preparasitic juveniles. We then selected 12 candidate effectors for functional characterisation including *in situ* hybridisation and silencing experiments. For plant nematologists, the localisation of the gene expression in the nematode secretory organs is a way to select candidate effectors secreted during parasitism. To date, *in situ* hybridisation analyses reported the localisation of about 80 *M. incognita* effector candidates in the salivary glands, the most important sources of effector secretion (Truong et al., 2015). Almost half of the effector candidates was expressed in the two SvGs, whereas the other half was expressed in the DG. In RKNs, the SvGs have been described as highly active during the early stages of the parasitism, while the DG becomes active during the development and maintenance of the feeding sites (Davis et al., 2000). Here, we showed that the validated differentially expressed genes are predominantly specifically expressed in oesophageal glands. We identified three genes expressed in the SvG (*Minc03314*, *Minc12024/Minc06089* and *Minc18636*) and four expressed in the DG (*Minc12754*, *Minc14652*, *Minc17611* and *MiSCR1* family). Interestingly, RNA seq data highlighted that 53% of the SvG-specific genes are overexpressed in J2s compared to J3-J4, whereas 80% of DG-specific genes are overexpressed in J3-J4 compared to J2s.

To confirm their role *in planta* as secreted effectors, we soaked the nematodes in siRNA to silence these genes and to determine their role in pathogenicity. Gene silencing is the unique available method to analyse the role of putative effectors in plant-parasitic nematodes (Gheysen & Vanholme, 2007; Rosso et al., 2009). To date, no transformation, nor mutagenesis could be applied to these obligate biotroph organisms with parthenogenetic reproduction. Two genes encoding small cysteine-rich secreted proteins and strongly expressed in early parasitic stages were studied (*Minc03314* and *MiSCR1* family). Only the silencing of the *MiSCR1* family, encoding a predicted secreted mature protein of 72 amino acids, showed a significant reduction of RKN reproduction, suggesting its important role in the early stages of giant cell formation. A high

cystein content is common in effectors of fungal pathogens in which disulfide bridges could enhance the stability of the protein and help it to function (Saunders *et al.*, 2012). The sequences required for the specific function could be provided by the variable regions, in which significant changes in amino acid sequences (except in Cys residues) could occur without altering the overall fold topology (Povolotskaya & Kondrashov, 2010). Moreover, Hacquard *et al.* 2012 suggested that the interaction between the C-terminal region of the SCR secreted proteins of the poplar leaf rust *Melampsora larici-populina* with host components could regulate the diversification of the gene family. Thus, a high content in Cys residues could play a disproportionately important role in the evolution of virulence effectors, leading to a very rapid diversification and contributing to the emergence of new virulences (Hacquard *et al.*, 2012).

However, among the eight candidates tested by siRNA soaking during my PhD, seven showed no effect on RKN infection. Although the silencing induced a reduction of transcript level of tested candidates from 40% to 80% 24h after soaking, no effect was observed in the nematode reproduction. Nevertheless, siRNA effect normally endures for several days depending on the gene, indicating that siRNA may not be an appropriate method to analyse the function of proteins involved in the later stage of the parasitism (Arguel *et al.*, 2012). Transgenic lines expressing a hairpin construct or an artificial miRNA in the infected roots could be a useful approach to investigate these effector candidates (see above in the *Toward new resistance strategies against RKN* part). *Arabidopsis* transgenic lines have been successfully constructed for several effectors (Michaël Quentin and Nhat My Truong, personal communication) and would allow the silencing of the nematode effector upon feeding on giant cells.

Alternatively, *Arabidopsis* lines overexpressing the RKN effector *in planta* may be useful to investigate their role during parasitism (Truong *et al.*, 2015). For example, in all *Arabidopsis* lines overexpressing Mi8D05 or Mi-CRT, susceptibility to *M. incognita* infection were reported to be higher than in control lines, suggesting the key role of these effectors in the parasitism (Xue *et al.*, 2013; Jaouannet *et al.*, 2013). A similar effect was reported in the transgenes lines over-expressing Mj-NULG1a with a larger number of galls formed in the *Arabidopsis* root (Lin *et al.*, 2013). Moreover, defense suppression essays could be used to demonstrate the function of RKN effectors in the plant immune signalling pathway (Lozano-Torres *et al.*, 2014; Niu *et al.*, 2016; Xie *et al.*, 2016; Zhuo *et al.*, 2016). Furthermore, we can also develop new screening strategies based for example on the visualisation of cytoskeleton reorganisation (using for example actin-binding proteins (ABPs) or microtubule-associated proteins (MAPs) fused to

fluorescent marker) or on plant gene silencing pathway. In collaboration with Dr Lionel NAVARRO and Dr Gersende LEPERE (ENS, Paris), we searched *M. incognita* proteome for secreted proteins containing glycine-tryptophan (GW) motifs. These proteins are predicted to interact with, and suppress the function of the plant ARGONAUTE 1 (AGO1) protein, a key component of the RNA-Induced Silencing Complex (RISC) as described for the *Pseudomonas syringae* pv. tomato bacterial effector HopT1-1 (Navarro *et al.*, 2008). One of the effector studied in this PhD work was identified, Minc17612. This DG-specific gene was thus cloned for an *in planta* silencing test. This test, that are performed at ENS Paris, use an *Arabidopsis* line expressing an artificial miRNA in the guard cells that targets a gene, *SULPHUR* (*SUL*), involved in the biosynthesis of the chlorophyll. This line shows chlorotic phenotype due to the extinction of the *SUL* expression. These plants were transformed with a construction allowing the specific expression of Minc17612 effector in the guard cells. Primary transformants were selected and are currently under multiplication. If the effector affects the plant gene silencing signaling pathway, e.g. via its interaction with AGO1, a reduction of the chlorotic phenotype will be observed in the transformed plants (Figure 22).

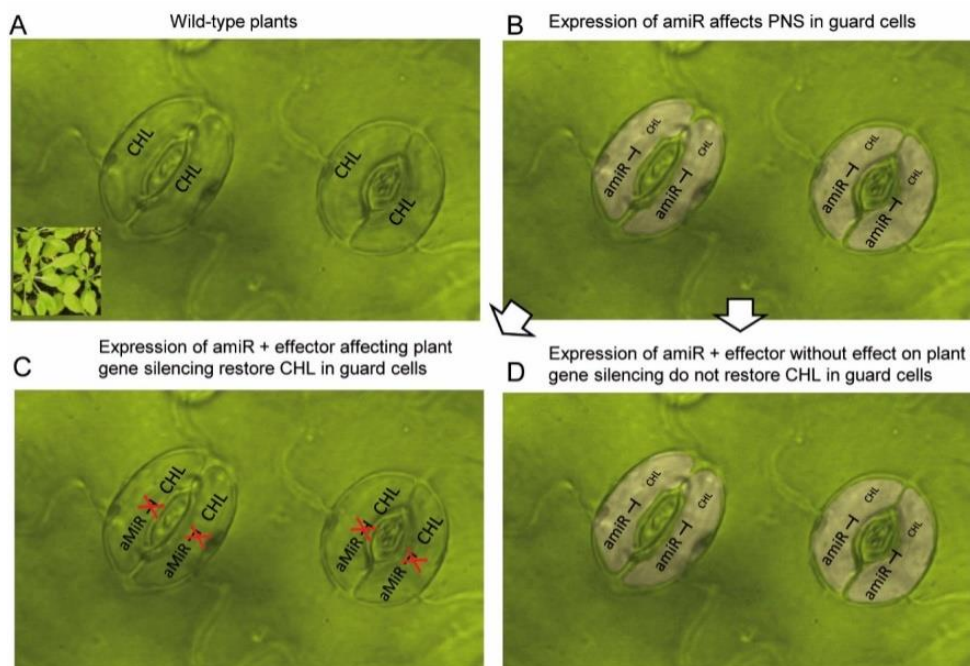


Figure 22: Plant gene silencing effector assay.

(A) wild type stomata and leaves (insert). The assay is based on the expression in the guard cells of an artificial microRNA (amiRNA) that targets a gene involved in chlorophyll (CHL) biosynthesis leading to chlorosis (B). The expression of the effector in guard cells may(C) or not (D) restore the level of chlorophyll (Navarro *et al.*, personal communication). Images were modified from Sarah M. Assmann web site (<http://sites.psu.edu/assmannlab/guard-cell-signaling-and-systems-biology/>).

The effectors required to be secreted *in planta*. Thus, a proof of their roles requires *in planta* subcellular localisation. Due to the lack of RKN transformation process, the unique strategy is based on immunolocalisation. Mi-CRT was the first protein shown to be secreted into the feeding site via the nematode stylet and to accumulate in large amounts at the cell wall of GCs (Jaubert *et al.*, 2005). Recently, two RKN effectors, Mi-EFF1 and Mj-NULG1a were reported to be injected into the cytoplasm for the GCs, and that these proteins then target the nuclei (Jaouannet *et al.*, 2012; Lin *et al.*, 2013). This analysis will be done for the most interesting candidates, such as MiSCR1. However, a simple method to have an idea of the *in planta* subcellular localisation is to use transient expression of effector fused to GFP in tobacco. The transient expression using infiltration of *Agrobacterium tumefaciens* into leaves represents a fast and convenient strategy, and has been successfully applied in nematodes and other pathogens (Elling *et al.*, 2007; Jones *et al.*, 2009; Blondeau *et al.*, 2015; Hewezi *et al.*, 2015; Petre *et al.*, 2015).

Finally, to identify the role of effector in the parasitism, it is important to find the plant target and figure out their interaction. The Y2H system was used to identify the plant targets of putative effectors in RKNs and CNs (Huang *et al.*, 2006a; Xue *et al.*, 2013; Hewezi *et al.*, 2015; Zheng *et al.*, 2015; Pogorelko *et al.*, 2016). For example, the interaction between Mi8D05 and the tomato aquaporin TIP2 illustrated that solute and water transport within GC could be regulated by RKN effectors to promote the parasitism (Xue *et al.*, 2013). Recently, the specific interactions of Hs25A01 with an Arabidopsis F-box-containing protein, a chalcone synthase and the translation inhibition factor eIF-2 β subunit, demonstrated their potential role for involvement in the observed changes in plant growth and parasitism (Zheng *et al.*, 2015). So far, several studied effectors were cloned in a yeast-two hybrid (Y2H) bait vector in order to perform a Y2H screen of a tomato prey library and identify their plant targets (Nhat-My Truong and Michaël Quentin, personal communication). A further study on these proteins could provide a better knowledge on RKNs GC formation.

2. Transcriptome profiling of the root-knot nematode *Meloidogyne enterolobii* during parasitism and identification of novel effector proteins

The aggressive RKN species *M. enterolobii* has been described as a new threat for global agriculture with its capacity to reproduce on commercial plant rootstocks resistant to

Meloidogyne spp. (Brito *et al.*, 2007; Cetintas *et al.*, 2007; Kiewnick *et al.*, 2009). To better understand the infection mechanism of this pest, we carried out a transcriptome approach focusing on two points of the nematode lifecycle: the pre-parasitic J2s and parasitic J3-J4s extracted from infected roots. Three biological replicates of each point were collected for Illumina sequencing.

J3-J4s were manually collected one by one after an enzymatic treatment to extract them from infected tomato roots. We chose this strategy to enrich on nematode sequences avoiding dilution with contaminating plant sequences. We first explored the genome size of *M. enterolobii*. Its measure via flow cytometry experiments was estimated at ~275 Mb. This genome size is in the range of those recently described for *M. incognita* (189 Mb), *M. javanica* (297 Mb) and *M. arenaria* (304 Mb) and reflects the complexity of apomictic RKN genomes (Blanc-Mathieu *et al.* 2016). The alternative would have been to collect galls, i.e. a mix of root and nematode tissues. This strategy was used to reveal the transcriptome of *M. graminicola* (Petitot *et al.* 2016). In the early stage of parasitism, from 2 dpi to 8 dpi, approximatively 97% of the reads belonged to the rice reference genome, but this number decreased to 50% at 12 dpi (J3-J4 stage) and to 13-21% at 16 dpi (female stage) (Petitot *et al.*, 2016). This dual RNA-seq yielded a total number of 174 million reads, 66 million reads from pre-parasitic J2s (2 libraries) and 108 million reads from parasitic stages (10 libraries) and allowed *de novo* assembly of the *M. graminicola* transcriptome. In our experiments, six libraries (3 from J2s and 3 from J3-J4s) yielded 520,733,592 raw reads.

However, the used of dissected J3-J4s, limited the quantity of available material. Moreover, the extracted total RNA is more fragmented and does not allow us to consider the use of a strategy using poly-A purification. Therefore, we searched a kit usable with limited input material and allowing to remove the ribosomal RNA. Indeed, a high percentage of ribosomal RNAs (rRNA: 5S, 18S and 28S) was observed in previously constructed *M. enterolobii* libraries (up to 95% in the worst library) that were not informative data. Nugen Ovation Universal RNA-seq system was chosen to produce amplified cDNA that meets the requirements for whole transcriptome analysis. This system requires a low input of total RNA (from 10-100 ng) for library construction. In a study comparing the different RNA-seq analysis methods for degraded or low input samples, Nugen technologies carried out with an input of 100 ng of fragmented RNA led to only 23.2% of rRNA observed in the sequenced library (Adiconis *et al.*, 2013). Ribodepletion was performed using 105 primers designed from the 18S and 28S rRNA consensus sequences.

However this strategy has not yielded the expected results. A high presence of rRNA, was still observed in the *M. enterolobii* libraries: from 34.42% of the reads in the J2-3 library to 88.1% in the J3-4-1 library. This result could be due to the limited and degraded input of total RNA extracted from parasitic J3-J4 samples. An alternative could be to use another kit, instead of Nugen kit, to improve the quality of the libraries. Ribo-Zero Gold Kit (Epicentre) was used as standard control in a study comparing different kits for ribodepletion and showed a good results in library quality (Shanker *et al.*, 2015). This kit was designed for known models such as human, mouse or *C. elegans*. Although the free-living nematode *C. elegans* is one of the most studied model in biological research while our non-model species *M. enterolobii* is rarely characterized, we could ask the manufacturer to optimise the kit with our nematode.

Finally, after removal of rRNA sequences, adaptor sequences, ambiguous reads and low-quality reads, we obtained a total of 174 million high-quality clean read pairs. These high-quality reads were assembled *de novo* and produced 127,355 contigs with an average length of 495 bp. We only got these data at the beginning of October 2016. Therefore, in this manuscript, we only reported a preliminary analysis of the *M. enterolobii* transcriptome. Actually, this study is performed in collaboration with Dr. Sebastian Kiewnick (Agroscope, Switzerland). Our sequencing data will be combined with their reads obtained from preparasitic J2s and eggs in order to improve the coverage of the transcriptome. A first analysis using Interproscan results in 103,075 proteins including 24,696 have functional annotation. Among them, 1,632 putative secreted proteins without transmembrane domain were identified, in which 280 showed at least one GO term. One of the most interesting points of this study is the comparison with other nematodes and in particular *Meloidogyne* species, and among them *M. incognita*. For further investigation of the *M. enterolobii* proteome, ortholog groups, as well as the species-specific proteins of *M. enterolobii*, will be identify by OrthoMCL analysis (Chen *et al.*, 2006) with other *Meloidogyne* spp. or plant-parasitic nematodes. A recent analysis on the putative secreted proteins of *M. enterolobii* predicted from 454-sequencing identified 679 conserved proteins between *M. enterolobii*, *M. incognita* and *M. hapla* and 701 proteins specific to *M. enterolobii* (Li *et al.*, 2016). In our study, blastP analysis of 106 *M. incognita* known putative effectors against *M. enterolobii* proteome reported the presence of 95% of these effectors in *M. enterolobii* transcriptome. These results figured out a high similarity between the effectomes of *Meloidogyne* spp. Three previously characterized RKN effectors were not detected in *M. enterolobii*, *M. hapla* and *M. javanica* transcriptomes or genome, but detected in *M. incognita*

and *M. arenaria*. They could constitute putative avirulence proteins with respect to the *Mi1.2*, *Me*, *N* or *R-Mia* resistance genes. Their absence may explain why *M. enterolobii* are not controlled by these resistance genes. However, this hypothesis should be tested to confirm the presence or absence of expression in the different *Meloidogyne* species, and also in virulent *M. incognita* populations. The expression of these genes when RKNs infect resistant plants may induce a hypersensitive response or their silencing in avirulent RKNs may allow the overcoming of the resistance.

Moreover, we will complete this analysis by carrying out the same approach as the transcriptomic analysis for *M. incognita*, i.e. we will focus on the putative secreted proteins without transmembrane domain that are differently expressed between pre-parasitic J2 and parasitic stage J3-J4 of lifecycle. The goal is to identify a set of effector candidates including common effectors with other *Meloidogyne* spp. and *M. enterolobii* specific-effectors. After *in silico* pipeline, qRT-PCR will be performed to validate profile expression of the candidates. A medium-term objective will be to perform function analysis of these *M. enterolobii* candidates. *In situ* hybridisation will be carried out to localise their expression in nematode organs. The putative role as effector of proteins found to be expressed in the salivary glands would be verified by functional analysis, by siRNA soaking or using transgenic lines of *A. thaliana* expressing a hairpin construct or an artificial miRNA as described above. *In planta* subcellular localisation will also be carried out to determine the cellular compartments where the newly identified nematode effectors would localise. It will be of course interesting to characterise the plant targets of these effectors. However, a first step could be to test whether the *M. incognita* and *M. enterolobii* effector orthologs may interact with similar targets using a pairwise Y2H test between *M. incognita* plant targets and *M. enterolobii* effectors. This analysis could help to develop resistance to both pathogens.

3. Toward new resistance strategies against RKN.

To date, with the restriction in using chemical nematicides due to their impact to health and environment, the requirements to find new effective ways of controlling RKNs in crop plants are more and more important. The identification and combination of natural nematode resistance genes represent a first step toward establishing control programs. In addition, new resistance strategies could be developed, based on the knowledges of the molecular interactions between

plants and nematodes, in particularly on the identification of nematode secreted effectors and their plant targets. During my PhD project, we focused on effectors of two *Meloidogyne* species with wide host ranges: *M. incognita* and *M. enterolobii*. The high similarity between effectomes of *M. incognita* and *M. enterolobii* gives hope to develop control strategies for both pathogens. In modern resistance breeding, effectors are emerging as tools to accelerate and improve the identification, functional characterization, and deployment of resistance genes. Effector-assisted breeding has contributed to classical resistance breeding as well as for genetically modified approaches (Vleeshouwers & Oliver, 2014)

Transgenic approaches for nematode control could be developed to target key nematode effectors by RNAi-based methods. siRNA can be delivered to nematodes by feeding on transgenic plants expressing a dsRNA (usually a hpRNA) or specific siRNA. This technology was used to demonstrate the function of several RKN candidate effectors (16D10, 8D05, Mi-CRT, fatty acid- and retinol-binding Mj-FAR-1 and Mj-NULG1a) in parasitism (Huang *et al.*, 2006a; Xue *et al.*, 2013; Iberkleid *et al.*, 2013; Lin *et al.*, 2013; Jaouannet & Rosso, 2013). Interestingly, the *in vivo* expression of 16D10 dsRNA in *Arabidopsis* resulted in resistance effective against the five major RKN species, *M. incognita*, *M. javanica*, *M. arenaria*, *M. chitwoodi* and *M. hapla* (Huang *et al.*, 2006b; Dinh *et al.*, 2014). Moreover, the *in planta* silencing of 16D10 could also be applied to crop plants such as grapes and potato, and this approach may provide interesting opportunities to provide crops with broad resistance to RKNs (Yang *et al.*, 2013; Dinh *et al.*, 2014). However, *in planta* siRNA approach implies the production of transgenic plants, that are genetically modified organisms (GMO), currently banned in EU.

Another approach for nematode control is to focus on the plant genes that are essential for disease development, i.e. giant cell formation in the case of RKNs. Mutation or loss of these susceptibility genes can therefore limit the ability of the pathogen to cause disease. Whereas classical resistance genes are typically dominant, resistance conferred by loss or alteration of susceptibility genes is generally recessive (De Almeida Engler *et al.*, 2005; Pavan *et al.*, 2010; van Schie & Takken, 2014). One of the best characterized susceptibility factors in plants are components of the translation initiation machinery, eukaryotic initiation factors 4E (eIF4E). These natural resistance alleles have been identified in numerous species, including lettuce, pepper, pea, tomato (Wang & Krishnaswamy, 2012). In *A. thaliana* lines, null mutations affecting eIF4E1 were reported to be involved in specific resistance to a wide range of potyviruses (Duprat *et al.*, 2002; Lellis *et al.*, 2002; Sato *et al.*, 2005).

The study of genes differentially expressed during Arabidopsis-RKN interaction led to the characterization of few genes essential for the development of giant cells induced by *M. incognita*, e.g. the microtubule-associated protein MAP65-3, the actin depolymerizing factor ADF9 and the phytosulfokine receptor PSKR1 (Caillaud *et al.*, 2008c; Clement *et al.*, 2009; Rodiuc *et al.*, 2016). The absence of MAP65-3 led to an incomplete formation of giant cells, they started to develop but accumulation of mitosis defects during nuclear division prevented the normal development of giant cells leading to the death of the nematode (Caillaud *et al.*, 2008c). In addition, the silencing of Arabidopsis cell cycle gene AtCDKA;1 led to a reduced susceptibility to RKN (Van De Cappelle *et al.*, 2008). A second strategy to identify new susceptibility genes is to characterize the direct targets of the effectors. The inactivation of the plant targets of RKN and CN effectors may lead to an increased resistance (Huang *et al.*, 2006a; Xue *et al.*, 2013; Hewezi *et al.*, 2015; Zheng *et al.*, 2015; Pogorelko *et al.*, 2016). However, this strategy usually requires a transfer of knowledge between model species such as Arabidopsis and crops. In recent years, TILLING (Targeting Induced Local Lesions IN Genomes), a new emerging technology that doesn't rely on genetic transformation techniques, has been applied to investigate the role of genes of interest, and also to develop new plant resistances. In this method, chemical mutagenesis was used for inducing variability and sensitive molecular screenings were developed to identify plants carrying the point mutations in the gene of interest (McCallum *et al.*, 2000). As an example, mutant tomato lines of eIF4E1 induced by TILLING were demonstrated to confer broad-spectrum resistance to potyviruses (Piron *et al.*, 2010; Gauffier *et al.*, 2016). Therefore, TILLING could be used to induced mutations or natural variants that enable the host protein to evade recognition by nematode effectors.

Furthermore, new emerging method such as CRISPR/Cas9 system could be a useful tool to improve plant resistance. The CRISPR/Cas9 system acts as an adaptive immune system to protect bacteria against invading foreign DNA, such as phages, by cleaving the nucleic acid by an RNA-guided DNA nuclease in a sequence-specific manner (Sorek *et al.*, 2013; Chaparro-Garcia *et al.*, 2015). Using this approach, transgenic *A. thaliana* plants with mutations in the PDS (phytoene desaturase) locus were generated (Nekrasov *et al.*, 2013). Recently, *N. benthamiana* plants expressing CRISPR/Cas9 exhibit delayed or reduced accumulation of viral DNA, abolishing or significantly attenuating symptoms of infection. Moreover, this system could simultaneously target multiple DNA viruses (Ali *et al.*, 2015). This developing technique of gene

editing could be a promising tool to modify host plant genes to confer nematode resistance, but there is still some uncertainty about the extent of off-target effects for this technology.

For conclusion, during my PhD project, we established a transcriptomic approach focused on the effectors playing a role in the parasitic stages of the nematode lifecycle. We figured out a new set of putative effectors of *M. incognita*, among which one was reported to play an important role in the early stages of parasitism. In parallel, we generated preliminary steps to identify the specific parasitism features of *M. enterolobii*. Although these studies need to be further developed, they yet provided new data that could enrich our knowledge on the interaction between plants and nematodes.

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ANNEXES

In these annexes are presented two key protocols for the functional analysis of RKN effectors and my Curriculum vitae.

Annex 1: *In situ* Hybridisation protocol for localisation of gene expression in nematode

Annex 2: siRNA soaking protocol and resistance test

Annex 3: Curriculum vitae

Annex 1: *In situ* Hybridisation protocol for localisation of gene expression in nematode

A. Material

Solution preparation:

Hybridization buffer (prepare in the hood)

Product	Store	Brand	REF N°	Lot N°	Master solution	V for 10mL
Deionized Formamide	4°C	SIGMA®	F9037	098K0662	99.5%	5mL
SSC buffer	RT	Ambion®	AM9770	0910007	20X	2mL
Borhinger blocking reagent	4°C	Roche®	11 096 176 00 1	14698921	10%	1mL
SDS	RT	GIBCO®	15553-035	1319588	10%	2mL
Denharts solution	-20°C	SIGMA®	D2532	078K6185	50X	200µL
EDTA pH8	RT	Ambion®	AM9261	0906009	0.5M	20µL
Salmon sperm DNA	-20°C	Invitrogen®	15632-011	675703	10mg/µL	200µL
tRNA from backer's yeast	-80°C	SIGMA®	R8759-500UN		500U/mL	62.5µL
Total volume						10.48mL

Stored at -20°C

10% Borhinger blocking reagent (Roche Cat # 1 096 176)

10 g blocking reagent powder in 100 ml of maleic acid buffer, pH7.5

Autoclave to dissolve

Store at 4°C

Caution: The blocking reagent is difficult to dissolve. Add 10g blocking powder into 70 ml of maleic acid buffer, heat to dissolve, and then transfer the solution into a scotch for autoclave.

Yeast tRNA (SIGMA Cat # R8759 type X-SA)

Dissolve 500 units in 1 ml of DEPC water

Aliquot and store at -80°C

20X SSC (pH7,2)

3M NaCl 43.83 g

0.3 M Na citrate 22 g

Autoclave and store at room temperature H₂O qsp 250 ml

Maleic acid buffer, pH 7.5

100 mM maleic acid final 11.61 g

150 mM NaCl final 8.76 g

Adjust pH to 7.5 by NaOH 5 g and then adjust pH to 7.5 by NaOH 10N

Autoclave and store at room temperature

H₂O qsp 1l

Alkaline phosphatase Buffer (AP Buffer) pH9,5

100 mM Tris-HCl pH9.5 final 50 ml [TrisHCl 2M pH 9.5]

100 mM NaCl final 5.85 g

50mM MgCl₂ final 10.17 g MgCl₂·7H₂O

Autoclave and store at room temperature H₂O qsp 1l

100X Denhardt's

2.5% Ficoll (400 kDa) 2.5 g

2.5% PVP 2.5 g

2.5% BSA (Fraction V) 2.5 g

Autoclave and store at room temperature H₂Oqsp 100 ml

Autoclave: razor blades and eppendorfs autoclaved 1 time

Use only filter tips from beginning to end of the experiment

Clean the pipettes and eppendorf tubes rack by ethanol and then by RNAseZap

Clean the workspaces by ethanol and then by RNAseZap

Wear gloves from beginning to end of the experiment!!

All solutions are RNase Free, never pipette in the Master solutions, pour into sterilized falcons.

Prepare the DIG probes 1-2 days before ISH

B. Protocol.

I. Probe synthesis for in situ hybridization.

Material: Forward and Reverse primers

My Taq polymerase, 10X Buffer with MgCl₂, 5X Buffer

10X DIG-dUTP/dNTP mix (Borhinger Cat#1277065)

1st tour: Matrix synthesis

PCR with miniprep product

Mix:		Cycle:	
Miniprep product	0.3 µl	95°C	4 min
Forward primer	2.5 µl	95°C	30s
Reverse primer	2.5 µl	54°C	30s x35
5X My Taq Buffer	10 µl	72°C	1 min
My Taq	0.5 µl	72°C	3 min
H ₂ O	34.2 µl	20°C	Pause

Check the amplification by migration with agarose gel.

If the signal is good, do the purification in using Qiagen Gel Extraction Purification Kit.

2nd tour: Probe synthesis

Mix:		Cycle:	
Reverse primer	8 µl	95°C	1 min
10X Buffer	2.5 µl	95°C	30s
10X DIG dUTP/dNTP	3 µl	56°C	45s x50
Matrix	4 µl	72°C	1 min
My Taq	0.5 µl	20°C	Pause
H ₂ O	18 µl		

(For negative control, use Forward primer)

Withdraw 5 µl of PCR product to check the amplification by migration with agarose gel. If the signal is good, do the purification in using Qiagen PCR Purification Kit.

II. Nematode fixation:

Wear the nitrile gloves to prepare « fixative buffer » (very toxic). Throw the tips into a recycle bin for solid phenol products.

Prepare the 1X PBS - dilution in 1/10 from Master solution: 10X PBS Buffer pH7.4 AM9624 Ambion® -lot0909011, store at RT.

- o Fixative buffer (prepare at the same day)

10% formalin in PBS 1X (40% formaldehyde = 100% formalin)

Formaldehyde 37Gew-% Lsg in water (Cat F1,558-7 Aldrich®) lotS37305-1081 ml

PBS 1Xqsp 10 ml

1- Suspend the nematodes in maximum 10 ml of fixative buffer, in a 1 falcon.

2- Over Night for the J2s / over the weekend at RT for parasitic stages.

Place methanol and acetone at -80°C

III. Nematode dissection:

1) Wear the gloves.

2) Wash the glass plate by dishwashing liquid. Clean it by distilled water, by RNaseZap and then by ethanol. Dry well.

- Centrifuge the tube containing nematode and fixative buffer at 3000 rpm, slow deceleration.

Use the 1.5 ml eppendorfs autoclaved 2 times

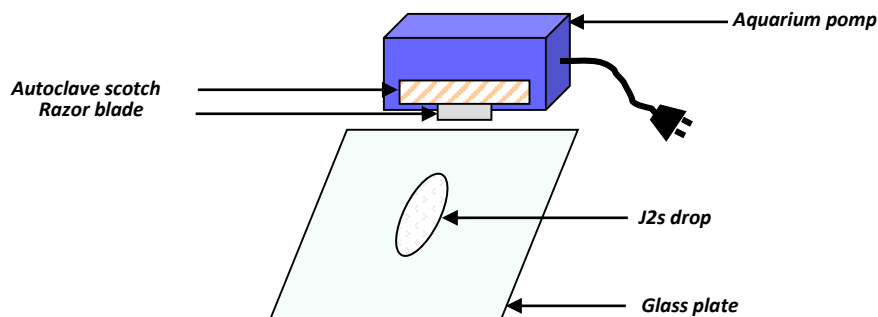
1- Centrifuge the tube containing nematode and fixative buffer at 3000 rpm, low deceleration.

Withdraw fixative buffer. The fixative buffer must be poured in a recycle bin for liquid phenol.

- 2- Transfer 1mL of fixative buffer containing nematode to an eppendorf. Centrifuge at 10000 rpm for 1 min - slow deceleration.
- 3- Keep 500 μ L of fixative buffer.
- 4- Spread this 500 μ L of fixative buffer containing nematodes on a glass plate, and then pass the machine (aquarium pump + razor blades autoclaved) over the drop for several times. Observe the nematode in microscope to know if the worms are well sectioned (2 - 4 sections).

Attention: for parasitic stages, don't chop them too much!

- 5- Recover the nematode sections in a 1.5ml eppendorf by deposit some drops of 1X PBS buffer on the glass plate.



- 6- Centrifugation (10000 rpm for 1 min– slow deceleration). Discharge the buffer.
- 7- Resuspend in 1ml of 1X PBS, and then centrifuge the tube at 10000 rpm for 1 min– slow deceleration.
- 8- Redo the wash in 1X PBS one more time. Totally, there are three washes in PBS 1X.

IV. Nematode permeabilisation:

- o Proteinase K (Am2546 Ambion® lot 09090011) Master solution=20mg/mL

Proteinase K master solution 50 µL

PBS 1X qsp 1mL (deduct the remaining volume of PBS from last wash)

Attention: Do not Vortex the proteinase K and keep it in a cold block

- 1- Resuspend the nematode palette in 1 ml of proteinase K solution [1mg/ml] in 1X PBS. Incubate the tube with agitation at 37°C for 1 hour in an appligene oven.
- 2- Centrifuge the tube at 10000 rpm for 1 min– slow deceleration. Discharge the buffer.
- ☑ Increase the temperature in the oven up to 50°C.
- 3- Wash the sections in 1 ml of 1X PBS and then centrifuge the tube at 10000 rpm for 1 min– slow deceleration. Discharge the buffer.
- 4- Totally, 3 washes in 1X PBS.
- 5- Keep the palette at -80°C for 15 min.
- 6- Add 1 mL of cold methanol (stored at -80°C from the 1st day).
- 7- Vortex to suspend the nematode palette, and then 2 min at RT.
- 8- Centrifuge the tube at 10000 rpm for 1 min– slow deceleration.
- 9- Discharge methanol.
- 10- Add 1mL of acetone (stored at -80°C from the 1st day).
- 11- Keep the tube at -80°C for 15min.

- Incubate the hybridization buffer at 50°C 5 min before centrifugation.
- 12- Centrifuge the tube at 10000 rpm for 1 min– slow deceleration.
- 13- Discharge acetone.

V. HYBRIDISATION

- 1- Wash the palette in 500 µl of hybridisation buffer.

- 2- Prehybridize the nematode palette in 500 μ l of hybridization buffer for 30 min at 50°C under agitation (applied oven)

During this time, denature the probes at 100°C for 5 min and then keep them in ice for 3 min.

Centrifuge the tube at 10000 rpm for 1 min– slow deceleration.

- 3- Suspend the nematode palette in a volume of V μ l of hybridization buffer ($V=100x \mu$ l and x =number of tested probes)
- 4- Aliquot the solution into eppendorfs RNase free tubes (100 μ l/tube/tested probe)
- 5- Adjust a desired volume of probe and hybridization buffer to obtain a final volume of 125 μ l and a dilution factor of the probe of 5X (in 2014, 10 μ l of probe)
- 6- Hybridization O.N. at 42°C with agitation (applied oven)
- 7- Centrifuge the tubes at 10000 rpm for 1 min– slow deceleration. Discharge the buffer.
- 8- 2 x 10 min in 1 ml of 4x SSC 0.1%SDS at RT with agitation. Centrifuge the tubes at 10000 rpm for 1 min– slow deceleration. Discharge the buffer.
- 9- 2 x 10 min in 1 ml of 0.1x SSC – 0.1% SDS at 50°C with agitation. Centrifuge the tubes at 10000 rpm for 1 min– slow deceleration. Discharge the buffer.

VI. REVELATION

- 1- Wash: 1x 30 sec in 1 ml of 1X maleic acid buffer.
- 2- Centrifuge the tubes at 10000 rpm for 1 min– slow deceleration. Discharge the buffer.
- 3- Incubate the nematodes 37°C for 30min (applied oven) in 1 ml of 1X Boehringer blocking reagent (10X solution diluted in 1X maleic acid: 450 μ L 1X maleic acid + 50 μ L Boehringer blocking reagent). Discharge the buffer.
- 4- Incubate the nematodes at 37°C for 3h in 1ml of the anti-DIG antibody in 1X Boehringer blocking reagent (Anti-Digoxigenin-AP-Fab fragments Labelling Mix-REF11093 274 910 Roche® lot 11787127 diluted at 1/500e in 1X Boehringer blocking reagent).
- 5- Centrifuge the tubes at 10000 rpm for 1 min– slow deceleration.
- 6- Discharge the mix of anti-DIG /Boehringer blocking reagent.
- 7- 3 washes of 15 min in 1 ml of 1X maleic acid buffer at 37°C.

- 8- 1 brief wash in 1ml of Alkaline Phosphatase Detection Buffer (AP Buffer)
- 9- Reveal the signal by incubating the nematodes O.N at RT without agitation in 1 ml AP Buffer + 3.75 μ l BCIP + 5 μ l NBT (BCIP Roche[®] REF 11 383 221 001, lot 13552029 ; NBT Roche[®] REF 11 383 213 001, lot 146 989)

Attention : Wear the Nitrile gloves, these two products are very toxic.

Modulate the revelation (O.N at 4°C) is preferable for some very highly expressed genes.

The next day, observe the signal on a slide. If it's ok, stop the coloration by one wash in 1 ml of RNase Free Water (Ref 10977-035 GIBCO[®] lot 712292) or 1X PBS.

Annex 2: siRNA soaking protocol and resistance test

Objective: silence the nematode gene by siRNA soaking

Materials: Ambion Silencer siRNA Construction Kit (AM1620), primers of siRNA sense and anti-sense, J2s nematode, 4-5 weeks old tomato plants, Volvic water

1. siRNA design.

1.1. Find 21 nt sequences in the target mRNA that begin with an AA dinucleotide.

Beginning with the AUG start codon of your transcript, scan for AA dinucleotide sequences. Record each **AA and the 3' adjacent 19 nucleotides as potential siRNA target sites**. This strategy for choosing siRNA target sites is based on the observation by Elbashir et al. (1) that siRNAs with 3' overhanging UU dinucleotides are the most effective. This is also compatible with using RNA pol III to transcribe hairpin siRNAs because RNA pol III terminates transcription at 4-6 nucleotide poly(T) tracts creating RNA molecules with a short poly(U) tail. In Elbashir's and subsequent publications, siRNAs with other 3' terminal dinucleotide overhangs have been shown to effectively induce RNAi. If desired, you may modify this target site selection strategy to design siRNAs with other dinucleotide overhangs, but it is recommended that you avoid G residues in the overhang because of the potential for the siRNA to be cleaved by RNase at single-stranded G residues.

In this step, we could use **RNAfold server** tool on <http://rna.tbi.univie.ac.at/> to predict minimum free energy structures and base pair probabilities from single RNA or DNA sequences. Copy and paste the sequence into the command box, and then choose the suitable options (for me, I choose default options) before proceeding the demand.

As results, the minimum free energy (MFE) structure will be shown as a secondary structure drawing. Moreover, a mountain plot representation of MFE structure, the thermodynamic ensemble of RNA structures, and the centroid structure will also be represented. **The siRNA target should be in the zone where MFE is high.**

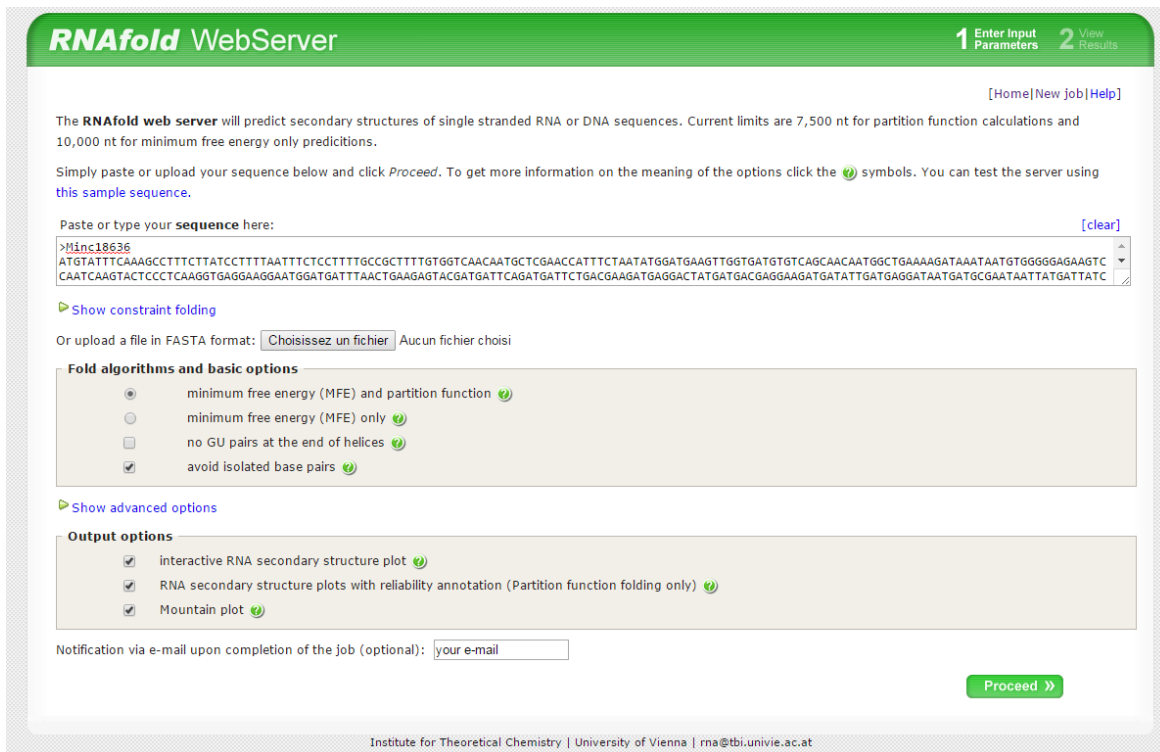


Figure 23: RNAfold Webserver interface

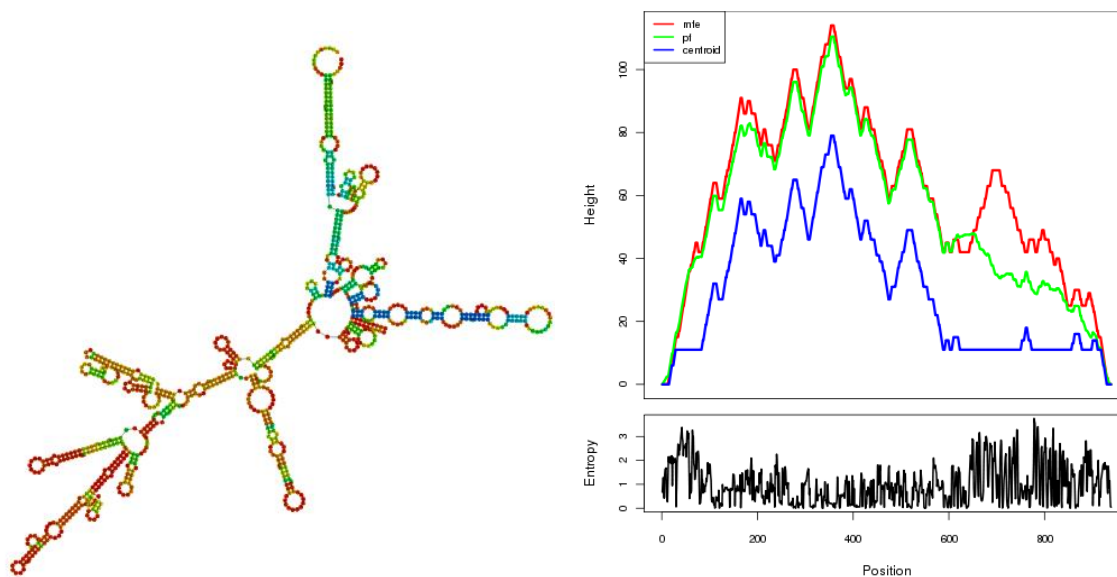


Figure 24: Secondary structure of the minimum free energy (MFE) and the thermodynamic of RNA structures

After that, design the siRNA with the help of RNAs server tool on <http://rna.tbi.univie.ac.at/> and <http://sidirect2.rnai.jp/> .

Caution: these tools are used only for suggestions. Don't copy and paste the sequences indicated in these tools.

For RNAxs server tool, in the Design Options, the Custom Sequence Rules should be 17 N + TT (NNNNNNNNNNNNNNNNNNTT) (if we choose more than 19nt, the tool won't work).

For sidirect2, in the Option, the GC content should be chosen between 30 and 50%.

After that, launch the tool and find the 21 nt sequences in the target mRNA that begin with an AA dinucleotide.

ma.tbi.univie.ac.at/RNAxs/RNAxshSJE15/18636_RNA/

RNA XS
1 2

1 Data Input
2 View Results

Hereunder you will find a detailed view of the 20 best siRNAs selected for 18636_RNA

A ranked list of all predicted siRNAs for 18636_RNA is available as text file [here](#)

Your results will be deleted on May 7, 2015. Find [here](#) a Tar Zip archive of your Results

Accessibility Plot siRNA:1 Pos:642

siRNA	1
Worst Rank	9
Position	642
Access 8nt	0.9709
Access 16nt	0.9274
Assymetry (S)	0.5000
Assymetry (E)	0.5000
Self Folding	1.0000
Free End	1.0000
Target Seq.	AAAAACAGAATTAAAAAA
siRNA Seq.	TTTTTTAATTCGTTTT
BLAST	NCBI BLAST

Accessibility Plot siRNA:2 Pos:657

siRNA	2
Worst Rank	10
Position	657
Access 8nt	0.2494
Access 16nt	0.1257
Assymetry (S)	0.5000
Assymetry (E)	0.5000
Self Folding	1.0000
Free End	1.0000
Target Seq.	AAAAACGTCGGCTAAAAAA
siRNA Seq.	TTTTTTAGCGGACGGTTTT
BLAST	NCBI BLAST

Figure 25 : siRNA target suggested by RNAfod Websever

Graphical view of effective siRNA candidates



Figure 26 : siRNA target suggested by sidirect2

Caution: The RNAs sever suggests only the siRNA target with 19 nt, while the sidirect2 suggests the one with 23 nt. It'd better to compare these two results to find a siRNA target of 21 nt.

1.2. Select 2-4 target sequences.

Research at Ambion has found that typically more than half of randomly designed siRNAs provide at least a 50% reduction in target mRNA levels and approximately 1 of 4 siRNAs provide

a 75-95% reduction. Choose target sites from among the sequences identified in Step 1 based on the following guidelines:

- Ambion researchers find that siRNAs with 30-50% GC content are more active than those with a higher G/C content.
- Since a 4-6 nucleotide poly(T) tract acts as a termination signal for RNA pol III, avoid stretches of > 4 T's or A's in the target sequence when designing sequences to be expressed from an RNA pol III promoter.
- Since some regions of mRNA may be either highly structured or bound by regulatory proteins, research at Ambion generally select siRNA target sites at different positions along the length of the gene sequence. They have not seen any correlation between the position of target sites on the mRNA and siRNA potency.
- The siRNA target sites should also be positioned in the qPCR amplicon of the interested gene to ensure the specificity of soaking.
- Compare the potential target sites to the appropriate genome database (human, mouse, rat, etc.) and eliminate from consideration any target sequences with more than 16-17 contiguous base pairs of homology to other coding sequences. The BLAST from NCBI server at: www.ncbi.nlm.nih.gov/BLAST and BLASTn from Nemesis server are suggested to be used.

Example of Template Oligonucleotide Design:

Target mRNA sequence: 5'- AACGAUUGACAGCGGAUUGCC-3' = AA + 19nt

The **antisense template** oligonucleotide will be designed as:

5'-AACGATTGACAGCGGATTGCC**CCTGTCTC**-3' = AA + 19nt + **CCTGTCTC** (8nt of T7 promoter)

After that, find the complementary sequence of 19nt antisense:

19nt antisense: CGATTGACAGCGGATTGCC => 19nt sense: GGCAATCCGCTGTCAATCG

The **sense template** oligonucleotide will be designed as:

5'-**AAGGCAATCCGCTGTCAATCGCCTGTCTC**-3' = AA + 19nt sense + **CCTGTCTC** (8nt of T7 promoter)

2. siRNA synthesis

Materials: Ambion Silencer siRNA Construction Kit (AM1620), primers of siRNA sense and anti-sense

Procedure:

Day 1:

- The primers of siRNA sense and anti-sense must be at the concentration of 100 μ M. Prepare 20 μ l for each oligonucleotide solution.

- **Hybridize each template oligonucleotide** (sense and anti-sense) separately to the T7 promoter primer:

+ In 2 separate tubes mix the following:

Amount	Component
2 μ L	T7 Promoter Primer (100 μ M)
6 μ L	DNA Hyb Buffer
2 μ L	either sense or antisense template oligonucleotide

+ Heat the mixture to 70°C for 5 min, then leave at room temp for 5 min.

- **Fill in with Klenow DNA polymerase:**

+ Add the following to the hybridized oligonucleotides:

Amount	Component
2 μ L	10X Klenow Reaction Buffer
2 μ L	10X dNTP Mix
4 μ L	Nuclease-free Water
2 μ L	Exo- Klenow

(Caution: Keep the tube of Exo- Klenow at -20°C and do not vortex it.)

+ Gently mix by pipetting or slow vortexing. Centrifuge briefly to collect the mixture at the bottom of the tube.

+ Transfer to 37°C incubator and incubate for 30 min.

The siRNA templates (20 μ l) can be used directly in a transcription reaction or stored at -20°C until they are needed for transcription.

- **Transcription**

+ For each siRNA, synthesize the sense and antisense RNA strands of the siRNA. For each transcription reaction, mix the following components in the order shown:

Amount	Component
2 μ L	sense or antisense siRNA template
4 μ L	Nuclease-free Water
10 μ L	2X NTP Mix
2 μ L	10X T7 Reaction Buffer
2 μ L	T7 Enzyme Mix

(Caution: Keep the tube of T7 Enzyme Mix at -20°C and do not vortex it.)

+ Gently mix contents thoroughly by flicking or brief vortexing and then microfuge briefly to collect the reaction mixture at the bottom of the tube.

+ Incubate transcription reactions for 2h at 37°C , preferably in a cabinet incubator (This will prevent condensation, which may occur if the tube is incubated in a heat block).

- Combine the sense and antisense transcription reactions into a single tube, mix gently and continue incubation at 37°C overnight. The overnight incubation will maximize the yield of RNA and facilitate hybridization of the sense and antisense strands of the siRNA, leading to a dsRNA ($V_f = 40 \mu\text{l}$).

Day 2: siRNA preparation/purification

- Digest the siRNA with RNase and DNase:

+ To the tube of dsRNA ($40 \mu\text{l}$), add the following reagents in the indicated order:

Amount	Component
6 μL	Digestion Buffer
48.5 μL	Nuclease-free Water (qsp 100 μL)
3 μL	RNase
2.5 μL	DNase

+ Mix gently, and incubate for 2 hrs. at 37°C .

- Preheat Nuclease-free Water to 75°C .

Remark: Before their first use, add 100% ethanol to the siRNA Binding and Wash Buffers as indicated in the bottle.

- Add 400 μL of siRNA Binding Buffer to the nuclease digestion reaction and incubate for 2–5 min at room temperature.

- Prewet a Filter Cartridge with 100 μL siRNA Wash Buffer and bind the siRNA:

+ For each siRNA preparation, place a Filter Cartridge in a 2 mL Tube (provided with the kit)

+ Apply 100 μL of siRNA Wash Buffer to the filter of the Filter Cartridge

+ Add the siRNA (500 μL) to a prewet Filter Cartridge and centrifuge at 10,000 rpm for 1 min

+ Discard the flow-through from the collection tube, and replace the Filter Cartridge in the 2 mL Tube.

- Apply 500 μL of siRNA Wash Buffer to the filter of the Filter Cartridge and centrifuge at 10,000 rpm for 1 min. Discard the flow-through from the collection tube, and replace the Filter Cartridge in the 2 mL Tube. Repeat the wash with a second 500 μL of siRNA Wash Buffer. Transfer the Filter Cartridge to a new 2 mL Tube.

- Add 100 μL of the preheated Nuclease-free Water to the filter of the Filter Cartridge and incubate at room temperature for 2 min.

- Centrifuge at 12,000 rpm for 2 min. The purified siRNA will be in the 2 mL Tube.

- Dose the siRNA concentration by Nanodrop (usually $\geq 400 \text{ ng}/\mu\text{l}$). Store at -20°C or -80°C .

3. Plant infection by nematode soaked by siRNA.

Materials: synthesized siRNA, J2s and 4-5 weeks old tomato plants, Volvic water

Procedure:

Day 1:

- Dilute the siRNA with Volvic water to the concentration of 50 ng/ μ l and to a final volume of 40 μ l.
- Concentrate the volume of water containing J2s using a 1 μ m filter (pluriStrainer, ref:43-50000-99, pluriSelect).
- Aliquot 10,000 J2s/condition (in a 1.5 ml tube)
- Discharge maximum water
- Add 40 μ l siRNA
- Incubate at RT for 1 h with agitation in the darkness: pack the tubes in aluminum paper, put them in a 50 ml falcon, and then put the falcon on a roller agitator.
- Add 1 ml Volvic water, and then centrifuge at 10,000 rpm for 1 min at RT. After that discharge maximum water (repeat this step 2 times).
- Add 100 μ l Volvic water and incubate the tube at RT for 16h.

Day 2: Infect the J2s soaked into plants

- The infection will be performed 16h after soaking.
- Add Volvic water to the tube containing J2s soaked by siRNA up to 1.5 ml.
- Take 3 x 10 μ l and count the J2 number in each sample.
- Calculate the necessary volume needed to withdraw to have 3750 J2s. The rest volume will be used for qPCR after discharging water and stored at -80°C. It'd better to keep the J2s 24h after soaking for qPCR. If possible, the number of J2s for qPCR should be more than 5000 to ensure the tRNA quantity after tRNA extraction.
- For each sample, add volvic water up to 25 ml.
- Took 500 μ l and count the number of J2s (expected to be ~ 75).
- The tomato plants will be infected by 150 J2s soaked/plant (1 ml).
- The number of galls and egg masses in the root will be counted 6 weeks after infection.

Caution: qPCR result is very important in this experiment. If we don't observe any reduction of gene expression after soaking, it is not necessary to count the galls and egg masses number.

Annex 3 : Curriculum vitae

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EDUCATION AND TRAINING

- 10/2013-now PhD student at Graduate School for Life and Health Sciences, Nice Sophia-Antipolis University, Nice, France.
Laboratory: Equipe Interactions Plantes-Nématodes (IPN), Institut Sophia Agrobiotech (ISA), UMR INRA-UNS-CNRS 400 route des Chappes 06903 Sophia Antipolis. Thesis director: Bruno Favery, (DR2 INRA).
- 09/2011-06/2012 Master on Physiology and Integrative Biology, Pierre and Marie Curie University, Paris, France.
- 09/2006-06/2011 Engineer on Food Technology, Hanoi University of Science and Technology, Hanoi, Vietnam.

WORK EXPERIENCES

- 10/2013-now PhD student. Project: “**Comprehensive transcriptome profiling of root-knot nematodes during plant infection and characterization of species-specific traits**”. *Plant-Nematode Interactions Team, INRA-CNRS-UNS, Institut Sophia Agrobiotech, Sophia-Antipolis, France.*
- 09/2012-09/2012 Research assistant on “**Bio-ethanol and butanol production**” projects. *Department of Food Technology, School of Biotechnology and Food Technology, Hanoi University of Science and Technology, Hanoi, Vietnam.*
- 01/2012-06/2012 Internship. Project: “**Gdf15 and proliferation of intercalated cells in the kidney collecting duct**”. *Renal Genomics, Physiology and Physiopathology Laboratory, Cordeliers Research Center, Paris, France.*
- 07/2010-06/2011 Internship. Project: “**Simultaneous Saccharification and Fermentation (SSF) of very high gravity (VHG) cassava mash for the production of bio-ethanol**”. *Department of Food Technology, School of Biotechnology and Food Technology, Hanoi University of Science and Technology, Hanoi, Vietnam.*

PUBLICATIONS

Truong, N. M.*, **Nguyen, C-N.***, Abad, P., Quentin, M., & Favery, B. (2015). Function of Root-Knot Nematode Effectors and Their Targets in Plant Parasitism. In *Advances in Botanical Research, Plant Nematode Interactions: A View on Compatible Interrelationships* (C. Escobar, & C. Fenoll, Eds.): pp. 293–324. ***Co first-authors** (Perr-journal, Impact Factor 2 years: 2,796)

Nguyen, C-N., Nottet, N., Perfus-Barbeoch, L., Danchin, E.G.J, Truong, N. M., Quentin, M., Magliano, M., Da Rocha, M., Abad, P., & Favery, B. Identification of parasitism effectors expressed during plant infection from the transcriptome of *Meloidogyne incognita* (submitted to *New Phytologist* on October, 2016).

Nguyen, C-N., Elashry, N., Perfus-Barbeoch, L., Braun-Kiewnick, A., Da Rocha, M., Pratx, L., Martinez-Jimenez, C., Danchin, E.G.J, Abad, P., Favery, B & Kiewnick, S., Transcriptome Profiling of the Root-Knot Nematode *Meloidogyne enterolobii* during plant parasitism and Identification of Novel Effector Genes (in preparation; 2nd chapter of the thesis results).

Nguyen, C-N., Le, T-M., Chu-Ky, S. (2014), Pilot scale simultaneous saccharification and fermentation at very high gravity of cassava flour for ethanol production. *Industrial Crops and Products*, 56, 160-165.

CONFERENCE PARTICIPATION

Nguyen C.N. (2016). Comprehensive Transcriptome Profiling of Root-knot Nematodes during Plant infection and Species-specific traits. "Doctoral School Days" ED85 01-02/09/2016, Nice, France (oral presentation)

Nguyen C.N., Quentin M., Perfus-Barbeoch L, Jaubert-Possamai S., P. Abad and Favery B. (2016), Transcriptomic Regulation in Root-Knot Nematodes. Bilateral Project SAKURA, 01-03/08/2016, Kumamoto, Japan (oral presentation)

Nguyen C.N., Quentin M., Danchin E.G.J, Perfus-Barbeoch L., Da Rocha M., Rancurel C., Jaubert-Possamai S. and Favery B. (2015), Comprehensive Transcriptome Profiling of Root-knot Nematodes during Plant infection and Identification of Species-specific traits. "Effectome", 16-18/09/2015, Lauret, France (oral presentation)

Nguyen C.N., Quentin M., Danchin E.G.J, Perfus-Barbeoch L., Da Rocha M., Rancurel C., Jaubert-Possamai S. and Favery B. (2015), Comprehensive Transcriptome Profiling of Root-knot Nematodes during Plant infection and identification of Species-specific traits. COST Action Fal208 "Pathogen-informed strategies for sustainable board-spectrum crop resistance" meeting, 26-28/08/2015, Kiel, Germany (poster).

Nguyen C.N., Quentin M., Danchin E.G.J, Perfus-Barbeoch L., Da Rocha M., Rancurel C., Jaubert-Possamai S. and Favery B. (2014), Transcriptomic Regulation in Root-Knot Nematodes, "Effectome", 07-11/10/2014, Lauret, France (poster).

Nguyen C.N., Quentin M., Perfus-Barbeoch L., Da Rocha M., Rancurel C., Jaubert-Possamai S. and Favery B. (2014), Transcriptomic Regulation in Root-Knot Nematodes, "6èmes journées des Doctorants SPE", 03-05/06/2014, Bordeaux, France (poster).

PERSONAL SKILLS AND COMPETENCES

Languages	Vietnamese: mother tongue English: Understanding: B2, Speaking: B2, Writing: B1 French: Understanding: B2, Speaking: B2, Writing: B2; working language
Technical skills	PCR, qPCR, gene silencing by siRNA, <i>in situ</i> hybridisation, Bioanalyser, microscopy, confocal, root-knot nematode production, HPLC, working with mice, fermentation, ethanol production, bioprocess engineering.
Computer skills	Microsoft Office, Corel Draw, AutoCad, Lightroom
Social skills	Organizational skills and teamwork: workshops organization, knowledge contest, camping, role play, etc.

HOBBIES

Football, History, Photograph, Singing, Travel, Martial Arts, Video Games