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Characterization of the post-transcriptional regulations involved in the formation of giant cells induced by root-knot nematode

Yara Nouredine

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THÈSE DE DOCTORAT

Caractérisation des régulations post-transcriptionnelles
intervenant lors de la formation des cellules géantes
induites par les nématodes à galles

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Resumé

Les nématodes à galles, du genre *Meloidogyne*, sont des vers microscopiques parasites des plantes qui infectent les racines de plus de 5 000 espèces de plantes et causent des pertes de rendement massives. Ces nématodes induisent la formation de galles en induisant la dédifférenciation de cellules racinaires en cellules nourricières géantes. La formation des cellules nourricières peut être divisée en deux phases : des mitoses successives sans cytokinèse au cours des dix premiers jours après infection (jai) puis de 10 à 21 jai, une phase d'endoreduplication et de forte croissance cellulaire. La formation de ces cellules est le résultat d'une reprogrammation massive de l'expression génique dans les cellules racinaires ciblées, comme le montrent les analyses transcriptomiques de galles. L'objectif de ma thèse était d'étudier des petits ARN non codants, les microARN, qui sont des régulateurs clés de l'expression génique chez les eucaryotes. Ces microARN agissent en induisant la dégradation ou l'inhibition de la traduction des ARN messagers (ARNm) ciblés. Au cours de ma thèse, le séquençage des petits ARN de galles de tomate induites par le nématode *Meloidogyne incognita* et de racines non infectées a permis d'identifier 174 microARN qui sont différentiellement exprimés dans les galles à 7 et/ou 14 jai. Les ARNm ciblés par les microARN dans ces galles ont été identifiés en intégrant les données de séquençage des microARN avec les données de transcriptome et d'un séquençage spécifique des ARNm clivés appelé dégradome. Cette analyse intégrative a permis la construction d'un réseau de régulation de l'expression génique agissant lors de la formation des cellules nourricières chez la tomate. Trois familles de microARN, miR167, miR398 et miR408, ont été sélectionnées pour les analyses fonctionnelles. La famille miR167 cible les transcrits d'*ARF8A* et *ARF8B*, codant des facteurs de réponse à l'auxine appartiennent à la voie de signalisation de l'auxine, un régulateur majeur dans l'interaction plante-nématode. En utilisant des lignées de tomates exprimant les deux promoteurs ARF8 fusionnés au gène rapporteur GUS, nous avons montré une forte activité des deux promoteurs dans les galles à 7 et 14 jai, confirmant les analyses transcriptomiques. Nous avons analysé l'effet d'une délétion CRISPR au sein des séquences codantes *ARF8A* et *ARF8B* sur l'infection par *M. incognita*. Ces deux lignées présentent une résistance accrue à l'infection en raison de défauts de formation des cellules nourricières. L'ensemble des résultats a montré que l'expression d'*ARF8A* et d'*ARF8B* est nécessaire pour la formation des cellules nourricières. Les familles de microARN conservées, miR398 et miR408, sont surexprimées dans les galles de tomate et d'*Arabidopsis thaliana*. Ces microARN et leurs cibles sont impliqués dans la signalisation du cuivre. Lors de carence en cuivre, l'expression des gènes *MIR398* et *MIR408* est activée par le facteur de transcription *SPL7*, réprimant alors l'expression de gènes codant des protéines liant le cuivre non essentiel au développement des plantes. En utilisant des lignées d'*A. thaliana* exprimant une fusion transcriptionnelle avec le gène GUS, nous avons montré que *MIR408* et *SPL7* étaient exprimés dans des cellules nourricières. Des tests d'infection réalisés avec des mutants *mir408* et *spl7*, ou des lignées exprimant des cibles mutées résistantes au clivage par miR398 ont montré une résistance accrue de ces lignées aux nématodes. L'apport de sulfate de cuivre, à une concentration inférieure aux concentrations toxiques, a induit une forte résistance à l'infection. L'ensemble de ces résultats montrent le rôle de l'homéostasie du cuivre dans la formation de cellules géantes via le module *SPL7*, miR408 et miR398. Pour conclure, les travaux présentés dans ce travail de thèse démontrent le rôle de ces trois familles de microARN et de leurs cibles dans la formation de cellules nourricières géantes induites par les nématodes.

Mots clés : microARN, Nématodes à galles, Tomate, *Arabidopsis thaliana*, Galles

Abstract

Root-knot nematodes (RKN), genus *Meloidogyne*, are microscopic plant parasitic worms which infect roots of more than 5 000 cultivated plant species and cause massive crop yield losses worldwide. Within host root, RKN induce the formation of root galls by inducing the dedifferentiation of root vascular cells into giant and multinucleated feeding cells. The formation of feeding cells can be split into two phases: successive nuclear divisions during the first ten days post infection (dpi), then from 10 to 21 dpi feeding cells nuclei undergo extensive endoreduplication and plant growth. These feeding cells supply water and nutrients essential for nematode development. The formation of these feeding cells is the result of an extensive reprogramming of gene expression in targeted root cells as shown by transcriptome analyses. However, few data are available on the regulation of gene expression in these structures. The objective of my PhD thesis was to study small non-coding RNAs, microRNAs, that are key regulators of gene expression. microRNAs act at the post-transcriptional level by inducing the degradation or inhibition of the translation of targeted messenger RNAs (mRNAs). During my PhD thesis, sequencing of small RNAs from tomato (*Solanum lycopersicum*) galls induced by *Meloidogyne incognita* RKN and uninfected roots identified 174 microRNAs that are differentially expressed in galls at 7 and/or 14 dpi. mRNAs targeted by microRNAs in tomato galls were then identified by integrating microRNA sequencing data with data from transcriptome analysis and from a specific sequencing of cleaved mRNAs named degradome approach. This integrative analysis built a microRNA-gene regulatory network acting during the formation of galls and feeding cells in tomato roots. Three microRNAs families, miR167, miR398 and miR408, were selected for functional analyses. MiR167 family targets the auxin-response factors *ARF8A* and *ARF8B*. These ARFs belong to auxin signaling pathway, a key hormone in plant-RKN interaction. Using tomato lines expressing the two *ARF8* promoters fused to GUS reporter gene, I showed a strong activity of both *ARF8* promoters in galls at 7 and 14 dpi, confirming the transcriptomic analyzes. Moreover, we analyzed the effect of a CRISPR deletion within *ARF8A* and *ARF8B* coding sequences on the infection by *M. incognita*. Both CRISPR lines showed a significantly increased resistance to nematode infection correlated with defects in feeding cell formation. Altogether, these result showed that *ARF8A* and *ARF8B* expression is required for successful tomato-RKN interaction. The two conserved microRNA families, miR398 and miR408, are upregulated in tomato and *Arabidopsis thaliana* galls. miR398 and miR408 and their targets have been previously described to be involved in the copper signaling pathway. *MIR398* and *MIR408* expression is activated in response to copper starvation by the *SPL7* transcription factor and mature miR398 and miR408 repress expression of genes encoding copper binding proteins non-essential for plant development. By using *Arabidopsis* lines expressing transcriptional fusion with GUS reporter gene, I showed that both *MIR408* and *SPL7* were expressed within nematode induced feeding cells. Moreover, infection assays with *mir408* and *spl7* mutants or lines expressing mutated targets resistant for miR398 cleavage showed an increased resistance of these lines to nematode infection. Finally, watering plants with copper sulfate, at concentration below toxic concentrations for the plants or for the nematodes, induced a strong resistance to nematode infection. Altogether, these results demonstrate the role of the copper signaling through activation of miR398 and miR408 by *SPL7* in the formation of giant feeding cells. To conclude, the work presented in this thesis demonstrates the important role of three microRNAs families and their targets in the formation of nematode-induced feeding cells.

Keywords: microRNAs, Root-knot nematodes, Tomato, *Arabidopsis thaliana*, Galls

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Abbreviations

- AGO: Argonaute protein
- ARF: Auxin Response Factors
- CDK: Cycline dependent kinase
- CN: cyst-nematodes
- CWDE: cell wall degrading enzymes
- DCL: Dicer-like protein
- DNA: Deoxyribonucleic acid
- dpi: Days post infection
- dsRNA: double-strand RNA
- ETI: Effector-Triggered Immunity
- GA: gibberellic acid
- GCs: Giant cells
- GFP: Green fluorescent protein
- GO: Gene Ontology
- GUS: β -glucuronidase
- HR: hypersensitive response
- IAA: indole-3-acetic acid
- J2: second stage juvenile
- KO: Knock-out
- mRNA: messenger RNA
- NC: Neighboring cells
- NGS: New Generation Sequencing
- PDs: Plasmodesmata
- PPN: plant-parasitic nematodes
- PTGS: Post Transcriptional Gene Silencing
- PTI: Pattern-Triggered Immunity
- RNA: Ribonucleic acid
- RNAi: RNA interference
- RISC: RNA-induced silencing complex
- RKN: root-knot nematodes
- ROS: reactive oxygen species
- SA: salicylic acid
- STTM: short tandem target mimic
- T-DNA: transfer DNA
- TGS: Transcriptional Gene Silencing

Preamble

Nematodes are roundworms living in various ecosystems. *Caenorhabditis elegans* is the most famous of the 26 000 nematode species described. Its genome was entirely sequenced in 1998, and this model organism, with its rapid reproduction and ease of generating mutants, enables the study of many cellular and molecular mechanisms. Studies with *C. elegans* allows major advances in the study of organ development and programmed cell death (S. Brenner, H. R. Horvitz and J. E. Sulston, Nobel Prize in Physiology or Medicine, 2002) and of the RNA interference (RNAi) – gene silencing by double-stranded RNA (A. Fire and G. Mello, Nobel Prize in Physiology or Medicine, 2006).

Despite these studies, the knowledge available on other nematodes, in particular animal and plant parasitic nematodes, remains limited. This is due to the difficulty of manipulating and genetically modifying these organisms. Sedentary endoparasitic nematodes are one of the most important crop pests that induce massive crop yield losses worldwide. Among them, root-knot nematodes (RKN) of the genus *Meloidogyne* are capable of infecting thousands of plant species and are considered as the most damaging genus. RKN are able to transform root cells into hypertrophied, hypermetabolic and multinucleate feeding cells that supply nutrients, essential for their development. Limitations of RKN control methods leads to an attempt for better understanding the development of the disease and the molecular dialogue between plants and RKN. The formation of the giant feeding cells implies a reprogramming of root cell gene expression. The aim of this thesis is to deepen our knowledge on the role of the small noncoding microRNAs, key regulators of plant gene expression, in the formation of giant feeding cells induced by *M. incognita*.

In the following introduction, I will first present the common characteristics of nematodes, then focus on RKN and feeding cell formation, and I will detail the reprogramming of plant gene expression that occurs within the feeding cells. Then I will present the plant small RNAs by focusing on the microRNAs and their role in plant development and plant response to abiotic and biotic stresses.

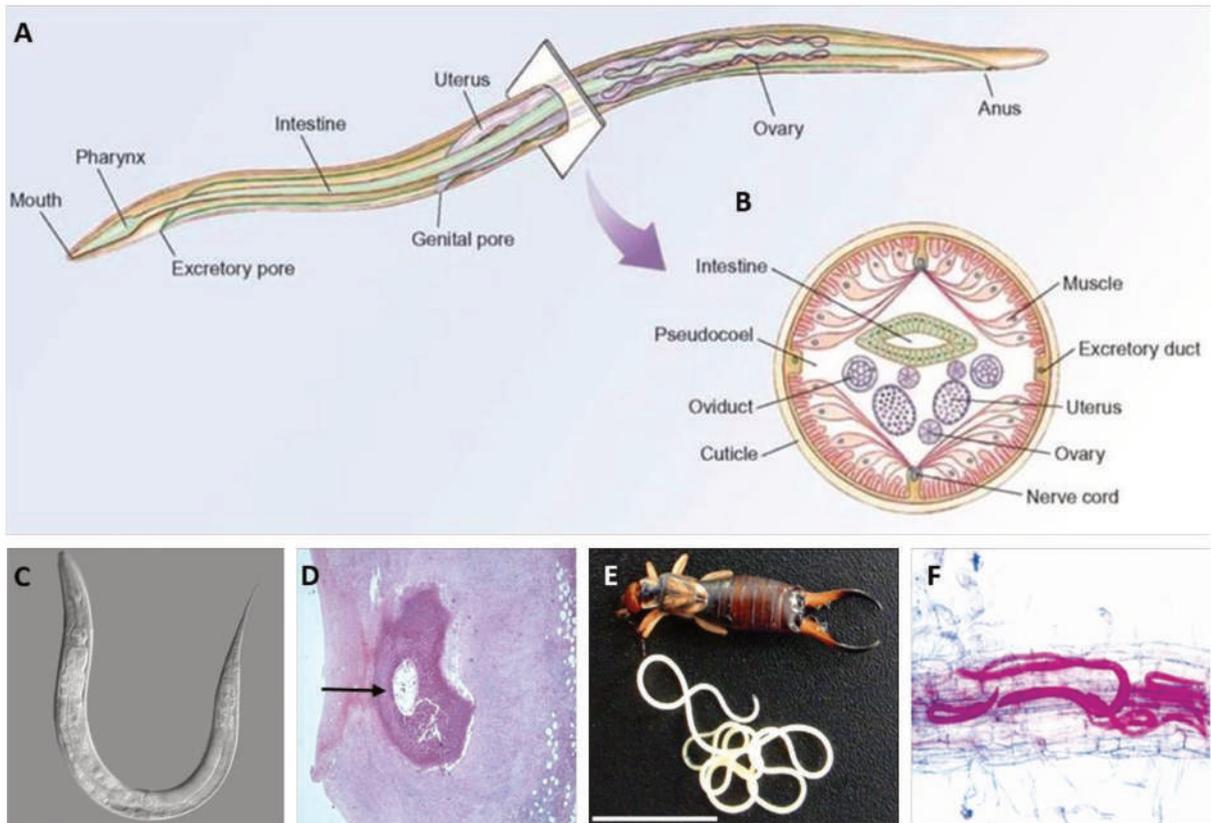


Figure 1. Schematic representation of a nematode and examples of free living and parasitic nematodes.

(A) Longitudinal and **(B)** cross section of an adult nematode (credit to: schoolbag.info). **(C)** Adult hermaphrodite of the nematode *C. elegans* (Photo by: courtesy Judith Kimble). **(D)** Histological section showing *Enterobius vermicularis* (black arrow) within intensely inflamed fibrotic tissue (Rajendran et al., 2015). **(E)** *Mermis nigrescens* and its host the European earwig (Presswell et al., 2015). **(F)** Stained root lesions nematodes (*Pratylenchus neglectus*) in a wheat root (Thompson et al., 2008).

Introduction

1. Generalities

Nematodes are round unsegmented worms. Nematodes etymology includes two ancient Greek words: “nêma”, meaning, “thread”, and “oidès” meaning, “like”. While different species of nematodes vary in size, from 0.1 millimeters in length up to 1 meter, they all tend to be thin (less than 100 micrometers thick). These pluricellular organisms are classified in the Nematoda phylum. Until now, more than 26 000 species of nematodes have been described (Zhang, 2013) including the best-known nematode: the model organism *Caenorhabditis elegans*. Their development is characterized by an embryonic phase followed by larval stages, which developed through cuticular molts in adult stage.

Nematodes are among the simplest organized animals. They are covered with a thick permeable cuticle, composed mainly of collagens, that enables the diffusion of water and gas exchange and compensates the lack of respiratory system (**Figure 1A-B**). Thanks to their cuticle that protects internal organs, nematodes are able to withstand extreme environments (Decraemer and Hunt, 2006). The nematode cuticle allows growth and expansion between molting periods. Nematodes have digestive, nervous and reproductive systems. The digestive system consists of a mouth connected to an esophagus (pharynx) which ends in an intestine and a rectum (Hussey, 1989). The nervous system controls mainly the somatic musculature and sensory perception through the chemoreceptor organs: amphids and phasmids. This system is composed of a nerve ring connected to several ganglia that is the coordinating center for the nervous system. The muscular apparatus is composed of longitudinal muscles under the cuticle and hypodermis that allow the nematode to move by undulations.

Nematodes occupy very diverse ecological niches on earth. They are spread in most ecosystems such as oceans and seas, fresh water, water films in the ground or in plants. Nematodes have varied lifestyles: parasitic and free living (Perry and Moens, 2011a). Free living nematodes, such as *C. elegans* (**Figure 1C**), reside in both aquatic and terrestrial habitats (Masler, 2013). They can feed on bacteria like *Cephalobus pseudoparvus* (Blanc et al., 2006), fungi like *Aphelenchus avenae* (Li et al., 2004) or other nematodes like *Mononchus aquaticus* (Grootaert and Maertens, 1976). Parasitic nematodes infect various types of organisms including humans, insects, and plants (**Figure 1D-F**).

Nematodes have been distributed into five major clades in the phylogeny of the phylum Nematoda based on the sequences of small subunit of ribosomal RNA (Blaxter and Koutsovoulos, 2015). Each of these clades contains species with diverse lifestyles and all includes parasitic species.

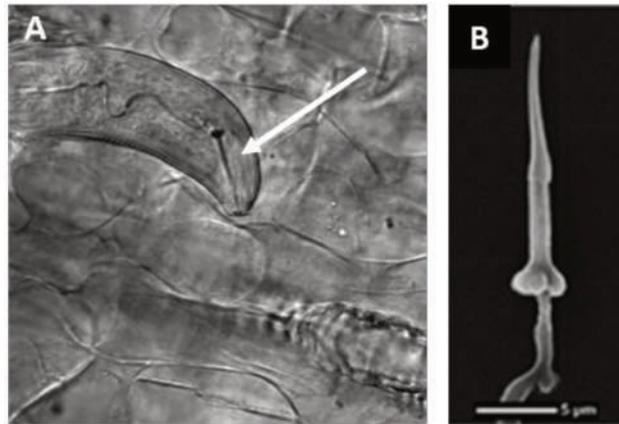


Figure 2. Stylet of plant parasitic nematodes. (A) *Meloidogyne incognita* second-stage juvenile (J2) and its stylet (arrow) (Favery et al., 2016). **(B)** Excised female root-knot nematode stylet, photographed using a scanning electron microscope (Pollok et al., 2016).

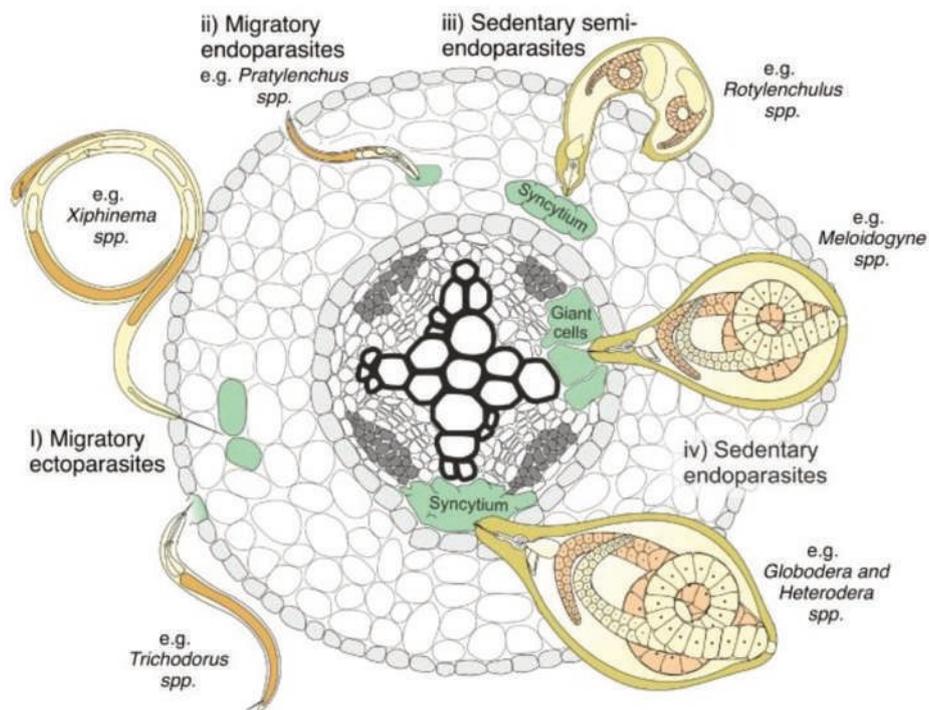


Figure 3. Schematic diagram of various plant-parasitic nematode lifestyles depicted on a cross section of a root. i) Ectoparasites migrate outside the plant for their entire life cycle and typically parasitize the outer layer of root cells (e.g. *Trichodorus* spp.), or feed from inner layers of the root (e.g. *Xiphinema* spp.). The endoparasites, however, spend at least part of their parasitic life cycle inside the host, and are further divided into migratory or sedentary. ii) Migratory endoparasites burrow inside the host and parasitise cells from within while migrating (e.g. *Pratylenchus* spp.). iii and iv) Sedentary (semi-)endoparasites induce the formation of ‘feeding sites’ inside the host, and withdraw nutrition in a non-destructive manner, for a prolonged period of biotrophy (e.g. the reniform, cyst, and root-knot nematodes) (Eves-van den Akker et al., 2021).

2. Plant-parasitic nematode

Plant parasitism appeared independently in three clades: in Dorylaimida (clade 1), Diptherophorina (clade 2) and in Tylenchomorpha (clade 4) (Holterman et al., 2006; Blaxter and Koutsovoulos, 2015). The Dorylaimida and Triplonchida are virus vectors. The Tylenchomorpha are the most important clade of plant-parasitic nematodes (PPN), both in term of species number and in terms of damage caused to the plant they parasitize. This clade contains the *Heteroderidae* family that is responsible for most of the economic damage. These major crop pests are currently the subject of a large number of studies aimed at elucidating the mechanisms of interaction between the nematode and the plant. All PPN have a mouth stylet, a hollow retractable needle (**Figure 2**). Nematodes use the stylet to perforate the cell wall of root cells, aspirate the cellular content and inject the esophageal secretions. The stylet is connected to the pharynx that is connected to the intestine. PPN are classified according to their life styles and feeding habits. PPN that stay outside the root and feed externally by inserting their stylets into root cells are classified as ectoparasites. Those that penetrate host root to feed from inner cell types are classified as endoparasites. According to their feeding habit, they are subclassified further into sedentary or migratory parasites (**Figure 3**).

2.1 Ectoparasitic nematodes

Ectoparasitic nematodes have a rather long thin stylet that enables them to dig deep plant cells. Migratory ectoparasites feed all along the root from multiple sites (e.g. genus *Xiphinema*, *Trichodorus*, *Longidorus*, *Macrotrophurus*, *Tylenchorhynchus*) and sedentary ectoparasites puncture a single specific root site (e.g. *Rotylenchus*). Several of these nematodes cause significant indirect damage (**Figure 4A, 4B**) through transmission of plant viruses like *Xiphinema* spp. which delivers the Grapevine Fanleaf Virus (GFLV) and leads to severe damages to grapevine (Andret-Link et al., 2017).

2.2 Endoparasitic nematodes

Endoparasitic nematodes spend most of their life cycle within host roots. They have a rather short and robust stylet to penetrate into the root. Endoparasitic nematodes are also subclassified according to their feeding habits: the migratory species move into the root or even the shoot and frequently cause cell necrosis like *Radopholus* spp. and *Pratylenchus* spp. The sedentary species, e.g. root-knot nematodes (RKN) and cyst-nematodes (CN), which feed from a single feeding structure and are the most damaging groups for agriculture (Jones et al., 2013) (**Figure 4C, 4D**). Sedentary endoparasitic nematodes establish an elaborate relationship with their host by setting up a complex feeding structure that provides nutrients required for nematode development. Infection by endoparasitic nematodes generally causes defects in root development, a reduction in water uptake, defects in leaf expansion, and in photosynthesis rate. Moreover, damages to plant tissues leave the plant more vulnerable to diseases caused by plant viruses, fungi and oomycetes (Jones and Goto, 2011).



Figure 4. Damages caused by different PPN species. (A) Ectoparasite nematode: *Longidorus africanus* damages on carrot field (Photo credit: Gerald Holmes). (B) Migratory endoparasite nematode: *Radopholus similis* damages on banana roots (Photo credit: Scot Nelson). Sedentary endoparasite nematode (C) Damages caused by *Heterodera glycines* in a soybean field (Photo credit: Manitoba pulse soybean growers) (D) *Meloidogyne incognita* damage on eggplant (INRA).

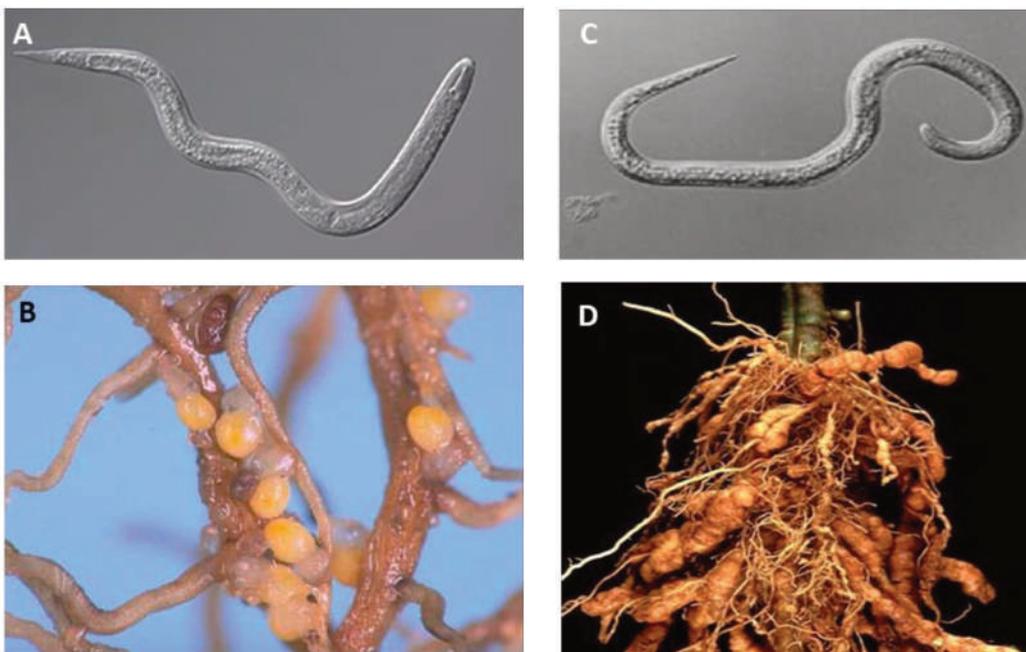


Figure 5. Heteroderidae family: CN & RKN. (A) Photo of a juvenile stage 2 larva (J2) of *Heterodera glycines* (Photo credit: Elizabeth Bush) (B) Photo of a juvenile stage 2 larva (J2) of *M. incognita* (Photo credit: INRAE) (C) Cyst of *H. glycines* on soybean roots (Photo credit: University of Kentucky, Department of Plant Pathology) (D) Root deformation "galls" induced by *M. incognita* on tomato roots (INRA).

2.3 Heteroderidae family

Among sedentary endoparasitic nematodes, the *Heteroderidae* family includes the most damaging species worldwide. Damages are estimated at more than 100 billion dollars per year (Trudgill and Blok, 2001). *Heteroderidae* is composed of two subfamilies: the cyst nematode (CN) (e.g. *Heterodera* and *Globodera* spp.) and the root-knot nematode (RKN) (*Meloidogyne* spp.) (De Ley and Blaxter, 2002). RKN can feed on almost all vascular plants (Jones and Goto, 2011) whereas CN show a more specific host preference (Mimee et al., 2015). RKN and CN have very different host ranges and generate varied symptoms on the host roots. They have a close development cycle and establish a close relationship with their host by inducing the formation of root feeding site.

2.3.1 Cyst nematodes

The CN includes eight *genera* (Turner and Rowe, 2006) but *Heterodera* and *Globodera* are the two most important economically *genera*. CN induce the formation of a feeding cell, named syncytium, within host root from which they withdraw nutrients required for their development. The second juvenile stage (J2) (**Figure 5A**) penetrates the root and migrates intracellularly to reach the vascular central cylinder causing cell necrosis on its pathway. The J2 selects one root cell into which it injects a cocktail of secretion produced in the esophageal glands. The targeted cell expands within the vascular tissue by a partial cell wall dissolution which will merge with the adjacent cells *via* cytoplasm fusion (Golinowski et al., 1996b; Belkhadir et al., 2006). This unique feeding structure is formed by the fusion of up to 200 root cells. Then the J2 moults in stage 3 (J3) and stage 4 (J4) juveniles then finally to the adult stage (male or female). CN have a sexual reproduction or “amphimixis”. After fertilization and egg development, the CN female dies and hardens forming a cyst visible at the root level (**Figure 5C**). This cyst contains up to 1 000 eggs from which new J2s will emerge.

2.3.2 Root-knot nematodes

RKN genus is formed by more than 90 obligatory endoparasites species (Jones et al., 2013). Infection by RKN larvae (**Figure 5B**) cause characteristic host root deformation named “knot” or “gall” (**Figure 5D**). Because of their extreme polyphagy, with a host spectrum including more than 5 000 plant species, and their wide geographical distribution, RKN are a major problem for worldwide agriculture (Castagnone-Sereno, 2006; Blok et al., 2008; Abad and Williamson, 2010). There are three types of reproduction in RKN species. The most polyphagous *Meloidogyne* species, e.g. *M. arenaria*, *M. incognita*, *M. javanica* and *M. enterolobii*, reproduce by mitotic parthenogenesis. The female produces a progeny, with no male participation in the reproduction (Castagnone-Sereno et al., 2013). Some species (e.g. *M. hapla*) reproduces by facultative meiotic parthenogenesis or amphimixis. Only few species have a strictly sexual reproduction.

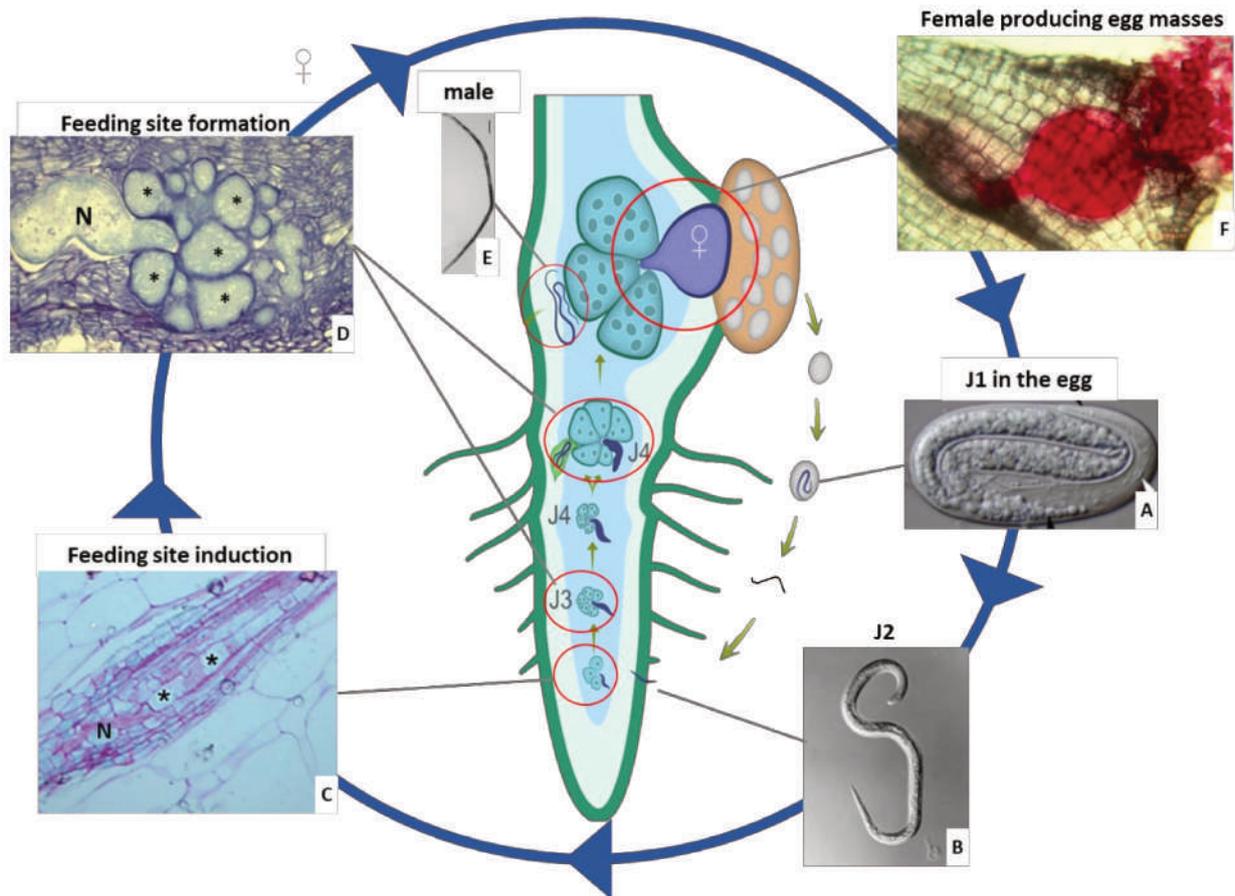


Figure 6. Life cycle of *M. incognita*. (A) J1 develops inside the egg into J2 until egg hatching that will release the J2 in the soil. (B) J2 penetrates into the root at the apex and migrates up into the vascular system. (C) In the differentiation zone of the root, the nematode (N) settles down and induces the dedifferentiation of 5 to 7 parenchyma cells into giant multinucleated and metabolically active feeding cells (*). (D) The J2 feeds from the giant feeding cells (*) and develops by successive molts (J3 & J4). (E) Males migrate outside the roots (F) while the adult female lays eggs on the root surface in gelatinous matrix. (Photo credit: INRAE)

2.3.2.1 *M. incognita* life cycle

M. incognita is the model species used to understand the molecular processes of plant-RKN interaction, for which the genome has been fully sequenced and annotated (Abad et al., 2008; Blanc-Mathieu et al., 2017). *M. incognita* life cycle (**Figure 6**) can be completed from three to eight weeks depending on the environmental conditions and host plants (Escobar et al., 2015, reviewed in Favery et al., 2016). The RKN life cycle starts with the first stage juvenile (J1) (**Figure 6A**) that develops inside the egg from which the J2 (**Figure 6B**) hatches and migrates within the soil towards host roots. Roots secreted compounds are detected by J2's chemosensory sensilla "amphids" and attract the J2 to the root (Perry and Moens, 2011). J2s were shown to be attracted by compounds from the seeds (Tsai et al., 2019), volatiles compounds (Williamson and Čepulytė, 2017) or compounds from root exudates (Oota et al., 2020). J2 penetrates at the elongation zone of the host root and migrates intercellularly towards the root apex, turns around to enter the central cylinder and migrates to the differentiation zone. When it reaches vascular cylinder, J2 injects secretions through the stylet within five to seven root parenchyma vascular cells. In response to these nematode signals, targeted cells dedifferentiate into giant, multinucleated, hypermetabolic feeding cells named giant cells (GCs) forming a feeding site that supplies nutrients required for nematode development (Escobar et al., 2015) (**Figure 6C-D**). Additionally, the neighboring cells surrounding the giant cells start to divide. Formation of giant feeding cells and division of neighboring cells result in the formation of a root-knot called gall, which is a characteristic symptom of RKN infection. Formation of this feeding site enables J2 sedentarization and development through three consecutive molts (J3, J4) towards adult stage. Since *Meloidogyne* reproduce by parthenogenesis, males are not necessary for reproduction and leave the roots. *M. incognita* males are thought to have a role in the control of nematode population when environmental conditions are not optimized (**Figure 6E**). The females – after asexual reproduction – produce eggs that they lay at the root surface in a gelatinous matrix called "egg mass" (**Figure 6F**), enabling a new cycle to proceed.

2.3.2.2 RKN control strategies

Until recently, the use of chemicals nematicides nematode were the nematode control strategies. Due to their high toxicity, chemicals like methyl bromide and nematicides based on carbamate have been banned in Europe since 2010. Other chemicals, belonging to fluoroalkenyl thioether group, have been shown to be effective against RKN with a lower impact on the environment but can disrupt other organisms (Kearn et al., 2014). Biological control is an alternative strategy that is ecofriendly and uses predators, parasites or pathogen of nematodes such as the bacteria *Pasteuria penetrans* or nematophagous fungi. However, there are still few commercial biological products successfully used in the field (Timper, 2011). While crop rotations are limited due to the wide host range of RKN, the use of plants with natural resistance genes, like *Mi* genes in tomato, is the most effective method to control RKN infestation and reduce yield losses (Ammiraju et al., 2003). However, some virulent

population of nematodes and/or new species (e.g. *M. enterolobii*) emerged all over the world that bypass these natural resistances (Jacquet et al., 2005). Development of novel and durable strategies to control RKN, it is therefore necessary. A better fundamental knowledge of the disease development and molecular mechanisms of susceptible plant-RKN interaction should leads to develop new strategies to control RKN.

3. Molecular mechanisms of plant-RKN susceptible interaction

3.1 RKN effectors

The formation of the feeding site is essential for RKN survival, and its formation is induced by molecules mainly synthesized in nematode salivary esophageal glands and secreted into the host plant via the stylet (**Figure 7A**). The esophageal gland secretions are released in spherical granules that vary in composition and size depending on nematode development stage and species (Hussey et al., 2002). They play a main role in different parasitism phases including invasion and the formation of the feeding site (Davis et al., 2004; Mitchum et al., 2013). Molecules synthesized in esophageal glands and secreted in plants are known as "effectors" (Toruño et al., 2016). Effectors are "the molecules of pathogens/parasites secreted in the host, which modify the structure and/or function of the host cell and which promote parasitism" (Hogenhout et al., 2009). Effectors produced from the two subventral glands are expressed in the first steps of infection while those secreted during parasitism are produced by the dorsal gland (Mitchum et al., 2013; Nguyen et al., 2018) (**Figure 7B, 7C**). Some effectors may also be produced in other secretory organs, including amphids like Mh-TTL2 effector (Gleason et al., 2017) or secreted through the cuticle like Mi-MIF2 effector (Zhao et al., 2019). So far, most of PPN effectors that have been identified are proteins or peptides (Mitchum et al., 2013; Quentin et al., 2013; Vieira and Gleason, 2019).

The identification of RKN effectors, understanding their functions and identifying their targets *in planta* remain a crucial challenge to better understand nematode parasitism and giant cell formation. Many approaches were developed to identify potential effectors involved in parasitism such as candidate gene strategy and the direct by proteomics analyses of esophageal secretions. Whole genome sequenced of two RKN *M. incognita* and *M. hapla* (Abad et al., 2008; Opperman et al., 2008) facilitated effector identification through bioinformatics analyses (Truong et al., 2015). Unlike *C. elegans*, PPN can't be genetically transformed. Therefore, the functional characterization of RKN effectors can still be a difficult step to bypass. Indeed, RKN effectors manipulate host cellular processes through interactions with plant target to favor parasitism. Currently, more than ten plant proteins targeted by RKN effectors have been identified and shown to contribute to feeding site formation in different plant species (reviewed Mejias et al., 2019).

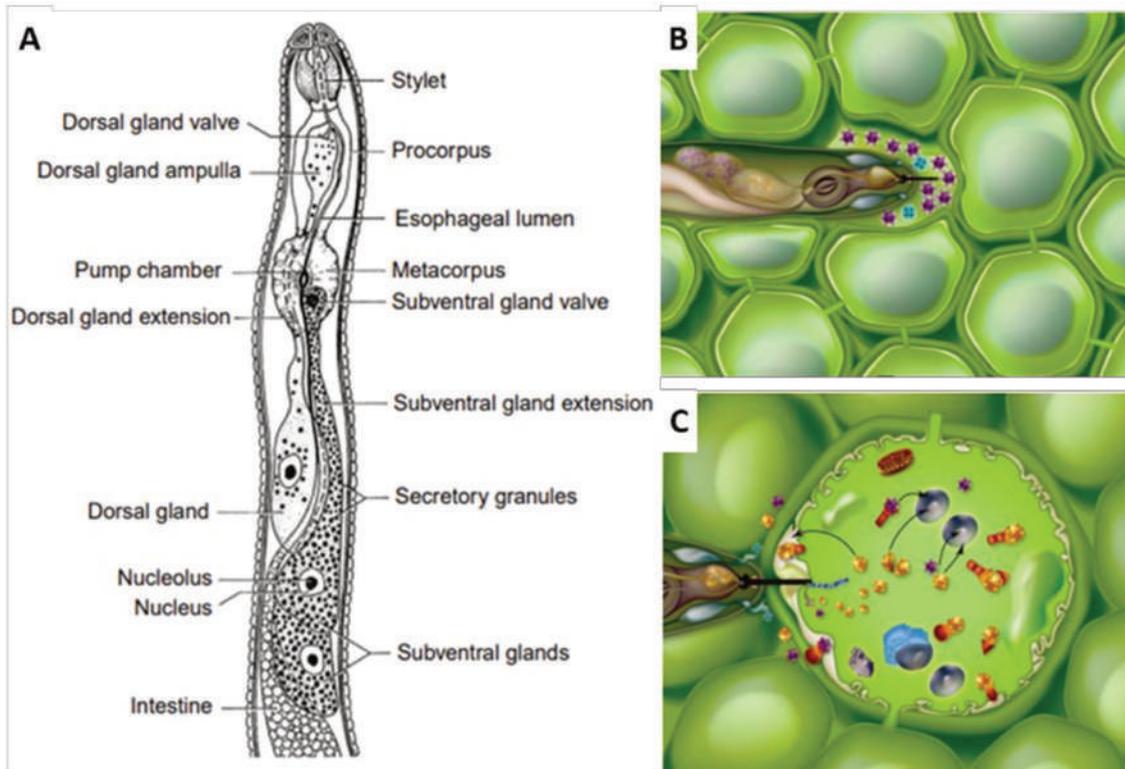


Figure 7. Esophageal secretions of *Meloidogyne*. (A) Secretory organs of a pre-infective RKN second-stage juvenile of *Meloidogyne*. The single dorsal and two subventral esophageal gland cells produce secretions that are introduced into host tissues through the stylet (Davis et al., 2004). Schematic representation of a J2 (B) secreting effectors in the intracellular space during the migration step and (C) during giant cell formation.

RKN effectors are major players in manipulating the physiology of the host plant. They can be classified depending on their role in parasitism.

3.1.1 Effectors involved in the migration and cell wall degradation

Cell wall is the first obstacle encountered by the free-living J2s. RKN needs to weaken the boundaries between the cells in order to facilitate their penetration and the inter-cellular migration through root tissue. The secretions of these cell wall degradation/modification enzymes (CWDE) helps i) the nematode along the penetration into the root and the vascular system and ii) the modification of cell wall during the formation of the feeding site (Bohlmann and Sobczak, 2014). Dozens of effectors secreted by RKN were shown to be CWDE including pectate lyases (Jaubert et al., 2002b), β -1,4-endoglucanase (Rosso et al., 1999; Ledger et al., 2006) and endo-1,4- β -xylanase (Mitreva-Dautova et al., 2006). Finally, *M. incognita* genome sequence revealed 81 genes encoding CWDE (Abad et al., 2008). These genes are mainly expressed in the subventral oesophageal glands (Jaubert et al., 2002a; Davis et al., 2004; Davis et al., 2011). Interestingly, it seems that these functions necessary for parasitism have been acquired several times independently through the phylum of phytoparasitic nematodes via horizontal transfers of genetic material of bacterial origin (Danchin et al., 2010; Danchin et al., 2017).

3.1.2 Effectors involved in the suppression of defense reactions

RKN facilitate their infection by suppressing plant immunity with effectors (Quentin et al., 2013). Mi-CRT protein produced in esophageal glands, was the first RKN proteins which secretion has been established *in planta* (Jaubert et al., 2005). Using *A. thaliana* lines overexpressing Mi-CRT, Jaouannet et al. (2013) have demonstrated that this effector is able to suppress the defense induced by the bacterial PAMP effector elf18 (Jaouannet et al., 2013). The silencing of this effector by RNAi leads to a decrease in infection rate in *A. thaliana*.

A similar study in 2016, suggested a role for Mi-Msp40 effector as an ETI suppressor (Niu et al., 2016). The overexpression of Mi-Msp40 in *A. thaliana* suppressed also the callose deposition in infected plants and increased susceptibility towards RKN infection. In addition, some RKN effectors have been shown to interact with host proteins to scavenge the ROS. The *M. javanica* effector Mj-TTL5 activates host ROS-scavenging system in *A. thaliana* to eliminate H₂O₂ by interacting with ferredoxin: thioredoxin reductase catalytic (FTRc), a component of host antioxidant system (Lin et al., 2016). RKN effectors can also participate in the final redox state of giant cells. Recently, effectors of *M. graminicola* Mg-MO289 and *M. incognita* Mi-PDII have been demonstrated to regulate redox state (Zhao et al., 2020; Song et al., 2021). Mi-PDII targets a stress-associated protein (SAP) to fine-tune SAP-mediated responses at the interface of redox-signaling, defense and stress acclimation in *Solanaceae* and *A. thaliana* (Zhao et al., 2020).

3.1.3 Effectors involved in the formation of giant cells

The formation of the feeding site and its maintenance is a critical step of plant-RKN interaction. Until now, only few RKN effectors have been demonstrated to be involved in feeding cell formation by i) their presence in the giant cell and/or ii) of the giant cell defect associated with the inactivation of the effector or its plant target.

Only seven RKN effectors have been shown to be secreted into the giant cells. Among them five target the nucleus of giant cells: *M. incognita* Mi-EFF1 (Jaouannet et al., 2012) and Mi-EFF18 (Mejias et al., 2021), *M. javanica* Mj-NULG1 (Lin et al., 2013) and *M. graminicola* Mg-GPP (Chen et al., 2017) and Mg16820 (Naalden et al., 2018). Knock down of Mg-GPP effector *in planta* in transgenic rice plants using RNAi reduced the number of female produced, suggesting that Mg-GPP plays a role in parasitism. Interestingly, subcellular localization assays showed that Mg-GPP can translocate from the endoplasmic reticulum to the nucleus. Furthermore, this effector suppress the cell death induced by ETI proteins Gpa2/RBP-1 in rice (Chen et al., 2017). The nuclear effector of *M. incognita* MiEFF18 has been demonstrated to target the plant core spliceosomal protein SMD1, a major component of the spliceosome, a complex involved in pre-mRNA splicing and alternative splicing (Mejias et al., 2021). *A. thaliana smd1b* mutants showed altered susceptibility to *M. incognita* infection and the giant cells formed on these mutants displayed developmental defects. This study revealed the importance of the regulation of gene expression by alternative splicing during the giant cell formation. Mi-16D10 effector have been shown to interact with two SCARECROW-like transcription factors, AtSCL6 and AtSCL21, that play a role in root development (Huang et al., 2006). The repression of Mi-16D10 in *A. thaliana* by RNAi *in planta* induces an important resistance towards RKN infection (Shivakumara et al., 2016). Leelarasamee et al., (2018) identified a *M. incognita* effector, Mi-PFN3, encoding a profilin, an actin-binding protein. Interestingly, the protoplasts of leaves overexpressing the effector Mi-PFN3 showed disrupted actin filaments, a process observed during giant cell formation. Mi-7E12 could be involved in the fragmentation of vacuoles observed in Giant cells (dos Santos de Lima e Souza et al., 2011).

RKN also secrete some effectors that mimics plant peptide hormones such as C-TERMINALLY ENCODED PEPTIDE (CEP)-like and INFLORESCENCE DEFICIENT IN ABSCISSION (IDA)-like peptides (Bird et al., 2015). IDA peptides are signaling peptides that control cell separation during cell division within the root apical meristem. The Mi-IDL1 effector is a peptide capable of mimicking the proteins of the IDA family (Kim et al., 2018). The repression of Mi-IDL1 *in planta* using RNAi showed fewer and smaller galls at 35 and 42 dpi compared to the control plants. These peptide mimics would play a role in the manipulation of hormone balance and in the formation of the feeding site.

3.2 RKN- induced feeding site

3.2.1 Morphology and ontogenesis of RKN-induced feeding site

RKN are able to induce the dedifferentiation of several root vascular cells into a sophisticated feeding structure that functions as a metabolically sink to supply water and nutrients required for nematode development. Giant feeding cells are multinucleate and hypertrophied (Bird, 1961; Huang and Maggenti, 1969) (**Figure 8A, 8B**). In *A. thaliana*, the volume of the giant feeding cell increases continuously and can reach a volume more than 400 times higher than normal root cell (**Figure 8C**) (Cabrera et al., 2015). Giant feeding cells are metabolically highly active. They are characterized by a dense cytoplasm containing abundant organelles and the multiplied fragmented vacuoles (Berg et al., 2009). Giant cells with the same phenotype are induced in more than 5 000 plant species which suggest that nematode manipulate conserved mechanisms. Elucidating all the processes involved in the transformation of initial root cells into a feeding cell remains a challenge.

The first sign of giant cell formation is nuclear division observed in cells around the J2 “head”. Cell plate alignment appeared to proceed normally, but cytokinesis was unsuccessful and binucleated cells formed subsequently in *Impatiens balsamina* (Jones and Payne, 1978). No wall breakdown was evident then or later. The number of nuclei increases by repeated mitoses without cytokinesis which leads to the multinucleated state of the giant cells (Jones and Payne, 1978; Caillaud et al., 2008c). The number of nuclei per giant cell increases rapidly until 10 days post infection (dpi), with a further small increases in the number of nuclei being observed at 15 dpi. The highest number of nuclei found in any single giant cell was 134 (in pea), with numerous giant cell having more than 75 nuclei each (Starr, 1993). Similar increase in the number of nuclei has been reported in giant cells from various plant hosts e.g pea, lettuce, tomato and broad bean (Starr, 1993). The formation of giant cells can be divided into two phases: a first phase of successive mitoses without cytokinesis followed by a phase of endoreduplication (DNA replication without nuclear division) (de Almeida Engler and Gheysen, 2013) (**Figure 9**) .

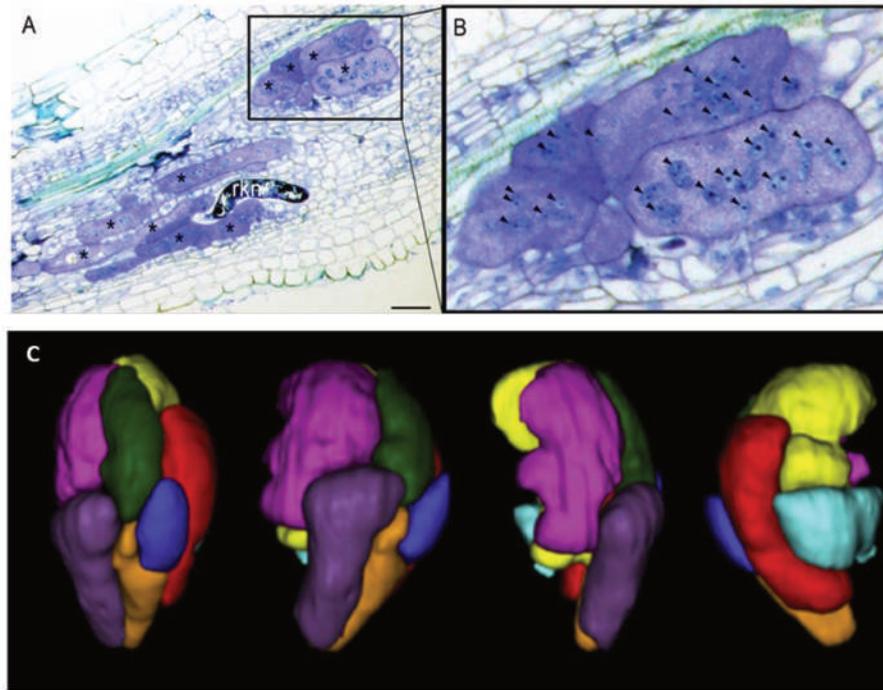


Figure 8. Giant cells induced by RKN. (A) Longitudinal sections of giant cells induced by *Meloidogyne chitwoodi* in pepper at 7 dpi (stained by toluidine blue). **(B)** Multiple nuclei (arrowheads) are visible in the magnification of the giant cells (Quentin et al., 2013). *, giant cell; n, nematode. Bars, 10 μ m **(C)** 360° rotating view of a 3D reconstruction of giant feeding cells induced by *M. javanica* at 7 dpi in *A. thaliana*. Each color represents one giant cell (Cabrera et al., 2015)

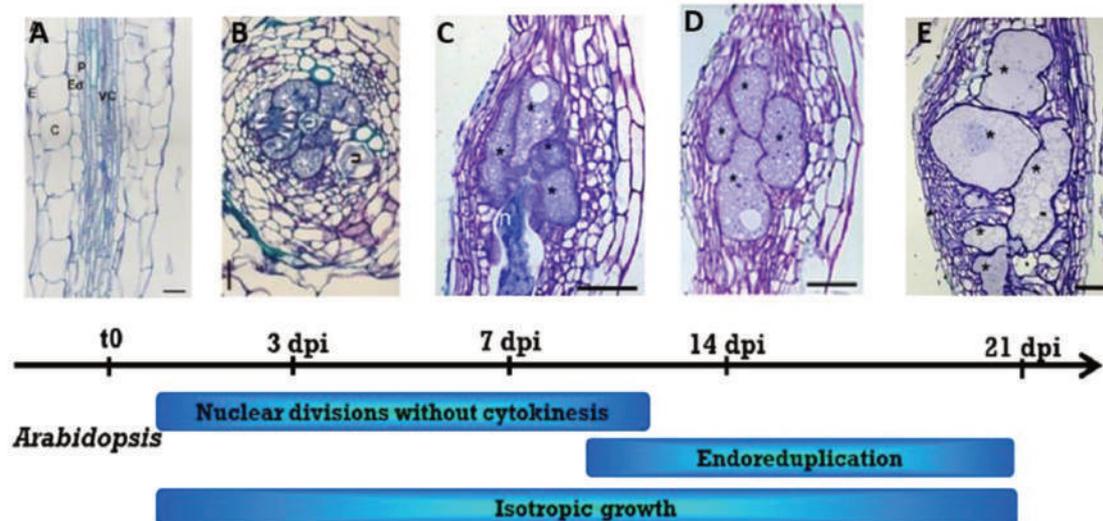


Figure 9. Kinetic of giant feeding cells development in *Arabidopsis thaliana*.

Longitudinal section (A, C, D and E) and cross section (B) of *A. thaliana* non infected roots (A) and galls at 3 (B), 7 (C), 14 (D) and 21 (E) dpi induced by *M. incognita*. Upon the injection of secretion by the nematode, five to seven parenchyma roots cell dedifferentiate into giant feeding cells. They undergo nuclear divisions without cytokinesis from few hours after infection to ten days post infection (dpi). From 10 to 21 dpi, feeding cells undergo endoreduplication. All along the development of galls, feeding cells enlarge through an isotropic growth. n, nematode; *, Giant cells; C, cortex, E, epidermis; P, pericycle; Ed, endodermis; VC, vascular cylinder. Bars (A) 25 μ m, (B-D) 50 μ m (de Almeida Engler et al., 2007; Coelho et al., 2017)

The nuclei of feeding cells are large with an irregular lobed shape and with large conspicuous nucleoli and an increase in ploidy (Escobar and Fenoll, 2015). The increase in the number of nuclei and the endoreduplication enable the isotropic growth of the giant cells. Giant cells are characterized by the development of cell wall ingrowths, typical of transfer cells that are in contact with the xylem and phloem vessel elements to increase the surface area of the associated membrane. Cell wall ingrowths (CWI) enable the nematodes to withdraw water and nutrients from the sap for its own development (**Figure 10A**) (Jones and Northcote, 1972b; Sobczak and Golinowski, 2011; Rodiuc et al., 2014). The sieve elements around giant cells are interconnected by plasmodesmata (PDs) (**Figure 10B, 10C**) (Jones and Goto, 2011; Bartlem et al., 2014). Giant cells are symplastically isolated from the surrounding tissue, and nutrient uptake depend on transport across membrane (Hoth et al., 2008).

3.2.2 Neighboring cells vs Giant cells

Cells surrounding the feeding giant cells and the nematode are named “neighboring cells” (NC) (**Figure 11A**). When feeding cells are induced, neighboring cells re-enter into cell division (Berg et al., 2009). The division of neighboring cells probably participates to the growth of giant cells by preventing the tissue around from breaking but also by initiating a *de novo* vascular differentiation process important to provide the elements necessary for the development and functioning of giant cells (Bartlem et al., 2014). A transcriptome analysis of microdissected giant cell and neighboring cells induced by *M. incognita* in *Medicago truncatula* showed that 740 genes are differentially expressed in neighboring cells at 7 dpi of which 498 genes are common with giant cells. The upregulated genes in neighboring cells are involved in the ‘cell’, ‘signaling’ and ‘cell-wall’ related pathway, suggesting that genes DE in neighboring cells are involved in cell to cell communication and signaling (Damiani et al., 2012). Among the genes specifically expressed in the neighboring cells and not in the giant cells are genes encoding small signaling peptides, named phytosulfokines (PSK), and their receptor PSKR1 (Rodiuc et al., 2016) (**Figure 11B**). In the absence of functional PSKR1, giant cells arrested their development and failed to fully differentiate. This result indicates that the observed restriction of PSK signaling to cells surrounding giant cells contributes to the isotropic growth and maturation of giant cells (Rodiuc et al., 2016).

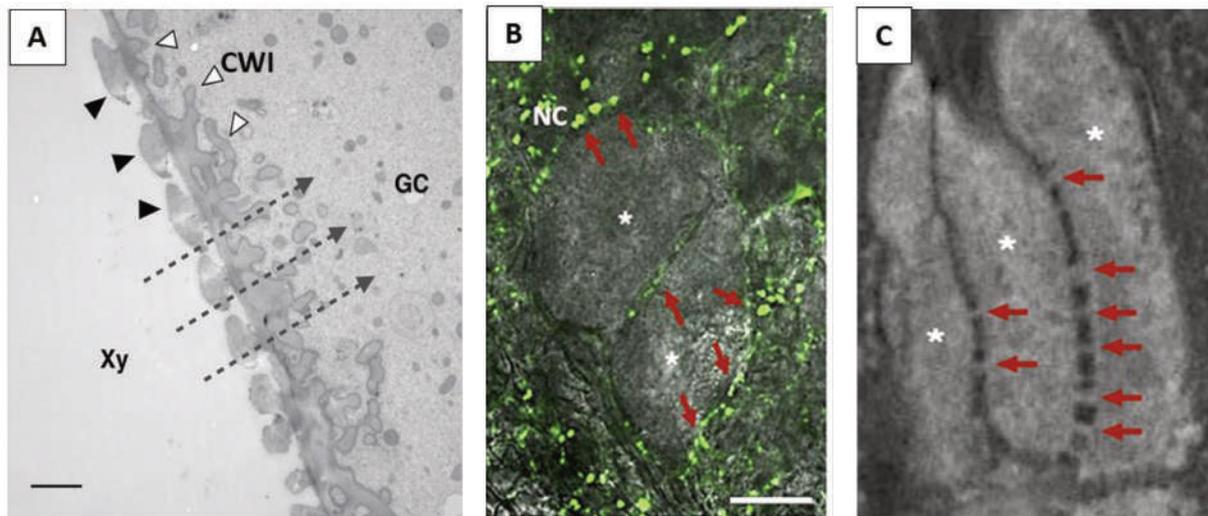


Figure 10. Cell wall ingrowths and Plasmodesmata of giant cell. (A) Electron micrograph of a cell wall interface between a xylem vessel (Xy) and a giant cell (GC) in *Lotus japonicus* at 3 weeks after inoculation with the root-knot nematode *Meloidogyne hapla*. Secondary wall depositions in the xylem vessel are marked (black arrowheads, three marked as representative). The presence of extensive wall ingrowths in the giant cells (white arrowheads, three marked as representative) is indicative of substantial water and solute movement across this interface. Scale bar=2 μ m (Bartlem et al., 2014) (B) *in vivo* localization of MP17PLRV-GFP (plasmodesmata localization marked by green fluorescence). Section of *Arabidopsis thaliana* giant cells containing numerous PD (red arrows). (C) Cleared whole-mount gall showing the complex network of PD (red arrows) between giant cells. *, Giant cells; NC, neighbouring cells. Bars = 50 μ m (Rodiuc et al., 2014)

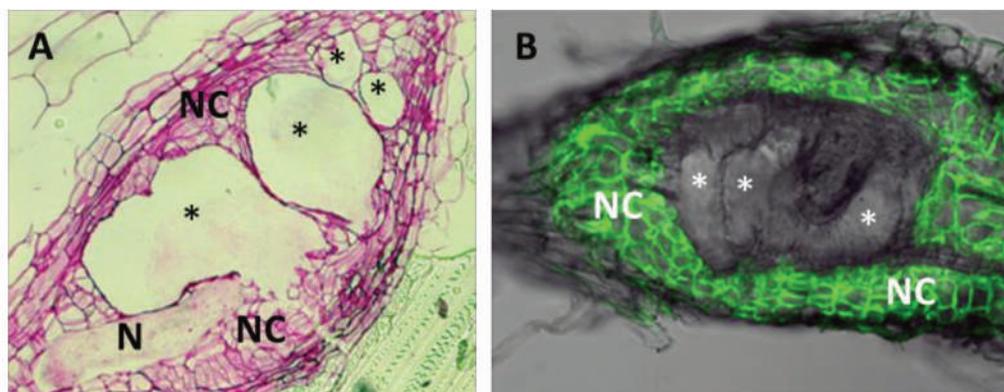


Figure 11. Neighboring cells. (A) Longitudinal section of *A. thaliana* galls induced by *M. incognita* at 14 dpi (stained with Ruthenium red). (B) Expression pattern of the GFP reporter gene under control of the PSKR1 promoter in galls induced by *M. incognita* in *A. thaliana* galls at 21 dpi. Shown are projections of serial confocal optical *in vivo* sections (Favery, unpublished).

NC: Neighboring cells; *: Giant cells; N: Nematode; Bars: 50 μ m (INRA)

3.2.3 Analysis of gene expression in galls and giant cells

One of the first method used to study the expression of “candidate” genes, believed to be involved in gall and giant cell formation was the transformation of plants with gene promoters fused to a GUS (β -glucuronidase) (Gheysen and Fenoll, 2002). This strategy was used to study the spatio-temporal expression of genes encoding key cell-cycle regulators: *CYCLINS* (CYCs) and *CYCLIN DEPENDANT KINASES* (CDKs) in *A. thaliana* (Niebel et al., 1996; de Almeida Engler et al., 2007). In *A. thaliana* lines expressing GUS under the control of the CDK *CDC2a* and the mitotic cyclin *CYC1* promoters showed a GUS signal in early stages (3 dpi) of gall formation indicating the early activation of these two genes. The use of inhibitors of the cell cycle such as oryzalin affected giant cell normal development demonstrating that the cell cycle is a key process in giant cell formation (de Almeida Engler et al., 2007). Though this candidate-approach was very useful to understand the role of known genes in giant cell formation, it was restricted to genes already described and doesn't enable the identification novel genes without a priori. In order to identify genes involved in giant cell formation without a priori, other approaches have been developed, such as promoter trap and transcriptomic analyses.

Promoter trap strategy consists on the random insertion of an *Agrobacterium* transfer DNA (T-DNA) carrying a GUS reporter and a selectable marker gene in the genome of a plant like *A. thaliana* (Favery et al., 1998). The mRNA of the reporter gene is transcribed only when the T-DNA is inserted within a transcriptionally active gene. In this case, the expression of the reporter gene reflects the expression of the gene in which the T-DNA was inserted. The screening of thousands of *A. thaliana* insertion lines following RKN infection and GUS staining of the galls resulted in the identification of several genes involved in giant cell formation such as the *RPE* gene coding a key enzyme of the pentose phosphate pathway (Favery et al., 1998) and the *MAP65-3* encoding a microtubule associated protein (Caillaud et al., 2008c). One of the interest of this approach as also the use of collections of T-DNA insertion lines, is to have access to insertion mutants in these genes in order to assess their role in the process studied. This strategy enabled to demonstrate the essential role of *RPE* and *MAP65-3* in the formation of giant cells in *A. thaliana* (Favery et al., 1998; Caillaud et al., 2008c).

To identify the genes that are differentially expressed (DE) in response to RKN infection, transcriptomic analysis was developed (**Table 1 and 2**). The first large-scale transcriptomic studies of plant-nematode interaction were realized by using microarrays. Microarrays are based on a hybridization of labelled nucleic acid samples to a very large set of nucleotide probes attached to a solid support (Zvara et al., 2015). Transcriptome analysis using *A. thaliana* whole genome microarrays was performed to study the gene expression in dissected galls induced by *M. incognita* (Hammes et al., 2005; Jammes et al., 2005). Jammes et al. (2005) showed that, 15% of protein-encoding genes were DE between uninfected roots and galls tissue at 7 and/or 14 dpi. These transcriptomic studies were developed in *A. thaliana*

but also in other plants like tomato and *Medicago truncatula* (Fuller et al., 2007; Ibrahim et al., 2011; Damiani et al., 2012; Bagnaresi et al., 2013) (**Table 1**). Development of New Generation Sequencing (NGS) facilitated transcriptome analysis for model and non-model plants. NGS identified 22% of genes encoding proteins DE in *A. thaliana* galls at 3, 5 and 7 dpi (Yamaguchi et al., 2017). NGS data of galls or whole infected roots are now available for *M. truncatula* (Postnikova et al., 2015), tomato (Shukla et al., 2018; Balestrini et al., 2019), sweet potato (Lee et al., 2019), eggplant (Zhang et al., 2021), *N. tabacum* (Li et al., 2018a), cotton (Kumar et al., 2019), rice (Kyndt et al., 2012; Petitot et al., 2017; Zhou et al., 2020), cucumber (Li et al., 2021), *Solanum torvum* (Sato et al., 2021) and mulberry (Shao et al., 2021) (**Table 2**). These studies showed a similar wide reprogramming of gene expression within the roots in response to RKN infection in multiple plant species. This RKN ability to induce similar giant cells in more than 5 000 plant species suggests that the plant molecular mechanisms manipulated by the RKN are conserved across the plant kingdom. Transcriptomic analysis of Portillo *et al.* (2013) in tomato compared to Barcala *et al.* (2010) in *A. thaliana* showed that 132 and 379 genes are differentially expressed genes at 3 and 7 dpi, respectively, in both plants. Using Gene Ontology (GO) algorithm to categorize the tomato and *A. thaliana* genes based on their functions, the authors showed that only 15 transcription factor families are co-regulated in both plants. They are involved in metabolism and defense processes such as MYB and WRKY transcription factor families or hormone signaling pathway like Auxin Response Factors (*ARF*) (Portillo et al., 2013).

Galls are mixed tissues composed of giant cells, neighboring cells, external root cell layers and nematode. In order to identify the genes specifically expressed in giant cells laser microdissection were developed (Fosu-Nyarko et al., 2009; Barcala et al., 2010; Ji et al., 2013; Portillo et al., 2013), that enabled to identify differences in expression patterns between whole gall and giant cells. For example, the comparison between genes expressed at 7 dpi in giant cells and in galls in rice roots infected by *M. graminicola* identified transcripts of genes coding proteins involved salicylic acid (SA), gibberellic acid (GA) and abscisic acid biosynthesis, induced in whole gall while they are downregulated in giant cells (Kyndt et al., 2012; Ji et al., 2013).

These transcriptomic analyses identified a wide range of genes DE between infected roots or galls and uninfected roots and shed light on the molecular mechanisms putatively involved in the giant cell formation. However, functional validations of these genes are necessary to understand their functions and the processes in which they are involved.

3.2.4 Key processes involved in formation of giant cells

Infection of plants by RKN supposes a tightly controlled reprogramming of plant cell machinery to enable proper development of feeding cells. Here, I will detail key processes involved in the formation of giant cells: modification of the cellular metabolism, cell cycle and cytoskeleton rearrangement, cell wall reorganization, defense suppression and modification of the phytohormone balance.

3.2.4.1 Metabolism

Giant cells are the unique source of nutrients for the nematodes. When parenchyma cells dedifferentiate to giant cells, their metabolism becomes over active (Machado et al., 2012). In addition to dense cytoplasm, cell wall ingrowths proliferate increasing the surface area of the transport of nutrients into the feeding cells (Jones and Northcote, 1972a; Berg et al., 2009; Sobczak et al., 2011; Rodiuc et al., 2014). Several studies focused on the regulation and the transport of water and solutes during the giant cell formation (Hoth et al., 2008; Hofmann et al., 2010; Absmanner et al., 2013b; Rodiuc et al., 2014). Metabolomic analysis of galls induced by *M. incognita* in *M. truncatula* showed an increased content in amino acids, in sucrose, glucose, malate and fumarate (Baldacci-Cresp et al., 2012). Moreover, many genes encoding proteins involved in key metabolism pathways of sugar, amino acid and water transporters are DE in galls (Jammes, 2005; Hammes et al., 2005; Barcala et al., 2010; Marella et al., 2013). As mentioned above, a key enzyme in the oxidative pentose phosphate pathway, RPE, has been showed to be essential for the early steps of giant cell formation in *A. thaliana* (Favery et al., 1998). Transcriptome analysis of tomato roots infected by *M. incognita* showed that genes encoding carbohydrate and sugar transporters (19 genes), lipid transporters (7 genes), aquaporins (13 genes), and peptide, nitrate and amino acid transporters (18 genes) were DE in galls mainly from 14 to 28 dpi (Shukla et al., 2018). An upregulation of genes encoding water channel proteins, such as aquaporins, e.g. *AtPIP2.5*, supports the idea of massive water import into the Giant cells (Favery et al., 2002; Hammes et al., 2005). Interestingly, the *M. incognita* effector Mi8D05 has been shown to interact with tomato aquaporin tonoplast intrinsic protein 2 (TIP2) showing that this process is also directly targeted by the nematode (Xue et al., 2013). Loss of function of some genes coding amino acid transporters like in the *Arabidopsis* mutants *app3* and *app6*, induced a decrease in the proportion of RKN juveniles that matured into females (Marella et al., 2013).

In *A. thaliana*, genes encoding main enzymes that cleave the sucrose, invertase CINV1 and sucrose synthases (SUS), SUS1 and SUS2, are upregulated in giant cells and galls at 3 dpi (Barcala et al., 2010). *A. thaliana* Knock-out (KO) mutant *sus1/sus4*, *cinv1* and *cinv1/cinv2* infected by *M. javanica* showed an increase in number of galls per root reflecting an increased susceptibility to RKN (Cabello et al., 2014). High-performance liquid chromatography (HPLC) was used to identify key sucrose transporter, SUT/SUC and SWEET, tonoplast monosaccharide transporter (TMT) and vacuolar glucose transporter (VGT) in tomato leaves and roots during early infection (from 12 to 72 hours post infection) by *M. incognita* (Li et al., 2018b). The

expression of three *SUTs*, 17 *SWEETs*, two *TMTs* and one *VGT1* showed an upregulation in roots at different times of nematode infection confirmed by qPCR, suggesting that transport of water and solutes occurred within the giant cell formation. Since *SUT1* gene in tomato has high homology with *SUC2* gene in *A. thaliana*, the authors used *Arabidopsis* KO, *suc2*, to evaluate its role in nematode infection (Hackel et al., 2006; Gong et al., 2015). *suc2* KO mutants did not affect the invasion of nematode at early stage of infection but delayed RKN development with a lower proportion of fourth-stage juveniles (J4s) than in the wild-type at 15 dpi. In contrast, the downregulation of *SUT1*, 2, 3, 4 and 5 were shown in galls at 3, 7 and 14 dpi in rice galls (Xu et al., 2021b). Overexpressing *GNS2* that encodes a callose synthase led to an increase in callose deposition, a reduced PD permeability and a decreased sucrose content in rice galls at 7 dpi induced by *M. graminicola* (Xu et al., 2021b). These results suggest that callose negatively affected sucrose supply and that plasmodesmata may mediate sucrose transport in galls.

3.2.4.2 Cell wall modification

During their formation, giant cells undergo considerable growth due to extensive cell wall modifications. In *A. thaliana*, the volume of giant cells can increase by 100 fold from 3 to 40 dpi (Cabrera et al., 2015). The plant cell wall is a complex structure formed by high molecular weight polysaccharides like cellulose and hemicellulose, structural proteins and aromatic substances. The expansion of giant cells requires both loosening and thickening of the cell walls of giant cells (Bohlmann and Sobczak, 2014; Escobar et al., 2015). Interestingly, CELL WALL DEGRADING ENZYMES (CWDEs) like cellulases, xylanases, pectate lyases, and expansins were found in *M. incognita* secretions (Jaubert et al., 2002a; Ledger et al., 2006; Bellafiore et al., 2008). Beyond the CWDEs produced by the RKN, microarrays analysis in *A. thaliana* and soybean (Jammes et al., 2005; Barcala et al., 2010; Ibrahim et al., 2011) showed that most of genes involved in cellulose synthesis and cell wall structural proteins, are upregulated, in plant-nematode interaction. *Arabidopsis* cell wall-related mutants were used to investigate the role of cell wall components in RKN parasitism (Bozbuga et al., 2018). Beta-galactosidase-5 (*bgal5*) and mannan synthesis related 1 (*msr1*) KO mutants, involved in the synthesis of galactan and mannan respectively, were infected by *M. incognita*. These two mutants showed a negative impact on RKN development: roots of mutant plants stained with acid fuchsin showed fewer nematodes than wild type plants at 21 dpi and smaller juveniles in *bgal5*, indicating that they are essential for giant cells function.

Expansins are proteins well known to be involved in plant cell wall expansion (Sobczak et al., 2011). In tomato, the gene coding for expansin *LeEXPA5* is expressed in neighboring cells (Gal et al., 2006). In transgenic tomato roots silenced by antisense construct for *LeEXPA5*, the number of eggs per egg masses produced by nematodes was significantly reduced at 42 dpi compared to the control lines. Moreover, cross sections of galls showed a significant reduction in the diameter of giant cells with smaller galls in *LeEXPA5*-silenced lines compared to control plants. This suggested that *LeEXPA5* is essential for a successful nematode infection.

3.2.4.3 Cell cycle regulation

Successive mitosis without cytokinesis generate multiple nuclei and lead to the multinucleate status of giant cells (Jones and Payne, 1978). An increase of DNA content has been also confirmed within these multiple nuclei, due to endoreduplication cycles that occur in later stage of giant cell development (Jones and Goto, 2011; de Almeida Engler and Gheysen, 2013). Transcriptomic analyses and cell biology studies showed that genes encoding mitotic cyclins (e.g. *CYCA1.2*) and cyclin dependent genes (*CDKs*) involved in the transition phases of the cell cycle are expressed in the early developing galls at 3 dpi in tomato (Niebel et al., 1996; Barcala et al., 2010), *A. thaliana* (Jammes et al., 2005) and rice (Ji et al., 2013). Moreover, two cell-cycle switch protein *CCS52A1* and *CCS52B* genes involved in the switch from the mitotic cycle to endoreduplication (Fülöp et al., 2005) are expressed in *A. thaliana* galls at 7 dpi (Favery et al., 2002; Jammes et al., 2005; De Almeida Engler et al., 2012).

In order to identify the function of these DE genes, several studies used chemical inhibitors of the cell cycle (e.g. colchicine, which inhibits microtubule polymerization and thus assembly of the mitotic spindle) or mutants disrupted for key regulators of cell cycle resulted in defects in giant cell formation (Wiggers et al., 2002; de Almeida Engler et al., 2007; de Almeida Engler and Gheysen, 2013). The RNAi silencing of *A. thaliana* cell cycle gene, *CDKA.1*, increases resistance towards RKN (Van De Cappelle et al., 2008). Overexpressing *KIP-RELATED PROTEIN 6 (KRP6)* in *A. thaliana* lines, an inhibitor of *CDK*, accelerates the entry into mitosis but delays mitosis progression confirming a role in the activation of the mitotic cell cycle. Moreover, the overexpression of *KRP6* lines infected by *M. incognita* showed an increase in mitotic activity in galls, resulted in more nuclei in giant cell and induces a strong decrease in the number of egg masses (Vieira et al., 2014; Vieira and de Almeida Engler, 2017). The overexpression of *DP-E2F-like (DEL)* in *A. thaliana*, a repressor of endoreduplication, leads to galls with smaller giant cells and less number of egg masses while the loss- function of this gene, *del1*, showed defects in giant cells containing little cytoplasm (De Almeida Engler et al., 2012). Similarly, the role of a negative regulator of DNA replication and transcription *ARMADILLO BTB ARABIDOPSIS PROTEIN 1 (ABAP1)* has been recently highlighted in *A. thaliana* galls at 7 and 14 dpi (Cabral et al., 2021). The knockdown or overexpression of *ABAP1* showed defects in giant cell formation and in nematode development indicating that the balance of *ABAP1* is needed for proper gall development. *WEE1*, a gene encoding for a protein kinase involved in the G2-to-M transition through the inhibition of activity of *CDKs*, has been shown to be expressed in *A. thaliana* galls at 3 dpi and localized to giant cell nuclei (Cabral et al., 2020). The knockdown of *WEE1* leads to increased mitotic activity in galls and represses the RKN infection and reproduction demonstrating a function for *WEE1* in DNA replication in galls.

3.2.4.4 Cytoskeleton reorganization

RKN induce changes in the organization of actin and microtubule cytoskeleton during giant cell development (De Almeida Engler et al., 2004; Caillaud et al., 2008a; Engler et al., 2010; Vinet and Zhedanov, 2010) (**Figure 12**). Transcriptomic analyses showed multiple genes encoding key factors of cytoskeleton reorganization differentially expressed in giant cells. An overexpression of α , β , and γ tubulins and actin genes like *ACT2* and *ACT7* was first observed in *A. thaliana* galls (De Almeida Engler et al., 2004). Moreover, *ACTIN DEPOLYMERIZING FACTOR 2* gene (*ADF2*) is overexpressed in *A. thaliana* giant cells at late stage of gall development 14 & 21 dpi (Clément et al., 2009). A role of cytoskeleton in giant cell formation was confirmed by using different pharmacological analyses. The treatment of *A. thaliana* infected roots with taxol, that stabilizes microtubules and makes them resistant to depolymerization, led to the arrest of proper giant cells development (De Almeida Engler et al., 2004). In cucumber roots, the treatment with Cytochalasin D, inhibitor of the rate of actin polymerization, reduced RKN parasitism with smaller galls and giant cells and with a significant reduction in the number of J2s compared with untreated plants at 21 dpi (Liu et al., 2016).

Regulators of the microtubule cytoskeleton are also important for the formation of giant cells. The Microtubule-Associated Protein65-3 (MAP65-3) protein in *A. thaliana* plays a key role in cell division and mainly in the organization of microtubules during mitosis and cytokinesis. The *map65-3* loss-function lines infected with *M. incognita*, showed defects in giant cells development: giant cells failed to establish a proper development and degenerate which leads to nematode death (**Figure 13**) (Escobar et al., 2011). In contrast, transgenic *A. thaliana* *FRAGILE FIBER 2* (*fra2*) mutant, altered in microtubule-severing katanin protein and displaying a loose in organization of microtubule, were more susceptible to RKN infection with an increase of number of females compared to control plants (Meidani et al., 2019). This difference observed in these mutants *map65-3* and *fra2* towards RKN parasitism is probably due to cellulose production defects observed in *fra2*. The *fra2* mutant cell walls are extremely fragile due to reduced cellulose production, thus could lead to an easiest nematode penetration. Interestingly, a *M. incognita* effector, Mi-PFN3, was showed to disrupt actin filament formation (Leelarasamee et al., 2018). The overexpression of this effector in *A. thaliana* plants led to an increase of susceptibility against RKN with an increase in the number of gall. Moreover, the protoplasts of leaves overexpressing the effector MiPFN3 fused to a red fluorescent protein (RFP) showed disrupted actin filaments.

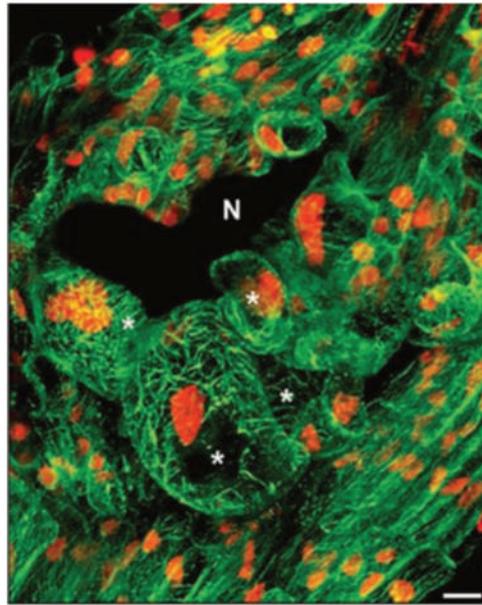


Figure 12. Reorganization of cytoskeleton in giant cell. *In vivo* microtubule organization in developing giant cells induced by *Meloidogyne incognita*. Microtubule arrays in multinucleate giant cells and surrounding cells co-expressing microtubule-binding domain (MBD)-GFP and nuclear histone H2B:YFP proteins. N, nematode; *, giant cells. Bar = 10 μ m (Caillaud et al., 2008a)

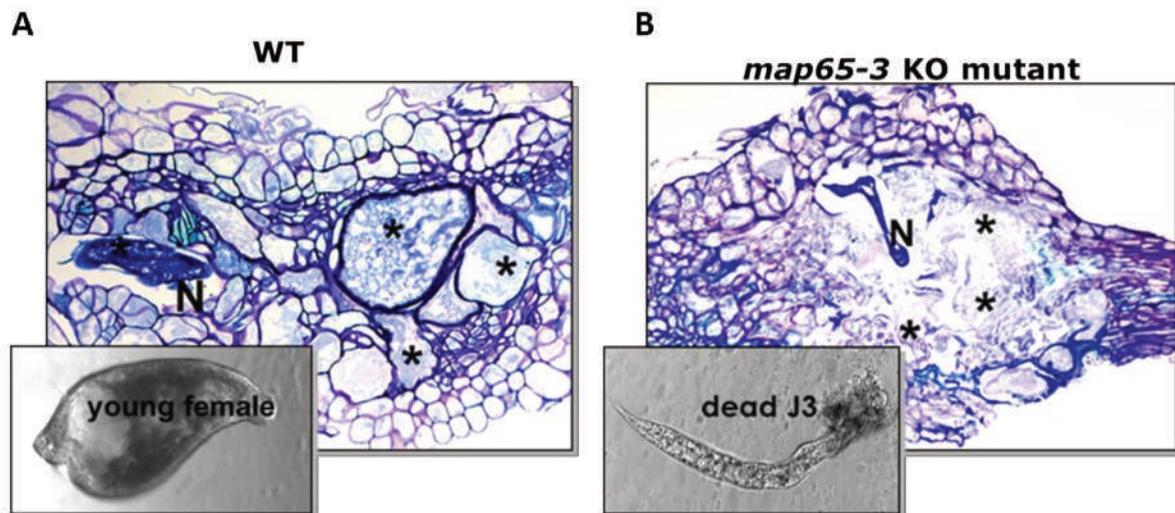


Figure 13. Defects in giant cell ontogenesis in the *map65-3* mutant. (A) Cross sections at 21 dpi through the galls of a wild-type plant; Giant cells were mature and nematode developed into the young female. (B) Cross sections at 21 dpi through the galls of *map65-3* mutant; Giant cells decayed and nematode development remained arrested at the third juvenile stage. *, Giant cells; N, nematode. Bars 40 μ m (Caillaud et al., 2008c)

3.2.4.5 Defense response

Transcriptomic analyses showed that most defense-associated genes are repressed in galls and/or giant cells. *WRKY* transcription factors, *PATHOGENESIS-RELATED (PR)* and lipoxygenases genes, are key markers and/or regulators of plant defense pathways in plant species like *A. thaliana*, tomato, *M. truncatula* and cotton (Jammes et al., 2005; Barcala et al., 2010; Damiani et al., 2012; Shukla et al., 2018; Kumar et al., 2019). The *WRKY* family of transcription factors, known to act downstream several plant hormones in the activation of plant defenses, are generally repressed (Jammes et al., 2005; Escobar et al., 2011). However, *WRKY25* has been shown to be induced in susceptible tomato upon *M. javanica* infection. Histological analysis of *WRKY45*:GUS lines showed *WRKY45* expression at early stage of infection (5 dpi) but also through gall formation and maintenance (15 and 28 dpi) (Chinnapandi et al., 2017). The overexpression of this gene was associated with a decrease in SA- (*PR1*) and jasmonic acid (JA)- defense marker genes and with an accelerated nematode infection and an increased in number of females and giant cell size in galls at 28 dpi.

Pathogenesis-related (PR) proteins, *PR-1* and *PR-2* salicylic acid (SA) marker genes have been shown to be induced in *A. thaliana* infected roots at 9 dpi and repressed at 14 dpi (Hamamouch et al., 2011). The expression of two lipoxygenases, *LOX3* and *LOX4*, were analyzed in *A. thaliana* infected by *M. javanica* (Ozalvo et al., 2014). Gene reporter GUS constructs showed local induction of *LOX3* expression all along galls formation while *LOX4* promoter was activated by RKN infection, although the GUS signal weakened as galls formation progressed. Intriguingly, the infection of *lox3* and *lox4* loss-function T-DNA mutants showed a decrease in number of females for *lox3* lines in comparison to control plants, whereas *lox4* lines showed an increase of susceptibility, with a higher in number of females and egg masses compared to control plants. This increase of susceptibility was also accompanied with an increase of JA and ethylene responsive genes 2 and/or 5 dpi. These results suggested that *LOX3* and *LOX4* interfere with different pathways that might affect the plant response to nematode infection while *LOX4* might control defense toward nematode infection.

Several hormones have been shown to be involved in defense response. Plant defense hormones, such as SA, JA and ethylene control various aspects of plant defense responses to PPNs. Recent reviews have described the complexity of JA- and SA-dependent pathway regulation during nematode infection, and the role of other phytohormones, such as gibberellin and abscisic acid (ABA), in regulating JA- or SA-dependent signaling (Gheysen and Mitchum, 2019; Sato et al., 2019). In tomato, transcriptomic analyses between RKN-resistant and susceptible tomatoes showed that several genes related to ethylene such as aminocyclopropane-1-carboxylic acid oxidase (*ACO*), ethylene-responsive transcription factors (*ERFs*), several genes involved in JA-synthesis and SA-responsive signaling were differentially expressed during disease development (Shukla et al., 2018). In *A. thaliana* galls, genes related to ethylene responses were mostly downregulated while only one gene related

SA was induced at 3 dpi (Barcala et al., 2010). Many transcripts involved in JA biosynthesis were shown to be repressed in rice giant cells (Ji et al., 2013).

Exogenous applications of defense related hormones were used to understand their role during RKN infection. In rice, the exogenous application of ethylene (ethephon) and JA (methyl jasmonate- MeJA) induced a strong systemic defense response against *M. graminicola* with an increased resistance reflected by a decrease in numbers of galls associated to upregulation of *PR1* gene (Nahar et al., 2013). Foliar application of MeJA significantly reduced the level of *M. graminicola* infection in rice (Verbeek et al., 2019). SA soil drenching reduced gall formations in tomato infected by *M. incognita* (Molinari, 2016) while foliar treatment with MeJA significantly reduced the infection of RKN (Fujimoto et al., 2011). Tomatoes inoculated by *M. incognita* and treated with SA showed an upregulation in the expression of *PR1* gene and a decreased number of galls (Bozbuga, 2020).

The overexpression of *NPR1*, an activator of SA signalization, in tobacco also led to a decreased number of galls and egg masses induced by *M. incognita* (Priya et al., 2011). Moreover, a *M. incognita* effector, Mi-CM-3, encoding a chorismate mutase, was shown to suppress plant immunity by regulating the SA pathway in infected *Nicotiana benthamiana*. The transient expression of this effector causes a reduction in SA level and increased susceptibility to nematode infection (Wang et al., 2018b).

Hydrogen peroxide (H₂O₂) and reactive oxygen species (ROS) act as signal molecules to activate plant immunity (Forman et al., 2010). Moreover, treatment with JA of tomato seedlings infected by *M. incognita* showed a 40 % decrease in H₂O₂ contents (Bali et al., 2018). In addition, JA treatment (100 nM) increased the activation of ROS-scavenging enzyme superoxide dismutase SOD activity in tomato treated and infected by *M. incognita* compared to JA- treated control (Bali et al., 2020).

Interestingly, ethylene signalization was shown to modulate the attraction of RKN to the plant. Root tomato exudates were significantly more attractive to the *M. incognita* in the knockdown *erf-e2* mutant (Dyer et al., 2019). The repression of *EIN2b* involved in ethylene signalization led to an increase in resistance in rice towards *M. graminicola* (Nahar et al., 2011)

3.2.4.6 Modulation of auxin and cytokinin phytohormones

Nematode interaction with their host implies the modulation of phytohormones balance to either suppress plant defenses (as described above) or promote the differentiation of their feeding sites (reviewed in Gheysen and Mitchum, 2019; Oosterbeek et al., 2021). Early studies highlighted the main role of auxin and cytokinin in giant cell formation (Balasubramanian and Rangaswami, 1962; Dimalla and van Staden, 1977). The presence of cytokinin and auxin-like compounds in *M. incognita* secretions was evidenced by mass spectrometry analyses of induced secretions, suggesting that these hormones could play crucial functions in feeding cell development (De Meutter et al., 2003). Auxin or indole-3-acetic acid (IAA), is a simple signaling molecule that plays a critical role in plant development and growth regulating cell division, elongation, and differentiation. Auxin is involved in a wide range of developmental processes e.g. organ differentiation and lateral root initiation (Quint and Gray, 2008; Gutierrez et al., 2012; Guilfoyle, 2015; Di et al., 2016; Majda and Robert, 2018). Auxin triggers cell or tissue specific responses through a well-studied signaling pathway. Inside the cell, auxin is bound by the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB) receptors. TIR1 proteins bind three different ligands: an SCF-TIR1 ubiquitin ligase complex, auxin and AUX/IAA proteins. The binding of all these three ligands at the same time triggers the ubiquitination of AUX/IAA proteins and their subsequent degradation by the 26S proteasome. AUX/IAA proteins repress the transcription of auxin-induced genes by building multimers with other AUX/IAA proteins and with *AUXIN RESPONSE FACTOR (ARF)* transcription factors. After the auxin-induced degradation of Aux/IAAs, ARF transcription factors are free to activate or repress the expression of genes to which promoters they are bound (reviewed in Chandler, 2016; Li et al., 2016b).

Microarray analyses of *A. thaliana* gall transcripts showed an early activation of genes responsible for auxin homeostasis and auxin-responsive genes, while repressors of auxin responses were downregulated (Hammes et al., 2005; Jammes et al., 2005; Barcala et al., 2010). The use of lines expressing the reporter *GUS* gene under the control auxin-responsive promoter *GH3* or the synthetic auxin responsive promoter DR5 showed activation of these two promoters in galls induced by RKN (Hutangura et al., 1999; Karczmarek et al., 2004; Absmanner et al., 2013). Within *A. thaliana* galls, DR5:GUS lines showed a strong signal in giant cells but also in neighboring cells at 4 dpi (**Figure 14**) (Cabrera et al., 2014b). Mutants in components of the auxin efflux carrier PIN-FORMEDs involved in the root-specific auxin transport, *pin2* and *pin3*, showed an increase of resistance towards RKN (Mazarei et al., 2003; Kyndt et al., 2016). PIN2 and PIN3 appear to be important for the delivery of auxin into the giant cells (Grunewald et al., 2009). In *A. thaliana*, two ARFs, ARF7 and ARF19, positively regulate lateral root formation through activation of the plant-specific transcriptional regulators *LATERAL ORGAN BOUNDARIES-DOMAIN*, and in particular LBD16. The loss of function of LBD16, induced a defect in giant cell formation demonstrating its role in giant cell formation (Cabrera et al., 2014b). The expression of this *LBD16* is also detected also in *M. javanica* and *M. arenaria* galls (Okushima et al., 2007; Lee et al., 2009). The auxin-cytokinin balance is considered the main hormonal control system in plant (Moubayidin et al., 2009).

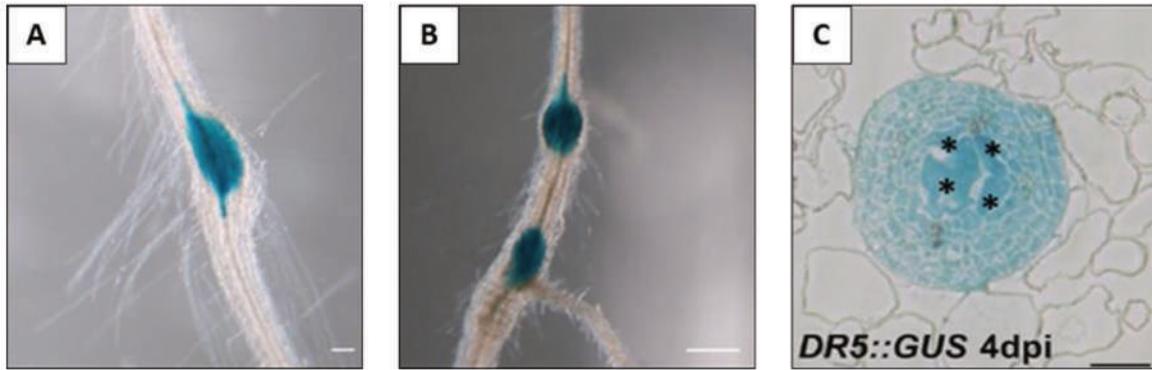


Figure 14. The synthetic auxin-inducible promoter, *DR5::GUS* activation patterns in *Arabidopsis thaliana* galls infected by *M. javanica*. Strong signal in the center of the gall at (A) 3 and (B) 7 dpi. (C) Semi-thin sections of galls from *DR5::GUS* at 4 dpi showed GUS staining in Giant cells (*) and in the small dividing cells inside the vascular cylinder. Bars: (A-B) 200 μ m; (C) 50 μ m. (Cabrera et al., 2014b)

Auxin induces cell division in the meristems while cytokinin control cell division and differentiation (Moubayidin et al., 2009; Bielach et al., 2012; Schaller et al., 2014). The activation of cytokinin-regulated genes has also been shown in early stages of gall formation, by using the cytokinin-responsive promoter *AAR5* (Lohar et al., 2004). *ARR5* expression was specifically absent in mature giant cells, although dividing cells around the giant cells continued to express *ARR5*. In this study, the authors also showed that the overexpression of the *CYTOKININ OXIDASES (CKX)*, gene coding for an enzyme that catalyzes the degradation of cytokinin, induced a resistance towards RKN. Even though these results support a role for cytokinin in gall formation, cytokinin signalization is still poorly described in plant response to RKN and the cytokinin responsive promoter *TCS* is not induced in galls. On the contrary, this cytokinin marker is activated in syncytia induced by cyst nematodes (Absmanner et al., 2013b) and the role of cytokinin is more described in syncytia (Ali et al., 2013).

Other phytohormones were also shown to be involved in formation of giant cells. In rice infected by *M. graminicola*, the application of low concentration of gibberellic acid (GA) enhanced nematode infection in rice (Yimer et al., 2018). Brassinosteroids are involved in response to *M. graminicola* (Nahar et al., 2013; Kyndt et al., 2017). The application of abscisic acid (ABA) increases the susceptibility of rice RKN infection. Strigolactones suppresses jasmonate accumulation and enhance *M. graminicola* infection in rice (Lahari et al., 2019).

4. MicroRNAs

As presented in the previous paragraphs, all transcriptome analyses performed in various plant species showed that the formation of galls and/or giant cells was the result of extensive reprogramming of plant cell transcription in host root. With the development of NGS, many genes with differentially expressed between galls and uninfected roots were identified in multiple plant species. On the contrary, how does this reprogramming of gene expression occur in root cells remains poorly understood and the regulators involved in this process are mainly unknown. During my thesis, I focused my investigations on the role in formation of giant cells of a family of small non coding RNAs, the microRNAs. As a part of miRNAs are highly conserved within the plant kingdom, they can be a part of the conserved mechanisms manipulated by RKN to induce the formation of the feeding site.

4.1 Generalities

Following transcription of eukaryotic protein-coding genes, the primary RNA transcript is processed to one or several mature messenger RNAs (mRNA). mRNAs are exported from the nucleus to the cytoplasm, where they are translated to amino acid sequence to form protein. Eukaryote cells have many sophisticated ways to control gene expression acting at different levels: chromatin, transcriptional level, RNA processing, RNA transport and translational controls. RNA interference (RNAi) known also as “gene silencing” was first discovered in

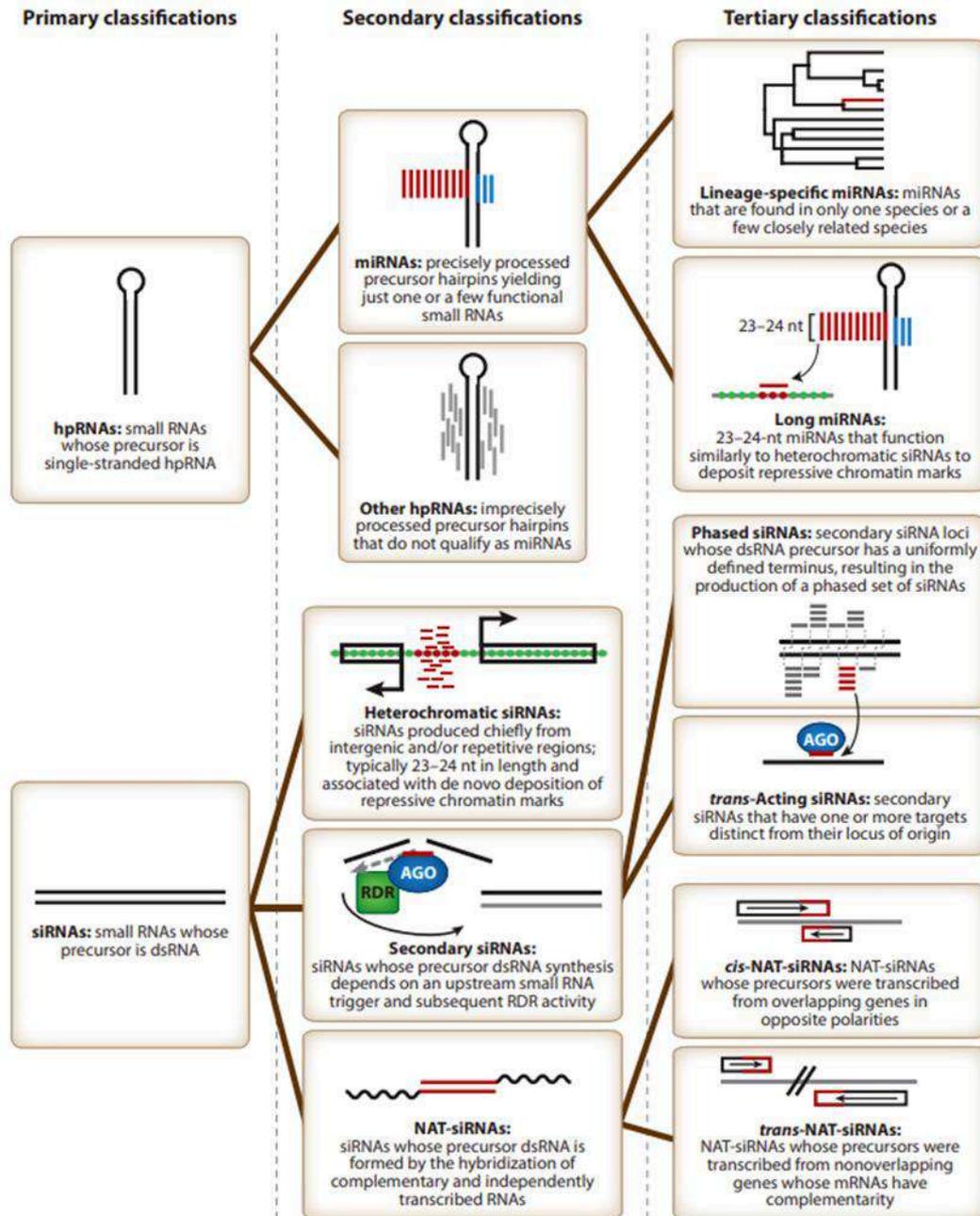


Figure 15. Hierarchical classification system for endogenous plant small RNAs. Thick black lines indicate hierarchical relationships. dsRNA, double-stranded RNA; hpRNA, hairpin RNA; miRNA, microRNA; NAT-siRNA, natural antisense transcript small interfering RNA; siRNA, small interfering RNA. (Axtell, 2013a)

plants in 1990 (Van Der Krol et al., 1990; Napoli et al., 1990). In order to enhance the purple color of petunia petals, a vector carrying the coding sequence of a gene involved in the biosynthesis of the purple pigment, was introduced to the plant. The overexpression of this gene caused a reverting phenotype with partially or completely white flowers due to the extinction of both exogenous and endogenous genes. In 1998, Dr Andrew Fire and Dr Craig Mello discovered in *C. elegans* a regulatory mechanism, named RNAi, that degrades mRNA from a specific gene. RNAi is activated when double-stranded RNA (dsRNA) molecules are detected in the cell. dsRNA was shown to have a silencing effect by targeting mRNA with complementary sequence (Fire et al., 1998; Tabara et al., 2002). RNAi was found in plants, animals, and humans and is widely used as a method to repress gene's expression in functional analysis. Since the discovery of RNAi, different classes of small non coding RNAs (ncRNAs), involved in various processes regulation of gene expression, have been identified. ncRNAs are classified depending on their size, their biogenesis and their regulation process. Small ncRNAs have a length of 18 to 30 nucleotides (nt). In plants, small ncRNAs are subclassified based on the biogenesis and /or functions: microRNAs (miRNA), hairpin RNAs (hpRNA), secondary siRNAs, natural antisense transcript siRNAs (reviewed in Axtell, 2013a; Borges and Martienssen, 2015) (**Figure 15**). Small ncRNAs regulate gene expression at the transcriptional level before gene transcription by DNA methylation or at the post-transcriptional level by cleaving mRNAs or inhibiting their translation. "Post Transcriptional Gene Silencing" (PTGS) is driven by miRNA and siRNA (Mallory and Bouché, 2008) while "Transcriptional Gene Silencing" (TGS) is driven by RNA (Ekwall, 2004). siRNAs have a very complex biogenesis and a mode of action. During my thesis I focused my studies on miRNAs, that I will be developed it in my introduction.

4.2 microRNAs and PTGS

4.2.1 Biogenesis and maturation of microRNAs

miRNAs are small ncRNAs of 20 to 22 nt in plants encoded by *MIR* genes often organized in multigene family within plant genomes (Jones-Rhoades, 2012). *MIR* genes are transcribed by RNA polymerase II (Pol II) (Kim et al., 2011) to generate a single-stranded primary transcript (pri-miRNA) (**Figure 16**). Several core activators interact with RNA Pol II such as NEGATIVE ON TATA LESS2 (NOT2), the putative MYB domain-containing DNA-binding protein CELL DIVISION CYCLE 5 (CDC5) and the Elongator complex (Wang et al., 2013; Zhang et al., 2013; Fang et al., 2015). The pri-miRNAs are polyadenylated at the 3' end, folds into a hairpin structure, thanks to a partial complementarity of its sequence and is then cleaved by the endonuclease DICER LIKE1 (DCL1) to generate a shorter stem loop precursors, the pre-miRNAs (Szarzynska et al., 2009). These pre-miRNAs are processed by the dicing complex composed of DCL1 and other core factors (HYPONASTIC LEAVES1 (HYL1) and SERRATE (SE)) (Kurihara and Watanabe, 2004; Fukudome and Fukuhara, 2017).

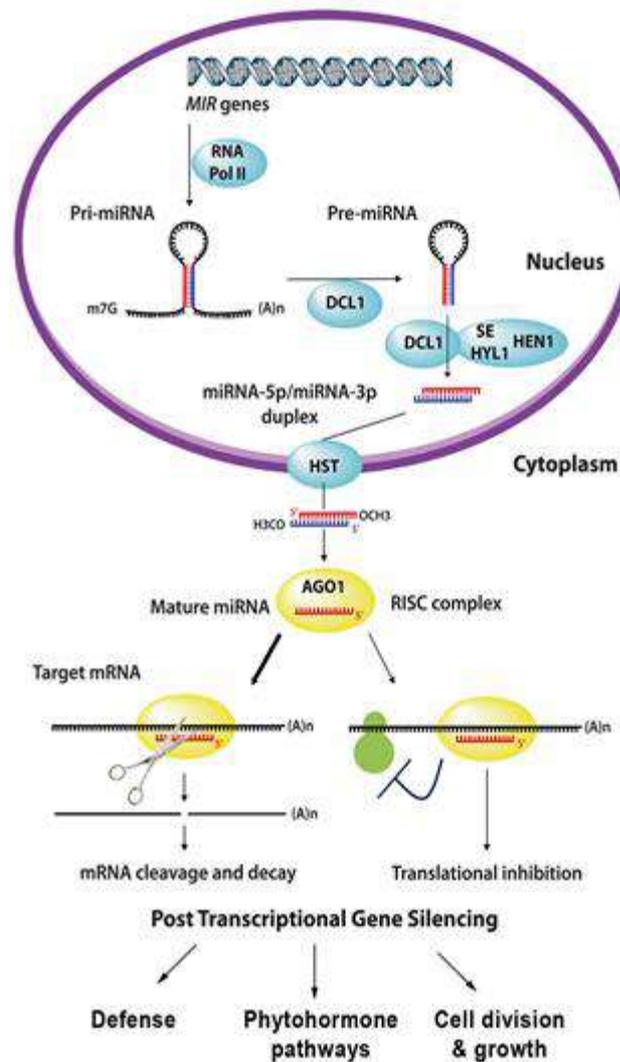


Figure 16. Simplified biogenesis and mechanisms of action of microRNAs in plants. The *MIR* genes are transcribed by RNA polymerase II (RNA Pol II) to generate single-stranded hairpin-containing primary transcripts (pri-miRNA). The pri-miRNA is then cleaved, in the nucleus, by Dicer-like 1, in association with hyponastic leaves 1 and serrate, to produce a precursor miRNA (pre-miRNA). The pre-miRNA is, in turn, cleaved by DCL1 and its cofactors, thus generating a duplex composed of the mature miRNA and its complementary strand. The HUA ENHANCER 1 protein then adds a methyl group to the OH end of each strand of the miRNA duplex, to protect against degradation. The miRNA duplex is then actively transported from the nucleus to the cytosol through interaction with the HASTY (HST) exportin. One of the two strands of the duplex is then loaded onto the ARGONAUTE-1 protein, the main constituent of the multiprotein RNA-induced silencing complex (RISC). The AGO1-associated strand guides the RISC to target mRNAs by sequence complementarity, resulting in target cleavage or the inhibition of protein synthesis. DCL1, Dicer-like 1; HYL1, hyponastic leaves 1; SE, serrate; HEN1, HUA ENHANCER 1 protein; HST, hasty; AGO1, argonaute 1. (Jaubert-Possamai et al., 2019)

Within the nucleus, DCL1 interacts with these cofactors to unit pre-miRNA in nuclear bodies, called dicing bodies (Song et al., 2007; Laubinger et al., 2008). DCL1 cleaves pre-miRNA into miRNA-5p/miRNA-3p mature duplex (Margis et al., 2006). This miRNA duplex is stabilized by HUA ENHANCER 1 (HEN1) that deposits a methyl group onto the OH group of each strand to protect it from degradation (Li et al., 2005; Yu et al., 2005). The duplex is then exported from the nucleus to the cytoplasm by HST (Mee et al., 2005).

4.2.2 Mode of action of microRNAs

ARGONAUTE-1 protein (AGO1) is the main protein of the RNA-induced silencing complex (RISC). AGO1 forms a complex with HEAT SHOCK PROTEIN (HSP90) that allows miRNA-5p/miRNA-3p to be incorporated (Iki et al., 2010). AGOs are the main silencing effectors that fold into a bilobal structure displaying a central groove for duplex binding (Mallory and Vaucheret, 2010). One strand of the miRNA-5p/miRNA-3p duplex, known as the guide strand, is loaded in AGO1 complex whereas the other strand is removed. The mature miRNA strand loaded in AGO1 guides RISC to a mRNA targeted by sequence complementarity resulting in the silencing of the corresponding gene (Yu et al, 2017).

Plant gene silencing by miRNAs occurs at two levels (reviewed in Voinnet, 2009; Yu et al, 2017) depending on the degree of sequence complementary between miRNAs and targeted mRNA (Hutvagner and Zamore, 2002; Franco-Zorrilla et al., 2007). When sequence complementarity between the miRNA and its target is perfect or nearly perfect, the RISC cleaves the targeted mRNA. When it has a reduced degree of complementarity, RISC represses mRNA translation by preventing ribosome access to mRNAs. In plants, repression of translation is less observed than mRNA cleavage. AGO1 mediates miRNA target cleaved, followed by the degradation of the cleavage fragments by exonucleases such as EXORIBONUCLEASE 4 (XRN4) (Souret et al., 2004). The cleavage zone is generally located at the center the miRNA sequence at position 10 or 11 (Palatnik et al., 2007) (**Figure 17**).

Most *MIR* genes are organized as multigenic families. The different *MIR* genes of a miRNA families show difference in the sequence of the precursor but identical, or nearly identical, sequence of the mature miRNA and share often the same targets (**Figure 17**) (Palatnik et al., 2007). Several miRNAs families are conserved within the plant kingdom, e.g. miR156, miR169, miR166 that were identified in more than 40 different plant species (Sunkar et al., 2008). However, the number of miRNAs specific for a plant species is higher than the number of conserved miRNAs (Cui et al., 2017). Several miRNAs/targets pairs are conserved between plant species (Cuperus et al., 2011; Jones-Rhoades, 2012), e.g. miR159 and *MYB33* transcription factor family in *A. thaliana*, tomato and rice (Allen et al., 2010; da Silva et al., 2017; Zhao et al., 2017). In some cases, the functions of these miRNAs and their targets can be also conserved. The regulation of two auxin response factors *ARF6* and *ARF8* by miR167 family are conserved in tomato and *A. thaliana*, and the cleavage by miR167 shows similar developmental functions in the two plants (Liu et al., 2014; Wang et al., 2015a).

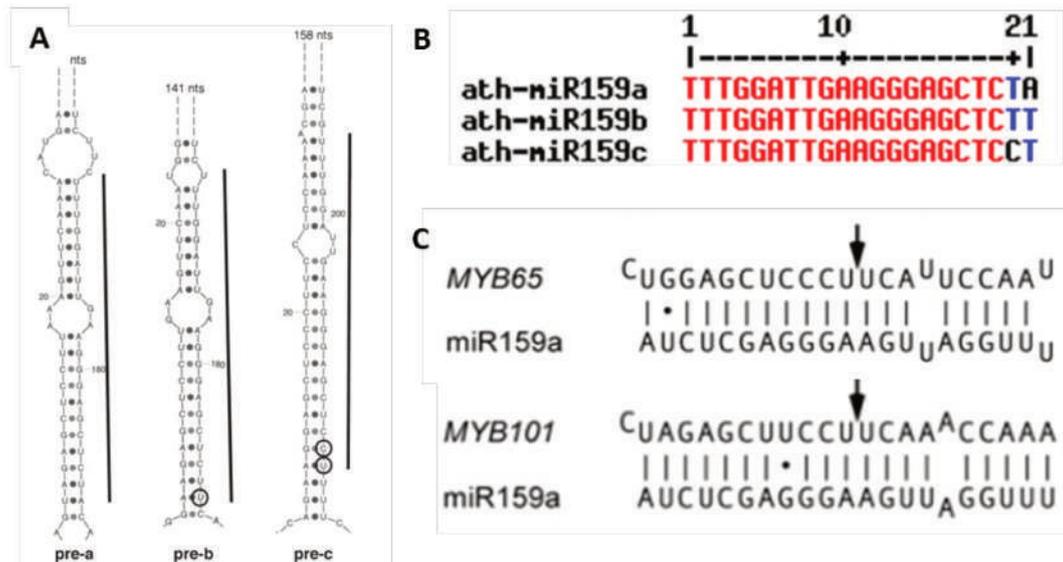


Figure 17. microRNA. (A) Proposed secondary structures for miR159a, b and c precursors. The vertical line indicates the position of the mature miRNA. miR159b and c differ from miR159a in the last one and two nucleotides at their 3' ends, respectively (black circles). The loop lengths of pre-miR159a, pre-miR159b and pre-miR159c not shown in the diagram are indicated (Reyes and Chua, 2007). **(B)** Alignment of mature sequences of miR159a, miR159b and miR159c. **(C)** Alignments of miR159a and their two targets *MYB101* and *MYB65*. The predominant cleavage sites of the targets found in wild-type are indicated by black. Targets are cleaved between positions 10 and 11 of the alignment (Palatnik et al., 2007).

miRNA/targets has complex regulations and are generally integrated in a complex network. While the same miRNA can regulate several targets, e.g. miR164 that targets the two genes *NAC1* and *CUC2* in *A. thaliana*, several miRNAs can regulate the same targets. miRNAs can also trigger the production of siRNAs, known as phased secondary siRNAs (phasiRNAs) that silence mRNA at PTGS level (reviewed in Rogers and Chen, 2013).

4.3 Role of microRNAs

microRNAs are major regulators of gene expression in plants and their roles have been described in various processes from plant development to response to biotic and abiotic stresses but also in trans-kingdom communication between pathogens and their hosts (Sunkar et al., 2007; Weiberg et al., 2015; Couzigou and Combier, 2016). NGS analysis of small RNAs identify many microRNAs DE in various biological conditions suggesting a role for these microRNAs in these processes. However, functional validation is needed to test the functions of these genes, in particular by using mutants of these miRNAs and their target. In this chapter, I will present plant miRNAs for which a role has been biologically validated.

4.3.1 MicroRNAs in plant development

microRNAs regulate shoot, leaf and root developmental processes. Some microRNAs can act individually while other miRNAs regulate coordinately their targets during plant developmental processes.

Multiple microRNAs and their targets were shown to regulate **leaf development**. Leaf development mostly relies on the function of the shoot apical meristem (SAM). It begins with the initiation of leaf primordia then polarity establishment till the leaf acquired its final shape and size (Laufs et al., 1998; Barton, 2010). miR394 and its target *LEAF CURLING RESPONSIVENESS (LCR)*; miR396/*GROWTH REGULATING FACTORS (GRFs)* and miR319/*TEOSINTE BRANCHED1, CYCLOIDEA and PROLIFERATING CELL NUCLEAR ANTIGEN BINDING FACTOR (TCP)* are involved in the control of SAM (Schoof et al., 2000; Efroni et al., 2008; Schommer et al., 2008; Liu et al., 2009; Baucher et al., 2013; Li et al., 2016a). Other microRNAs control leaf polarity like miR164 /*CUP-SHAPED COTYLEDON2 (CUC2)*, miR165/miR166 /*HOMEODOMAIN LEUCINE ZIPPER (HD-ZIP)* and miR390/*TAS3-tasiRNA* (Peaucelle et al., 2007; Huijser and Schmid, 2011; Moon and Hake, 2011; Rubio-Somoza and Weigel, 2011; De Felippes et al., 2017).

An example of coordination of several miRNAs is the interplay between miR396 and miR319 and their targets in the control of cell proliferation and leaf shape in *A. thaliana* (Schommer et al., 2014), also with miR164 in the regulation of the shape and the size of the limbus (Rubio-Somoza and Weigel, 2011) and with miR156 in the switch from the juvenile to the adult phases of vegetative development (Rodriguez et al., 2016) (**Figure 18**). miR396 regulates leaf morphology by targeting *GROWTH REGULATION FACTORS (GRF)* transcription factor family (Liu et al., 2009; Baucher et al., 2013). *GRFs* are highly expressed in leaf meristematic tissue

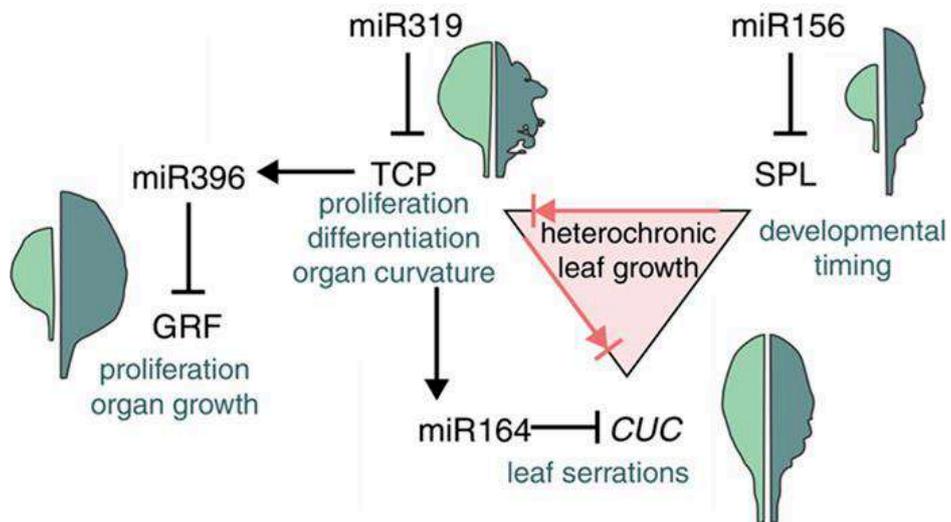


Figure 18. Interplay of evolutionary conserved microRNA-transcription factor networks during leaf development. The functions associated to each miRNA and their targets network are indicated as well as silhouettes of leaves representing the phenotypes observed in *Arabidopsis thaliana* after modifying each network. The pink triangle refers to the protein-protein interactions between the miRNA targets of the different networks. *GRF*, GROWTH REGULATING FACTORS; *CUC*, CUP-SHAPED COTYLEDON2; *TCO*, TEOSINTE BRANCHED1, CYCLOIDEA and PROLIFERATING CELL NUCLEAR ANTIGEN BINDING FACTOR; *SPL*, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (Rodriguez et al., 2016).

and positively regulate leaf size by promoting cell proliferation (Kim et al., 2003). Overexpression of miR396 induces the silencing of six *GRF* genes and leads to the formation of narrow leaves with a reduced size like the *grf* KO mutants (Horiguchi et al., 2005; Jeong and Byung, 2006). The expression of *MIR396* is activated by *TCP4* which is targeted by miR319 that is involved in leaf shape development (Palatnik et al., 2007; Schommer et al., 2014). *A. thaliana* transgenic plants overexpressing miR319 have increased leaf size, and a similar phenotype is observed in the *tcp* KO mutants (Schommer et al., 2008). Significant changes in *TCP4* transcripts levels affect organ curvature. *TCP4* directly activates the promoters of miR396 and miR164. miR164 targets *CUC* transcription factors whose activities contribute positively to the generation of leaf serrations. miR156 targets *SPL* transcription factors which promote the phase change from the juvenile to the adult phases of vegetative development and also to reproductive development; but they also influence leaf growth itself. The miR156, miR319 and miR164 networks are further interconnected by the protein-protein interactions of their targets. During the development of the younger leaves miR156 levels are high and therefore *SPL* levels are low, and TCP proteins form dimers with CUC proteins which in turn leads to smooth margins. Later on in development, when miR156 level goes down, *SPL* level increases and the *TCP-CUC* dimers are replaced by *TCP-SPL* dimers. The released *CUC* proteins dimerize and leaf serrations are formed.

The flowering stage, is regulated by an important number of miRNAs and their targets to synchronize flowering (Spanudakis and Jackson, 2014; Teotia and Tang, 2015; Samad et al., 2017). We can cite for example, miR172/*APETALA2-like (AP2)* (Zhu and Helliwell, 2011; Teotia and Tang, 2015; Tripathi et al., 2018; Ó'Maoiléidigh et al., 2021), miR156/*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* (Chuck et al., 2007; Efroni et al., 2008; Yamaguchi and Abe, 2012; Hong and Jackson, 2015; Teotia and Tang, 2015; Tripathi et al., 2018) and miR319/*TCP* (Schommer et al., 2008). As described above for leaf development, these microRNAs act coordinately to regulate transition from vegetative to flowering phase. miR156 family targets 11 of the 17 *SPL* genes. Among these genes, *SPL3*, *4*, and *5* promote floral transition (Chuck et al., 2007; Xie et al., 2012; Hong and Jackson, 2015; Teotia and Tang, 2015). Tomato plants overexpressing SlymiR156a showed a delay in flowering and an extended juvenile phase (Zhang et al., 2011b). miR172 repress translation of *AP2*, a floral repressing transcription factor inducing defect in floral organ identity and mimicking the phenotype of the loss-of-function *ap2* mutants (Chen, 2004; Teotia and Tang, 2015). Different studies highlighted the complex organization of miR172/*AP2* and miR156/*SPL* module in stabilizing the floral state (**Figure 19**). In *A. thaliana*, miR156 and miR172 act together: in vegetative phase, miR156 maintains the juvenile phase by repressing *SPL15* which inhibits floral transition. While in the flowering phase, *TCP15* activates *MIR172* expression that represses *AP2* expression that induces the activation of floral transition (Teotia and Tang, 2015; Tripathi et al., 2018; Lian et al., 2021; Ó'Maoiléidigh et al., 2021).

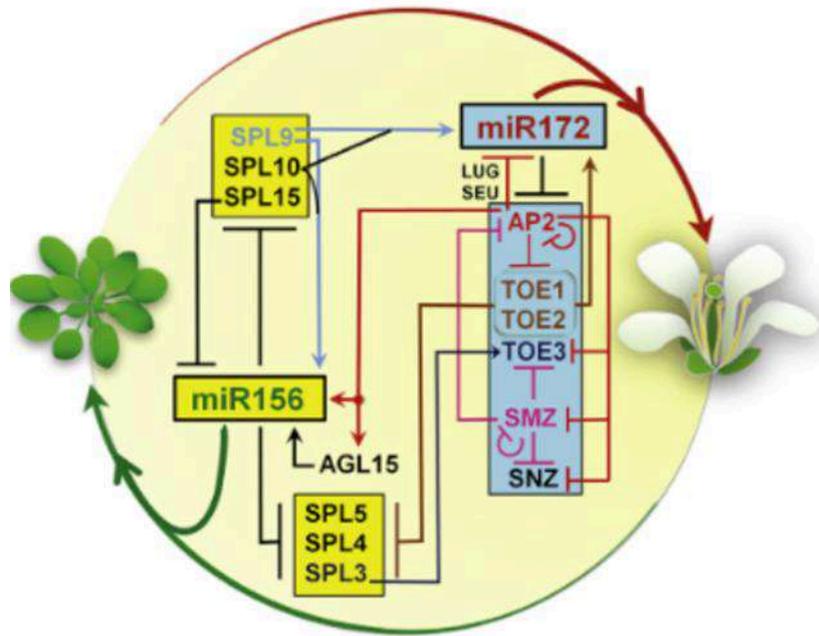


Figure 19. Schematic representation of the interactions between miR172 and miR156 and their targets during flower transition; Crosstalk between miR156 and miR172 modules are shown along with the regulatory networks and feedback regulation of the target genes. miR156 is regulated by positive and negative feedback loop of *SPL9* and *SPL15*, respectively; and positively regulated by *AP2* and *AGL15*. miR172 is regulated by the positive feedback loop of *TOE1/2* and negatively by *AP2* through *LUG* and *SEU*. *TOE1/2* represent the expression of *SPL3/4/5* genes. *SPL3* positively regulates the expression of *TOE3*. *AP2* and *SMZ* repress their own expression and also of other miR172 target genes. *AGL15*, *AGAMOUS LIKE15*; *AP2*, *APETALA2*; *LUG*, *LEUNIG*; *SEU*, *SEUSS*; *SMZ*, *SCHLAFMUTZE*; *SNZ*, *SCHNARCHZAPFEN*; *SPL*, *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE*; *TOE1-3*, *TARGET OF EAT1-3*. (Teotia and Tang, 2015)

The root growth is sustained by the root apical meristem (RAM) which contains undifferentiated stem cells able to divide and differentiate to produce the different tissues of the root (Petricka et al., 2012). The function of miR160 (Mallory et al., 2005; Wang et al., 2005) and miR396 (Rodriguez et al., 2010; Bazin et al., 2013) has been established in RAM. It is important to mention that most miRNAs that are involved in root development are involved also in auxin and cytokinin hormone pathway. These two hormones are well known modulators of root formation and the cross-talk between them regulates RAM maintenance and lateral root emergence (Bishopp et al., 2011; Bielach et al., 2012). Thus, miR156 and its target *SPL10* control root meristem activity and root-derived *de novo* shoot regeneration via cytokinin in *A. thaliana* (Barrera-Rojas et al., 2020). *Arabidopsis* plants overexpressing miR156a showed a reduced meristem size. The opposite root phenotype was observed in knock down miR156a expressing short tandem target mimic (STTM) that act as molecular sponge to sequester the microRNA and block its action to cleave the targets. Interestingly, CRISPR/Cas9-derived *spl10-2* mutant (35-bp deletion in *SPL10*) displayed shorter meristems whereas *rSPL10* lines that resist to miR156a cleavage showed the opposite phenotype suggesting that miR156 contributes to modulation of root meristem activity by targeting *SPL10*. Cytokinin controls root meristem size by activating the transcription factors of type-B *ARABIDOPSIS THALIANA RESPONSE REGULATORS* (*ARRs*) (Mason et al., 2004). *ARR5* fused to the gene reporter GUS showed a strong expression in root explants of plants overexpressing miR156a. Conversely, weaker GUS staining was observed in *rsp10* mutants resistant to cleavage. Moreover, medium supplement with different cytokinin presented an important regenerative capacity for controlling root explants, but it did not improve the low regenerative capacity of plants overexpressing miR156 neither for *rsp10*. All together, these results indicated that the meristem activity is regulated by miR156-*SPL10* module probably through the reduction of cytokinin responses, via the modulation of *ARR* expression (Barrera-Rojas et al., 2020).

Moreover, miR393 (**Figure 20**), miR390 (Chen et al., 2011; Lu et al., 2018), miR164 (Li et al., 2012; Geng et al., 2020), miR167 (Gutierrez et al., 2009), miR476 (Xu et al., 2021a) or miR847 (Wang and Guo, 2015) have been shown to be involved in the formation of lateral and adventitious roots. Several of these miRNAs regulate lateral root formation *via Auxin Response Factor* (*ARFs*). *ARFs* are key transcriptional factors regulators of auxin signaling that bind to auxin response elements (AuxREs) included in promoters of numerous auxin-responsive genes (Reviewed in Guilfoyle and Hagen, 2007; Chandler, 2016; Li et al., 2016b). miR390 induces the production of tasiRNAs that silence *ARF2*, *ARF3* and *ARF4* removing the repression of lateral root growth (Marin et al., 2010). miR160 is known to repress *ARF17* (negative regulators of adventitious rooting) while miR167 repress the two targets *ARF6* and *ARF8* (positive regulators of adventitious rooting). A complex regulation and interaction between the three *ARFs* (*ARF6*, *ARF8* and *ARF17*) and miR160/miR167 was observed using different *grf* mutant lines (Gutierrez et al., 2009). Thus, the balance between these two miRNAs control lateral root development (Couzigou and Combier, 2016).

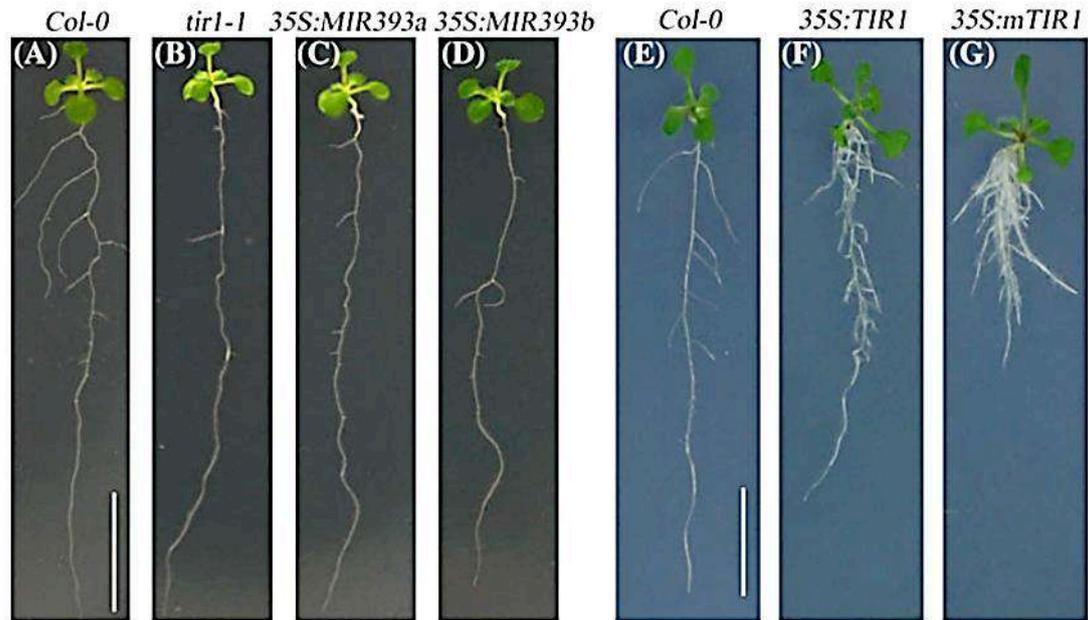


Figure 20. Regulation of miR393 and its target in root development. *A. thaliana* lateral roots (**A and E**) Col-0 compared with mutants (**B**) *tir1-1* and mutants overexpressing (**C**) miR393a, (**D**) miR393b, (**F**) *TIR1* and (**G**) miR393-resistant form of *TIR1* (*mTIR1*). Overexpressing (**F**) *TIR1* and (**G**) *mTIR1* displayed shorter primary roots, and more lateral roots while (**B**) *tir1* and (**C and D**) miR393a/b overexpression displayed slightly longer primary root and fewer lateral roots, compared to wild type (**A and E**) Col0. *TIR1*, *TRANSPORT INHIBITOR RESPONSE1*. Bars = 10 mm (Chen et al., 2011)

4.3.2 MicroRNAs in plant response to abiotic stress

Plants are subjected to different environmental challenges such changes in temperature, soil water potential and nutrients. Induced phenotypic and physiological changes help the plant to adapt and survive in response to environmental changes (**Figure 21**) (Singh et al., 2020). Interestingly, some miRNAs respond to different abiotic stresses such as miR408 in response to cold, drought and copper stresses (Ma et al., 2015).

Under drought or salinity stresses, several miRNAs are differentially expressed and can positively or negatively regulate plant tolerance to drought stress (Song et al., 2013; Fang et al., 2014; Ma et al., 2015) or salinity stress (Jung and Kang, 2007; Song et al., 2013; Zhou et al., 2013; Iglesias et al., 2014; Kong et al., 2014). miR169 is a negative regulator of drought stress. In *A. thaliana*, plants overexpressing miR169 have an increase in susceptibility towards drought stress. Mutant overexpressing miR169 showed increased in size of the stomatal aperture that mimics the phenotype observed in miR169 target mutant *nfya5* (*NUCLEAR FACTOR Y SUBUNIT A5*) (Li et al., 2008). miR394 and its target *LEAF CURLING RESPONSIVENESS (LCR)* are involved in plant responses to both drought and salinity stresses (Song et al., 2013). Using RT-PCR and GUS reporter fusion, miR394a and miR394b were showed to be induced under saline and drought stresses in *A. thaliana*. Conversely, plants overexpressing miR394b and *lcr* mutant showed a tolerance to drought stress. On the other hand, treatment with 100 mM NaCl showed a decrease in the germination of plants overexpressing miR394a and *lcr* seeds compared to wild type. These results suggest that both miR394 and *LCR* are critical for plant response to salt and drought stresses.

NGS showed that several microRNAs like miR156, miR396 and miR402, are differentially expressed under heat or cold stress suggesting that these microRNAs play important roles in the regulation of heat or cold tolerance in plants (Zhou et al., 2008; Kim et al., 2010; Wang et al., 2012b; Guan et al., 2013; Dong and Pei, 2014; Li et al., 2014; Stief et al., 2014; Wang et al., 2014a; Ma et al., 2015; Zhang et al., 2016b; Zhou et al., 2016; Ding et al., 2017; Mangrauthia et al., 2017; Song et al., 2017; López-Galiano et al., 2019; Tiwari et al., 2020). *A. thaliana* plants overexpressing miR397a improves plant tolerance under cold stress. The transcript levels of *COLD REGULATED (COR15A)*, *(COR47A)* and *RESPONSIVE TO DEHYDRATION (RD29A)* plants have been shown to be higher in 35S:miR397 than in wild-type plants after cold treatment for over 48 h at 4°C. These results showed that miR397 is involved in the cold signaling pathway and enhanced cold responsive gene expression that may contribute to tolerance to cold stress.

Finally, several miRNAs and their targets have been shown to be differentially expressed in response to nutrients deficiency : Nitrogen, Phosphorus, Sulfur and Copper (Yamasaki et al., 2007; Gifford et al., 2008; Hsieh et al., 2009; Liang et al., 2010; Zhao et al., 2011; Jagadeeswaran et al., 2014). The role of miRNAs that respond to copper will be discussed later in the results (chapter 2).

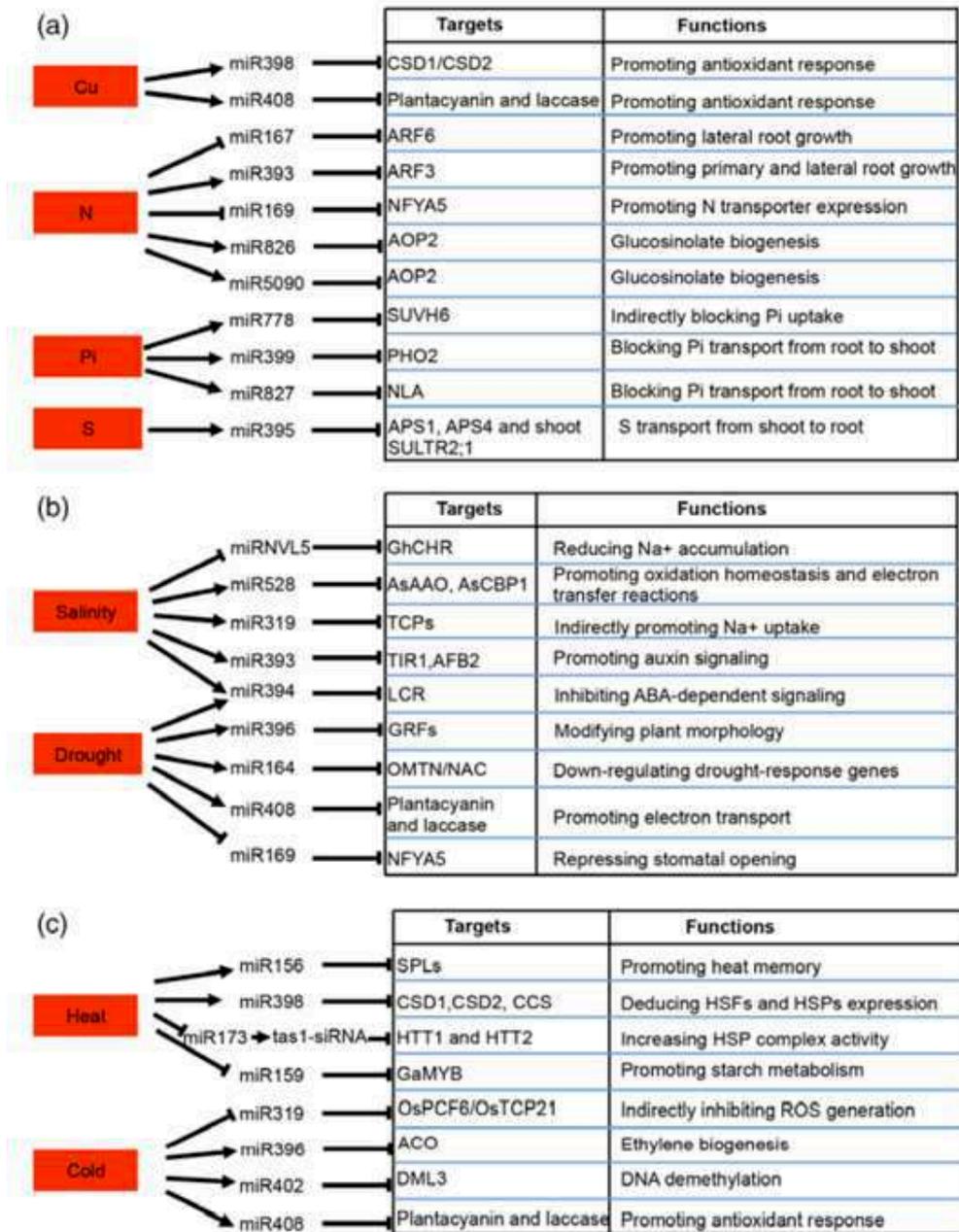


Figure 21. Functions of miRNAs and their targets under different abiotic stresses (a) nutrients stresses, (b) drought and salinity stresses, (c) temperature stress. (Li et al., 2017)

4.3.3 MicroRNAs in plant-microorganism interactions

Plants are constantly challenged by various pathogens, like bacteria, viruses, oomycetes, fungi, nematodes but also in interaction with beneficial symbiotic microorganisms, like nitrogen-fixing bacteria and arbuscular mycorrhizal fungi. Several studies assessed the role of important components of the RNAi machinery, in particular DCLs and AGOs, in plant-microorganisms interactions (Blevins et al., 2006; Zhang et al., 2006; Navarro et al., 2008; Li et al., 2010; Medina et al., 2017). These studies demonstrated that miRNAs are involved in plant response to microorganism infection. In addition, several effectors of plant pathogens have been described as targeting the silencing pathway, e.g. in virus, bacterial, fungi infection and RKN (Navarro et al., 2008; Qiao et al., 2013; Csorba et al., 2015; Ye and Ma, 2016; Yin et al., 2019; Mejias et al., 2021).

In **symbiotic interactions**, small RNA sequencing approach identified microRNAs differentially expressed in nitrogen fixing nodules in several host plants like soybean (Subramanian et al., 2008; Wang et al., 2009; Joshi et al., 2010; Turner et al., 2012; Yan et al., 2015; Yan et al., 2016), *M. truncatula* (Lelandais-Brière et al., 2009), *Lotus japonicus* (De Luis et al., 2012; Holt et al., 2015) and common bean (Formey et al., 2016). The function of several miRNAs in plant response to nitrogen-fixing bacteria was demonstrated in soybean (miR167; Wang et al., 2015), in *L. japonicus* (miR171 & miR397; De Luis et al., 2012), soybean (miR393; Subramanian et al., 2008) and *M. truncatula* (miR169; Lelandais-Brière et al., 2009). Several miRNAs, including miR156, miR167 and miR168, were found up-regulated in tomato roots in response to colonization by the endophyte *Pochonia chlamydosporia* (Pentimone et al., 2018). In soybean, a role for the module miR167-ARF6/8 was established in nodule formation. A strong expression is observed for miR167c in mature soybean root nodules (Wang et al., 2009; Wang et al., 2015). Soybean plants overexpressing miR167c showed increase in number and length of lateral roots and an increase in numbers of nodule when infected by *Bradyrhizobium japonicum*. STTM knockdown of miR167c showed the opposite phenotype of the overexpression of miR167c. RNAi of the two miR167 validated targets, *ARF8a* and *ARF8b* (Wang et al., 2015), also produced more nodules in roots while plants overexpressing *ARF8a* showed less number of nodules than control plants infected by *B. japonicum*. These results suggest that the regulation of *ARF8A* and *ARF8B* by miR167 regulates soybean nodulation and lateral root development. The role of miR172 was also established in nodule formation in common bean infected by *Rhizobium etli*: miR172 is overexpressed during nodule development and cleaves the target *NODULE NUMBER CONTROL1 (NNC1)* to regulate nodule formation (Wang et al., 2014b; Nova-Franco et al., 2015) (**Figure 22**).

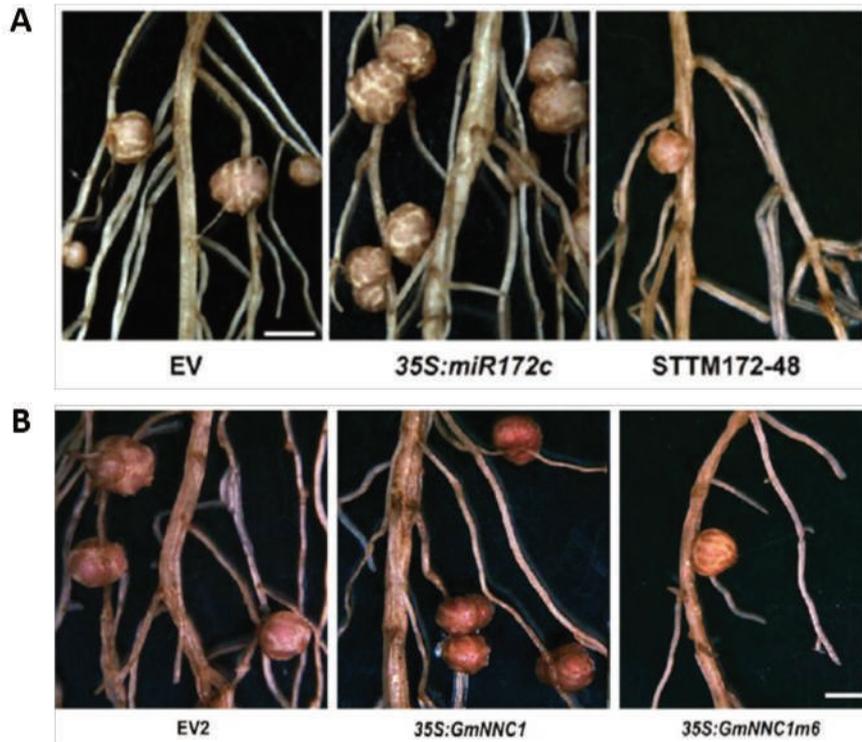


Figure 22. miR172c regulates the number of soybean nodule through inhibition of its target gene *NNC1*. (A) Overexpression of miR172c (35S:miR172c) roots developed more nodules than empty vector (EV) control roots while number of nodules in the knockdown of miR172c (STTM172-48) was reduced. (B) Overexpression of *NNC1* (35S:GmNNC1) did not significantly affect nodulation; the overexpression of the mutant *NNC1* resistant to cleavage (35S:GmNNC1m6), reduced the number of nodules compared to the empty vector (EV2) control roots; Bar = 3 mm (Wang et al., 2014b)

Plant viruses cause serious damages in plant and affect crop production (Scholthof et al., 2011; Wang et al., 2012a). The infection with viruses regulates the accumulation of small RNAs in plants which can affect either plant defense or virus pathogenicity (Prasad et al., 2019). Virus dsRNAs are recognized and cleaved by DCLs to produce primary virus-derived small interfering RNAs (vsiRNAs). Plants have developed antiviral defense systems such as RNAi that targets viral RNAs (Ruiz-Ferrer and Voinnet, 2007). The components of miRNAs pathways are involved in plant defense towards viruses (Blevins et al., 2006; Csorba et al., 2007; Havelda et al., 2008; Prasad et al., 2019). A recent study, using microarray analysis, revealed 129 miRNAs differentially expressed in *N. benthamiana* infected by Beet necrotic yellow vein virus (BNYVV) (Liu et al., 2020). Functional analysis showed a role for miR398 induction in the defense response towards BNYVV.

The role of small RNAs in plant defense towards **bacterial infection** was described precisely in response to *Pseudomonas* genus, like miR160 (Fahlgren et al., 2007), miR164 (Lee et al., 2017), miR167 (Fahlgren et al., 2007; Li et al., 2010), miR393 (Fahlgren et al., 2007 ; Zhang et al., 2011a), miR398 (Jagadeeswaran et al., 2009) and miR482 (Shivaprasad et al., 2012). A recent study highlighted the role of miR167 and its targets, the two auxin response factors *ARF6* and *ARF8* and salicylic acid (SA) in defense against *Pseudomonas syringae* pv. tomato DC3000 in *A. thaliana* (Caruana et al., 2020). The overexpression of miR393 in *A. thaliana* restrict the development of *Pseudomonas*, while the overexpression of one of its target resistant to cleavage, *AUXIN SIGNALING F-BOX PROTEIN (AFB1)*, increase the sensitivity of the plant to bacterial infection (Navarro et al., 2006). A recent study identified the miRNAs and their targets expressed in susceptible and resistant ginger transcriptomes in response to the soil borne bacteria, *Ralstonia solanacearum*, infection (Snigdha and Prasath, 2021). Some studies also showed the involvement of small RNAs during the interaction of plants with *Agrobacterium tumefaciens*. In *N. tabacum*, miR393 is induced by the gall-forming bacteria (Pruss et al., 2008).

Infection of mutants of PTGS pathway demonstrates a role of small RNAs in plant response to **fungi and oomycetes** (Ellendorff et al., 2009; Weiberg et al., 2013). In cotton infected by *Verticillium dahlia*, miR164 and its target *NAC100* plays a role in plant resistance to fungal infection (Hu et al., 2020a). miR164 is known to target *NAC* genes and participate in plant development and defense (Mallory et al., 2004; Sieber et al., 2007). Indeed, plants overexpressing STTM miR164 showed more severe disease symptom compared to wild type plants, while overexpressing miR164 plants were more resistant to the infection. In response to oomycete infection, overexpression of miR393 in *A. thaliana* leads to a resistance towards *Hyaloperonospora arabidopsidis* and *Phytophthora capsici* (Robert-Seilaniantz et al., 2011; Hou et al., 2019). Wong et al. (2014) identified an overexpression of miR166, miR393, miR1507, miR2109 and miR3522, and a downregulation of miR168, miR319 and miR482 in soybean infected by *Phytophthora sojae* (Wong et al., 2014). STTM knock down of miR393 in soybean leads to a susceptibility towards *Phytophthora* infection suggesting that miR393 is a positive regulator of soybean defense towards oomycete infection.

miRNAs have also been described as a key regulator in the plant-**nematode** interactions. I wrote as a co-first author, a review entitled “MicroRNAs, New Players in the Plant–Nematode Interaction” published in *Frontiers in Plant Science* (Jaubert-Possamai, Nouredine, Favery, 2019) This review summarizes the current knowledge about the function of plant miRNAs in plant response to RKN and CN. In this review, we listed the microRNAs that were DE in the two types of feeding sites, galls/giant cells and syncytia, induced by RKN and CN, respectively. Expression profile of most conserved miRNAs (except miR390) in feeding site had different expression profiles according to the type of feeding structures, the kinetic points and/or the plant species. These differences in giant cell and syncytia can be explained by their distinct ontogenesis, but also in the different biological material and sequencing analyzes.

This mini-review was published in 2019. New studies have been published since then describing miRNAs differentially expressed in response to RKN and CN.

Sequencing of small RNAs from control and infected roots of resistant and susceptible soybean cultivars at early stage (1 and 5 dpi) of infection induced by *Heterodera glycines* identified 14 known and 26 novel microRNAs differentially expressed (Lei et al., 2019). The expression of 19 over 24 miRNAs were confirmed using RT-qPCR.

In cotton-RKN interaction, Cai et al. (2021) identified 266 miRNAs including 193 known miRNAs and 73 novel miRNAs expressed in cotton roots 2 months after infection with *M. incognita* (Cai et al., 2021). Among them, 50 miRNAs were DE: 28 miRNAs were up-regulated and 22 miRNAs down-regulated in galls. In a first attempt to understand the functions of these miRNAs, the targets of miRNAs were predicted by using “TargetFinder” algorithm and degradome sequencing. Degradome is a biological identification of microRNA targets that consists in the specific sequencing of cleaved mRNA products. This approach is based on the difference between the 5' end of cleaved mRNA and intact mRNA. RNA adapters specifically binds to the 5' monophosphate end of cleaved mRNA. The ligation products are then retrotranscribed, the cDNAs are amplified by PCR and then sequenced. 87 gene targets were identified to be targeted by 57 miRNAs. Based on GO (gene ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis, miRNA targets were categorized in different processes including those associated with organism responses to the environmental stresses.

Interestingly, in galls of rice infected by *M. graminicola*, 25 long non-coding RNAs (lncRNAs), 3739 siRNAs and 16 miRNAs differentially expressed 3 dpi were recently identified in comparison to control roots (Verstraeten et al., 2021). The expression of three miRNAs (miR408-3P, miR3979-3P and miR850.1) was confirmed using RT-qPCR. In addition, miRNA targets were identified in galls using the degradome approach.

All these sequencing data enable to identify microRNAs that are DE in galls and therefore that could regulate gene expression in plant-nematode interactions. However, most of these studies lack functional validation of these miRNAs. So far the function of only four and three microRNAs has been biologically validated in plant response to RKN and CN infection, respectively. These DE microRNAs and the key role of important components of the RNAi machinery (DCLs and AGOs), are a strong elements showing that microRNAs are key regulators of gene expression in galls.



MicroRNAs, New Players in the Plant–Nematode Interaction

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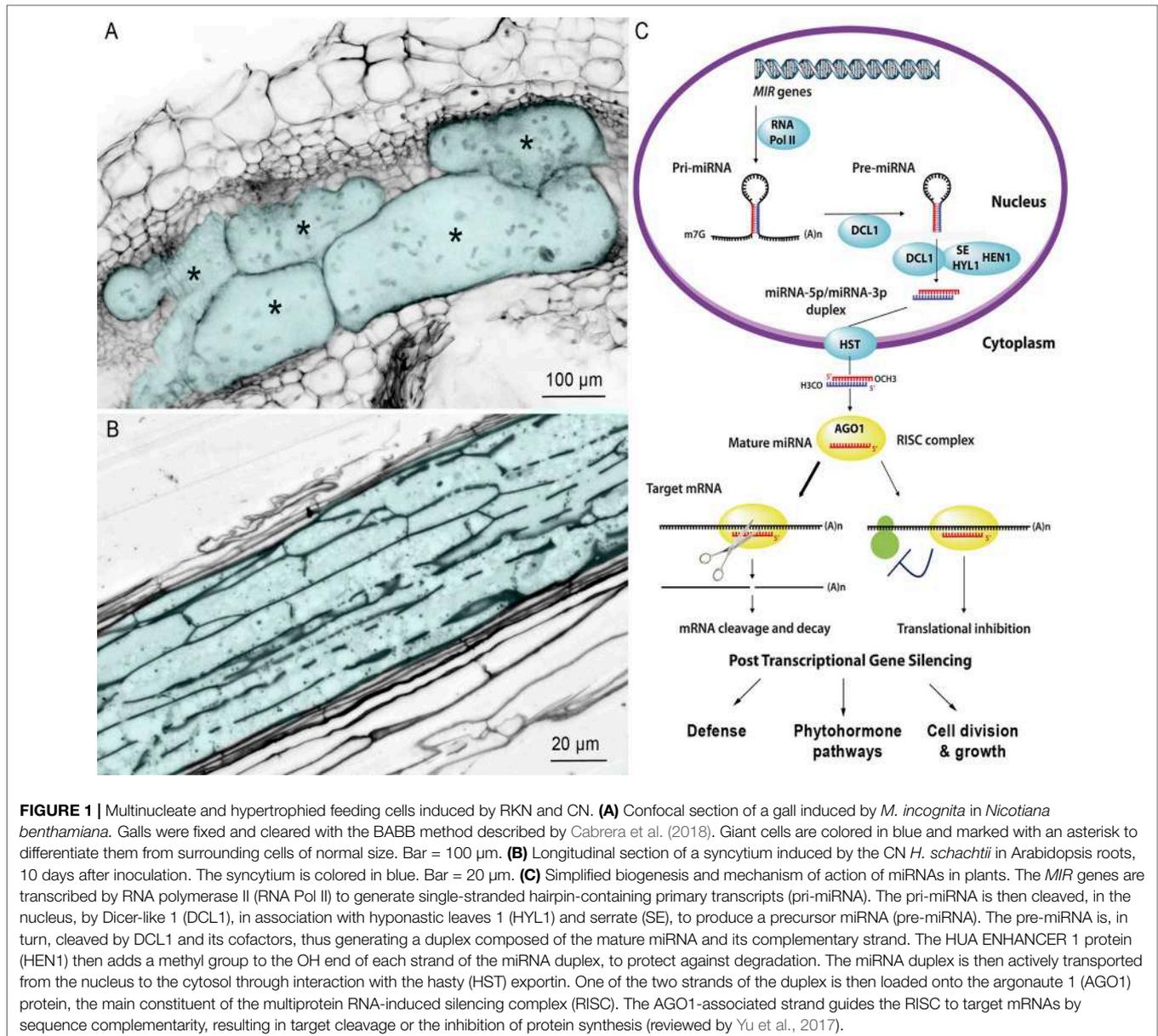
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Plant-parasitic root-knot and cyst nematodes are microscopic worms that cause severe damage to crops and induce major agricultural losses worldwide. These parasites penetrate into host roots and induce the formation of specialized feeding structures, which supply the resources required for nematode development. Root-knot nematodes induce the redifferentiation of five to seven root cells into giant multinucleate feeding cells, whereas cyst nematodes induce the formation of a multinucleate syncytium by targeting a single root cell. Transcriptomic analyses have shown that the induction of these feeding cells by nematodes involves an extensive reprogramming of gene expression within the targeted root cells. MicroRNAs are small noncoding RNAs that act as key regulators of gene expression in eukaryotes by inducing the posttranscriptional silencing of protein coding genes, including many genes encoding transcription factors. A number of microRNAs (miRNAs) displaying changes in expression in root cells in response to nematode infection have recently been identified in various plant species. Modules consisting of miRNAs and the transcription factors they target were recently shown to be required for correct feeding site formation. Examples include miR396 and *GRF* in soybean syncytia and miR159 and MYB33 in *Arabidopsis* giant cells. Moreover, some conserved miRNA/target modules seem to have similar functions in feeding site formation in different plant species. These miRNAs may be master regulators of the reprogramming of expression occurring during feeding site formation. This review summarizes current knowledge about the role of these plant miRNAs in plant–nematode interactions.

Keywords: root-knot nematodes, cyst nematodes, galls, syncytium, microRNAs, siRNAs

INTRODUCTION

Sedentary endoparasitic nematodes are the most damaging plant-parasitic nematodes (PPNs) that cause massive crop yield losses worldwide (Blok et al., 2008). There are two main groups of PPNs: the root-knot nematodes (RKNs) of the genus *Meloidogyne* and the cyst nematodes (CNs) of the genera *Heterodera* and *Globodera* (Jones et al., 2013). After penetrating the root and migrating to the vascular cylinder, mobile second-stage juvenile (J2) selects one (CNs) or a few (RKNs) initial root cells, into which it injects a cocktail of secretions that transform these cells into hypertrophied multinucleate feeding cells that supply nutrients required for nematode development: the giant cells induced by RKNs (**Figure 1A**) or the syncytium induced by CNs (**Figure 1B**).



Common and Specific Processes Involved in Feeding Site Formation

Both hypertrophied and multinucleate feeding cells are highly active metabolically and have a dense cytoplasm, with a large number of organelles and invaginated cell wall (**Figure 1A, B**) (Grundler et al., 1998; Sobczak and Golinowski, 2011; Favery et al., 2016). They accumulate sugars and amino acids (Hofmann et al., 2010; Baldacci-Cresp et al., 2012). The nuclei and nucleoli of both giant cells and syncytia are larger than normal root cells, due to endoreduplication (de Almeida Engler and Gheysen, 2013). However, these two feeding structures have very different ontogenies. RKN J2 selects five to seven parenchyma cells and induces their dedifferentiation into giant cells through successive mitosis without cytokinesis (Caillaud et al., 2008b). Expansion of giant cells by isotropic

growth (Cabrera et al., 2015) together with hyperplasia of the root cells surrounding the giant cells results in a swelling of the root, known as a gall, the characteristic symptom of RKN infection. By contrast, CN J2 targets a single initial root cell. This cell expands within the vascular tissue by progressive cell wall dissolution and incorporation into the syncytium of adjacent cells *via* cytoplasm fusion (Golinowski et al., 1996; Grundler et al., 1998).

Studies of the feeding site formation have greatly benefited from whole-transcriptome analyses. Such analyses were initially developed in the model host plant *Arabidopsis thaliana* and were then extended to various crop species (Escobar et al., 2011; Favery et al., 2016; Yamaguchi et al., 2017). All these analyses showed that feeding site formation involves an extensive reprogramming of gene expression within the root

cells targeted by the nematodes. These analyses suggested that CNs and RKNs establish feeding sites by recruiting and/or manipulating several plant functions, including plant defense and phytohormone pathways (Gheysen and Mitchum, 2019), cell wall modification (Sobczak and Golinowski, 2011), cytoskeleton (Caillaud et al., 2008a), and the cell cycle (de Almeida Engler and Gheysen, 2013). These analyses also revealed the conservation of some nematode-responsive genes within the plant kingdom (Portillo et al., 2013).

MicroRNAs Are Key Regulators of Gene Expression

Plant miRNAs are 20- to 22-nucleotide-long noncoding RNAs (Bartel, 2004) that regulate gene expression through posttranscriptional gene silencing. Plant miRNA precursors are produced from *MIR* genes and are processed by several proteins, including Dicer-like 1 (DCL1), to generate a mature miRNA duplex. One strand of the duplex is loaded into the RNA-induced silencing complex (RISC), in which its sequence complementarity directs gene silencing (Figure 1C) (Yu et al., 2017). Perfect miRNA/mRNA complementarity generally induces cleavage of the mRNA at nucleotide position 10 or 11 (Franco-Zorrilla et al., 2007; Bartel, 2009). However, in some cases, such as the miR172/*APETALA2* module in *Arabidopsis*, the miRNA inhibits mRNA translation (Chen, 2004; Zhang and Li, 2013). Interestingly, the miRNA target may activate the expression of its regulator miRNA, e.g. *CUC2* and *MIR164a* (Nikovics et al., 2006). Therefore, regulation of genes by miRNA does not always imply a negative correlated expression between mature miRNA and the targeted transcripts. Plant *MIR* genes are often organized into multigene families in which the sequences of the precursors differ, but the mature sequences are almost identical, suggesting that they share some target mRNAs (Palatnik et al., 2007). Moreover, many *MIR* families are conserved between evolutionarily distant plant species, either targeting conserved genes or having different targets in different plant species (Jones-Rhoades, 2012). Small regulatory RNAs are major regulators of gene expression in plant development and in responses to various microorganisms such as beneficial mycorrhizal fungi (Bazin et al., 2013) and fungal (Park et al., 2014) or bacterial pathogens (Navarro et al., 2006). Plant miRNA may regulate the plant defense or the neoformation of specific structures during plant–microbe interactions (Comber et al., 2006; Park et al., 2014; Lee et al., 2015). Plant-parasitic nematodes induce the neoformation of feeding structures within host roots by inducing an extensive reprogramming of gene expression in the targeted root cells. The role of small noncoding RNAs in the plant–nematode interaction was established with the increased resistance to RKN and CN of *A. thaliana* mutants disrupted for miRNA or siRNA pathway (Hewezi et al., 2008; Medina et al., 2017; Ruiz-Ferrer et al., 2018). The development of sequencing technologies has made it possible to initiate studies of the role of plant miRNAs in this process in various plant species. This review provides an overview of current knowledge about of the conserved and species-specific plant miRNAs involved in responses to RKNs and CNs.

Plant MicroRNAs Responding to RKNs

The identification of novel and differentially expressed (DE) miRNAs involved in plant response to nematodes is based principally on the sequencing of small RNAs (< 35 nt) from infected and uninfected root tissues. If three independent replicates *per* sample are available, the comparison can be performed directly, by digital expression profiling. Otherwise, sequencing identifies the miRNAs expressed in the samples analyzed, and the levels of these miRNAs are then compared between samples by reverse transcriptase–quantitative polymerase chain reaction (RT–qPCR). The miRNAs involved in the gall formation induced by RKN have been investigated in *Arabidopsis* dissected galls and uninfected roots, 3 (Cabrera et al., 2016), 7, and 14 dpi (Medina et al., 2017). This approach identified 62 miRNAs as DE in galls induced by *Meloidogyne javanica* at 3 dpi, and 24 miRNAs as DE in galls induced by *Meloidogyne incognita* at 7 and/or 14 dpi. Only two DE miRNAs with the same expression profile were common to these three stages of gall formation: miR390, which is upregulated in galls, and miR319, which is repressed in galls. Using RT–qPCR, identified 17 miRNAs as DE in tomato galls at one or more of the five developmental stages analyzed (Kaur et al., 2017), while Pan et al. (2019) identified 16 miRNAs as DE in whole cotton roots infected by *M. incognita* at 10 dpi (Table 1). A comparison of susceptible and resistant tomato cultivars identified five RKN-responsive miRNAs in the WT and/or the jasmonic acid–deficient *spr2* mutant at 3 dpi (Zhao et al., 2015). Some conserved miRNA families present similar expression profiles in galls from different plant species at similar time points. For example, the evolutionarily conserved miR159 is upregulated in *Arabidopsis*, tomato, and cotton galls at 10 to 14 dpi, and miR172 is upregulated in *A. thaliana* and tomato at 3 to 4 dpi (Table 1). The genes targeted by miRNAs have been identified by *in silico* prediction (Zhao et al., 2015; Cabrera et al., 2016; Pan et al., 2019) or by 5′ RNA ligase-mediated (RLM)–rapid amplification of cDNA ends (RACE) sequencing (Kaur et al., 2017). The expression profiles of genes predicted or known to be targeted by miRNAs were analyzed by transcriptomic analysis or RT–qPCR. A negative correlation between the levels of several DE miRNAs and their targeted transcripts, for miR156/SPB or miR159/MYB, for example, was observed in galls from *Arabidopsis*, tomato, and cotton (Zhao et al., 2015; Cabrera et al., 2016; Pan et al., 2019).

Multiple miRNAs have been shown to be DE, but the functions of only four plant miRNAs in plant–RKN interactions have been validated to date. Functional validation involves the characterization of expression profile, often with reporter gene lines or by *in situ* hybridization, and analyses of the infection status of plants with modified expression or functions for either miRNAs (e.g. overexpression, KO or buffering “target mimicry” lines) or their targets (e.g. overexpression of a miRNA-resistant form, with a mutation in the miRNA target site or knockout lines). For example, miR319 is upregulated in tomato galls at 3 dpi, whereas its target, *TCP4* (*TEOSINTE BRANCHED 1/CYCLOIDEA PROLIFERATING FACTOR 4*), is downregulated (Zhao et al., 2015). Tomato plants overexpressing a miR319-resistant *TCP4* have fewer galls and higher levels of endogenous JA, whereas the opposite effect is observed in lines overexpressing *Ath-MIR319*. These results

TABLE 1 | List of functionally validated miRNAs differentially expressed in response to RKN and/or CN.

miRNA	Host plant	Infected material	Nematode species ^a	miRNA regulation ^b					References
				3 or 4	7	10	14	27-30	
miR159	Arabidopsis	Galls	<i>M. javanica</i>						Cabrera et al., 2016 Medina et al., 2017 Zhao et al., 2015 Kaur et al., 2017 Koter et al., 2018; Świącicka et al., 2017 Pan et al., 2019
		Galls	<i>M. incognita</i>						
	Tomato	Roots							
		Roots							
		Roots	<i>G. rostochiensis</i>						
Cotton	Roots	<i>M. incognita</i>							
miR172	Arabidopsis	Galls	<i>M. javanica</i>						Díaz-Manzano et al., 2018 (pre-miRNA) Cabrera et al., 2016 (mature) Hewezi et al., 2008 Hewezi et al., 2008 Díaz-Manzano et al., 2018 Kaur et al., 2017 Koter et al., 2018 Díaz-Manzano et al., 2018
		Galls	<i>M. javanica</i>						
		Roots	<i>H. schachtii</i>	172c	172c				
	Tomato	Roots	<i>H. schachtii</i>		172a				
		Galls	<i>M. javanica</i>						
		Galls	<i>M. incognita</i>						
	Pea	Roots	<i>G. rostochiensis</i>						
Galls	<i>M. javanica</i>								
miR319	Arabidopsis	Galls	<i>M. javanica</i>						Cabrera et al., 2016 Medina et al., 2017 Zhao et al., 2015 Koter et al., 2018 Pan et al., 2019
		Galls	<i>M. incognita</i>						
	Tomato	Roots	<i>M. incognita</i>						
		Roots	<i>G. rostochiensis</i>						
Cotton	Roots	<i>M. incognita</i>							
miR390	Arabidopsis	Galls	<i>M. javanica</i>						Cabrera et al., 2016 Cabrera et al., 2016 Pan et al., 2019 Díaz-Manzano et al., 2018
		Galls	<i>M. incognita</i>						
	Cotton	Roots	<i>M. incognita</i>						
		Tomato and pea	Galls	<i>M. incognita</i>					
miR396	Arabidopsis	Roots	<i>H. schachtii</i>	396a	396a				Hewezi et al., 2008; Hewezi et al., 2012 Hewezi et al., 2008; Hewezi et al., 2012 Zhao et al., 2015; Kaur et al., 2017 Świącicka et al., 2017 Pan et al., 2019 Noon et al., 2019
		Roots	<i>H. schachtii</i>	396b	396b				
	Tomato	Roots	<i>M. incognita</i>						
		Roots	<i>G. rostochiensis</i>						
	Cotton	Roots	<i>M. incognita</i>						
Soybean	Roots	<i>H. glycines</i>							
miR827	Arabidopsis	Roots	<i>H. schachtii</i>						Hewezi et al., 2016
	Cotton	Roots	<i>M. incognita</i>						Pan et al., 2019
miR858	Arabidopsis	Galls	<i>H. schachtii</i>						Piña et al., 2017

^anematodes species: RKN in yellow, CN in pink.

^bexpression pattern between 3 and 27-30 dpi; up-regulated in infected material in red; down-regulated in infected material in green.

suggest that the miR319/TCP4 module is essential in tomato galls by modulating the JA biosynthesis induced by RKN invasion (Zhao et al., 2015). miR159 is a conserved family of miRNAs upregulated in *Arabidopsis* galls at 14 dpi (Medina et al., 2017). Studies on transgenic GUS lines demonstrated the posttranscriptional regulation of MYB33, the main target of miR159, in *Arabidopsis* galls at 14 dpi. The *mir159abc* triple loss-of-function mutant displays enhanced resistance to RKN, with decreased numbers of galls and egg masses, demonstrating the role of the miR159 family in the response of *Arabidopsis* to *M. incognita*, probably through the regulation of MYB33. Furthermore, *in situ* hybridization has shown that *miR159* is also expressed in tomato giant cells (Medina et al., 2017) and a conserved upregulation of miR159 associated with a downregulation of MYB transcription factors has also been observed in galls from tomato (3 dpi and 13-15 dpi; Zhao et al., 2015; Kaur et al., 2017) and cotton (10 dpi; Pan et al., 2019). These results suggest that the function of the miR159/MYB module may be conserved in the galls *Arabidopsis*, tomato and cotton (Medina et al., 2017). The conserved auxin-responsive miR390 family is overexpressed in *A. thaliana* galls at 3, 7, and

14 dpi (Cabrera et al., 2016; Medina et al., 2017). In *Arabidopsis*, the cleavage of *TAS3* transcripts by miR390 generates secondary siRNAs (tasiRNAs) that induce post-transcriptional repression of the auxin-responsive transcription factors *ARF2*, *ARF3*, and *ARF4* (Marin et al., 2010). Cabrera et al. (2016) demonstrated the coexpression of *MIR390A* and *TAS3* in galls and giant cells at 3 dpi and the post-transcriptional regulation of *ARF3* by tasiRNAs in galls, in experiments comparing *ARF3* sensor lines sensitive or resistant to cleavage by tasiRNAs. Studies of *miR390a* and *tas3* loss-of-function mutants reported the production of fewer galls, suggesting that the miR390/*TAS3*/*ARF3* regulatory module is required for correct gall formation (Cabrera et al., 2016). Finally, a role for the regulatory gene module composed by miR172 and the two transcription factors TOE1 (target of early activation tagged 1) and FT (flowering locus T) has been demonstrated in root galls during the formation of giant cells in *Arabidopsis* (Díaz-Manzano et al., 2018). The role for the miR172/TOE1/FT module has been first described during *Arabidopsis* flowering (Aukerman and Sakai, 2003). In *Arabidopsis* root, the 3' strand of mature miR172 has been shown to be downregulated in galls at 3 dpi, whereas the

pri-miR172 precursor is induced, and its target *TOE1* repressed, according to transcriptome data for microdissected *A. thaliana* giant cells at the same time point (Barcala et al., 2010). Consistent with the negative regulation of *FT* by *TOE1*, an induction of *FT* was observed in galls at 3 dpi. *Arabidopsis* plants expressing miR172-resistant *TOE1* or KO for *FT* were less susceptible to RKNs and had smaller galls and giant cells. Like miR390, miR172 is an auxin responsive microRNA. Auxin is a crucial signal for feeding site formation and parasitism. An enhanced auxin response has been observed in RKN feeding sites (Hutangura et al., 1999) and auxin has been identified in the secretion of RKNs (De Meutter et al., 2005). The function of miR390 and miR172 in the feeding site is probably a part of the auxin response.

Plant Small Noncoding RNAs Responding to CNs

The identification and analysis of miRNAs involved in plant-CN interaction are based on the same approaches that the ones described above. Sequencing identified 30 mature DE miRNAs in *Arabidopsis* syncytia induced by *Heterodera schachtii* at 4 and 7 dpi, and qPCR analyses revealed inverse expression profiles for six miRNAs and their targets (Hewezi et al., 2008). A recent analysis of syncytia from tomato plants infected with *Globodera rostochiensis*, performed at 3, 7, and 10 dpi, identified between 200 and 300 miRNAs at each stage as DE (Koter et al., 2018). Reverse transcriptase-qPCR analyses revealed inversely correlated expression patterns for six miRNAs and their targets (Koter et al., 2018). Moreover, the expression of eight tomato miRNAs regulating defense-related proteins was specifically analyzed by qPCR at 3 and 7 dpi; an inverse correlation between the expression of these miRNAs and their targets in response to CN infection was observed (Świąćicka et al., 2017). Finally, several studies have analyzed expression of soybean miRNAs in response to infection with *Heterodera glycines* by comparing expression levels in resistant and susceptible cultivars (Li et al., 2012; Xu et al., 2014; Tian et al., 2017). Tian et al. (2017) identified 60 miRNAs from 25 miRNA families as DE relative to uninfected roots in susceptible and/or resistant cultivars and validated the expression profiles of most of these miRNAs by qPCR. While most of the miRNAs identified by Tian et al. (2017) are upregulated in resistant lines relative to susceptible lines, the majority of miRNAs were downregulated in the study performed by Li et al. (2012). These discrepancies may reflect differences in resistance between these soybean cultivars or a technical bias related to the number of replicates analyzed in these two studies. A comparison of the expression profiles of conserved miRNAs in response to CN infection identified some miRNAs as DE, with the same expression profile, in several plant species. miR396b and the miR167 family were downregulated in *Arabidopsis* roots infected by *H. schachtii* at 4 and 7 dpi (Hewezi et al., 2008) and in tomato syncytia induced by *G. rostochiensis* at 3 and 7 dpi (Świąćicka et al., 2017) (**Table 1**).

Three miRNAs DE in syncytia were validated by functional approaches. In *Arabidopsis*, miR396 was repressed at the onset of syncytium formation in roots infested with *H. schachtii* and upregulated at later stages, whereas its target transcription

factors, the growth-regulating factors (GRF) *GRF1*, *GRF3*, and *GRF8*, displayed the opposite pattern (Hewezi et al., 2012). *Arabidopsis thaliana* mutants overexpressing miR396 have smaller syncytia and greater resistance to CN. These results suggest that the coordinated regulation of miR396 and *GRF1* and *GRF3* is required for correct syncytium development in *Arabidopsis*. Interestingly, a repression of the miR396 family associated with an upregulation of soybean *GRF* genes was observed in soybean syncytia induced by *H. glycines* at 8 dpi (Noon et al., 2019). A combination of 5' RLM-RACE and a reporter gene approach demonstrated that the *GRF6* and *GRF9* genes were targeted by miR396 in syncytia. Transgenic soybean lines overexpressing pre-miR396 and *GRF9* RNAi lines displayed similar decreases in the number of *H. glycines* females per root, reflecting an increase in resistance to CN. These results indicate that the miR396/GRF module is essential for *H. glycines* infection, and this role is conserved in *Arabidopsis* and soybean. Furthermore, the use of a reporter gene strategy made it possible to demonstrate an inverse correlation in the expression profiles of the conserved miR827 and its known target *NLA* (nitrogen limitation adaptation) during syncytium development in *Arabidopsis* (Hewezi et al., 2016). The overexpression of miR827 increased susceptibility to *H. schachtii*, whereas the expression of a miR827-resistant *NLA* decreased plant susceptibility. These results show that miR827 downregulates *Arabidopsis* immunity to *H. schachtii* by repressing *NLA* activity in the syncytium (Hewezi et al., 2016). Finally, a role for the miR858/MYB83 module has been established in *Arabidopsis* syncytia induced by *H. schachtii*, in which an inverse correlation of transcript levels was observed between miR858 and its target MYB83 at 7, 10, and 14 dpi (Piya et al., 2017). Modulation of the expression of these genes through gain- and loss-of-function approaches altered the *Arabidopsis* response to nematode infection, demonstrating a role for this module in syncytium formation.

Conclusions and Perspectives

The results presented provide the first insights into the function of miRNAs in the plant response to nematode infection. Except for miR390, expression profile of most miRNAs in feeding site shows heterogeneity (**Table 1**), with different expression profiles according to the type of feeding structures, the plant species, and/or the phase of development. Difference of expression in giant cell and syncytia may be explained by their distinct ontogenesis. Whether these variations of expression of plant miRNAs are directly induced by the nematode or are the results of modification of plant hormonal balance is a question that still needs to be investigated. The identification of the targets of these DE miRNAs and the biological pathways they regulate would improve our understanding of feeding cell development. Moreover, resistance genes of the nucleotide binding site-leucine-rich repeat (NBS-LRR) family genes are known to be targeted by miRNAs and phased siRNAs (reviewed by Fei et al., 2016). An inverse correlation on several tomato NB-LRR transcripts and their miRNA regulators has been evidenced after infection by CN (Świąćicka et al., 2017).

A better understanding of the role of miRNA in PPN feeding sites may lead to new methods of control for these organisms.

Most studies to date have focused on miRNAs, but few studies investigating the siRNAs expressed in roots infected with PPNs in *Arabidopsis* (Hewezi et al., 2017; Medina et al., 2018; Ruiz-Ferrer et al., 2018) have highlighted an overrepresentation in galls of 24 nt siRNAs known to be associated with RNA-directed DNA methylation. Two first studies of changes in DNA methylation have been performed in *A. thaliana* and soybean plants infected with CN (Rambani et al., 2015; Hewezi et al., 2017). These studies support a role for changes in DNA methylation in plant responses to PPN infection. Future combined studies of small RNAs, methylome and transcriptome should result in an integrative understanding of the epigenetic regulation of feeding site formation. Several intriguing questions remain unanswered: i) How do PPNs modify the expression of small RNA genes in the plant genome? ii) Do the small RNAs produced by nematodes play a role in the plant and vice versa? Genomes of several PPN species are now available (Cotton et al., 2014; Eves-van den Akker et al., 2016; Blanc-Mathieu et al., 2017; Masonbrink et al., 2019) and should be used to investigate the small RNAs produced by the nematode during parasitism. Finally, cross-kingdom RNAi (reviewed by Weiberg and Jin, 2015) probably also occurs during

interactions between plants and PPNs. Integrative analyses of the small RNAs from both side of the interactions should shed light on this molecular dialog.

AUTHOR CONTRIBUTIONS

YN, BF, and SJ-P organized the study and wrote this manuscript. All authors have read and approved the manuscript.

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- Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Objectives

My thesis aims to understand how microRNAs regulate the reprogramming of gene expression involved in the formation of feeding sites induced in *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) roots by *M. incognita*. Previous transcriptomic analysis has shown that the formation of RKN feeding sites is the result of a wide reprogramming of gene expression. Until now, we lack knowledge on how these genes are regulated. I choose to focus on microRNAs, as conserved key regulators of gene expression.

NGS approach was previously developed to identify the small RNAs expressed in *A. thaliana* galls induced by RKN and uninfected roots at two key stages in the kinetics of giant cell formation: 7 days post infection (dpi) which corresponds to the phase of successive mitoses without cytokinesis and 14 dpi, the phase of endoreduplication. Bioinformatics and statistical analysis of the NGS data identified 24 microRNAs from *A. thaliana* DE in galls at 7 and/or 14 dpi (Medina et al., 2017). A comparable analysis was carried out during my thesis on a plant of agronomic interest, the tomato *S. lycopersicum*. Illumina sequencing was performed enabling the analysis of mRNA and small RNAs (<200 nt) expressed in galls 7 and 14 dpi and in uninfected tomato roots. Data from small RNA sequencing were used to predict *MIR* genes in the tomato genome. Statistical analysis identified 174 mature microRNAs DE at 7 and/or 14 dpi in galls. To understand the function of these miRNAs and identify their targets in the galls, I performed a specific sequencing of cleaved mRNA: “degradome”. An integration of the results of the transcriptome and degradome analyzes identified the miRNAs DE in galls and their targets with anti-correlated expression profiles and enable the selection of miRNA/targets couples for initiating functional analysis.

During my thesis, I focused on three miRNA/target couples. In the first chapter, I will present the first couple that I characterized on in tomato: miR167 and its targets the auxin-response factors *ARF8A* and *ARF8B*. This couple is associated with the auxin signaling knowing to play a major role in the formation of feeding sites.

In the second chapter, I will present two other miRNA/target couples, miR408 and miR398, that are conserved microRNAs, and their targets associated to copper signaling. These two miRNAs families are among the very few conserved miRNAs that present the same expression profiles in *A. thaliana* and tomato galls.

The work presented in my thesis highlights the key role of three miRNAs families and their targets in the formation of nematode-induced feeding cells: the *miR167/ARF8* pair associated with auxin signaling and the *SPL7/miR408-UCC2/miR398-CSD1-BCBP* module involved in copper signaling pathway.

Results

CHAPTER 1: miR167-ARF8, an auxin responsive module involved in the formation of galls induced by root-knot nematodes in tomato

Article 1 :

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miR167-ARF8, an auxin responsive module involved in the formation of galls induced by root knot nematodes in tomato. *In preparation*

This first part of my work, consisted in identification of small RNAs from tomato *Solanum lycopersicum* uninfected roots and galls induced by *M. incognita* at 7 and 14 dpi. Illumina sequencing enabled the analysis of messenger RNAs (mRNA) and small RNAs (<200 nt) expressed in galls and uninfected tomato roots. Statistical analyzes (DSeq2 and EdgeR) identified 1,958 differentially expressed genes (DEGs) at 7 dpi, 3,468 DEGs at 14 dpi and 1,239 DEGs at both 7 and 14 dpi. Data from small RNA sequencing were used to predict *MIR* genes in the tomato genome (V3.0) by using three prediction algorithms: MirCat, Shortstack and MirDeep plant. DSeq2 statistical analysis identified 174 DE mature microRNAs at 7 and/or 14 dpi. To understand the function of these miRNAs, the degradome was sequenced and analyzed by CleaveLand algorithm to identify the targets of the miRNAs in the galls. Predicted targets were classified into five categories based on the abundance of reads at the predicted cleavage site relative to the overall profile of reads across the entire transcript. We have limited our analysis to categories 0 and 1 which correspond to the most robust predictions. An integration of the results of the transcriptome and degradome sequencing identified 12 robust miRNA/target pairs in tomato galls. miR167/ARF8 pair was selected further for functional analysis. In galls, miR167 is downregulated and targets the auxin-response factors *ARF8A* and *ARF8B*. This downregulation is correlated with the upregulation of its targets in galls. These *ARFs* belong to auxin signaling pathway, a key hormone in plant-RKN interaction. Using tomato lines expressing the two *ARF8* promoters fused to GUS reporter gene, we showed a strong activity of both *ARF8* promoters in galls at 7 and 14 dpi, confirming the results of the transcriptomic analyzes. Moreover, we analyzed the effect of a CRISPR deletions within *ARF8A* and *ARF8B* coding sequences on the infection by *M. incognita*. *arf8a*, *arf8* and *arf8ab* mutants showed a significantly decrease of susceptibility to nematode infection correlated with defects in feeding cell formation. Altogether, these result showed that *ARF8A* and *ARF8B* expression is required for successful tomato-RKN interaction. In order to identify the genes targeted by *ARF8A* and *ARF8B*, mRNA from galls of WT and *arf8a* and *arf8b* has been sent for sequencing. Since the sequencing is still *in progress*, the results of this article will be discussed later in the general discussion of the thesis. Once we will get the sequencing data, the article will be submitted to *New Phytologist*.

miR167-ARF8, an auxin responsive module involved in the formation of galls induced by root-knot nematodes in tomato

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Abstract

- Root-knot nematodes (RKN), genus *Meloidogyne*, induce the dedifferentiation of root vascular cells into giant and multinucleated feeding cells. The formation of these feeding cells is the result of an extensive reprogramming of gene expression in targeted root cells as shown by analyses of transcriptomes from galls or giant cells from various plant species.
- Small RNAs (<35nt) and messenger RNAs from tomato (*Solanum lycopersicum*) galls and uninfected roots were sequenced using the Illumina technology. *De novo* prediction of miRNAs in tomato genome (V3.0) was then performed by using three bioinformatic algorithms (MirCat, Mirdeep Plant, Shortstack). Identification of microRNAs expressed in galls and uninfected roots followed by statistical analyses (Dseq) identified 174 miRNAs genes that are differentially expressed in galls at 7 and/or 14 days post infection (dpi).
- mRNA targeted by microRNAs in tomato gal at 7 and 14 dai were then identified by a specific sequencing of mRNA cleaved degradation products by using the degradome approach.
- Integrative analyses combining smallRNAs, degradome and transcriptome highlighted the role of a transcription factor auxin response factor 8 (ARF8) in the formation of giant cells.

Key words: root-knot nematodes, galls, microRNAs, tomato, auxin, ARF8

Introduction

Root-knot nematodes (RKNs) are major crop pests causing massive loss of yields worldwide estimated at millions of euros per year (Blok et al., 2008; Abad and Williamson, 2010). These microscopic worms of genus *Meloidogyne* have a wide host spectrum, including more than 5,000 plant species, as well as a wide geographical distribution. Upon root infection, these obligatory plant parasites induce the neof ormation of a specialized feeding site critical for nematode survival. After penetration and migration into the roots, the second stage RKN juveniles (J2) injects a cocktail of molecules into five to seven root parenchyma cells (Favery et al., 2016). In response to RKN signals, targeted root parenchyma cells dedifferentiate into giant multinucleated and hypermetabolic feeding cells. These “giant cells” formed the feeding site that supplies nutrients required for nematode development (Favery et al., 2020). The dedifferentiation into giant cells involves a first phase of successive mitoses without cytokinesis followed a second phase of endoreduplication (de Almeida Engler and Gheysen, 2013). In parallel to the formation of the feeding cells, the root cells surrounding the feeding site, named neighboring cells start to divide. This whole process results in a root swelling named gall, which is the characteristic symptom of RKN infection. Several biological processes are involved in the formation of the feeding site like the cell cycle (de Almeida-Engler et al., 2011), metabolic reprogramming (Marella et al., 2013), cytoskeleton organization (Caillaud et al., 2008b), or auxin signaling (Gheysen and Mitchum, 2019). Auxin or indole-3 Acetic Acid (IAA) is a major plant hormone that play a key role in root development by regulating cell division and the establishment/maintenance of root primordia (De Smet et al., 2007; Weijers and Wagner, 2016). The formation of RKN-induced feeding site has been shown to involved auxin peak (Karczmarek et al., 2004; Absmanner et al., 2013a) and gall transcriptome analyses showed that auxin biosynthesis and auxin-responsive genes were upregulated in *A. thaliana* early galls while the genes coding for repressors of the auxin response genes were repressed (Barcala et al., 2010). Moreover, application of auxin indole acetic acid (IAA) compound to tomato roots induces in a concentration-dependent weight increase of galls induced by *M. javanica* (Glazer et al., 1986).

Multiple transcriptome analyses were performed on infected roots, galls or specifically on feeding cells, from various plant species firstly by microarrays and more recently by RNA sequencing. Four time points of feeding site formation were investigated by these transcriptome analyses: the early phase of feeding site formation at 3 days post infection (dpi), 7dpi that corresponds to the multiple mitoses without cytokinesis, 14 dpi that corresponds to the

endoreduplication phase and finally 21 dpi when the feeding cells are matured and fully functional. All these analyses showed similar massive reprogramming of plant gene expression in response to nematode infection with approximately 10% of protein coding genes which expression is modified in response to nematode infection. How this reprogramming occurs and how these genes are regulated is still poorly understood. MicroRNAs are small non coding RNAs that are major repressors of gene expression in eukaryotes. Within plant genome, microRNAs are encoding in by *MIR* genes, often organized as multigene families, that are transcribed in a single stranded RNA precursor which folds into a typical hairpin structure. This hairpin precursor is processed to generate a duplex of mature 20-22 nt microRNA. One of these two mature strands is then loaded into the ARGONAUTE-1 protein, and guides the RNA silencing complex (RISC) to target messenger RNAs by miRNA/mRNA sequence complementarity. mRNA targeting by a microRNA induce its degradation or the inhibition of its translation depending on the mRNA/miRNA sequence complementarity. Several recent articles identified the microRNAs expressed in galls (Jaubert-Possamai et al., 2019) induced by RKN in *Arabidopsis thaliana* (Cabrera et al., 2016; Medina et al., 2017), tomato *Solanum lycopersicum* (Zhao et al., 2015; Kaur et al., 2017), in cotton *Gossypium hirsutum* (Pan et al., 2019; Cai et al., 2021) or in *Oryza sativa* (Verstraeten et al., 2021). However, the role of only four microRNAs has been validated by functional analyses: miR390/tasiRNA/ARF3 module (Cabrera et al., 2016), the miR159/MYB33 pair (Medina et al., 2017), or miR172/TOE1/FT module (Díaz-Manzano et al., 2018) in *Arabidopsis* or the miR319/TCP4 pair in tomato (Zhao et al., 2015) .

In this article, we investigated the gene regulation network of plant response to RKN by integrating transcriptome, microRNome and degradome sequencing of tomato uninfected roots and galls induced by the RKN *M. incognita* at two key time points of the gall development: 7 and 14 days post inoculation (dpi). We identified twelve miRNA/targeted transcripts couples that are robust candidates to regulate gall formation. Among these candidates, the role in gall formation of the auxin responsive miR167/*ARF8* transcripts pair was confirmed by functional analyses.

Materials and methods

Biological Materials, Growth Conditions

For *in vitro* experiments, seeds of *Solanum lycopersicum* cv Micro-Tom and of transgenic lines (pARF8A:GUS and pARF8B:GUS) (provided by Mohamed Zouine; ENSAT, Toulouse) were surface-sterilized with chlorine solution (44% active chlorine) and washed three times with 1 ml of milli-Q water. 10 to 15 sterile seeds were sown on a Gamborg B5 (Duchefa Biochemie) agar plates (1x Gamborg B5; pH = 6.4; 1% Sucrose; 0,7% Agar), placed at 24°C for 48 hours for germination, and finally transferred in a growth chamber (8h light; 16h dark, 20°C). *M. incognita* strain Moreleos[®] J2s were sterilized with HgCl₂ (0.01%) and streptomycin (0.7%) as described before (Caillaud and Favery, 2016). One to two weeks after germination, roots were inoculated with 1,000 sterile J2s resuspended in phytigel (5%) per petri dishes.

In soil infection assay

Tomato (*Solanum lycopersicum* cv Micro-Tom) plants of Wild type and CRISPR lines (*ARF8A*, *ARF8B* and *ARF8AB*) (provided by Mohamed Zouine; ENSAT, Toulouse) were sown in pots filled with a mix 2:3 soil and 1:3 of sand, kept at 4°C for 48 hours, then transferred in a growth chamber (16h light and 8h dark, at 24°C). Seedlings were individually transferred in pots filled with a mixture of sand 50% and soil 50% and kept in the growth chamber. Two weeks after germination, each plant was inoculated, with 200 J2s resuspended in water . Infection rate was evaluated six weeks after inoculation. The root system of each plant was collected, rinsed with tap water, weighted and stained for 30 s. in eosin solution (0.5%). Galls and egg masses were counted for each root under the binocular magnifier MZFLIII (Leica). Mann–Whitney *U*- tests ($\alpha = 2.5\%$) were performed to determine the significance of the differences in the numbers of egg masses and galls per root observed between mutants and WT.

BABB clearing

For giant cell area measurements, galls were collected 21 days post-infection (dpi), cleared in benzyl alcohol/benzyl benzoate (BABB) as previously described (Cabrera et al., 2018; Mejias et al., 2021) and examined under an inverted confocal microscope (model LSM 880; Zeiss). The mean areas of giant cells in each gall, for wild type and CRISPR lines, for two biological replicates, were measured with Zeiss ZEN software. The impact of the mutation on the giant cell surface was analyzed using a Mann & Whitney Test.

RNA extraction

Total RNAs, including small RNAs (< 200 nt), were isolated from *in vitro* galls or uninfected roots at 7 and 14 dpi. Approximately 40 galls or uninfected roots devoid meristems were independently frozen into powder by using a tissue lyser (Retsch; MM301) at 30 Hertz frequency for 30 seconds with 4 mm tungsten balls (Retsch; MM301). Total RNAs were extracted from these samples with the miRNeasy Mini Kit (Qiagen), according to the manufacturer's instructions, with three additional washes in RPE buffer.

RNA sequencing

Small RNA libraries were generated by ligation, reverse transcription and amplification (11 cycles) from total RNAs (1 µg), with the reagents of the NEBNext Small RNA Library Prep Set for Illumina. Libraries were then quantified with the Bioanalyzer High Sensitivity DNA Kit (Agilent) and sequenced at the Nice-Sophia Antipolis functional genomics platform (France Génomique, IPMC, Sophia Antipolis, France). The full raw sequencing data were submitted to the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>).

PolyA-RNA libraries were generated from 500 ng of total RNA using Truseq Stranded mRNA kit (Illumina). Libraries were then quantified with Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) and pooled. 4nM of this pool were loaded on a Nextseq 500 High output Flowcell and sequenced on a NextSeq 500 platform (Illumina) with 2 × 75bp paired-end chemistry.

miRNAs Analysis

For each library, adapters were trimmed and reads matching ribosomal RNA, mitochondrial RNA and repeat sequences were removed by performing Blast analyses with the sequences listed in the Rfam database (Nawrocki et al., 2015). The STAR 2.5 aligner (: --twopassMode Basic --alignEndsType EndToEnd) was then used to align the trimmed reads (Dobin et al., 2013) on a virtual concatenated genome generated from the *S. lycopersicum* genome (V3.01, annotation V3.2) and the *M. incognita* genome (Blanc-Mathieu et al., 2017). Each read was attributed to the *S. lycopersicum* and/or *M. incognita* genome on the basis of the best alignment obtained. Low-quality mapped reads were removed. The htseq-count package version 0.9.1 (Anders et al., 2014) was used to count reads mapping perfectly onto the *S. lycopersicum* genome. The counts for protein coding genes from each replicate were used for differential expression analysis with the R package. EdgeR version 3.4.1 (Robinson et al., 2009) and DSeq2 (Anders and Huber, 2010) Differentially expressed miRNAs, identified with a false discovery rate of 5% (adjusted pvalue<0.05; Benjamini-Hochberg adjustment).

De novo microRNA encoding genes were predicted in tomato genome V3.0 by using three algorithms MirCat (Paicu et al., 2017), Shortstack (Axtell, 2013b) and MirDeep plant (Yang and Li, 2011) with default parameters. The HTSEQCOUNT package (Anders et al., 2015) was used to count reads mapping perfectly onto the predicted *S. lycopersicum* mature microRNA 5P or 3P sequence. Reads mapping to multiple loci were counted for each of the loci concerned. The counts for mature miRNAs (5P and 3P) from each replicate were used for differential expression analysis by using DSeq2 statistical analysis (Anders and Huber, 2010). Mature miRNAs with an adjusted p value below 0.05 were considered as differentially expressed.

Transcriptome Analysis

GO analyses of genes differentially expressed in galls were performed by using over-representation test from PANTHER analysis tools (Mi et al. NAR 2013) with a Fisher's exact test, a FDR threshold of 0,05 and by selecting "Biological Process » as GO category.

Degradome analysis

Degradome libraries were constructed from total RNAs extracted from galls at 7 and 14 dpi by Vertis Biotechnologie (Freising, Germany) using the parallel analysis of RNA ends (PARE) protocol described by German *et al.* (2009). The PARE libraries were sequenced on an Illumina High Sequencing 2000 platform. The full raw sequencing data were submitted to the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). To identify miRNA targets, degradome reads were analyzed and classified by using the CleaveLand 4.0 (Addo-Quaye et al., 2009) algorithm with default parameters. All hits are classified into five categories based on the abundance of the diagnostic cleavage tag relative to the overall profile of degradome tags matching the targets.

GUS staining analysis

We localized the promoter activity in tomatoes transgenic lines expressing a reporter gene GUS fused to the promoter of the two tomato genes *ARF8A* and *ARF8B* (pARF8A:GUS and pARF8B:GUS). We inoculated 21-day-old seedlings *in vitro*, as described above. We collected inoculated roots and washed them in water 7 and 14 dpi. GUS staining was performed as previously described (Favery et al., 1998), and the roots were observed under a Zeiss Axioplan 2 microscope. Stained galls were dissected, fixed by incubation in 1% glutaraldehyde and 4% formaldehyde in 50 mM sodium phosphate buffer pH 7.2, dehydrated, and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer's

instructions. Sections were cut and mounted in DPX (VWR International Ltd, Poole, UK), and observed under a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany).

Results

Gall formation is the result of a massive reprogramming of gene expression of the root cells

Transcripts level between tomato galls and uninfected roots were compared with two statistical methods: DSeq2 (Anders and Huber, 2010) and EdgeR (Robinson et al., 2009). 19,918 genes were considered for DSeq2 and EdgeR statistical analyses. Genes found as differentially expressed by the two methods with an adjusted pvalue below 0.05 were selected as differentially expressed genes (DEG). 1,958 DEGs were found at 7 dpi (**Supplemental table S1**) and 3,468 DEGs at 14 dpi (**Supplemental table S2 and figure 1A**). 1,239 DEGs at both 7 and 14 dpi including 625 down regulated and 600 upregulated genes at both time points and 14 DEGs with an anti-correlated expression fold change at 7 and 14 dpi were identified. 719 genes were found differentially expressed specifically in galls at 7 dpi including 327 upregulated and 392 down regulated genes. 2,229 genes were found specifically differentially expressed in galls at 14 dpi including 1006 upregulated and 1223 down regulated genes. Gene Ontology (GO) analysis of the DEGs in galls at 7 and/or 14 dpi showed an over-representation of the genes associated with biological processes described previously as involved in the formation of giant cells (**Supplemental table S3**) like i) «cell division» including multiple categories linked to cytokinesis and cell wall biogenesis, ii) «response to auxin», iii) «response to endogenous stimulus» (including response to hormone and to cytokinin) and iv) «response to abiotic stress». As previously shown for various plant species, this analysis confirms that formation of galls and feeding cells is the result of a massive reprogramming of gene expression of the root cells.

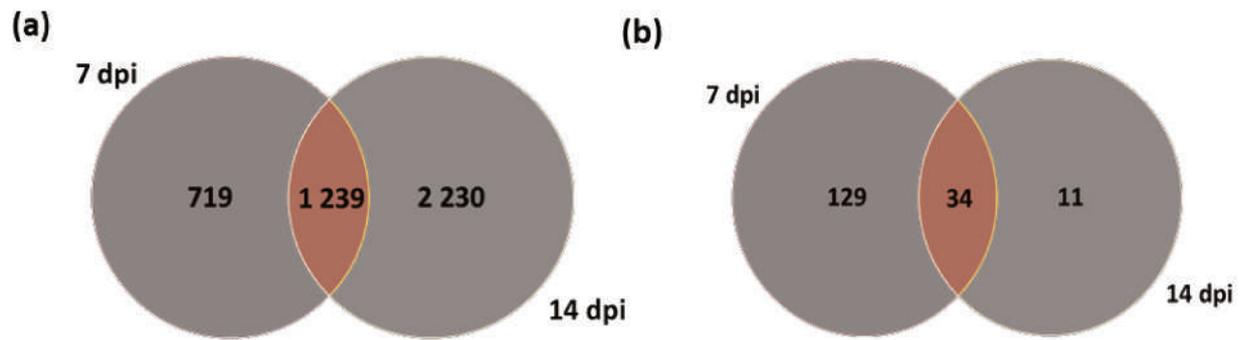


Fig.1 Venn diagram of (A) the protein-coding genes and (B) the miRNAs differentially expressed at 7 and/or 14dpi in tomato galls.

microRNAs regulate gene expression in galls

To understand how is regulated the massive reprogramming of gene expression observed in tomato galls, we investigated microRNAs expression in galls. First, we identified tomato miRNAs differentially expressed in galls using Illumina sequencing technology. Small RNA libraries of tomato galls and uninfected roots were constructed, from three independent replicates at two points of gall development: 7 and 14 dpi. These libraries were sequenced generating a total of 333,949,327 raw reads (**Supplemental table S4**). Reads were cleaned and mapped to a virtual genome constructed from the *S. lycopersicum* (genome V3.0; ITAG3.3) concatenated with *M. incognita* (genome V2.0; Blanc-Mathieu et al., 2017) to reflect the dual composition of root galls. A *de novo* prediction of microRNAs was performed by integrating the results from three prediction algorithms: MirCat (Paicu et al., 2017), Shortstack (Axtell, 2013b) and MirDeep plant (Yang and Li, 2011). The sequence homology between newly predicted miRNA mature sequences and mature miRNA sequences listed in miRBase 22.1 was analyzed by using SSearch algorithm (Kozomara et al., 2019).

Expression level of microRNAs in galls and in uninfected roots was compared by DSeq2 statistical analyses (Anders and Huber, 2010). 174 mature microRNAs (5P and/or 3P) corresponding to 148 *MIR* genes were identified as differentially expressed (DE) between uninfected roots and galls at 7 and/or 14 dpi (**Supplemental table S5**). From the 174 mature microRNAs DE in galls, 129 were identified as specifically DE at 7 dpi, 11 were specifically DE at 14 dpi and 34 mature microRNAs were DE in galls at 7 and 14 dpi (**figure 1B**). These 148 *MIR* genes DE in galls include 65 known *MIR* genes listed in miRbase (Kozomara et al., 2019) and 73 novel *MIR* genes. The 65 known *MIR* genes DE in galls are organized in 20 miRNA families. 14 miRNA families are conserved between Tomato and other plants, while six miRNA families are specific to tomato.

solyc	annotation	Degradome Category	logFC 7dpi	padj 7 dpi	logFC 14dpi	padj 14dpi	miRNA family	miRNA genes	LogFC 7dpi	padj 7 dpi	logDC 14dpi	padj 14 dpi
Solyc06g069710.3.1	NAC domain protein	0	-0.45430566	0.26681253	-2.04657016	1.0354E-06	mir164	SL3.0ch01_423 SL3.0ch09_148_3 SL3.0ch09_355	1.27038737 1.2697662 1.26980068	0.0073152 0.0073152 0.0073152	2.07315374 2.07392488 2.07352134	0.0658435 0.0658435 0.0658435
Solyc07g056630.3.1	NAC domain-containing protein	0	-0.53927717	0.0422403	-0.63035885	0.26930184	mir164	SL3.0ch01_423 SL3.0ch09_148_3 SL3.0ch09_355	1.27038737 1.2697662 1.26980068	0.0073152 0.0073152 0.0073152	2.07315374 2.07392488 2.07352134	0.0658435 0.0658435 0.0658435
Solyc03g031970.3.1	Auxin response factor	0	0.31969156	0.18150265	0.84298618	0.00148765	mir167	SL3.0ch06_17 SL3.0ch09_408 SL3.0ch09_413	-0.94277542 -0.94385004 -0.94285095	0.00271051 0.00271051 0.00271051	-1.88252148 -1.88289304 -1.88281562	0.03299542 0.03299542 0.03299542
Solyc02g037530.3.1	auxin response factor 8B	0	0.82002435	0.00019817	0.96491062	0.00113185	mir167	SL3.0ch06_17 SL3.0ch09_408 SL3.0ch09_413	-0.94277542 -0.94385004 -0.94285095	0.00271051 0.00271051 0.00271051	-1.88252148 -1.88289304 -1.88281562	0.03299542 0.03299542 0.03299542
Solyc06g082260.3.1	Laccase	0	-0.31973181	0.06688516	-1.25798519	0.00573385	mir397	SL3.0ch09_418	-0.94282309	0.00271051	-1.88276331	0.03299542
Solyc01g067740.3.1	Superoxide dismutase [Cu-Zn] 1	0	-0.83570314	0.04335707	-1.50747013	0.00016079	mir398	SL3.0ch07_274_3	2.26982258	6.3496E-05	2.58381874	0.00264634
Solyc02g069260.3.1	Argonaute2a	0	0.45704662	0.18150265	1.15984653	0.00099252	mir403	SL3.0ch01_435	0.99077701	0.11152967	1.75884846	0.06735039
Solyc04g074740.3.1	Blue copper protein, putative	0	-0.79014988	0.03120593	-0.69445941	0.15688356	mir408	SL3.0ch01_419 SL3.0ch01_346	1.38644055 3.74140804	0.02901224 3.2152E-13	2.17848304 3.28861783	0.03299542 0.01652169
Solyc03g044710.3.1	Patatin	0	1.10309353	0.00286947			mir7981	SL3.0ch09_239	-0.98388516	0.00383257	0.78365455	0.16433171
Solyc02g055090.3.1	Patatin	0			2.09389482	0.00397442	mir7981	SL3.0ch09_239	-0.98388516	0.00383257	0.78365455	0.16433171
Solyc09g007260.3.1	AP2-like ethylene-responsive transcription factor	0	0.6361042	0.06880036	1.26240935	0.00013009	mir172	SL3.0ch04_250	0.53362924	0.14311798	-1.28373321	0.04686608
Solyc06g075510.3.1	APETALA2e	0	0.99584968	0.0365922	1.53210758	4.45E-05	mir172	SL3.0ch04_250	0.53362924	0.14311798	-1.28373321	0.04686608

Table 1: The twelve miRNA/mRNA pairs showing an anti-correlation of their expression profile.

Degradome data identified twelve genes targeted by miRNAs. *S. lycopersicum* (Solyc) genes expression level were compared using two statistical methods (Dseq2 and EdgeR). Dseq2 Analysis identified the expression level of miRNAs. Gal/Root expression fold change at 7 and 14 dpi (LogFC) and adj pvalue are indicated for genes and miRNAs. Red: Overexpression; Green: Repression; Grey: no DE.

Integration of data from transcriptome, small RNAs and degradome sequencing to build a gene-microRNA regulation network in gall

Once the miRNAs expressed in galls have been identified, the transcripts cleaved by the microRNAs in galls were identified by degradome sequencing (German et al., 2009) of mRNA extracted from galls at 7 (G7) and 14 dpi (G14). CleaveLand pipeline (Addo-Quaye et al., 2009) was used to analyse degradome sequencing data and predict mRNA cleaved by miRNAs in galls. We restricted our analysis to the highest confidence targets by selecting CleaveLand categories 0 & 1 with a degradome pvalue below 0.05. 153 transcripts were identified as targeted by microRNAs in galls (**Supplemental table S6**) including 58 targets shared by the two libraries G7 and G14, while 45 targets were identified specifically in the G7 library and 50 were only found in the G14 library. 111 targets were identified for 135 known miRNAs belonging to 39 miRNAs known families. Out of the 298 novel miRNAs identified in galls, 46 were found to target 47 transcripts in galls at 7 and/or 14 dpi.

To build a gene-miRNAs regulation network involved in the formation of galls, data from transcriptome, microRNAs and degradome sequencing were integrated. From the 153 transcripts were identified as targeted by a microRNAs expressed in galls, 32 were shown as DE in galls by transcriptome analysis. 19 targeted genes were DE in galls at 7 and 14 dpi including 11 upregulated and eight downregulated genes. Five targeted genes were specifically DE at 7 dpi including three transcripts upregulated and two downregulated in galls. At 14 dpi, only eight transcripts identified as targets were DE including three upregulated and five downregulated. Since most plant miRNAs silence gene expression through the cleavage of targeted transcripts, a negative correlation of expression profiles between the microRNA its targeted gene is usually expected. We identified twelve miRNA/mRNA pairs that show negative correlation of their expression level (**Table 1**). These twelve miRNA/mRNA pairs are the most robust candidates to be involved in the formation of galls.

ARF8 auxin-related transcription factors are involved in tomato-RKN interactions

Among the twelve negatively correlated microRNAs/mRNA pairs, two of the strongest candidate to be regulated by a microRNA are the *AUXIN RESPONSE FACTORS 8A* and *8B* and their common regulator miR167. These two genes belong to ARF transcription factors that relay auxin signaling at the transcriptional level by regulating the expression of auxin-responsive genes (Guilfoyle and Hagen, 2007). Gall transcriptomic analyzes showed that *ARF8B* is overexpressed in tomato galls at 7 and 14 dpi and *ARF8A* is overexpressed at 14 dpi (**Table 1**). *ARF8A* and *ARF8B* were identified as cleaved by miR167 in galls by degradome

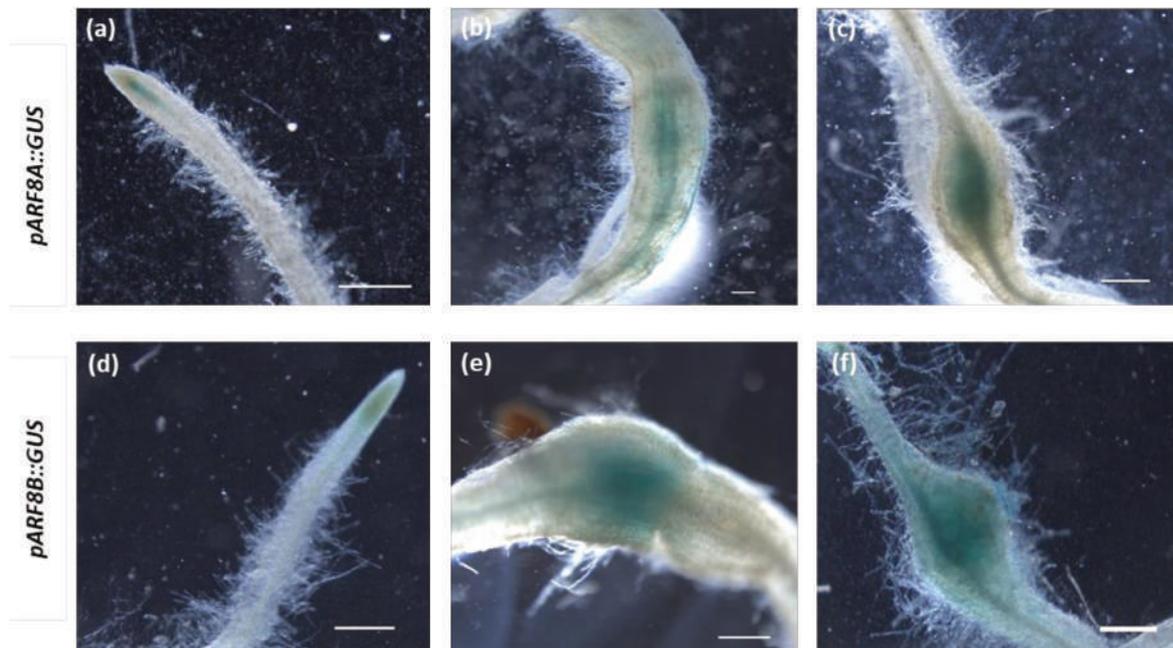


Fig. 2 Expression patterns of *pARF8A::GUS* and *pARF8B::GUS* in galls and uninfected tomato roots. Activity of *pARF8A* was observed in (a) uninfected roots and galls from tomato induced by *Meloidogyne incognita* at (b) 7 days post inoculation (dpi) and (c) 14 dpi. Activity of *pARF8B* was observed in (d) uninfected roots and in galls at (e) 7 dpi and (f) 14 dpi. Bars: 500 μ m

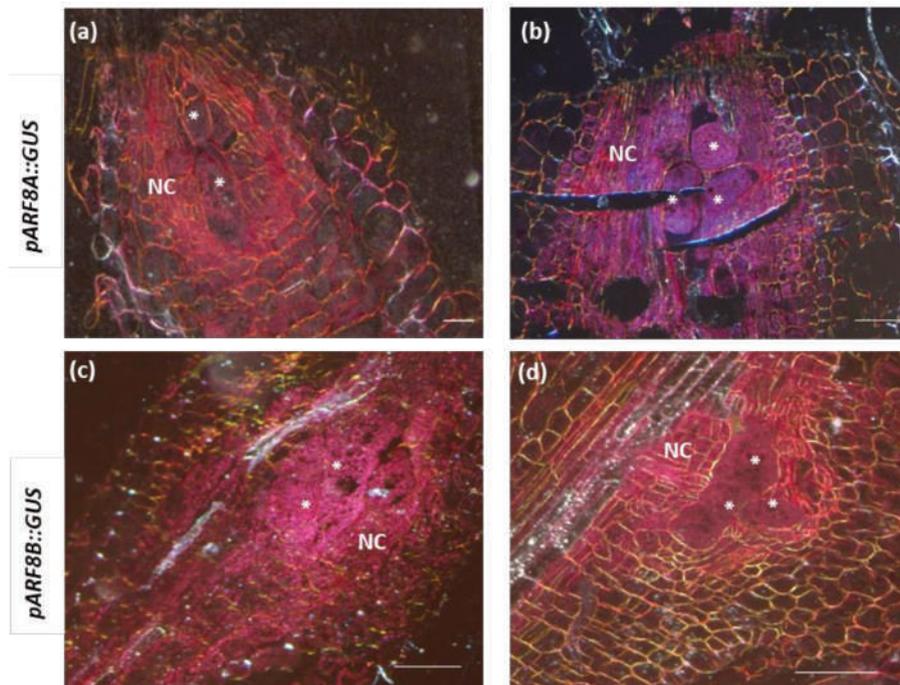


Fig. 3 The activity of *pARF8A::GUS* and *pARF8B::GUS* was observed in tomato galls sections of 5 μ m. Activity of *pARF8A* was observed in giant cells and neighboring cells (a) 7 dpi and (b) 14 dpi. Activity of *pARF8B* was observed in in giant cells and neighboring cells at (c) 7 dpi and (d) 14 dpi. *, giant cells; NC: neighboring cells; Bars: (a) 100 μ m (b-d) 50 μ m.

sequencing. Four *MIR167* genes were identified in tomato genome all sharing the same mature sequence and all are down regulated in galls while both *ARF8* genes were found to be upregulated. *ARF8A* and *ARF8B* transcripts were previously shown to be cleaved by miR167 in tomato (Liu et al., 2014) and this regulation is conserved in *Arabidopsis* (Wu et al., 2006). The down regulation of miR167 and the upregulation of *ARF8A* and *ARF8B* observed in galls suggest that, by repressing *MIR167* expression, RKN infection prevents *ARF8* silencing by miR167 that occurs in uninfected root.

The tissue expression of *ARF8A* and *ARF8B* in roots infected by RKN was further investigated *in vivo*, by analyzing the activity of both *ARF8A* and *ARF8B* promoters in transgenic tomato lines expressing promoter-GUS fusions. A strong GUS signal was observed in galls 7 and 14 dpi of two *pARF8B::GUS* and *pARF8A::GUS* lines (Bouzroud et al., 2018) and in root tips from uninfected roots (**Figure 2A-F**). Histological sections of the galls showed a strong GUS signal within the feeding giant cells and in neighboring cells at 7 and 14 dpi for both *ARF8A* and *ARF8B* lines (**Figure 3A-D**). The strong activity of both promoters *ARF8A* and *ARF8B* observed in galls *in vivo* confirm the results of transcriptomic data.

To investigate the role of both *ARF8A* and *ARF8B* in gall development, we analyzed the effect of CRISPR deletions within *ARF8* coding sequences on the infection by *M. incognita* (*arf8a*^{CR-2} (deletion 2nt), *arf8b*^{CR-11} (deletion 11nt) and *arf8ab*^{CR-2,4}, (double mutant) (all provided by M. Zouine, ENSAT, Toulouse). All these CRISPR lines did not show any root phenotype compared to wild type (**Supplemental figure S2**). Infection rate of these CRISPR lines inoculated with *M. incognita* was quantified by counting the galls and egg masses produced by the adult females at the root surface. A strong and significant decrease, of approximately 50%, in the number of galls and egg masses was observed for the *arf8a*^{CR-2}, *arf8b*^{CR-11} and *arf8ab*^{CR-2,4} lines in comparison to wild type plants. These results showed that disruption of *ARF8* leads to resistance and therefore demonstrated that *ARF8A* and *ARF8B* genes are involved in the plant-RKN interaction (**Figure 4A**). To check whether the increased resistance of the *arf8a*^{CR-2}, *arf8b*^{CR-11} and *arf8ab*^{CR-2,4} lines was due to defects of giant cells, the feeding site surface was measured directly with a confocal microscope, after gall BABB clearing. A comparison of the mean surface areas of the giant cells in each gall showed that giant cells from CRISPR lines were approximately 30% smaller than those from control plants (**Figure 4B**). Altogether, these results showed that *ARF8A* and *ARF8B* expression is required for the proper formation of feeding site during tomato-RKN interaction.

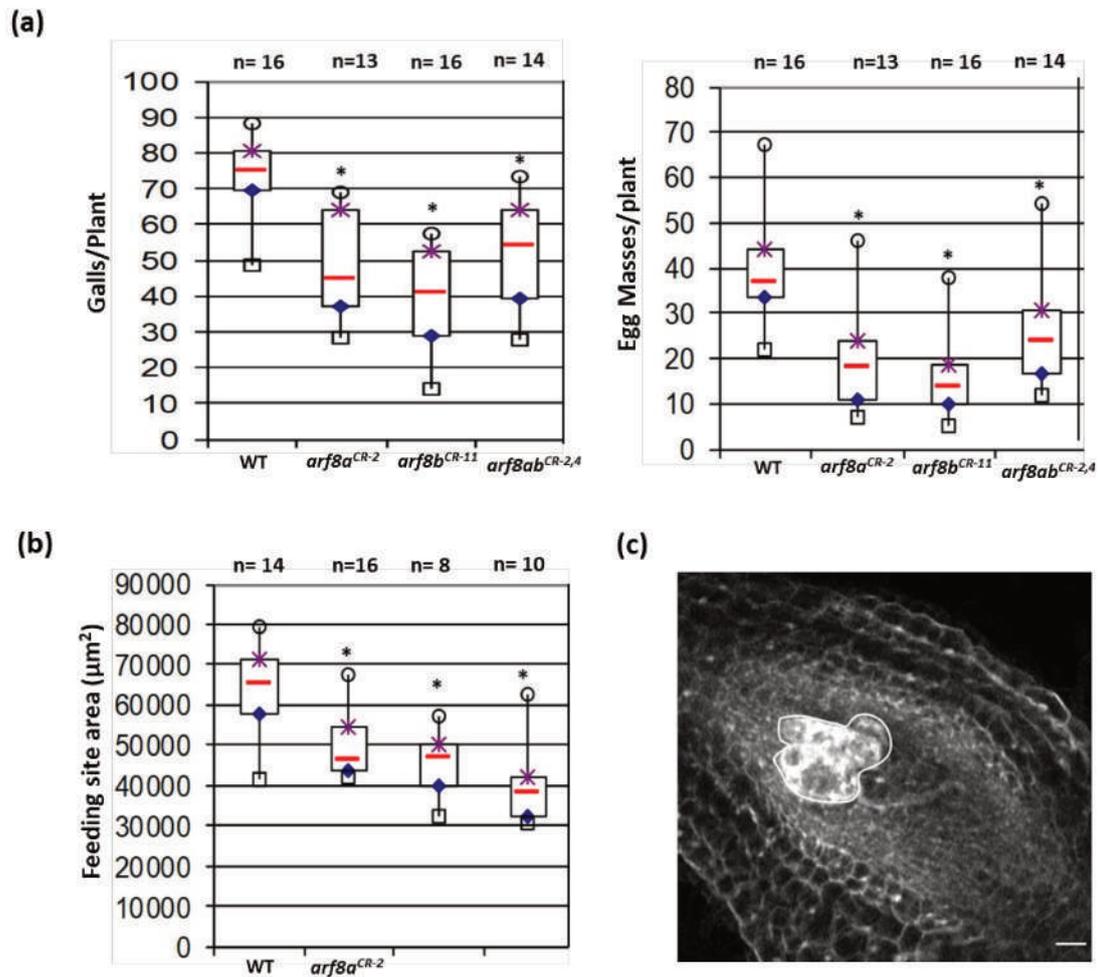


Fig. 4 Infection assay with CRISPR *arf8* lines. (a) The single mutants *arf8a*^{CR-2} and *arf8b*^{CR-11} and the double mutant *arf8ab*^{CR-2,4} infected with *M. incognita* is more resistant than wild-type to RKN as shown by the significant smaller mean number of galls and egg masses per plant in the two independent infection tests. (b) The effect of *arf8a*^{CR-2} and *arf8b*^{CR-11} and *arf8ab*^{CR-2,4} on the development of giant feeding cells was further evaluated by measuring the size of the feeding site (µm²) produced in galls from each CRISPR line compared to galls in WT at 21 dpi, by the BABB clearing method (Cabrera et al., 2018). (c) Galls collected at 21 dpi *in vitro* to measure the surface of the feeding site (white line) using BABB clearing method. Box plots were realized with the results of infection assays. Mann–Whitney U-tests were performed for statistical analysis in each experiment. n' denotes the number of plants tested. *, P < 0.05. Open squares, minimum values; open circles, maximum values; red lines, median values; blue diamond, first quartile; purple star, third quartile.

ARF8A and ARF8B regulated genes in galls

In order to identify the targets of *ARF8A* and *ARF8B*, mRNA from galls of WT and *arf8a^{CR-2}* and *arf8b^{CR-11}* has been sent for sequencing. Still the sequencing is still *in progress*. The results of this article will be discussed later in the general discussion of the thesis.

Author contributions

Y.N., B.F and S.J.P. designed the study, performed the experimental work and wrote the manuscript. All authors analysed and discussed the data. Y.N., B.F and S.J.P. designed the study. M.Q., J.M. and P.A. participated in the writing of the manuscript. Y.N. and C.M. produced biological material for sequencing. M.dR. and C.M. analysed NGS data. M.Z. designed the CRISPR lines.

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Supplemental Data Set: (Online version for the tables)

Supplemental table S1: Protein coding genes DEGs in galls at 7 dpi

Supplemental table S2: Protein coding genes DEGs in galls at 14 dpi

Supplemental table S3: Gene Ontology (GO) analysis of the DEGs in galls at 7 and/or 14 dpi

Supplemental table S4: raw reads

Supplemental table S5: microRNAs DE in galls at 7 and/or 14 dpi

Supplemental table S6: 153 transcripts targeted by miRNAs in galls at 7 and/or 14 dpi

Supplemental figure S1: Infection test of CRISPR *arf8* lines

Supplemental figure S2: Weight root of CRISPR *arf8* lines

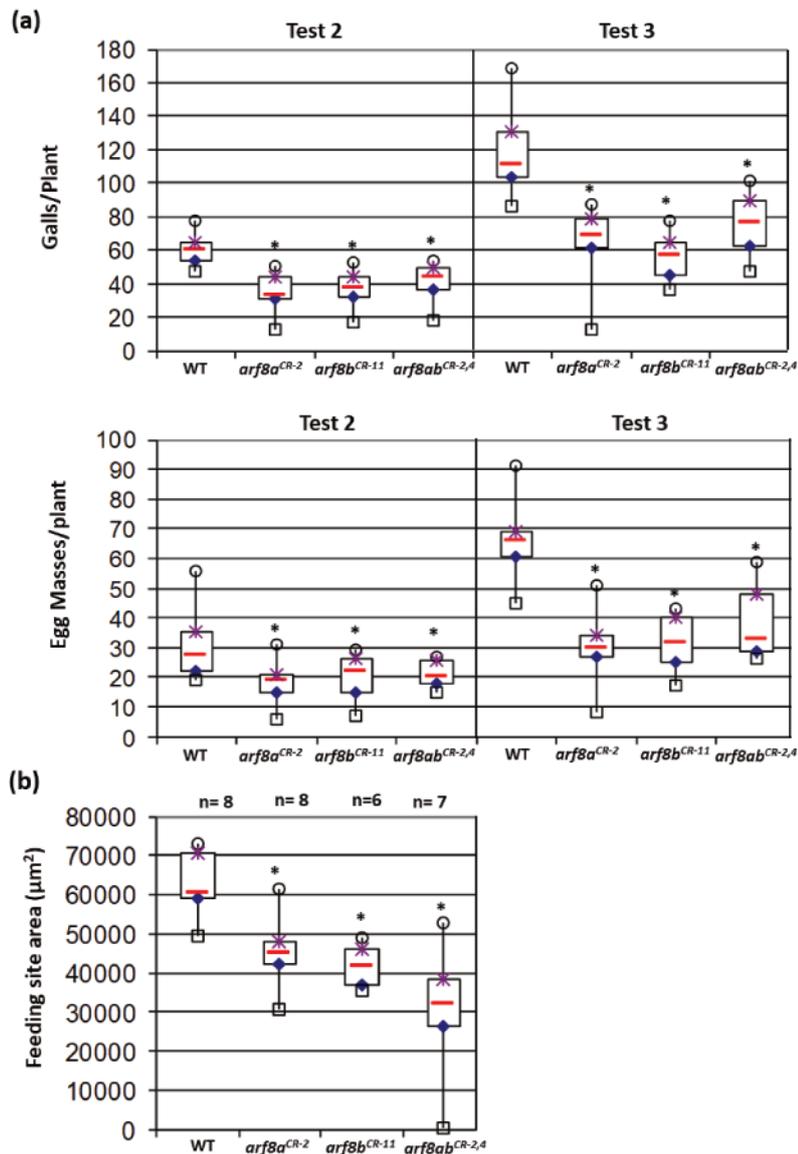


Fig. S1 Two other infection assays with CRISPR *arf8* lines. (a) The single mutants *arf8a*^{CR-2} and *arf8b*^{CR-11} and the double mutant *arf8ab*^{CR-2,4} infected with *M. incognita* is more resistant than wild-type to RKN as shown by the significant smaller mean number of galls and egg masses per plant in the two independent infection tests. Number of plants per test: (Test 2: WT, n= 8; *arf8a*^{CR-2}, n=17; *arf8b*^{CR-11}, n=11; *arf8ab*^{CR-2,4}, n=6) (Test 3: WT, n= 11; *arf8a*^{CR-2}, n=9; *arf8b*^{CR-11}, n=10; *arf8ab*^{CR-2,4}, n=7). **(b)** The effect of *arf8a*^{CR-2} and *arf8b*^{CR-11} and *arf8ab*^{CR-2,4} on the development of giant feeding cells was further evaluated by measuring the size of the feeding site (µm²) produced in galls from each CRISPR line compared to galls in WT at 21 dpi from test 2, by the BABB clearing method (Cabrera et al., 2018). Box plots were realized with the results of infection assays. Mann–Whitney U-tests were performed for statistical analysis in each experiment. n' denotes the number of plants tested *, P < 0.05. Open squares, minimum values; open circles, maximum values; red lines, median values; blue diamond, first quartile; purple star, third quartile.

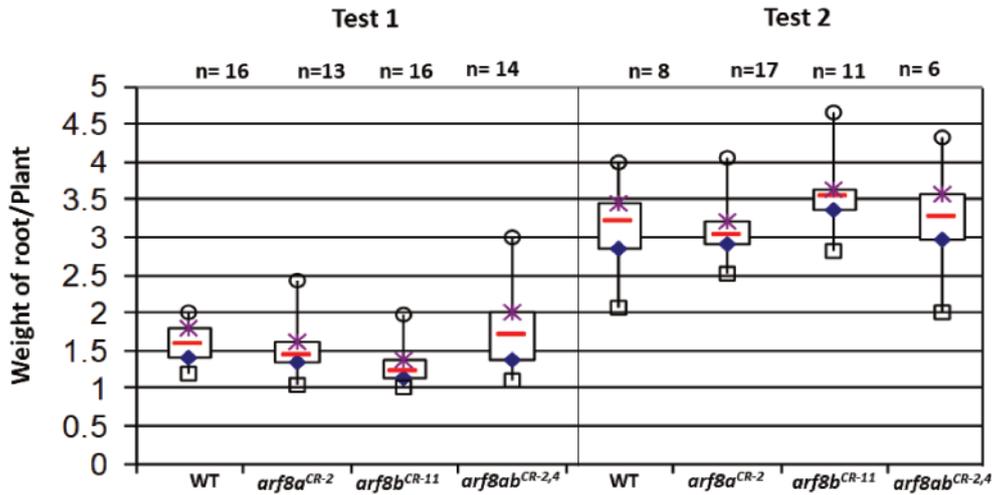


Fig S2 The weight of infected roots from *arf8a*^{CR-2} and *arf8b*^{CR-11} and *arf8ab*^{CR-2,4} lines was compared to the weight of roots from wild type plants seven weeks after infection. No difference in weight was observed by Mann & Whitney statistical tests. Number of plants per test: (Test 1: WT, n= 16; *arf8a*^{CR-2}, n=13; *arf8b*^{CR-11}, n=16; *arf8ab*^{CR-2,4}, n=14) (Test 2: WT, n= 8; *arf8a*^{CR-2}, n=17; *arf8b*^{CR-11}, n=11; *arf8ab*^{CR-2,4}, n=6). Box plots were realized with the results of infection assays. Mann–Whitney U-tests were performed for statistical analysis in each experiment. *, P < 0.05. Open squares, minimum values; open circles, maximum values; red lines, median values; blue diamond, first quartile; purple star, third quartile.

CHAPTER 2: Copper microRNAs govern the formation of giant feeding cells induced by the root knot nematode *Meloidogyne incognita* in *Arabidopsis thaliana*

Article 2:

Noureddine *et al.*, (2021) in revision in *New Phytologist*

Yara Noureddine¹, Martine da Rocha¹, Sébastien Thomine², Michaël Quentin¹, Pierre Abad¹, Bruno Favery¹, and Stéphanie Jaubert-Possamai^{1*}. Copper microRNAs govern the formation of giant feeding cells induced by the root knot nematode *Meloidogyne incognita* in *Arabidopsis thaliana*.

This second part of my work consist in studying the role of two copper-miRNAs, miR408 and miR398, in the formation of giant cells in *A. thaliana* induced by *M. incognita* interaction. MiR408 and miR398 families and their targets are known to be involved in the copper signaling pathway. In response to copper starvation, the expression of *MIR398* and *MIR408* genes is activated by the *SPL7* transcription factor, and they repress the expression of genes encoding copper binding proteins non-essential for plant development. These two conserved microRNA families are upregulated in tomato and *A. thaliana* galls. In this study, I have used *Arabidopsis* lines expressing transcriptional fusion with GUS reporter gene. I showed that both *MIR408* and *SPL7* were expressed within nematode induced feeding cells. Moreover, infection assays with mir408 and *spl7* KO mutants or lines expressing mutated targets resistant for miR398 cleavage showed a decreased of susceptibility towards nematode infection. Moreover, watering plants with copper sulfate, at concentration below toxic concentrations for the plants or for the nematodes, induced a strong resistance to nematode infection. Altogether, these results demonstrate the role of disruption of copper homeostasis through activation of miR398 and miR408 by *SPL7* in the formation of giant feeding cells. This article is in revision in the journal *New Phytologist*.

Copper microRNAs govern the formation of giant feeding cells induced by the root knot nematode *Meloidogyne incognita* in *Arabidopsis thaliana*

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Short title: Copper miRNAs in plant-nematode interactions

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Abstract (169 words)

miR408 and miR398 are two conserved microRNAs which expression is activated by the SPL7 transcription factor in response to copper starvation. We identified these two microRNAs families as upregulated in *Arabidopsis thaliana* and *Solanum lycopersicum* roots infected by root-knot nematodes. These endoparasites induce the dedifferentiation of a few root cells and the reprogramming of their gene expression to generate giant feeding cells. By combining functional approaches, we deciphered the signaling cascade involving these microRNAs, their regulator and their targets. *MIR408* expression was located within nematode-induced feeding cells in which it co-localised with *SPL7* expression and was regulated by copper. Moreover, infection assays with *mir408* and *spl7* KO mutants or lines expressing targets rendered resistant to cleavage by miR398 demonstrated the essential role of the *SPL7/MIR408/MIR398* module in the formation of giant feeding cells. Our findings reveals how perturbation of plant copper homeostasis, *via* the *SPL7/MIR408/MIR398* module, governs the formation of nematode-induced feeding cells.

Introduction (1124 words)

MicroRNAs are small non-coding RNAs that regulate the expression of protein-coding genes, mostly at the post-transcriptional level, in plants. They are major post-transcriptional regulators of gene expression in various biological processes, including plant development (Li and Zhang, 2016), responses to abiotic stresses (Barciszewska-Pacak et al., 2015), hormone signalling (Curaba et al., 2014), and responses to pathogens or symbiotic micro-organisms (Weiberg and Jin, 2015; Hoang et al., 2020). MicroRNAs and short interfering RNAs (siRNAs) were recently shown to play a key role in plant-pathogen crosstalk through trans-kingdom RNAi processes (Weiberg et al., 2013; Cai et al., 2018a; Dunker et al., 2020). MicroRNAs are produced by the cleavage of long double-stranded RNA precursors by the DICER RNase, generating 20-22 nucleotides miRNA duplexes composed of a mature (5P) and a complementary (3P) strand. One of the two strands is then incorporated into the RNA-induced silencing complex (RISC) to guide the major RISC protein, ARGONAUTE1 (AGO1), to the targeted messenger RNA (mRNA) on the basis of sequence complementarity. The hybridization of AGO1-bound miRNAs to their targets induces predominantly targeted mRNA degradation in plants, but it may also lead to an inhibition of mRNA translation (Axtell, 2013a).

The miR408 and miR398 microRNA families are conserved so-called “copper microRNAs”, due to their involvement in the plant response to copper deficiency (Yamasaki et al., 2007; Zhang et al., 2014). Copper is an essential nutrient for plants, due to its function as a cofactor for many proteins. Copper proteins are involved in electron transport chains or function as enzymes in redox reactions. In plants, copper is involved in respiration, photosynthesis, ethylene perception, the metabolism of reactive oxygen species and cell wall remodelling (reviewed in Burkhead et al., 2009). Copper microRNAs accumulate in response to copper deficiency and their synthesis is repressed when copper concentrations are sufficiently high (Yamasaki et al., 2009). The underlying mechanism has been described in *Arabidopsis thaliana*, in which the regulation of *MIR408*, *MIR398B* and *MIR398C* by copper levels was shown to be mediated by the SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE7 (*SPL7*) transcription factor (Yamasaki et al., 2009). The *A. thaliana* genome contains a single copy of *MIR408*, and three *MIR398* genes: *MIR398A*, *-B* and *-C*. At high copper concentrations, the DNA-binding activity of the *SPL7* transcription factor is repressed, preventing the induction of transcription for downstream genes, such as *MIR408* or *MIR398B* and *MIR398C*, but not *MIR398A* (Yamasaki et al., 2009; Sommer et al., 2011). In the presence of low concentrations of copper, *SPL7* activates the expression of copper-responsive microRNAs that target and

repress the expression of genes encoding copper-binding proteins. These proteins are replaced by proteins that do not bind copper, to save copper resources for the functions for which this element is essential, such as photosynthesis (reviewed in Burkhead et al., 2009). For example, the mRNA for the cytosolic COPPER/ZINC (Cu-Zn) SUPEROXIDE DISMUTASE, CSD1, which can be replaced by an iron (Fe)-dependent SOD, is targeted by the copper microRNA miR398. In addition to their regulation as a function of copper levels through the activity of SPL7, MIR408 and the MIR398 family are also regulated by several environmental cues and abiotic stresses, such as light, which regulates MIR408 activity through the ELONGATED HYPOCOTYL5 (HY5) or PHYTOCHROME INTERACTING FACTOR1 (PIF1) transcription factors in *A. thaliana* (Jiang et al., 2021), and salinity, oxidative and cold stresses, which have been shown to induce miR408 in *A. thaliana* (Ma et al., 2015), or cadmium treatment in *Brassica napus* (Fu et al., 2019). The miR408 and miR398 families have been widely analysed in plant responses to abiotic stresses, but little is known of their role in plant responses to biotic stresses. In sweet potato, MIR408 has been associated with plant defences, as it is repressed by jasmonic acid (JA) and wounding, and miR408-overexpressing plants have attenuated resistance to insect feeding (Kuo et al., 2019). Moreover, miR398 has been shown to regulate cell death in response to the causal agent of barley powdery mildew, *Blumeria graminis* (Xu et al., 2014).

Root-knot nematodes (RKN) of the genus *Meloidogyne* are obligatory sedentary plant parasites capable of infesting more than 5,000 plant species (Blok et al., 2008; Abad and Williamson, 2010). The RKN larvae penetrate the roots, in which they induce the de-differentiation of five to seven parenchyma root cells and their reprogramming into the multinucleate, hypertrophied feeding cells that form the feeding site. These metabolically overactive feeding cells provide the nutrients required for RKN development (Favery et al., 2020). During the dedifferentiation of vascular cells and their conversion into ‘giant’ feeding cells, the cells surrounding the feeding site begin to divide again. The growth of the feeding cells and the division of the surrounding cells lead to a root swelling known as a gall. The feeding cell induction occurs in the first three days after root infection. Feeding cell formation can be split into two phases. Firstly, the cells undergo successive nuclear divisions coupled with cell expansion until ten days post infection (dpi) in *A. thaliana* (Caillaud et al., 2008). In the second phase, from 10 to 21 dpi, the successive nuclear divisions stop and the nuclei of the feeding cells undergo extensive endoreduplication (Wiggers et al., 1990; de Almeida Engler and Gheysen, 2013). The dedifferentiation of vascular cells and their conversion into giant cells result from an extensive reprogramming of gene

expression in root cells, in response to RKN signals. In *A. thaliana*, the expression of approximately 10% of protein-coding genes is modified in galls induced by RKN (Cabrera et al., 2014; Yamaguchi et al., 2017; Jammes et al., 2005; reviewed in Escobar et al., 2011). The sequencing of small RNAs identified 24 mature microRNAs differentially expressed between *A. thaliana* galls induced by *M. incognita* and uninfected roots at 7 and 14 dpi (Medina et al., 2017). The miR408 and miR398 families of copper-responsive microRNAs were found to be upregulated in galls at 7 and/or 14 dpi.

In this article, we showed a conserved upregulation of these two microRNA families in *Arabidopsis* and tomato galls. Moreover, we found that the upregulation of miR408 in response to nematode was required for successful infection. Our findings highlighted a strong activity of *MIR408* promoter (*pMIR408*) in early galls that is i) driven by the modulation of environmental copper levels, ii) colocalised with strong *SPL7* expression. Moreover, we also demonstrated the involvement of this transcription factor in giant cell formation. In addition, we showed that the silencing of *CSD1* and *BLUE COPPER BINDING PROTEIN (BCBP)* transcripts by miR398 is involved in gall development. Finally, the watering of *Arabidopsis* with copper sulphate solutions at concentrations below the toxicity thresholds for nematode and plant development greatly decreased the RKN infection and impaired feeding cell development.

Results

The copper microRNA miR408 is crucial for the *Arabidopsis*-*Meloidogyne* interaction

Our previous analysis of microRNAs expressed in *Arabidopsis* galls induced by *M. incognita* revealed an upregulation of mature miR408 in *Arabidopsis* galls at 7 and 14 dpi, whereas miR398b/c was specifically upregulated in galls at 14 dpi. Sequencing of small RNAs from uninfected roots and galls of *Solanum lycopersicum* showed that these two microRNA families were also upregulated in tomato galls at 7 and 14 dpi (Table 1 and supplemental Table S1). Therefore, these microRNAs are among the very few conserved microRNAs which expression profile is conserved in *Arabidopsis* and tomato galls. We investigated the role of miR408 in gall development using two previously described *Arabidopsis* KO mutant lines: *miR408-1* and *miR408-2* (Maunoury and Vaucheret, 2011). The KO lines and corresponding wild-type plants

miRNA family	miRNA name	Plant species	miRNA mature sequence	logFC G/R 7dpi	adj. pvalue	logFC G/R 14dpi	adj. pvalue
mir408	SL3.0ch01_346	<i>S. lycopersicum</i>	TGCACAGCCTCTCCCTGGCT	3,741	0,000	3,289	0,017
	SL3.0ch01_419	<i>S. lycopersicum</i>	TGCACTGCCTCTCCCTGGCT	1,287	0,029	3,133	0,033
	Ath-miR408	<i>A. thaliana</i>	ATGCACTGCCTCTCCCTGGC	2,042	0,042	2,411	0,001
mir398	SL3.0ch12_14_3	<i>S. lycopersicum</i>	ATGTGTTCTCAGGTTACCCCT	3,272	0,009	2,166	0,066
	SL3.0ch05_225	<i>S. lycopersicum</i>	TTGTGTTCTCAGGTCACCCCT	0,290	0,863	3,430	0,015
	SL3.0ch11_437	<i>S. lycopersicum</i>	TATGTTCTCAGGTCGCCCTG	2,144	0,112	1,759	0,067
	ath-miR398b-c	<i>A. thaliana</i>	TGTGTTCTCAGGTCACCCCTT	2,464	0,119	3,287	0,000

Table 1: Expression profile of miRNAs of the miR408 and miR398 families in *Arabidopsis* and tomato galls. Expression level of mature sequence of the miR408 and miR398 families upregulated in *A. thaliana* and tomato (*S. lycopersum*) galls were identified by small RNA Illumina (for tomato samples) or SOLID (for *Arabidopsis* samples) sequencing, followed by DESeq 2 statistical analysis. MicroRNAs significantly upregulated in galls in comparison to uninfected roots (adjusted pvalue <0.05) are indicated in red.

were inoculated with *M. incognita* second stage juveniles (J2s) and their susceptibility was quantified by counting the galls and egg masses produced by adult females at the root surface.

The two KO lines for *MIR408* had 40 to 50% fewer galls and egg masses than the wild type ($p < 0.05$; Figure 1 and Supplemental Data Set S1). The roots of these KO lines were of similar weight and global architecture to those of wild-type plants (Supplemental Figure S1 and Supplemental Data Set S1). We then investigated the effect of the *MIR408* mutation on feeding site development, by comparing the area of feeding cells within galls collected from KO and wild-type plants (Figure 1A-C and Supplemental Data Set S1). Both KO mutants had a significantly smaller feeding site area than the wild type. Overall, these results demonstrate that *MIR408* is involved in feeding cell development in the *Arabidopsis*-nematode interaction, and that the lower susceptibility of the *mir408* KO lines is due to defects of feeding site formation.

We investigated the mechanisms by which miR408 regulates feeding cell formation in galls, by identifying the targets of miR408. The psRNA target algorithm (Dai et al., 2018) predicted 101 genes as putative targets of miR408 in *Arabidopsis* (Supplemental Data Set S2A). The expression profiles of these genes in galls at 7 dpi and 14 dpi were obtained from previous transcriptome analyses (Jammes et al., 2005). Only seven of the 101 putative targets were differentially expressed in galls at 7 and/or 14 dpi, and only two putative targets were repressed: a gene encoding a copper-binding protein, UCLACYANIN2 (*UCC2*, *At2g44790*), which is known to be cleaved by miR408 in senescing leaves and siliques (Thatcher et al., 2015), and a gene encoding a PHOSPHATASE 2G (*PP2CG1*, *At2g33700*) (Supplemental Data Set S2B).

***MIR408* induction in galls is driven by modulation of copper level**

We investigated the induction of *MIR408* in response to nematode infection, by inoculating plants expressing p*MIR408*::GUS with *M. incognita* J2s (Zhang and Li, 2013) *in vitro* in the presence of normal copper levels (0.1 μM CuSO_4) or with a high copper concentration (Gamborg B5 plus 5 μM CuSO_4). In the presence of normal concentrations of copper, we observed a strong GUS signal in developing galls at 3 and 7 dpi (Figure 2A-B). This signal had decreased in intensity by 14 dpi (Figure 2C) and disappeared completely from fully developed galls at 21 and 28 dpi (Supplemental Figure S2). On gall sections, the GUS signal was localised in the giant feeding cells and neighbouring cells, at the 3 dpi, 7 dpi and 14 dpi time points (Figure 2D-F). By contrast, in plants grown in the presence of high copper concentrations, the GUS signal was much weaker in galls at 3 and 7 dpi (Figure 2G-H), and undetectable in galls

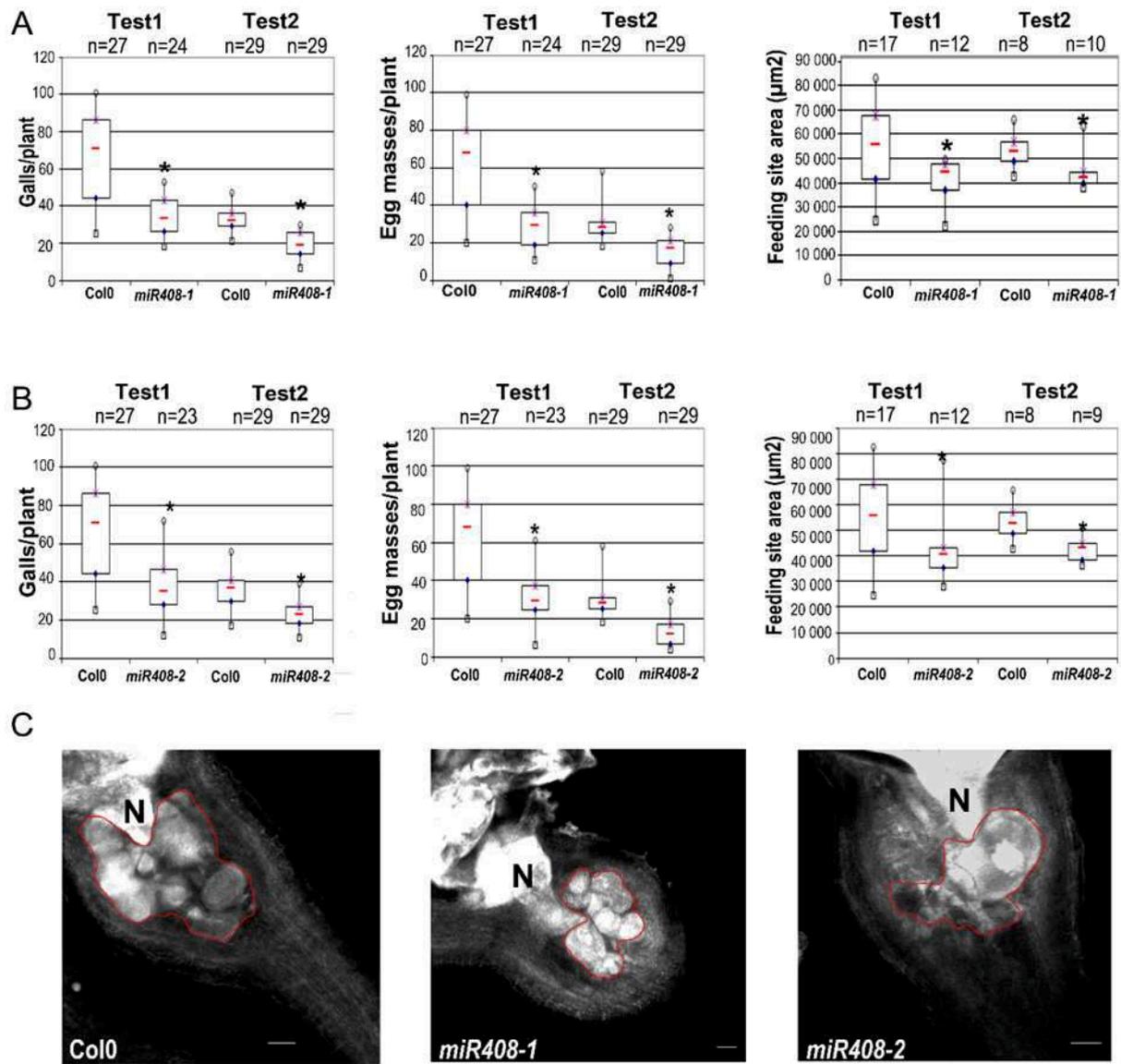


Figure 1: The miR408 KO lines were significantly less susceptible to *M. incognita* than the wild type. A-B, The susceptibility of the two miR408 KO lines, *miR408-1* (A) & *miR408-2* (B), and Col0 wild type to *M. incognita* was evaluated by counting the number of galls and egg masses per plant in two independent infection assays in soil. The effect of miR408 mutation on the development of giant feeding cells was further evaluated by measuring the size of the feeding site produced in each KO line and comparing it to that in Col0. (C) Galls were collected seven weeks post *in vitro* infection to measure the area (μm^2) covered by the giant cells by the BABB clearing method (Cabrera *et al.*, 2018). The impact of plant genotype was analysed in Mann and Whitney tests. *, $P < 0.05$. Open squares, minimum values; open circles, maximum values; red lines, median values; blue diamond, first quartile; purple star, third quartile. Bars 50 μm .

at 14 dpi (Figure 2I). The repression of *MIR408* expression in galls by high copper concentrations indicates that *MIR408* expression within *Arabidopsis* galls is regulated by modulation of copper levels.

***SPL7* is an activator of *MIR408* transcription in galls**

The regulation of *MIR408* by modulation of copper levels has been shown to be mediated by the *SPL7* transcription factor (Zhang et al., 2014; Bernal et al., 2012). Transcriptome analyses from *Arabidopsis* galls showed that *SPL7* was expressed in galls at 7, 14 and 21 dpi (Jammes et al., 2005). We further investigated the expression of *SPL7* within galls, by inoculating a *pSPL7::GUS Arabidopsis* line (Araki et al., 2018) with *M. incognita* in the presence of a normal copper concentration. *SPL7* promoter activity was observed within the gall from 3 to 14 dpi, with lower levels at 14 dpi (Figure 3A-C). As observed for *pMIR408::GUS*, sections of *pSPL7::GUS* galls revealed a GUS signal in the giant feeding cells and neighbouring cells (Figure 3D). We investigated the putative function of *SPL7* in plant responses to RKN, by inoculating the *Arabidopsis spl7* KO mutant described by Zhang et al. (2014) with *M. incognita* J2s. *SPL7* knockout led to the production of smaller numbers of galls and egg masses per plant than were observed for the wild-type (Figure 3E-F and Supplemental Data Set S3). This knockout had no effect on root weight (Supplemental Figure S3 and Supplemental Data Set S3). Measurements of the area of the feeding site within galls revealed defects of feeding site formation in the *spl7* KO mutants, resulting in smaller giant cells than were observed in wild-type plants (Figure 3G and Supplemental Data Set S3). Overall, these results demonstrate the requirement of miR408 and *SPL7* for the development of giant cells. The upregulation of mature miR408 observed in galls suggest an induction of *MIR408* expression driven by *SPL7* due to a decrease in copper availability within the gall.

micro398, a second copper-responsive microRNA family involved in the *Arabidopsis-Meloidogyne* interaction

MiR408 is not the only copper-responsive microRNA differentially expressed in galls. The expression of *MIR398B* and *MIR398C*, from the conserved miR398 family, has also been shown to be induced in response to copper deficiency, via *SPL7* activity (Araki et al., 2018). We previously described an induction of the mature miR398b and miR398c in *Arabidopsis* galls at 14 dpi (Medina et al., 2017). Three targets of the miR398 family have been biologically

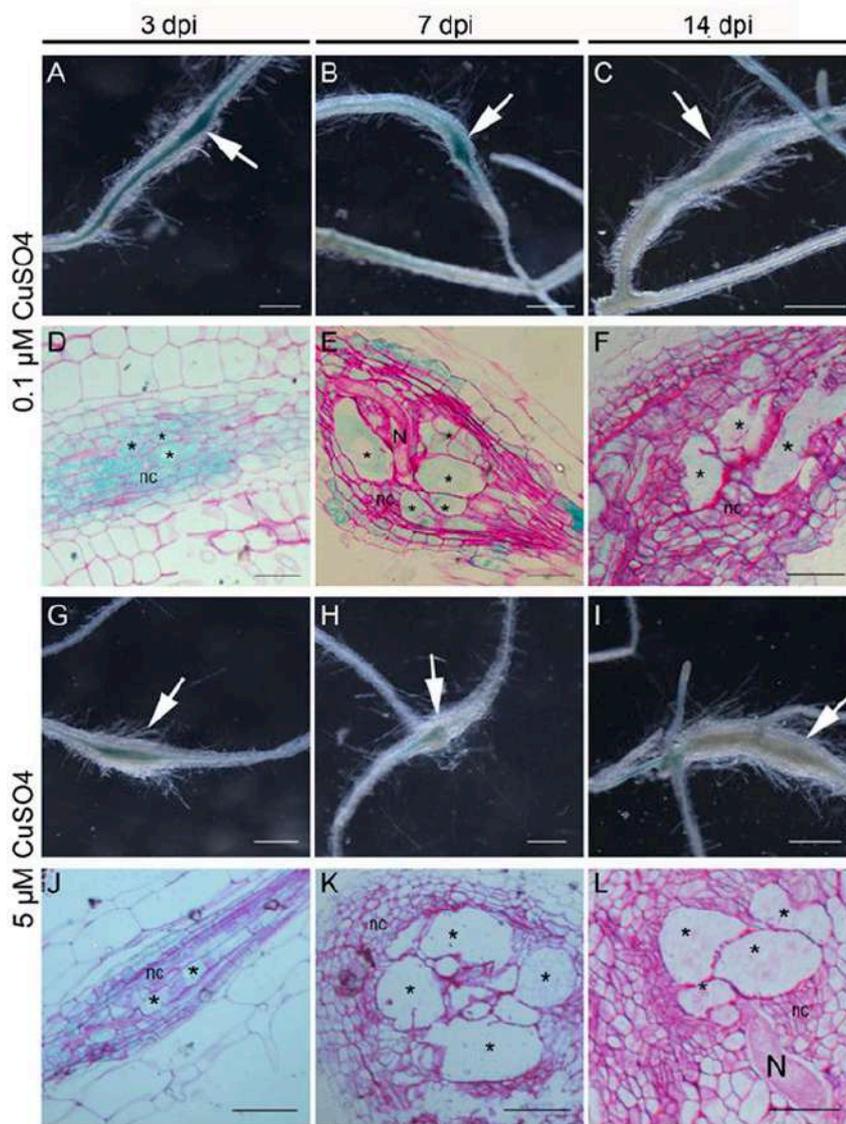


Figure 2. Copper modulates *MIR408* promoter activity in galls. A-H, The activity of the *MIR408* promoter was analysed in galls induced by *M. incognita* in *Arabidopsis* expressing the p*MIR408*::GUS construct grown in the presence of normal concentrations of copper (0.1 μ M CuSO₄) (A-F) or in the presence of high concentrations of copper (5.0 μ M CuSO₄) (G-L). A-C, a strong GUS signal was observed in galls 3 days post infection (dpi) (A), 7 dpi (B) and 14 dpi (C) in plants grown with 0.1 μ M CuSO₄. D-F, Section of gall at 3 dpi (D), 7dpi (E) and 14 dpi (F) showing the GUS signal in giant cells and in the cells surrounding the giant cells. G-I, a weaker GUS signal was observed in galls from plants grown with 5.0 μ M CuSO₄ analysed at 3 dpi (G) and 7 dpi (H) and no GUS signal was observed in galls at 14 dpi (I). J-L, Section of gall at 3 dpi (J), 7dpi (K) and 14 dpi (L). Galls are indicated with an arrow; N, nematode; (*) giant feeding cells; nc, neighbouring cells. Bars 500 μ m (A-C; G-I) or 50 μ m (D-F; J-L).

validated: the *At1g08830* and *At2g28190* transcripts encoding two copper superoxide dismutases, *CSD1* and *CSD2*, respectively, and the *At5g20230* transcript encoding the blue copper binding protein (BCBP), identified as a non-canonical target of miR398 (Brousse et al., 2014). We investigated the role of miR398 further, by infecting *Arabidopsis* lines expressing modified versions of *CSD1* (*mcsd1*) or *BCBP* (*mbcbp*) mRNAs rendered resistant to cleavage by miR398 (Beauclair et al., 2010; Brousse et al., 2014). The target mRNA levels is therefore artificially increased in these plants. The prevention of *CSD1* transcript cleavage by miR398 had no effect on root weight (Figure S4), but led to lower levels of nematode infection, with the mutant having less galls and egg masses than the wild type (Figure 4 and Supplemental Data Set S4). The *mbcbp* line also had fewer egg masses than the wild-type (Figure 4 and Supplemental Data Set S4). No defect of feeding cell formation, such as slower feeding cell growth, was observed in either of these lines (Figure 4 and Supplemental Data Set S4). The specific decrease in egg mass production by females provides evidence for a role for miR398 in the functionality of feeding cells, although the mutations in the *mcsd1* and *mbcbp* lines did not affect feeding site size. These findings demonstrate that the cleavage of *CSD1* and *BCBP* transcripts by miR398 is required for plant-RKN interaction.

Modulation of copper levels is essential for plant-RKN interaction

To study further the effect of copper on nematode infection, we analysed the direct effects of copper on nematode survival and gall development. Free-living *M. incognita* J2s were incubated in several concentrations of copper sulphate (50 μ M to 2 mM) used in previous studies assessing the effect of copper on plant development (Schulten et al., 2019). As a negative control, J2s were incubated in tap water. Living J2 counts after 24 hours in the copper sulphate solution showed that copper was non-toxic at a concentration of 50 μ M (Supplemental Figure S5 and Supplemental Data Set S5). By contrast, toxic effects were observed for all other concentrations tested (0.5 mM, 1 mM and 2 mM). We then analysed the effect of copper on gall formation in Col0 and *pMIR408::GUS* plants grown in soil watered with 50 μ M CuSO₄. We also minimised J2 exposure to copper in the soil, by beginning to water plants 50 μ M CuSO₄ two days after inoculation, after the J2s had already penetrated the roots. Watering with 50 μ M CuSO₄ repressed pMIR408 activity, confirming the effects of such treatment in galls (Supplemental Figure S6 and Supplemental Data Set S6). Watering with 50 μ M CuSO₄ had no visible effect on root weight and architecture (Supplemental Figure S7 and Supplemental Data Set S7), but it resulted in a strong and significant decrease in the number of galls and egg masses

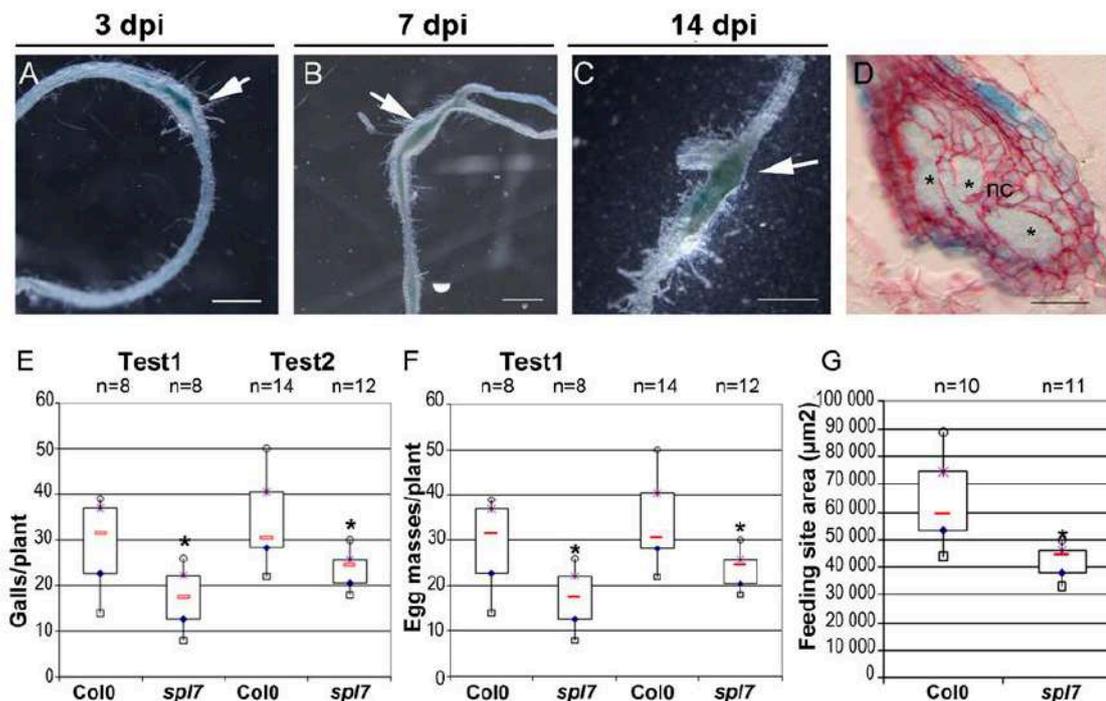


Figure 3. *SPL7* is induced and required for *M. incognita* infection and giant cell formation in *Arabidopsis*. A-D, The activity of the *SPL7* promoter (*pSPL7*) was studied in galls induced by *M. incognita* from *A. thaliana* expressing the *pSPL7::GUS* construct, at 3 days post inoculation (dpi)(A), 7 dpi (B) and 14 dpi (C). D, GUS activity was observed within 5.0 µm-thick gall sections at 7 dpi. E-G, the KO *spl7* line (SALK093849c) was infected with *M. incognita* J2. This line was significantly less susceptible to RKN than Col0, as shown by the smaller mean number of galls (E) and egg masses (F) per plant in two infection assays. (G), the effect of *spl7* mutation on the development of feeding cells was further evaluated by measuring the size of the feeding site produced. Galls were collected seven weeks post *in vitro* infection for measurement of the area (µm²) covered by the giant cells, by the BABB clearing method (Cabrera et al., 2018). Mann–Whitney tests were performed for statistical analysis in each experiment; significant differences relative to Col-0: *, *P* < 0.05; Open squares, minimum values; open circles, maximum values; red lines, median values; blue diamond, first quartile; purple star, third quartile. Galls are indicated with an arrow; (*) giant feeding cells; nc, neighbouring cells. Bars 50 µm (A-C) or 500 µm (D).

relative to control plants watered with tap water (Figure 5). These results demonstrate that perturbation of plant copper homeostasis governs the formation plant-RKN interaction.

Discussion

RKN induce the formation of similar giant feeding cells in thousands of plant species. The conservation of the ontogeny and phenotype of nematode-induced feeding cells between species, strongly suggests that the plant molecular mechanisms manipulated by RKN are widely conserved across the plant kingdom. Previous transcriptome analyses on various plant species have shown that the development of galls in roots infected by RKN is associated with a massive reprogramming of gene expression (reviewed in Escobar et al., 2011). MicroRNAs are small non-coding RNAs that regulate gene expression at the post-transcriptional level, and some microRNA families, such as the miR156 and miR167 families, are widely conserved in plants (Chavez Montes Nature Communications 2014). The role for microRNAs in controlling gene expression during the formation of galls was recently reported in *Arabidopsis* (reviewed in Jaubert-Possamai et al., 2019), for the conserved microRNAs miR390, miR172 and miR159 (Escobar et al., 2015; Medina et al., 2017).

miR408 and miR398: two copper-responsive microRNA families activated in *Arabidopsis* galls induced by *M. incognita*

A previous analysis of the levels of mature microRNAs in galls at 7 and or 14 dpi and in uninfected roots showed that mature mi408 and mir398b/c were induced in galls in response to *M. incognita* (Medina et al., 2017). A combination of *in silico* predictions of the transcripts targeted by miR408 and previous transcriptional analyses of galls and uninfected roots identified two putative targets downregulated in galls the genes the *UCLACYANIN-2* (*UCC2*) and the *PHOSPHATASE* (*PP2CG*). The cleavage of *UCC2* transcripts by miR408 has been biologically validated in *Arabidopsis* and rice (Thatcher et al., 2015; Zhang et al., 2017). Moreover, several targets of miR398 have been biologically validated, including the cytosolic *CSD1* and chloroplastic *CSD2*, and the non-canonical target *BCBP* (Beauclair et al., 2010; Brousse et al., 2014). Our analysis, thus, identified several biologically validated and conserved targets that may be considered robust candidates for mediating the functions of miR398b/c and miR408 in galls.

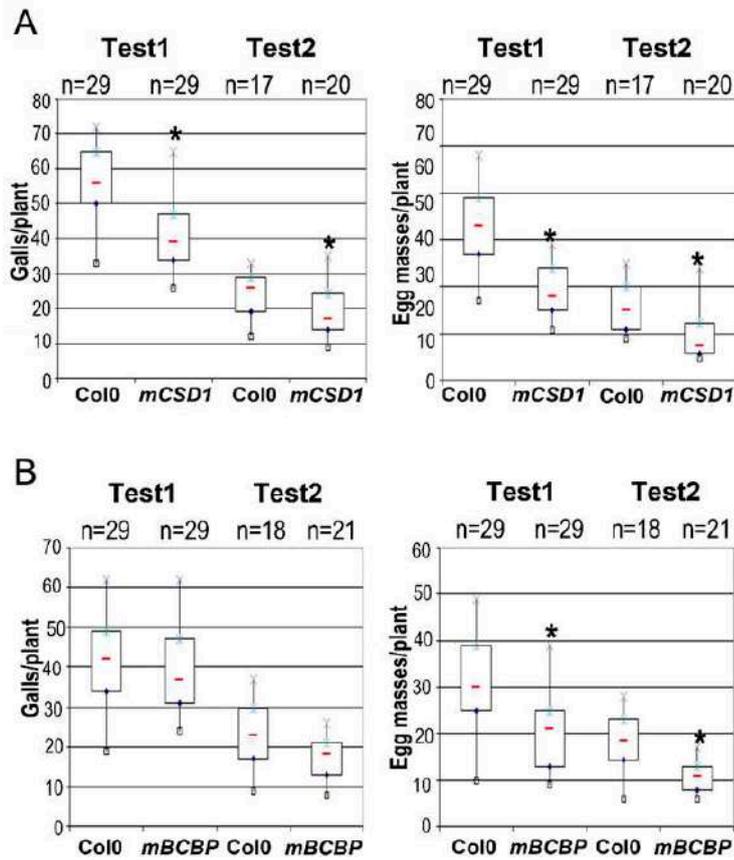


Figure 4. The miR398-resistant *mcsd1* and *mbcbp* mutant lines had smaller numbers of egg masses. The susceptibility of the *mcsd1* (A) and *mbcbp* (B) lines and of wild-type Col0 plants was evaluated by counting the number of galls and egg masses per plant in two independent infection assays in soil. The impact of the plant genotype on the number of galls and egg masses relative to Col0 was analysed in Mann and Whitney statistical tests. *, $P < 0.05$. Open squares, minimum values; open circles, maximum values; red lines, median values; blue diamond, first quartile; purple star, third quartile.

The inactivation of miR408 in T-DNA mutant lines, or of miR398b/c function in transgenic plants expressing mutated *CSDI* or *BCBP* resistant to miR398 cleavage, led to decreases in both the parameters used to assess parasitic success (the number of galls and the number of egg masses per root). The smaller number of galls in the mutant lines demonstrates the involvement of the miR398 family and miR408 in the early plant response to RKN. Moreover, the smaller feeding sites observed in the two *miR408* KO lines and the *spl7* mutant demonstrate that this miR408 and *SPL7* are involved in the formation of the giant cells, which are essential for nematode growth and development. Females are unable to develop normally if the feeding cells are too small, as already reported in some *Arabidopsis* mutants, such as lines with a knockout of *PHYTOSULFOKINE RECEPTOR1 (PSKR1)* (Rodiuc et al., 2016). Only a few genes and plant functions have been demonstrated to be essential for the formation of giant feeding cells (Favery *et al.*, 2020). In the absence of changes in giant cell size in the *mcsd1* and *mcbcbp* mutants, we hypothesise that the miR398-regulated *CSDI* and *BCBP* genes may play a role in giant cell functioning, potentially in reactive oxygen species (ROS)-related redox regulation and signalling (Zhao et al., 2020). Further studies will be required to determine their precise roles in the plant-RKN interaction.

SPL7 is a regulator of miR408 and miR398 in galls

In *A. thaliana*, it has been shown that *MIR408*, *MIR398B* and *MIR398C* are activated by the same transcription factor, *SPL7*, the activity of which is dependent on copper levels (Yamasaki et al., 2009; Araki et al., 2018). We confirmed the activity of the *SPL7* promoter and *MIR408* in feeding cells and neighbouring cells. The co-expression of *SPL7* and *MIR408* within developing galls, and the similar nematode infection phenotype, with feeding site formation defects, strongly suggest that *SPL7* is responsible for activating *MIR408* transcription in galls, as already reported in leaves and the root vasculature (Yamasaki et al., 2009; Araki et al., 2018). We therefore hypothesised that the expression of *MIR408* and *MIR398B* and *-C* is activated by the *SPL7* transcription factor in response to a decrease in copper concentration within galls. Other transcription factors, such as *HY5* and *PIF1*, which are known to regulate the expression of *MIR408* in response to light stress (Zhang et al., 2014; Jiang et al., 2021), are expressed in galls and could also play a role in the regulation of *MIR408* expression. However, the strong repression of *MIR408* by excess copper observed in galls suggests that *MIR408* upregulation in galls is predominantly driven by copper and *SPL7*.

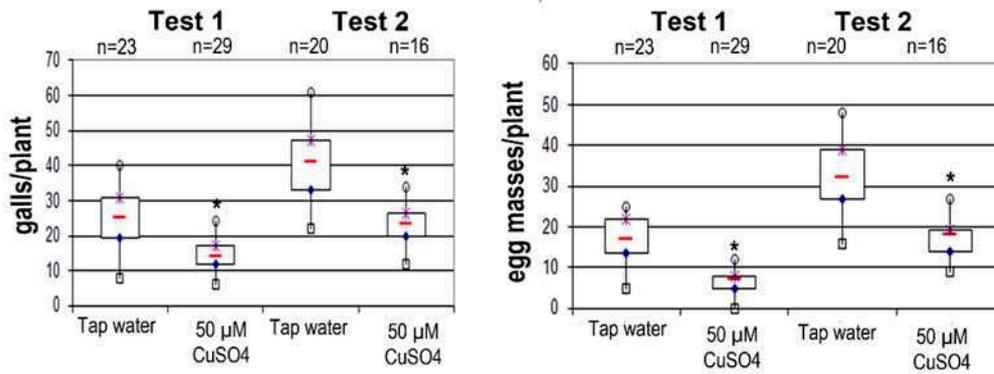


Figure 5. Plants watered with copper sulphate solution were significantly less susceptible to root knot nematodes. The effect of physiological concentrations of copper on *M. incognita* infection was evaluated by counting galls and egg masses in Col0 plants watered with a copper sulphate solution at a non-toxic concentration (50 µM) and comparing the results to those for Col0 watered with tap water. The impact of plant genotype on the numbers of galls and egg masses relative to Col0 was analysed in Mann and Whitney statistical tests. *, $P < 0.05$. Open squares, minimum values; open circles, maximum values; red lines, median values; blue diamond, first quartile; purple star, third quartile.

Modulation of copper levels, a key conserved factor for gall formation

Infection assays with *A. thaliana* plants watered with a copper sulphate solution at a concentration non-toxic for plants and nematodes, showed a strong decreased RKN infection rates and resulted in defective feeding site formation. Together with the upregulation in galls of two microRNA families known to be induced by copper deficiency, the miR408 and miR398 families, and the regulation of *MIR408* expression by copper, this finding suggests that copper content decreases in the galls induced by RKN infection. This hypothesis is supported by the downregulation of the *COPT2* gene, encoding a copper importer, in the *Arabidopsis* gall transcriptome (Jammes et al., 2005). Assays in RKN-infected susceptible tomato roots have also demonstrated a decrease in copper concentration (Lobna et al., 2017).

The *SPL7/MIR408-UCC2/MIR398-CSD1* copper signalling cascade may be a key factor in gall formation, conserved across the plant kingdom. MiR408 and miR398 have been identified in more than 40 plant species (Griffiths-Jones et al., 2007) and *SPL7* is widely conserved throughout the plant kingdom (Yamasaki et al., 2009). Moreover, the targeting of *UCLACYANIN* by miR408 and of *CSD1* by miR398 is conserved in both dicotyledonous and monocotyledonous plants (Zhang et al., 2017; Thatcher et al., 2015). A role for *UCLACYANINS* in the formation of a lignified nanodomain within the Casparian strips known to form an endodermal barrier in *Arabidopsis* roots has recently been described (Reyt et al., 2020). Casparian strip defects have been observed in the endodermis bordering the giant cell area within sorghum galls induced by *M. naasi* (Ediz and Dickerson, 1976). Moreover, *Arabidopsis* mutants with disrupted Casparian strips are particularly susceptible to RKN (Holbein et al., 2019). The infection of plants with nematodes may, therefore, provides a unique model for investigating the role of copper modulation, *via* miR408 and its *UCLACYANIN2* target, in the formation of Casparian strips.

Methods

Biological material, growth conditions and nematode inoculation

Seeds of *A. thaliana* Col0 and mutants *miR408-1*(SALK_038860), *miR408-2* (SALK_121013.28.25.n), *spl7* (SALK_093849), *mcsd1* and *mbcbp* (Beauclair et al., 2010), *pmiR408::GUS* (Zhang et al., 2013) and *pSPL7::GUS* (Yamasaki et al., 2009) were surface-sterilised and sown on Gamborg B5 medium agar plates (0.5 x Gamborg, 1% sucrose, 0.8%

agar, pH 6.4). The plates were incubated at 4°C for two days, then transferred to a growth chamber (20°C with an 8 h light/ 16 h darkness cycle). *M. incognita* strain “Morelos” was multiplied on tomato plants in a growth chamber (25°C, 16 h light/8 h darkness). For the RKN infection of plants in soil, two-week-old plantlets grown *in vitro* were transferred to a mixture of 50% sand (Biot B5)/50% soil in a growth chamber (21°C, 8 h light/16 h darkness). For studies of the effect of copper on gall development on plants *in vitro*, *Arabidopsis* plantlets were sown and cultured *in vitro*, as described above, on Gamborg B5 medium supplemented with 50 µM CuSO₄.

Root knot nematode infection assay

For nematode infections *in vitro*, J2s were surface-sterilised with HgCl₂ (0.01 %) and streptomycin (0.7 %), as described by Caillaud and Favery (2016). We inoculated each 25-day-old seedlings grown individually *in vitro* with 200 sterilised J2s resuspended in Phytigel (5 %). Infection assays were performed on *Arabidopsis* mutants and a wild-type ecotype in soil. We inoculated 20 to 30 two-month-old plantlets with 150 J2s per plant and incubated them in a growth chamber (21°C, 8 h light/16 h darkness). Seven weeks after infection, the roots were collected, washed in tap water and stained with eosin (0.5 %). Stained roots were weighed and galls and egg masses were counted on each root under a binocular microscope. Mann and Whitney tests (2.5 %) were performed to determine the significance of the observed differences in the numbers of egg masses and galls per root.

Small RNA sequencing from galls and uninfected tomato roots

Biological material, RNA extraction, small RNA sequencing, read mapping and statistical analysis are presented as supplemental material.

BABB clearing

Feeding site development was evaluated by the BABB clearing method described by Cabrera et al., (2018). Briefly, the area occupied by the giant cells was measured on galls collected 14 dpi, cleared in benzyl alcohol/benzyl benzoate (BABB) and examined under an inverted confocal microscope (model LSM 880; Zeiss). Zeiss ZEN software was used to measure the

area occupied by the giant cells in each gall, on two biological replicates. Data were analysed in Mann and Whitney tests.

Copper treatment

M. incognita eggs were collected as previously described (Caillaud and Favery, 2016) and placed on a 10 µm-mesh sieve for hatching in tap water. Free-living J2s were collected from the water with a 0.5 µm-mesh sieve. We evaluated the toxicity of copper to J2s by incubating freshly hatched J2s in solutions of copper sulphate of various concentrations for 24 hours. The numbers of living or dead J2s were then determined by counting under a binocular microscope. We investigated the effects of copper on plant-nematode interactions in *Arabidopsis* Col0 grown in soil. *Arabidopsis* Col0 plantlets were prepared and inoculated as previously described for in-soil infection. Half the plants were watered with 50 µM CuSO₄ two days after inoculation with J2s and then once per week for the next seven weeks. Control Col0 plants were watered with tap water in place of copper sulphate solution, at the same frequency. Seven weeks after inoculation, the plants were collected, their roots were washed and weighed, and the numbers of galls and egg masses on the roots were counted, as described above.

Studies of promoter-GUS fusion gene expression

We localised the promoter activity of *MIR408* and *SPL7* in *A. thaliana* lines expressing various fusions of the GUS reporter gene to promoters from these genes (ref & Supplemental Table S7). We inoculated 21-day-old seedlings in soil and *in vitro*, as described above. We collected inoculated roots and washed them in water, 3, 7, 14 and 21 dpi. GUS staining was performed as previously described (Favery et al., 1998), and the roots were observed under a Zeiss Axioplan 2 microscope. Stained galls were dissected, fixed by incubation in 1% glutaraldehyde and 4% formaldehyde in 50 mM sodium phosphate buffer pH 7.2, dehydrated, and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer's instructions. Sections were cut and mounted in DPX (VWR International Ltd, Poole, UK), and observed under a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany).

Bioinformatic analysis

We used psRNA target with default parameters for the prediction of miR408 targets (Dai, Zhuang and Zhao 2018).

Author contributions

Y.N., B.F and S.J.P. designed the study and performed the experimental work. All authors analysed and discussed the data. S.J.P and B.F. designed the study and wrote the manuscript. M.Q. and P.A. participated in the writing of the manuscript. S.T. participated in the copper studies. MdR analysed NGS data. The author responsible for distributing materials integral to the findings presented in this article, in accordance with the policy of the journal (<https://academic.oup.com/plcell>) is: Stephanie Jaubert Possamai (Stephanie.jaubert@inrae.fr).

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Supplemental Data Set

Supplemental Data Set 1: Infection assays of miR408-1 and miR408-2 ko lines infected with *M. incognita*.

Supplemental Data Set 2: Prediction of Arabidopsis miR408 targets with psRNA targets.

Supplemental Data Set 3: Infection assays of *spl7* ko line infected with *M. incognita*.

Supplemental Data Set 4: Infection assays of *mcsd1* and *mbcbp* mutated lines infected

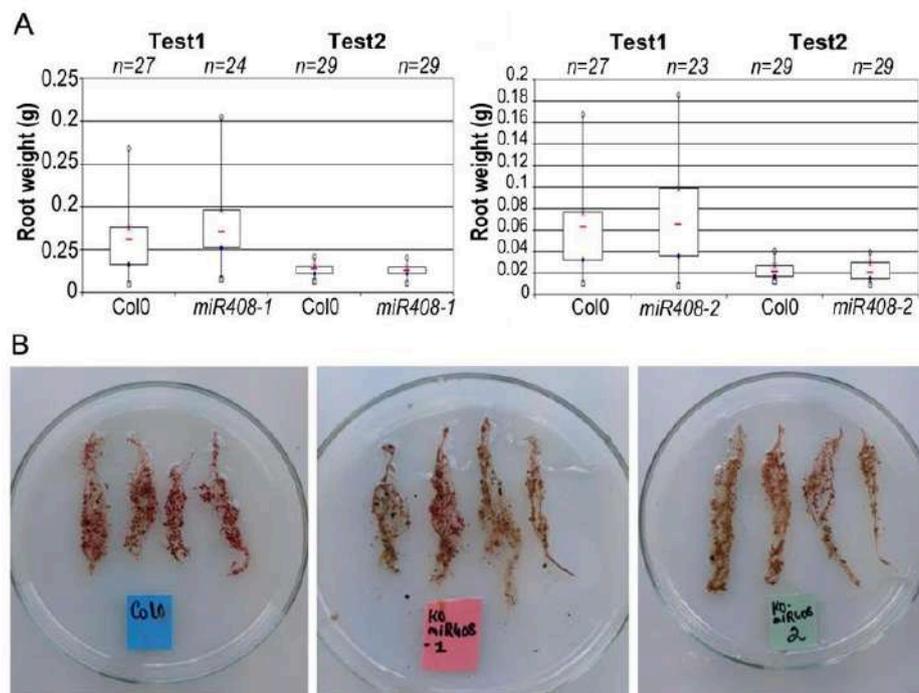
with *M. incognita*

Supplemental Data Set 5: Nematode (J2) survival after being incubated for 24 hours in a CuSO₄ solution.

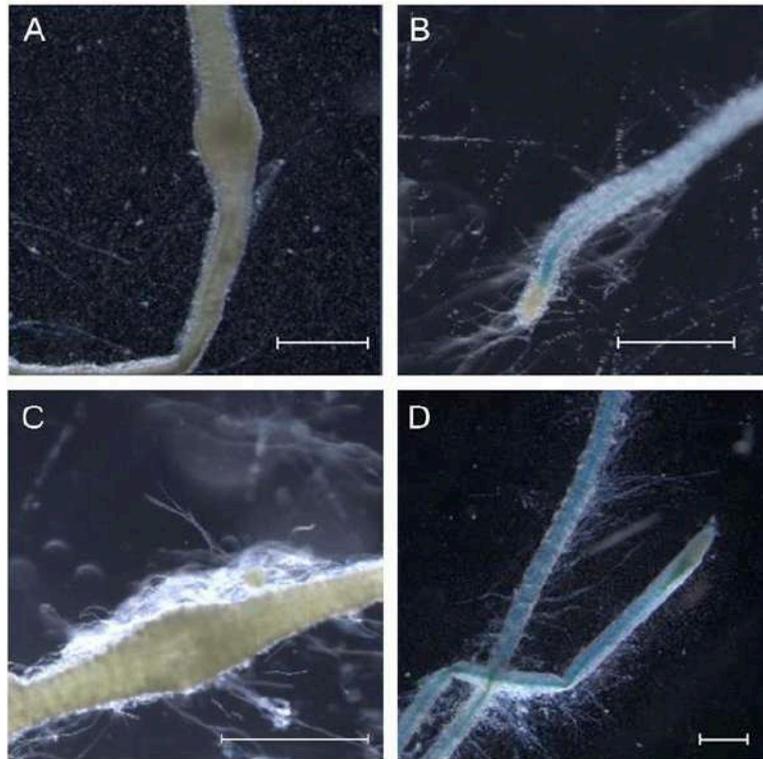
Supplemental Data Set 6: Infection assays of *spl7* ko line infected with *M. incognita*.

Supplemental Data Set 7: Infection assays of Col0 plants infected with *M. incognita* and watered with 50 μ M CuSO₄ solution.

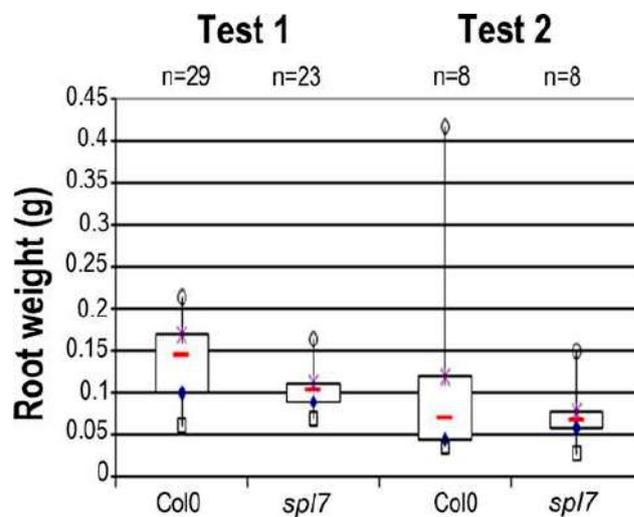
Supplemental Table S1: Expression level of mature sequence of the miR408 and miR398 families upregulated in *A. thaliana* and tomato (*S. lycopersum*) galls



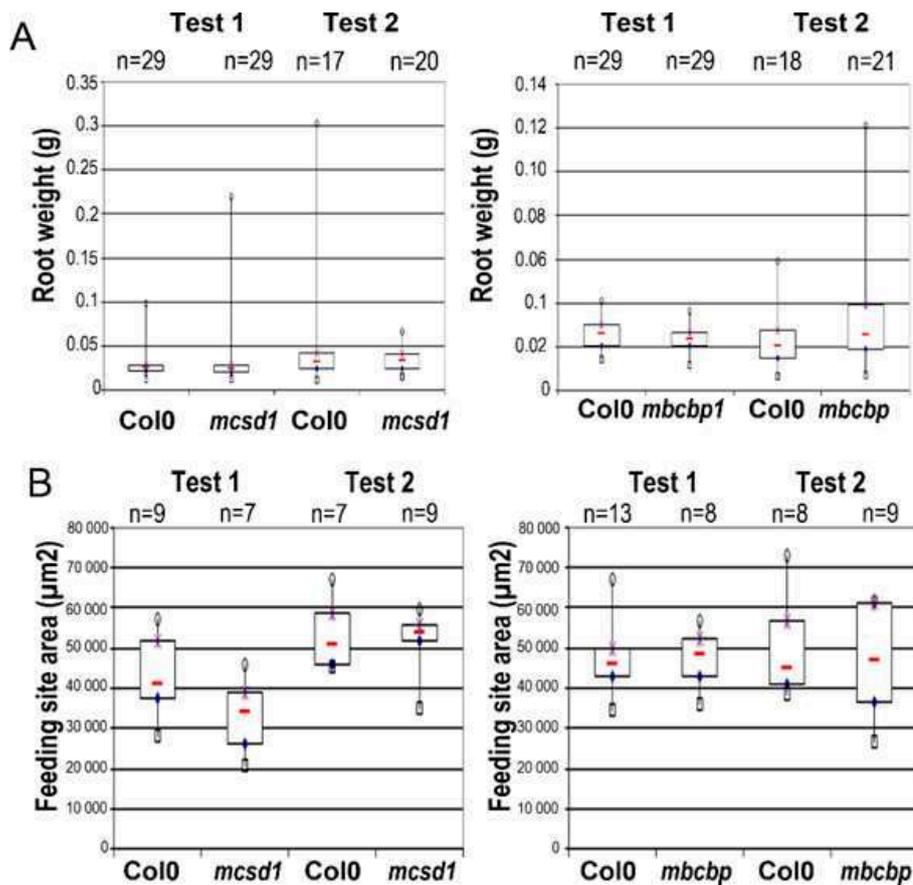
Supplemental Figure S1. Root phenotype of the *miR408-1* and *miR408-2* KO lines. A, Weight of infected roots from the two *miR408* KO lines relative to that of wild-type Col0 plants, seven weeks after infection. Mann and Whitney tests showed that there was no significant difference in weight between the roots of Col0 plants and those of the two KO lines. B, Images of four root systems per line. Open squares, minimum values; open circles, maximum values; red lines, median values; blue diamond, first quartile; purple star, third quartile.



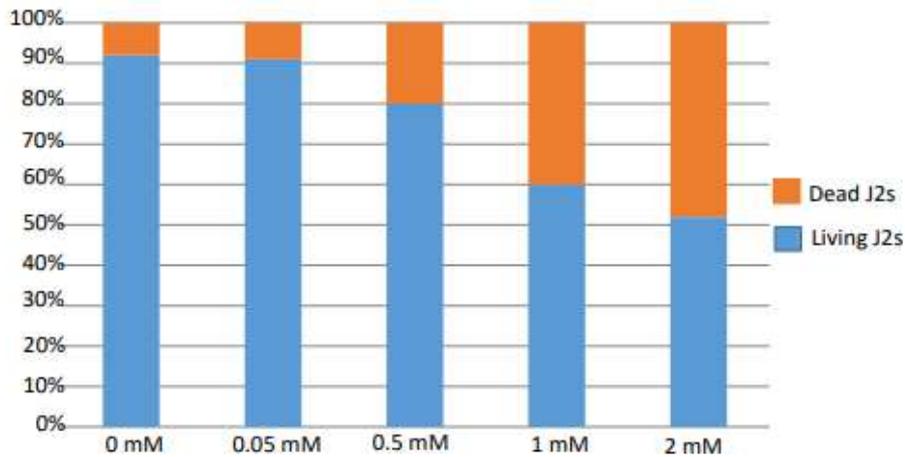
Supplemental Figure S2. Localization of *pMIR408* activity in fully developed galls. A, C, MIR408::GUS activity was observed in galls from *A. thaliana* induced by *M. incognita* examined at 21 dpi (A) and 28 dpi (C). B, D, a GUS signal was observed in root tips from uninfected plants at the same stage. Bars: 500 μ m.



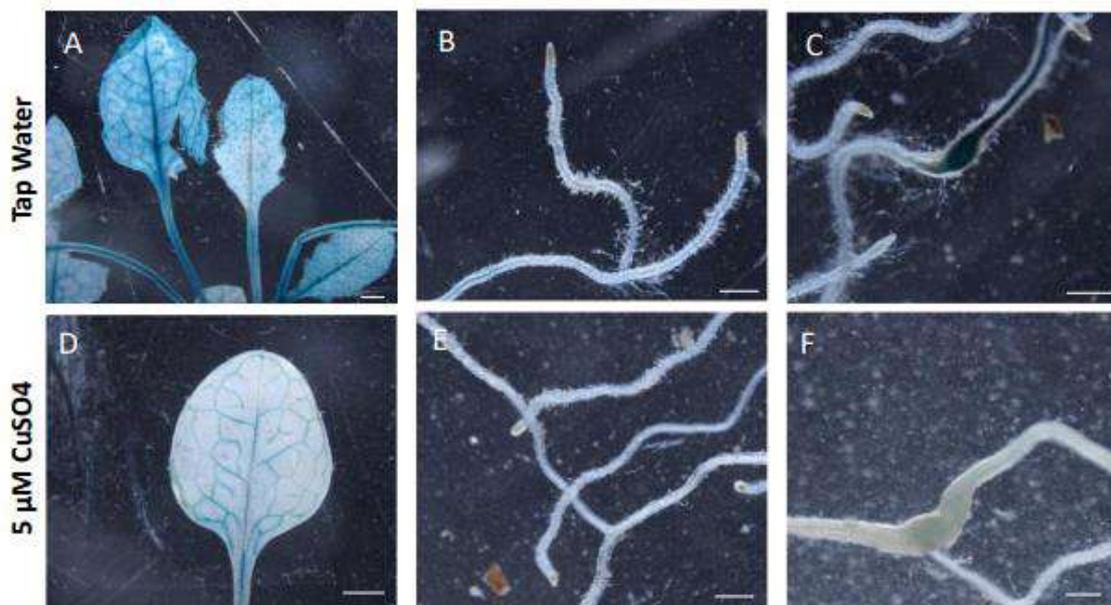
Supplemental Figure S3. Root phenotype of the *spl7* KO line. Weight of infected roots from *spl7* KO lines relative to the weight of roots from infected Col0 plants seven weeks after infection, as assessed by Mann & Whitney statistical tests. No difference in weight was observed between the roots of the Col0 wild type and the KO line.



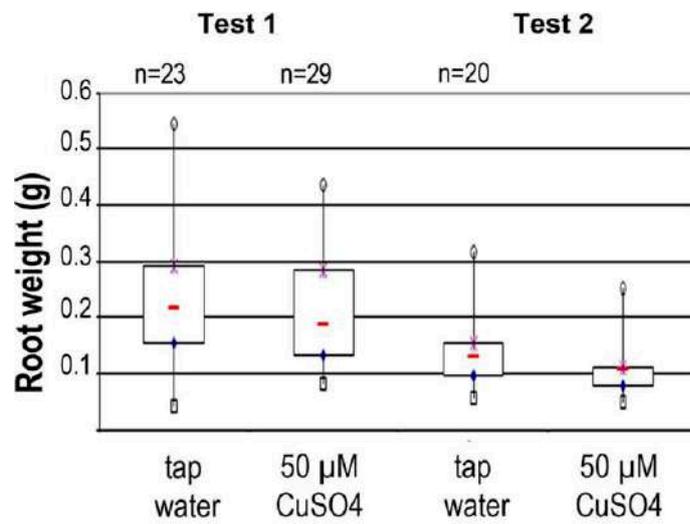
Supplemental Figure S4. Root phenotype and area covered by the feeding site in the *mcsd1* and *mbcbp* lines. (A), Weight of infected roots from the *mcsd1* and *mbcbp* lines relative to that for Col0 plants, seven weeks after infection, as assessed by Mann & Whitney statistical tests. No difference in weight was observed between the roots of the wild-type Col0 and those of the two mutant lines. (B), The effect of mCSD1 and mBCBP mutations on the development of giant feeding cells was evaluated further, by measuring the size of the feeding site produced in each KO line and compared it with that in wild-type roots. Galls were collected seven weeks after *in vitro* infection for measurement of the area (μm^2) covered by the giant cells by the BABB clearing method (Cabrera et al., 2018). The impact of plant genotype on the area of giant cells was analysed in Mann and Whitney tests. *, $P < 0.05$. Open squares, minimum values; open circles, maximum values; red lines, median values; blue diamond, first quartile; purple star, third quartile.



Supplemental Figure S5. Assessment of copper toxicity in *M. incognita* J2s. Living and dead J2s were counted after 24 hours of incubation in various concentrations of copper sulphate.



Supplemental Figure S6. Repression of *pMIR408* activity in galls by watering with CuSO_4 , assessed at 14 dpi. A-C, Activity of *pMIR408::GUS* in the leaves of plants watered with tap water (A), in the root tips (B) and in galls (C). Activity of *pMIR408::GUS* in the leaves of plants watered with $50 \mu\text{M}$ CuSO_4 (D), (e) in root tips (E) and in galls (F).



Supplemental Figure S7. Root phenotype of plants watered with 50 μM copper sulphate. Weight of infected roots from plants watered with 50 μM copper sulphate was compared to the weight of roots from infected plants watered with tap water seven weeks after infection. No difference in weight was observed between the two treatments as assessed by Mann & Whitney statistical tests.

miRNA family	miRNA name	Plant species	miRNA mature sequence	Base mean G 7dpi	Base Mean R7dpi	logFC G/R 7dpi	adj. pvalue	Base mean G 14dpi	Base mean R 14dpi	logFC G/R 14dpi	adj. pvalue
miR408	SL3.0ch01_346	<i>S. lycopersicum</i>	TGCACA GCCCTCTCCCTGGCT	190.931157621242	14.572605027777	3.141	0.000	169.917951763724	17.2014654672013	3.269	0.017
	SL3.0ch01_419	<i>S. lycopersicum</i>	TGCACTGCCCTCTCCCTGGCT	40.40.9930128031	1656.5630688061	1.237	0.029	2176.28163480235	480.434726753192	3.133	0.033
	Ath-miR408	<i>A. thaliana</i>	ATGCACTGCCCTCTCCCTGGC	20642.5866335013	5067.26816498732	2.042	0.042	13085.3889541097	2746.36032511984	2.411	0.001
miR398	SL3.0ch12_14_3	<i>S. lycopersicum</i>	ATGTGTTCTCAGGTTACCCCT	6707.53918689119	2254.58026280341	3.272	0.009	4689.15660323759	1044.66293889578	2.166	0.066
	SL3.0ch05_225	<i>S. lycopersicum</i>	TTGTGTTCTCA GGTCA CCCCT	162.472551824703	144.468350467079	0.290	0.863	1688.1238660157	157.150115939722	3.430	0.015
	SL3.0ch11_437	<i>S. lycopersicum</i>	TATGTTCTCAGGTGGCCCTG	19433.5756367137	9779.29785028973	2.144	0.112	11410.6351654164	3371.26716225445	1.759	0.067
	ath-miR398b-c	<i>A. thaliana</i>	TGTGTTCTCAGGTCA CCCCTT	25237.7043496972	4539.02505137682	2.464	0.119	28637.8650925948	3347.47844474666	3.287	0.000

Table S1.

Expression level of mature sequence of the miR408 and miR398 families upregulated in *A. thaliana* and tomato (*S. lycopersum*) galls were identified by small RNA Illumina (for tomato samples) or SOLID (for *Arabidopsis* samples) sequencing followed by DESeq 2 statistical analysis. MicroRNAs significantly upregulated in galls in comparison to uninfected roots (adjusted pvalue <0.05) are indicated in red. The sequence and base mean of mature microRNAs are indicated.

Supplemental material

Biological Materials, Growth Conditions

Seeds of *Solanum lycopersicum* cv St Pierre were surface-sterilized with chlorine solution (44% active chlorine) and washed three times with 1 ml of milli-Q water. 10 to 15 sterile seeds were sown on a Gamborg B5 medium agar plates (0.5 x Gamborg, 1% sucrose, 0.8% agar, pH 6.4), placed at 24°C for 48 hours for germination, and finally transferred in a growth chamber (8h light; 16h dark, 20°C). *M. incognita* strain Morelos' J2s were sterilized with HgCl₂ (0.01%) and streptomycin (0.7%) as described before (Caillaud & Favery, 2016). One to two weeks after germination, roots were inoculated with 1,000 sterile J2s resuspended in phytigel (5%) per petri dishes.

RNA extraction

Total RNAs, including small RNAs (< 200 nt), were isolated from in vitro galls or uninfected roots at 7 and 14 dpi. Approximately 40 galls or uninfected roots devoid meristems were independently frozen into powder by using a tissue lyser (Retsch; MM301) at 30 Hertz frequency for 30 seconds with 4mm tungsten balls (Retsch; MM301). Total RNAs were extracted from these samples with the miRNeasy Mini Kit (Qiagen), according to the manufacturer's instructions, with three additional washes in RPE buffer.

RNA sequencing

Small RNA libraries were generated by ligation, reverse transcription and amplification (11 cycles) from total RNAs (1 µg), with the reagents of the NEBNext Small RNA Library Prep Set for Illumina. Libraries were then quantified with the Bioanalyzer High Sensitivity DNA Kit (Agilent) and sequenced at the Nice-Sophia Antipolis functional genomics platform (France Génomique, IPMC, Sophia Antipolis, France).

miRNAs Analysis

For each small RNA library, adapters were trimmed and reads matching ribosomal RNAs, mitochondrial RNAs and repeat sequences were removed by performing Blast analyses with the sequences listed in the Rfam database (Nawrocki et al., 2015). The Bowtie aligner was then used to align the trimmed reads (Langmead et al., 2009) on a virtual concatenated genome generated from the *Solanum lycopersicum* genome (V3.0) and the *M. incognita* genome (V2.0; Blanc-Mathieu et al., 2017). Each read was attributed to the *S. lycopersicum* and/or *M. incognita* genome on the basis of the best alignment obtained. Reads with identical best alignment hits for the two genomes were attributed to both the nematode and the plant. If one read aligned to multiple genomic locations, a single read was attributed to each locus. Lowquality mapped reads were removed and reads corresponding to molecules of between 20 and 24 nt in size were retained for further analysis. De novo microRNA encoding genes were

predicted in tomato genome V3.0 by using three algorithms MirCat (Paicu et al., 2017), Shortstack (Axtell,2013) and MirDeep plant (Yang & Li, 2011) with default parameters. The HTSEQCOUNT package (Anders et al., 2014) was used to count reads mapping perfectly onto the predicted *S. lycopersicum* mature microRNA 5P or 3P sequence. Reads mapping to multiple loci were counted for each of the loci concerned. The counts for mature miRNAs (5P and 3P) from each replicate were used for differential expression analysis by using DSeq2 statistical analysis (Andres & Huber, 2010). Mature miRNAs with an adjusted p value below 0.05 were considered as differentially expressed.

Complementary results

In addition to the results presented in the article submitted to The Plant Cell, additional analyses were performed. The expression profiles of key genes of Casparian Strip (CS) was investigated in available *A. thaliana* transcriptome and measurement of copper and other micronutrients has been performed by Dr. Sébastien Thomine at I2BC institute, Gif-sur Yvette, France.

1) Expression profile of genes involved in the formation of CS

In order to identify the expression profile for the genes involved in the CS strip formation, we analyzed in the transcriptomic data of *A. thaliana* galls at 3, 5 and 7 dpi (Yamaguchi et al., 2017) (**Table 3**). These data showed a global repression of most genes involved in the formation and lignification of CS. Surprisingly, *MYB36*, the transcription factor that orchestrates the CS formation, is upregulated in galls at 3, 5 and 7 dpi, while most of *MYB36* targets are downregulated. These results suggest that RKN induce a defect in CS formation through a disruption of copper homeostasis.

2) Measurement of Copper and other nutrients

The nutrient content (copper, iron, calcium, magnesium, manganese and zinc) in dissected galls, whole infected and uninfected roots of tomato and *A. thaliana* was performed by Microwave Plasma-Atomic Emission Spectroscopy (MP-AES).

Since sampling tomato galls *in vitro* is easy, we first measure the level of micronutrients in galls and uninfected roots collected at 14 dpi *in vitro* (**Table 4a**). A reduction of copper level in tomato galls compared to uninfected roots was observed in the three independent replicates. This result supports our hypothesis of a decrease of copper level in galls. A reduction of Fe, Mn and Zn was observed in tomato galls compared to the roots. Then the level of nutrients was measured in whole infected (RI) and uninfected roots (RNI) *in soil*, from 3 independent replicates. A similar reduction of copper level is observed in whole infected roots compared the uninfected roots (**Table 4b**).

For the measurement in *A. thaliana*, we didn't measure nutrients level in galls *in vitro* due to the need of important quantities of material for the experiment. We measured the level of micronutrients in galls and uninfected roots collected at 14 dpi *in soil*, from three independent replicates (**Table 5a**). A slightly reduction of copper level was observed in galls compared to uninfected roots in the first and third replicate. Since the expression of *MIR408* decreased in galls at 14 dpi (*mir408::GUS*), this suggested that the copper level decrease at 14 dpi. Therefore, we measured the copper at earlier stage of gall development. Since the experiment required important quantities of material, we have collected whole infected roots at 4 dpi *in soil*. Surprisingly, we observed an increase of copper level in whole infected roots (RI)

compared the uninfected roots (RNI) (**Table 5b**). This results suggest that the decrease of copper in *A. thaliana* infected by RKN occurs specifically in galls. However, the disruption of copper in *A. thaliana* galls should be more investigated.

Table 3 Expression profile of *A. thaliana* genes involved in the formation of the Casparian strip. The accession number of each gene is indicated. Log Fold Change (LogFC) of galls versus roots. Green: Repression. Red: Overexpression. Grey: No differentially expressed.

Accession numbers	Genes	LogFC G/R		
		3 dpi	5 dpi	7 dpi
<i>AT5G57620</i>	<i>MYB36</i>	1.426958097	2.85992883	2.04477592
<i>AT2G36100</i>	<i>AtCASP1</i>		-1.23628349	-2.43959916
<i>AT2G27370</i>	<i>At CASP3</i>		-1.20148756	-2.52356238
<i>AT3G11550</i>	<i>AT CASP2</i>		-1.12596114	-2.45809689
<i>AT5G06200</i>	<i>CASP4</i>		-2.12021478	-0.55032069
<i>AT5G15290</i>	<i>CASP5</i>		-4.83139598	-3.12361091
<i>AT2G32300</i>	<i>UCC1</i>			-1.81533822
<i>AT2G44790</i>	<i>UCC2</i>			-0.796376
<i>AT2G28670</i>	<i>ESB1</i>			
<i>AT5G50150</i>	<i>LOTR</i>			
<i>AT1G61590</i>	<i>SGN1</i>	-0.885013288		-5.85324429
<i>AT4G20140</i>	<i>SGN3</i>	-0.563039621		-0.93063269
<i>AT5G03540</i>	<i>ATEXO70A1</i>	-0.331663576	-0.53983707	-0.55032069
<i>AT1G64060</i>	<i>RBOHf</i>			
<i>AT5G42180</i>	<i>PER64</i>		-1.44517059	-2.4952277

Table 4 Measurement of nutrients in Tomato. (a) Measurement of nutrients in tomato galls and uninfected roots collected *in vitro* at 14 dpi (b) Measurement of nutrients in and whole infected roots (RI) and uninfected roots (RNI) collected *in soil* at 14 dpi.

(a)

	Ca	Cu	Fe	Mg	Mn	Zn
Roots 1	2230	6.3	228	1836	996	137
Galls 1	3584	3.9	88	4066	178	83
Roots 2	3957	2.9	311	2730	1063	146
Galls 2	2363	1.5	103	1749	185	61
Roots 3	2149	3.0	1269	1221	548	273
Galls 3	2713	1.7	143	3048	360	87

(b)

	Ca	Cu	Fe	Mg	Mn	Zn
RNI 1	12940	33.4	1504	14275	615	69.0
RI 1	5316	10.1	720	4372	89	20.4
RNI 2	13760	29.9	2396	12916	591	72.5
RI 2	13349	23.2	2554	11821	222	49.2
RNI 3	9439	42.5	1325	9715	666	67.0
RI 3	16874	24.5	2725	6543	376	54.6

Table 5 Measurement of nutrients in *A. thaliana*. (a) Measurement of nutrients in *A. thaliana* galls and uninfected roots collected *in soil* at 14 dpi (b) Measurement of nutrients in *A. thaliana* in whole infected roots (RI) and uninfected roots (RNI) collected *in soil* at 4 dpi.

(a)

	Ca	Cu	Fe	Mg	Mn	Zn
Roots 1	12554.66	39.05	1889.7	3172.08	924.81	225.4
Galls 1	14256.4	33.87	1520.76	4001.83	297.09	202.09
Roots 2	19481.662	27.28	2430.33	3394.7	3504.645	190.9
Galls 2	15882.17	26.98	2124.29	3783.46	2384.28	177.59
Roots 3	25169.89	36.12	4829.11	4411.05	4121.32	160.54
Galls 3	15760.16	20.03	1627.41	3482.34	2120.82	151.66

(b)

	Ca	Cu	Fe	Mg	Mn	Zn
RNI 1	14025	14.4	1668	2255	129	66.5
RI 1	12963	31.7	1574	3114	829	83.7
RNI 2	8580	10.1	1602	1530	141	47.1
RI 2	18961	32.8	2176	4497	943	82.8
RNI 3	14001	16.4	1649	2410	192	56.6
RI 3	16669	33.6	2129	3091	979	84.2
RNI 4	15684	17.0	2026	2456	192	70.8
RI 4	16387	33.1	1917	3290	1299	90.8
RNI 5	10849	13.3	1435	2144	258	65.5
RI 5	16936	36.9	3027	3765	928	87.6
RNI 6	12713	14.3	993	1904	199	56.8
RI 6	17396	27.2	2289	3447	695	74.6

Discussion

1. Sequencing analysis in tomato

Understanding the transcriptional modifications that result in the formation of giant feeding cells, enables to understand the molecular dialogue between the plant and the nematode. The development of NGS made transcriptomic analysis possible in different non model plant species (reviewed in Favery et al., 2016). All the different transcriptomic analyses presented in the introduction, showed the profound molecular changes observed in forming giant cells: approximatively 15 % of genes are differentially expressed in galls compared to the uninfected roots. Although the biological functions of these genes are starting to be deciphered, little is known about the regulators of gene expression reprogramming. The regulation of gene expression by microRNAs involved in giant cell formation induced by RKN was first evidenced in 2015 (reviewed in Jaubert-Possamai et al., 2019). My PhD aimed to characterize the microRNAs involved in the formation of giant feeding cells induced by *M. incognita* in a plant of agronomic interest: tomato (*Solanum lycopersicum*). This characterization was performed at two key kinetic points of formation of giant feeding cells, which corresponds to the first phase of successive mitosis without cytokinesis (7 dpi) and a later phase of endoreduplication and cell growth (14 dpi).

1.1 Identification of miRNAs differentially expressed in tomato galls

Illumina sequencing was performed enabling the analysis of messenger RNAs (mRNA) and small RNAs (<200 nt) expressed in galls and uninfected tomato roots 7 and 14 dpi. Bioinformatics and statistical analysis identified 174 microRNA differentially expressed between galls and uninfected roots at 7 and/or 14 dpi. A comparable analysis was carried out in 2017 in our lab with *A. thaliana* (Medina et al., 2017). Surprisingly, we found only two miRNAs (miR408 and miR398) that share the same expression profile in *A. thaliana* and tomato galls. In the literature, comparison of small RNA sequencing in different plant species (Jaubert-Possamai et al., 2019) identified very few miRNAs with the same profile expression. miR408 and miR827 upregulated in our tomato data, were also upregulated in cotton galls at 10 dpi (Pan et al., 2019). This few similarities of conserved miRNA expression profile seems to rely on the phase of feeding site development and the biological material.

Two genome wide analyses of RKN-responding microRNAs in tomato have been previously published (Zhao et al., 2015; Kaur et al., 2017). Zhao *et al.*, (2017) identified miRNAs expressed in whole root infected by *M. incognita* at early stage of gall formation (from 6 hours post infection until 3 dpi), in wild-type plants and in *spr2* (suppressor of prosystemin-mediated response 2, JA-deficient) mutant disrupted in the acid jasmonic pathway and resistant to RKN. On the other hand, the work of Kaur *et al.*, (2017) identified miRNAs expressed in whole root infected by *M. incognita* at five stage of nematode

infection with stage 2 (5, 6 and 7dpi) and stage 3 (12, 13, 14 dpi) that overlap with our analyses. The biological material (whole root vs hand dissected galls) and/or the timing of infection kinetic and/or the number of replicates of these studies are different from our analysis. It is therefore difficult to compare the data between these three studies. Despite this, some RKN responsive tomato microRNAs are shared by our analyses and Kaur *et al.*, like the miR164 family which is upregulated in galls at 14 dpi, or miR169 which is upregulated at 7 dpi.

Many analyses of miRNAs involved in plant-CN interaction were also performed in different plant species (Hewezi *et al.*, 2008; Świącicka *et al.*, 2017; Tian *et al.*, 2017; Koter *et al.*, 2018; Noon *et al.*, 2019). CN and RKN are closely related nematode that induce feeding sites within host plant. As RKNs have a large range of hosts, similar giant cells are observed in almost all vascular plants while host range of CN is more restrained. The formation of feeding sites by RKN and CN differ in several points. Following intercellular migration, RKN J2 selects five to seven parenchyma cells and induces their dedifferentiation into giant feeding cells through successive mitosis without cytokinesis. By contrast, CN J2 targets a single initial root cell that expands within the vascular tissue by progressive cell wall dissolution and incorporation into the syncytium of adjacent cells via cytoplasm fusion (Golinowski *et al.*, 1996; Grundler *et al.*, 1998). While endoreduplication occurs in giant cells and syncytia, the successive mitoses without cytokinesis is specific of giant feeding cells (Huang and Maggenti, 1969; De Almeida Engler *et al.*, 2004). In contrast, cell fusions following partial cell wall dissolution are only observed in syncytia. Although they differ in their ontogeny, syncytium and giant cells have similar phenotype. Both of these feeding sites are hypertrophied and multinucleated, highly active metabolically, have a dense cytoplasm (Sobczak and Golinowski, 2011; Favery *et al.*, 2016) and accumulate sugars and amino acids (Hofmann *et al.*, 2010; Baldacci-Cresp *et al.*, 2012). Cell wall composition of syncytium and giant cells appears similar and its mainly composed of polysaccharides (Rodiuc *et al.*, 2014). In addition, both feeding sites develop cell wall ingrowth to facilitate the uptake of solutes (Offler *et al.*, 2003). Around both syncytia and giant cells, sieve elements are interconnected by PDs at early stage of feeding site formation. On the contrary of giant cells, symplastic transport enables movement of nutrients into syncytia (Hoth *et al.*, 2005; Hoth *et al.*, 2008). Expression profile of conserved miRNAs DE between giant cell or syncytia and giant cells have different expression profiles. The difference of miRNAs expression profile can be explained by the distinct ontogenesis of these two feeding sites (reviewed in Jaubert-Possamai *et al.*, 2019). However, we find two miRNAs families with the same expression profile in giants cells and syncytium in tomato: miR396 and miR167 families that are downregulated in our data and in syncytium induced in tomato infected by *Globodera rostochiensis* (Świącicka *et al.*, 2017; Koter *et al.*, 2018).

1.2 Degradome analysis

A degradome sequencing was performed from gall RNAs to understand the function of the miRNAs DE in tomato galls. From 153 transcripts identified as targeted by microRNAs in galls only 12 were selected as robust candidates. However, this is a very stringent selection and expression profile of microRNAs and their biologically validated targets are not always negatively correlated. Firstly, some transcripts can be targeted by several microRNAs with different expression pattern. MiR159 and miR319 are closely related microRNAs that share some targets like *MYB* and *TCP* (Palatnik et al., 2007). Gall degradome data showed that these two microRNA families shared four targets in galls 7 and 14 dpi but displayed different expression profile in galls. On the other hand, one miRNA can target multiple genes. For example, the genes of miR396 family that are downregulated in galls, targets different genes, *GFR* and basic helix-loop-helix (*bHLH*), that are also repressed. Moreover, a feedback regulation of a miRNA by its own target is common in plant like the negative feedback loop involving miR172 that is positively regulated by the transcription factors *TARGET OF EARLY ACTIVATION TAGGED (TOE1 and TOE2)* it targets (Wu et al., 2009). Therefore, additional DE miRNAs/mRNA target pairs identified by degradome analyses may be involved in plant RKN interaction despite the absence of negatively correlated expression profile.

From these 12 miRNA/target pairs, three families of microRNAs were selected for functional analysis: miR167, miR408 and miR398. MiR408 and miR398 were selected due to the conservation of their expression profiles in *A. thaliana* and in tomato while miR167 was selected based on its role in auxin signaling, a main process for the formation of giant cells.

2. miR167/ARF8: an auxin responsive module involved in gall formation

2.1 Auxin and ARFs

Among the various phytohormones, auxin plays a role in the formation of giant feeding cells (reviewed in Gheysen and Mitchum, 2019). As presented in introduction auxin peaks have been showed in formation of RKN induced feeding site. Likewise, auxin-mimicking compounds have been found in nematode secretions (De Meutter et al., 2003; De Meutter et al., 2005). This phytohormone regulates several mechanisms in plants such as cell division, organ differentiation, embryogenesis or lateral root initiation (Quint and Gray, 2008; Majda and Robert, 2018).

Auxin regulates a large number of genes that are involved in plant growth and development processes in many plant species (Zouine et al., 2014; Guilfoyle, 2015). Auxin signaling pathway is a complex mechanism involving multiple gene families. *A. thaliana* contains 6 auxin receptors of the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING

F-BOX PROTEIN (TIR1/AFB) family, 29 AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) repressors, and 23 ARFs transcription factors (Remington et al., 2004) The distribution of auxin is achieved via the auxin transporters such as PINFORMED1 (PIN) and AUXIN1/LIKE AUX1 (AUX/LAX) family (Bainbridge et al., 2008; Adamowski and Friml, 2015) (**Figure 23 A**). ARFs mediate auxin signaling by directly transmitting auxin response through the activation or repression of auxin-induced genes (Liscum and Reed, 2002). ARFs are a large multigene family conserved across plant kingdom that is well described in various plant species such as *A. thaliana* (23 genes) (Hagen and Guilfoyle, 2002), *S. lycopersicum* (22 genes) (Zouine et al., 2014), *Oryza sativa* (25 genes) (Wang et al., 2007) or *Glycine max* (51 genes) (Van Ha et al., 2013).

In high level of auxin, ARF8 together with ARF5 ARF6, ARF7, and ARF19 (class II of ARF) activate transcription of auxin-responsive genes (Tiwari et al., 2003) (**Figure 23B, 23C**). In *A. thaliana* roots, the role of ARF8 have been described in the formation of lateral roots (Gifford et al., 2008) and adventitious roots (Gutierrez et al., 2009). Recently, the role of ARF6 and ARF8 was attributed to cambium establishment and maintenance (Ben-Targem et al., 2021). *arf6arf8* double mutant displayed decrease in xylem occupancy and absence of fiber accumulation until very late stages of plant growth. This regulation is via a cross-talk between GA and auxin.

2.2 Regulation of ARF8 in tomato galls

Gall transcriptomic analyzes showed that *ARF8B* is overexpressed in tomato galls at 7 and 14 dpi while *ARF8A* is overexpressed at 14 dpi. Using tomato transgenic lines expressing the promoter of *ARF8A* and *ARF8B* fused to *GUS*, we confirmed RNAseq results and located the expression of both genes in giant cells and neighboring cells at 7 and 14 dpi.

Degradome analysis identified *ARF8A* and *ARF8B* as cleaved by miR167 in galls. In tomato, four *MIR167* genes sharing the same mature sequence were identified. They are all down regulated in galls at 7 and 14 dpi. In *A. thaliana* and in tomato, *ARF6* and *ARF8* have been shown to be targeted by miR167 (Wu et al., 2006; Liu et al., 2014). In tomato, the two *ARF8* and two *ARF6* genes are cleaved by miR167 (Liu et al., 2014). The overexpression of miR167 in tomato, leads to a downregulation of *ARF8* and *ARF6* genes which caused defect in flower maturation and fertility. *ARF6* and *ARF8* are closely related putative ARFs with both independent and cooperative functions in plant development (Nagpal, 2005; Gutierrez et al., 2009; Gutierrez et al., 2012; Liu et al., 2014; Wang et al., 2015a). However, they are expressed differentially throughout plant development (Rademacher et al., 2011; Vernoux et al., 2011).

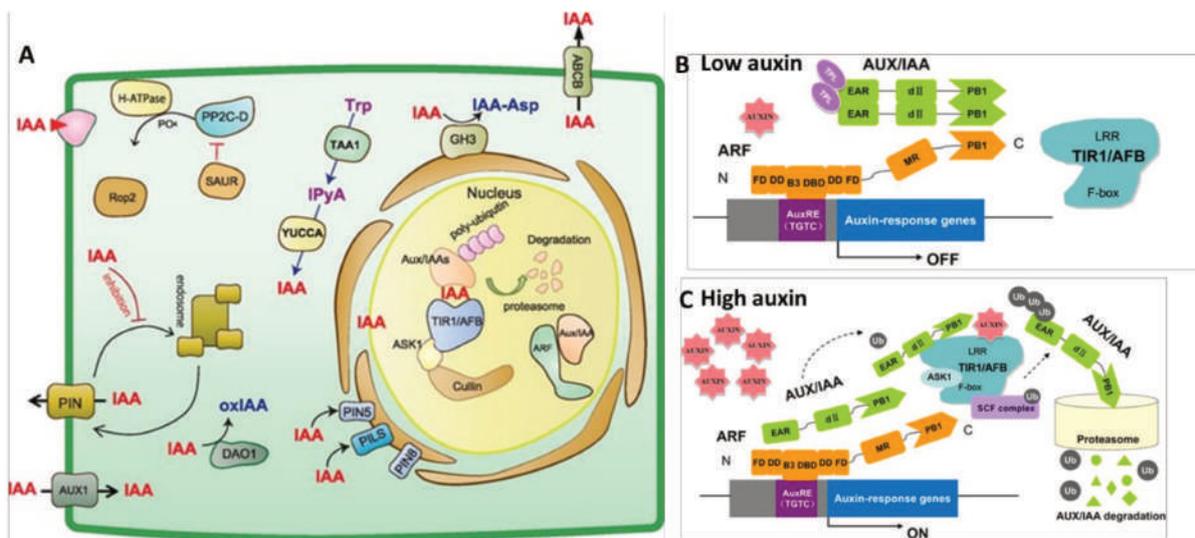


Figure 23. Auxin signaling in plant cell

(A) Cellular model of auxin biosynthesis, transport and signaling. IAA is biosynthesized from tryptophan (Trp) via indole 3-pyruvic acid (IPyA) by TAA1 and YUCCA in the IPyA pathway. IAA is incorporated by AUX1/LAX1 symporters and exported by PIN and ABCB proteins. ER-localized PIN and PLS carrier proteins regulate the homeostasis of cellular IAA. Auxin blocks the clathrin-mediated endocytosis of PIN from the plasma membrane. The SCFTIR1/AFB, E3 ubiquitin ligase complex is composed of SKP (ASK1), Cullin and F-box protein (TIR1/AFB). Auxin induces the ubiquitination of Aux/IAA proteins via SCFTIR1/AFB. The degradation of Aux/IAA repressor recovers ARF activity to activate the transcription of auxin-responsive genes. The SAUR proteins induced by auxin inhibit PP2C-D phosphatases, resulting in the activation of H⁺-ATPase to induce a rapid auxin response such as turgor-induced growth. IAA is converted to IAA-aspartate (IAA-Asp) by the auxin-induced GH3 enzyme IAA-amino acid conjugate synthase. IAA is oxidized to 2-oxo-IAA (oxIAA) by DAO1 dioxygenase (Fukui and Hayashi, 2018). **(B)** At low auxin levels, the Aux/IAA proteins form multimers with ARFs and recruit TPL to the chromatin. **(C)** High levels of auxin promote ubiquitination and degradation of Aux/IAs through SCFTIR1/AFB and the 26S proteasome (Li et al., 2016b)

qRT-PCR analysis showed that *ARF6B* displayed a low expression in all tomato tissues, including the root (Zouine et al., 2014). In tomato roots, the level expression of *ARF6A*, *ARF8A* and *ARF8B* is very low, with a higher expression of *ARF6A*, probably due to the cleavage by miR167 (Liu et al., 2014; Zouine et al., 2014). However, neither *ARF6A* nor *ARF6B* have been identified as targeted by miR167 in degradome. Therefore, I propose a model of ARF8 regulation in tomato roots and galls (**Figure 24**). In roots, *ARF8* transcripts are cleaved by miR167. During RKN infection, the inhibition of miR167 in galls prevents the cleavage of *ARF8*, together with auxin peak, its allows an overexpression of *ARF8*.

Although the repression of miR167 prevents post-transcriptional silencing of *ARF8A* and *ARF8B* in galls, how the expression of these transcription factors is activated in galls needs to be further investigated. Recently the transcriptional regulation of *ARF8* by a complex network of multiple activating and repressing transcriptional factors has been shown in *A. thaliana* figure (Truskina et al., 2021). This work showed that most TFs regulating *ARF8* expression are involved in plant development such as WUSCHEL but many *ARF8* regulators are associated with biotic and abiotic stress. The authors proposed that *ARF8* may act as an environmental hub mediating auxin responsiveness. The formation of feeding sites by RKN interferes with plant developmental processes, suggesting that nematode may hijack these mechanisms. In *A. thaliana*, miR167/*ARF8* controls the balance between initiating and emerging lateral roots in relation to nitrogen availability (Gifford et al., 2008). Indeed, several genes involved in lateral root formation were shown to be required for the formation of giant cell. The transcription factor, LBD16 that is activated by auxin is a perfect example on the role of auxin gene in lateral root development and also in gall formation (Cabrera et al., 2014b).

2.3 ARF8 is involved in plant response to microorganisms

In order to understand if *ARF8* plays a role in nematode parasitism, we used tomato lines with CRISPR deletion within *ARF8A*, *ARF8B* and *ARF8AB* coding sequence (provided by M. Zouine). *arf8a*, *arf8b* and *arf8ab* lines showed a resistance towards nematode infection. Moreover, phenotyping of giant cells in cleared galls showed a reduced size of giant cells within the two crisper lines. These defects associated to CRISPR lines confirmed that expression of *ARF8A* and *ARF8B* genes play a key role in the tomato-RKN interaction and are necessary for a proper formation of giant cell.

During biotic stresses, different ARFs, including *ARF8*, have shown to be regulated in leaves under different biotic stress such as Flagelline and *Pseudomonas syringae* (Bouzroud et al., 2018). A recent study highlighted the role of miR167 and its two targets *ARF6* and *ARF8* against *P. syringae* pv. tomato DC3000 in *A. thaliana* (Caruana et al., 2020). In this study, the authors showed that *A. thaliana* plants overexpressing miR167a had smaller stomatal apertures with less symptoms of infection by *P. syringae*. *arf6 arf8* double mutant were also resistant to *P. syringae*, with the same phenotypes as plants overexpressing miR167, suggesting that miR167 modulates defense through these two targets. These results suggest that the resistance observed in plants overexpressing miR167 is due to the suppression of

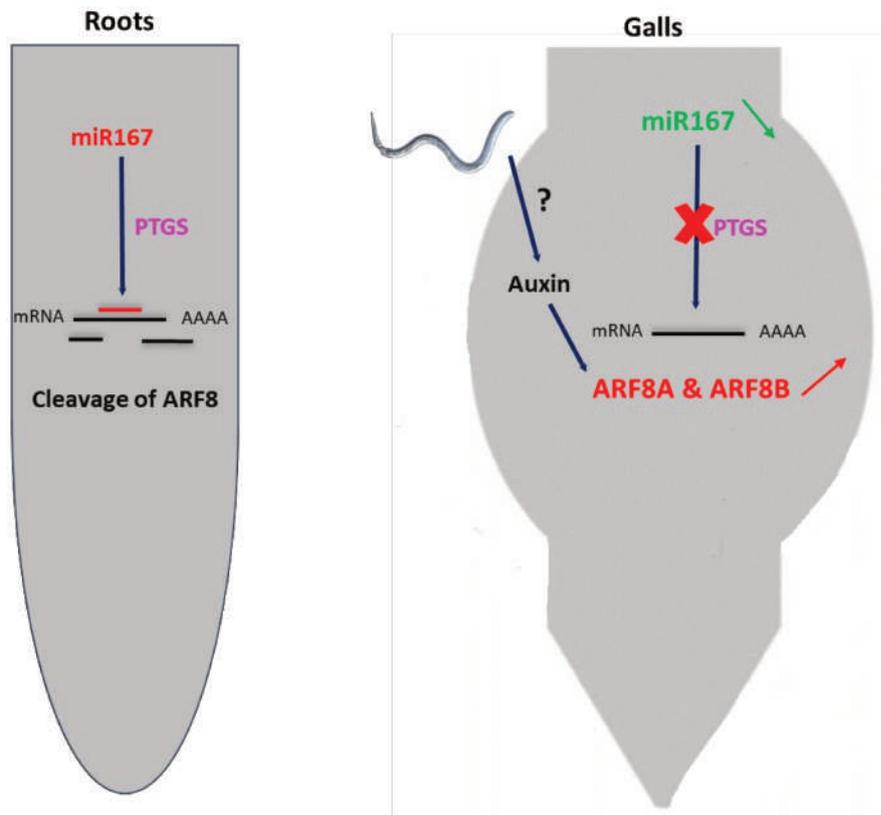


Figure 24. A model of *ARF8* regulation in tomato roots and galls

In roots, *ARF8* transcripts are cleaved by miR167. During RKN infection, the inhibition of miR167 in galls prevents the cleavage of *ARF8*, together with auxin peak, its allows an overexpression of *ARF8*.

auxin responses. In soybean, a role for the module miR167-ARF6/8 was established in nodule formation. The regulation of *ARF8A* and *ARF8B* by miR167 regulates soybean nodulation and lateral root development in soybean infected by *Bradyrhizobium japonicum* (Wang et al., 2009; Wang et al., 2015). These different studies suggest that the regulation miR167/*ARF8* it is key actor in the response to the environmental stresses but also in response to pathogen infection, therefore a role in defense in giant cells. We can suggest that the induction of *ARF8* in tomato galls, is in favor for nematode parasitism to bypass plant defense.

2.4 miRNAs responsive to auxin in plant-RKN interaction

In previous studies, two other auxin-responsive microRNAs, miR390 and miR172, have been shown to be involved in the formation of feeding site induced by RKN (Cabrera et al., 2016; Díaz-Manzano et al., 2018). miR390 is overexpressed in *A. thaliana* galls at 3, 7 and 14 dpi. miR390 induces the cleavage of the lncRNA encoded by the *TAS3* genes resulting in secondary siRNAs production (tasiRNA). TasiRNAs induce the cleavage of mRNAs of *ARF2*, *ARF3* and *ARF4* (Marin et al., 2010). KO of either *MIR390* or *TAS3* gene, leads to the reduction of the number of galls, suggesting that the miR390/*TAS3*/*ARF2-3-4* module is also required for the formation of the gall induced by *M. javanica* (Cabrera et al., 2016). Moreover, the role of miR172 and the two transcription factors *TOE1* (*TARGET OF EARLY ACTIVATION TAGGED 1*) and *FT* (*FLOWERING LOCUS T*) has been demonstrated in the formation of giant cells in *A. thaliana*. In roots, the 3' strand of mature miR172 has been shown to be downregulated in galls at 3 dpi, whereas the pri-miR172 precursor is induced, and its target *TOE1* is repressed (Barcala et al., 2010). Consistent with the negative regulation of *FT* by *TOE1*, an induction of *FT* was observed in galls at 3 dpi. *A. thaliana* plants expressing miR172-resistant *TOE1* or KO for *FT* were less susceptible to RKNs and had smaller galls and giant cells (Díaz-Manzano et al., 2018). Since miR167 is not the only miRNA that responds to auxin, we can suggest that the other miRNAs can interfere in auxin response in galls.

3. Copper microRNAs

The two copper microRNA families upregulated in galls, miR398 and miR408, are the only conserved miRNAs that have the same profile expression in our data (tomato and *A. thaliana*) galls. This study highlighted the role of copper in the plant response to nematode.

3.1 Copper in plants

Copper is an essential micronutrient necessary for human, animals and for plant growth, and it is required for different physiological and biochemical processes (Yruela, 2005; Garcia et al., 2014). It has been shown that animals use copper as an antimicrobial weapon. Microbes have also developed mechanisms to counteract the toxic effects of copper (Samanovic et al., 2012). In plants, copper plays a critical role in mitochondrial respiration, cell wall metabolism and remodeling, hormone signaling and in response to oxidative stress (Pilon et al., 2006). Copper is a cofactor of different enzymes such as laccase, cytochrome c oxidase and superoxide

dismutase (SODs). However, the deficiency or the excess of copper is very toxic for the plants. Symptoms of copper deficiency can be spotted by the whitening and curling of leaves, reduction in plant growth, decrease in cell wall formation and lignification in several tissues (Tiffin, 1972; Martin and Marschner, 1988). Excess of copper can also interfere with plant growth and development and causes reduction in plant biomass, leaf chlorosis, necrosis and also inhibited root growth (Martin and Marschner, 1988; Prasad and Strzałka, 1999; Navarri-izzo et al., 2006). The excess of copper causes the generation of reactive oxygen species (ROS) a signaling molecule involved in the regulation of various physiological and developmental processes and in defense against pathogen (Hänsch and Mendel, 2009; Karuppanapandian et al., 2011). Therefore, it is crucial for plants to maintain copper homeostasis.

Copper homeostasis is mainly regulated by the copper-responsive transcription factor SPL7 (Yamasaki et al., 2009). In low copper condition, SPL7 binds to DNA and activates the transcription of copper-responsive microRNAs: miR397, miR398, miR408, and miR857 (Yamasaki et al., 2007). These microRNAs repress the expression of genes encoding non-essential copper-binding proteins such as SODs and laccases; therefore, their expression is repressed to preserve copper for the copper binding proteins involved in most vital functions for the plant like photosynthesis. In contrast, when the plant is in excess of copper, it prevents the binding of SPL7 to DNA and the expression of copper-responding miRNAs enabling expression of genes coding copper binding proteins (Yamasaki et al., 2009; Zhang and Li, 2013; Shahbaz and Pilon, 2019).

3.2 Conservation of copper module

Our sequencing results support a conserved role for modulation of copper homeostasis in plant response to root-knot nematodes in *A. thaliana* and tomato. Sequencing analyzes show that miR408 and miR398 families are overexpressed in *A. thaliana* but also in tomato galls. The identification of the targets of these two microRNAs in the degradome analyses of tomato galls and *in silico* (psRNA target) in *A. thaliana*, associated with the integration of transcriptomic data in these two plants identified conserved targets for these microRNAs. *COPPER SUPEROXIDE DISMUTASE1 (CSD1)* and *UCLACYANIN 2 (UCC2)*, targets of miR398 and miR408 respectively, are both repressed in galls of both plant species. In a collaboration with Dr. Sébastien Thomine (I2BC institute, Gif-sur Yvette, France), we have measured copper level in tomato galls versus uninfected roots. A decrease of copper level has been observed in galls collected at 14 dpi *in vitro* and in whole infected roots *in soil* in comparison to uninfected roots (Table 4). Another study in 2017 showed a reduction of copper in tomatoes in whole roots infected by *M. javanica* in comparison to uninfected roots (Lobna et al., 2017). Resistance to RKN was correlated with a higher root copper content in resistant tomatoes cultivars in comparison to the susceptible one (Lobna et al., 2017). These results support our hypothesis that the induction of miR408 and miR398 in tomato galls is due to a reduction of copper level in galls in comparison to uninfected roots during susceptible response.

3.3 Copper in biocontrol

Treatment with high level of copper is used to protect the plants against pathogen infection. Copper-based bactericides and fungicides such as Bordeaux mixture, are extensively used in agriculture. In plant response to pathogen, pepper plants treated with copper (50 μM) showed a resistance to *Verticillium dahlia* fungi (Chmielowska et al., 2010). Maize plants treated with copper (10 - 80 μM) were more resistant towards *Spodoptera frugiperda* infection (Winter et al., 2012). The concentrations of copper used in these studies are ten times higher than the concentration (5 μM) we used to water plants during infection test. Copper treatment is known to enhance ROS level and to increase peroxidase enzyme and laccase activity increasing lignin content (Kuc and Preisig, 1984; Díaz et al., 2001). Lignin is a cross-linked phenolic polymer material that forms key structural materials in the supporting tissues of plants such as vascular plants (Printz et al., 2016). The complex structure of lignin provides the mechanical support, water transmission, as well as blocking the growth of pathogen and infection. The polymerization of lignin is catalyzed by peroxidase in the presence of H_2O_2 and by laccases in the presence of O_2 (Sterjiades et al., 1992). Soybean plants cultured in various concentrations of CuSO_4 showed a decrease in H_2O_2 level with increase in POX activity and lignin content after 72 hours on 5 μM copper medium (Lin et al., 2005). Same results have been observed in *Raphanus sativus* plants cultured on 4 μM of copper medium (Chen et al., 2002). In these studies, the treatment with copper affected plant growth and photosynthesis, while in our cases plant treated with copper did not show any difference in root development compared to the plants treated with water. The plants were treated two days after infection to let the nematode penetrate to the roots and plants were treated one time per week, in contrast to these studies plants were grown on medium containing different concentrations of copper.

3.4 miR398 and ROS

miR398b/c is a copper-miRNA which expression is regulated by SPL7 and it is overexpressed in *A. thaliana* galls. *CSD1* and 2, *BLUE COPPER BINDING PROTEIN (BCBP)* and *COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE (CCS)* are biologically validated targets of miR398 (Jones-Rhoades and Bartel, 2004; Beauclair et al., 2010; Brousse et al., 2014). The role of *BCBP* is still unknown, *CCS*, *CSD1* and *CSD2* are related to the redox process in the activation of primary defense response of plants. *CCS1* delivers the copper to *CSD1* and *CSD2*. *CSD1* and *CSD2* are superoxide dismutase (SODs) enzymes expressed in response to stresses that generate ROS (Abdel-Ghany et al., 2005; Sunkar et al., 2006).

In low copper conditions, miR398 is induced and it is involved in the degradation of *CSD1* and *CSD2* mRNA to restrict copper for plastocyanin (Yamasaki et al., 2007). The expression of *CSD1* and *BCBP* is repressed in *A. thaliana* galls, while *CSD2* is not differentially expressed. We showed that *A. thaliana* expressing gene with a mutation at the cleavage site, *mcsd1* and *mbcbp*, had fewer egg masses than the wild-type. These findings demonstrate that the cleavage of *CSD1* and *BCBP* transcripts by miR398 is required for plant-RKN interaction.

ROS act as signal molecules to activate plant immunity. Plants produce ROS, such as hydrogen peroxide (H_2O_2), to defend itself from pathogens (Forman et al., 2010). However, plants need to maintain redox homeostasis by balancing ROS production and removal while by ROS-scavenging enzymes like SOD (Eves-van Den Akker et al., 2014; Camejo et al., 2016). SODs aids the scavenging of ROS by converting O^{2-} into H_2O_2 .

miR398 has been mainly studied in the plant response to abiotic stress. In response to oxidative stress, *MIR398* is transcriptionally repressed to prevent the cleavage of *CSD1* and *CSD2* transcripts leading to an accumulation of *CSD1* and *CSD2* mRNAs that scavenge superoxide radicals (Sunkar et al., 2006; Jagadeeswaran et al., 2009).

Recently, miR398 was shown to be induced in *Nicotiana benthamiana* infected by Beet necrotic yellow vein virus (BNYVV) (Liu et al., 2020). TRV-induced gene silencing (VIGS) of miR398 target *UMECYANIN* (Cu-superoxide dismutase in *A. thaliana*), showed a higher concentration of O^{2-} in silenced plants that in control plants and a lower accumulation of BNYVV in the systemic leaves, suggesting that the induction of miR398 during BNYVV infection is favorable for the activation of plant defense and it inhibits the O^{2-} scavenging activity of plants. In contrast, in our results the induction of miR398 and the cleavage of its target it's in favor of RKN parasitism.

The role of ROS towards nematode infection have been described in resistant and susceptible cultivars. Mi-resistant tomato cultivar infected by *M. incognita* produced a higher level of ROS (Melillo et al., 2006). Siddique et al., (2014) have showed that CN infection causes a localized ROS burst in *A. thaliana*. In a recent paper, Chopra *et al.*, have used two mutants upon CN and RKN infection: *RESPIRATORY BRUST OXYDATIVE HOMOLOGUES* mutants (*RbohD/F*), that generate ROS in response to pathogens and that interacts with *WALLS ARE THIN1* (*wat1*) mutant, an auxin transporter. Interestingly, these two mutants lead to a decrease in susceptibility to CN but not to RKN. Authors have suggested that *RbohD/F*-mediated ROS production and activated *WAT1* is needed for a syncytium establishment and suggesting that CN use the host's ROS for their own benefit (Chopra et al., 2021). Since ROS are involved in numerous processes throughout the plant life cycle such as germination root, shoot and flower development (reviewed in Mittler, 2017; Mhamdi and Van Breusegem, 2018) we can suggest that RKN also take advantage of ROS for gall potentially *via* miR398 induction.

The family of miR398 includes three genes, *MIR398A*, *MIR398B* and *MIR398C*. The sequences of the precursor of *MIR398A* doesn't contain the GTAC motif necessary for SPL7 binding in copper deficiency. Only *MIR398B* and *MIR398C* respond to copper deficiency while the level of *MIR398A* doesn't change in all copper conditions (Yamasaki et al., 2009). So the induction of *MIR398A* observed in *A. thaliana* galls is not due to copper response and should involve different pathway, that needs to be further investigated.

3.5 miR408/Uclacyanin/Casparian Strip

Sequencing of small RNAs showed an upregulation of miR408 in *A. thaliana* galls at 7 and 14 dpi. The localization of miR408 expression during the *A. thaliana* - *M. incognita* interaction using promoter GUS fusion confirmed the strong expression in galls observed in the sequencing analyzes. Infection tests using *Arabidopsis* KO mutant lines: *miR408-1* and *miR408-2* (Maunoury and Vaucheret, 2011) showed a decrease in susceptibility of these two KO lines associated with a defect of feeding site formation. These results demonstrated the key role of miR408 in the development of the feeding cells during *Arabidopsis*-nematode interaction.

Using psRNA target, an algorithm that identifies plant small RNA targets based on sequence complementary (Dai and Zhao, 2011), we predicted more than 100 targets of miR408 with only two targets downregulated in *A. thaliana* galls: *UCC2* and *PHOSPHATASE 2C (PP2C)*. The cleavage of *UCC2* transcripts by miR408 has been biologically confirmed in *A. thaliana* and rice (Zhou et al., 2010; Thatcher et al., 2015). *UCC* are a sub-family of phytoeyanins family, a plant-specific blue copper protein. This family is characterized by a copper binding site and is associated with lignified tissues (Drew and Gatehouse, 1994; Nersissian et al., 1998). *UCC* are anchored to the cell surface and associate with the cell wall. In rice, role of *UCC8* was described in the fertility, pollen tube formation and growth (Zhang et al., 2018) and also in regulating rice photosynthesis and grain yield (Zhang et al., 2017). Recently, a study highlighted a role for *UCC1* and *UCC2* in the formation of lignified nanodomain within Casparian strips (CS) in *A. thaliana* (Reyt et al., 2020). CS are belt-like lignin structures surrounding endodermal cells, that seals the apoplastic way in and out of the endodermis to control the uptake of water and solutes (Alassimone et al., 2010; Naseer et al., 2012; Doblaz et al., 2017). In Reyt's article, the authors generated CRISPR CAS9 double mutant of the two genes of uclacyanin, *ucc1ucc2*. Interestingly, this double mutant showed a strong increase of permeability using Apoplastic tracer and the loss of function of *UCC1* and *UCC2* reduced lignification in central of CS, suggesting a role of these two genes in the lignification of CS.

In 2015, a study has showed that *UCC1* is downregulated in the transcriptomic data in *A. thaliana* of *myb36* mutants compared to the control plants (Kamiya et al., 2015). The transcription factor MYELOBLASTOSIS PROTO-ONCO GENE 36 (MYB36) is the main regulator of several genes necessary in the position of CS in the endodermis. The formation of CS required CASPARIAN STRIP MEMBRANE DOMAIN PROTEINS (CASPs). There are five genes of this family in *A. thaliana* and the double mutant *cas1cas3* leads to a defect in CS formation (Roppolo et al., 2011). The formation of CS required also an association with CASPs and lignin deposition. The deposition of lignin in CS involved many signaling pathway like the kinase SCHENGEN (SGN1 &3) and their ligands CASPARIAN STRIP INTEGRITY FACTOR 1& 2 (CIFs) (Nakayama et al., 2017). This deposition required the polymerization of lignin thanks to the peroxidase PER64 and the enhanced suberin 1 (ESB1) (Hosmani et al., 2013; Lee et al., 2013) (Figure 25).

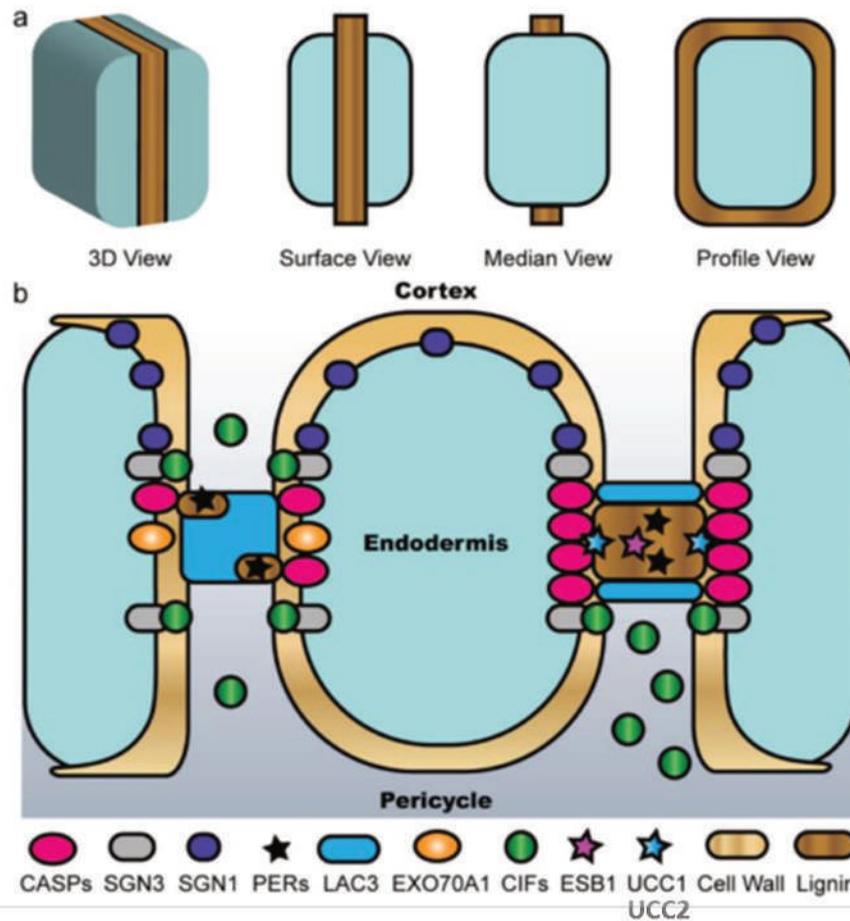


Figure 25. Schematic representation of the multi-protein framework for CS development. (a) The morphology of CS observed from different views. (b) Model for how the wall domain coordinates with the membrane domain to spatially define CS. Before the diffusion barrier is established, as shown on the left portion, SGN3 and SGN1 recognize the CIF peptides. As shown on the right portion, LAC3 participates in forming the cell wall domain, which together with EXO70A1 help focalize CASPs to demark the CS membrane domain and recruit PER64 for further lignification. ESB1 and UCC1 are involved in this scenario with unknown functions. CASPs: CASPARIAN STRIP MEMBRANE DOMAIN PROTEINS; SGN: SCHENGEN; PERs: peroxidase; LAC3: laccase; ESB1: enhanced suberin 1; UCC: Uclacyanin. (adapted from Zhuang and Li, 2020).

esb1 and *casp1casp3* mutants shows a strong increase of permeability PI (Apoplastic Tracer), the same result was observed in *ucc1.ucc2* mutant (Reyt et al., 2020). LOTR1 (lord of the rings) is a member of an uncharacterized family and LOTR2 is a subunit of the exocyst complex: LOTR2/ EXO70A1 transiently accumulates on CS membrane domain and guide CASP localization (Kalmbach et al., 2017). The analysis of the expression profile in *A. thaliana* galls of the different genes involved in the formation of CS showed that *MYB36* is upregulated in galls at 3, 5 and 7 dpi while most of *MYB36* targets were are downregulated (*CASPs*, *SGN3*, *SGN1*). Earlier studies in plant-nematode interaction have shown that the endodermis bordering the giant-cell in barley infected with *Meloidogyne naasi*, lacked a CS (Ediz and Dickerson, 1976). More recently, transgenic *A. thaliana* mutants, with defects in CS strip and lignin deposition (e.g. *cap1-1casp3-1*), displayed increased in susceptibility towards *M. incognita* infection (Holbein et al., 2019). Finally, a recent article has shown that the root of the rice cultivar resistant towards *M. graminicola* infection presented a thicker CS lining compared to the susceptible rice cultivar (Singh et al., 2021). These different studies showed that CS formation is altered in galls induced by nematode infection. Moreover, in another pathogen infection, *Plasmodiophora brassicaei*, that also induces galls, transcriptome analysis showed several genes involved in CS formation (e.g. *CASP1*, *CASP3*, *UCC1*, *UCC2*, *MYB36*, *PER64*) were downregulated in *A. thaliana* galls (Liégard et al., 2019).

Moreover, using psRNA target, we found that *UCC1* is targeted by miR2934. *UCC1* is downregulated in *A. thaliana* galls at 7 and 14 dpi, while miR2934 is upregulated at 14 dpi. The role of this miRNA is still unknown (Borges et al., 2011). Additionally, we have found that *CASP1* that is downregulated in *A. thaliana* galls at 7 dpi is also predicted to be cleaved by miR390b that is upregulated in galls at 7 & 14 dpi. These results suggest that genes involved in CS formation and lignification, could be posttranscriptional repressed by miRNAs, like the cleavage of *UCC2* transcripts and its downregulation by miR408 in galls.

The next question that needs to be answered is how nematode can use the CS defect for its own benefit? We can think that since giant cells remain symplasmically isolated (Hoth et al., 2008) and since solutes are unloaded from sieve elements into the apoplast from which the nutrients are further transported into the symplasmically isolated giant cells, defects in CS formation would lead to an outflow of solutes, which may leads to a better nematode development (Bartlem et al., 2014; Holbein et al., 2019). So far the role of copper in CS regulation is not well known. Interestingly, pharmacological treatments using copper chelator, leads to a defect in CS with a disorganization of lignin deposition (Zhuang et al., 2020). This can suggest the role of copper in CS formation and lignification.

A recent article has shown that two auxin, auxin phenylacetic acid (PAA) and indole-3-acetic acid (IAA), plays a role in the formation of CS (Cook et al., 2021). PAA and IAA treatment induces a decrease in the expression level of the *CASP* and *ESB* gene families but didn't affect *MYB36* level expression. Moreover, a reduction in auxin level has been observed in the CS mutants *esb1* and *casp1casp3* but not in *myb36* mutant suggesting that a functional *MYB36* is necessary for the repression of auxin biosynthesis in CS deficient plants. The authors

proposed a model on the role of auxin in regulation CS genes (**Figure 26**). The contradictory in the expression profile of *MYB36* (upregulation) and *CASP & ESB* (downregulation) observed in *A. thaliana* galls, could be therefore explained by a role of auxin.

3.6 How does the nematode induce copper decrease in galls?

All the results obtained on copper module points a reduction of copper level in galls. The question of how nematode induce this decrease of copper level in galls, needs to be further investigated. One hypothesis could be that nematode secretions/effectors would manipulate copper homeostasis of feeding cells. Indeed, some nematode effectors have a copper-binding domain. A *M. graminicola* effector, Mg-MO289, has been recently identified to interact with rice copper metallochaperone heavy metal-associated plant protein (OsHPP0₄) (Song et al., 2021). Mg- OsHPP0₄ is involved in copper binding as Cu metallochaperones. HPP0₄ transport Cu to Cu binding enzymes such as COPPER/ZINC-SUPEROXIDE DISMUTASE 2 (Cu/Zn-SOD) that plays an important role in scavenging superoxide radical (O²⁻) produced by ROS (Pilon et al., 2006). The treatment of plants with copper for 24 hours showed less accumulation of O²⁻ in HPP0₄ and Mg-MO289-transgenic lines than the plants treated with water, suggesting that OsHPP0₄ and Mg-MO289 can promote O²⁻ decrease by boosting the rice Cu/Zn-SOD activity. The authors suggest a role of this effector by eliminating O₂⁻ and suppressing plant immunity. Nematode secretions can therefore play a role in the manipulation of copper in galls induced by RKN.

I propose a model on copper regulation in galls, integratory auxin, key regulator of plant development (**Figure 27**). RKN secretion induce a decrease in copper level in galls, that increase the activity of SPL7 and therefore induce the expression of *MIR398* and *MIR408*. The genes involved in CS formation, repressed by auxin, all along with the cleavage of *UCC2* by miR408, leads probably to a defect in CS formation and lignification. The cleavage of miR398 targets, *CSD1* and *BCBP*, needs to be further investigated.

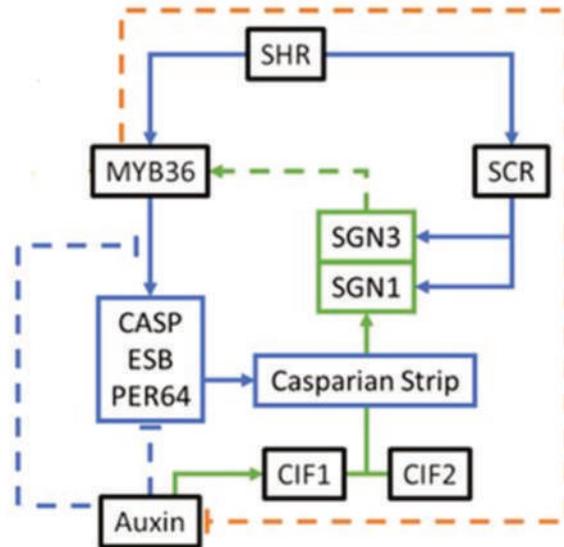


Figure 26. Hypothetical model integrating auxin and endodermal barrier regulation. Colors describe subsections of the regulatory network; Blue: Casparian strip development/ root development, Green: CIF peptide hormone pathway. Orange: uncharacterized CS/ auxin feedback loop. Arrows and flat ends indicate direction of influence (promotion or repression, respectively) and dashed lines remain to be characterized. CASP: Casparian strip membrane protein, CIF1/2: Casparian strip integrity factor, ESB: enhanced suberin, PER64: peroxidase64, SCR: scarecrow, SHR: shortroot, SGN1/3: Schengen receptor kinases1/3 (Cook et al., 2021).

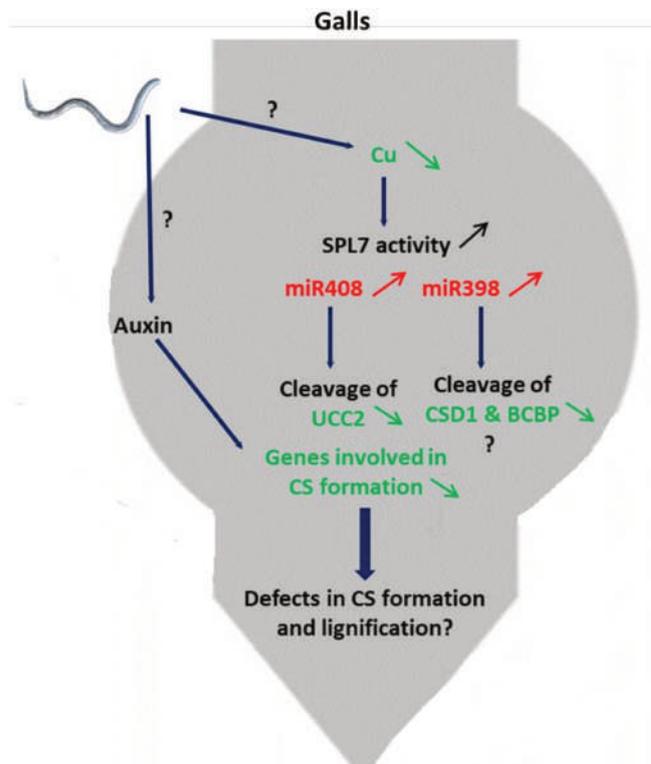


Figure 27. A proposed model on copper module regulation in galls. Cu: copper; CS: Casparian strip. Red: upregulation; Green: Downregulation.

Perspectives

During my PhD, I performed the functional analysis of two miRNAs and/or their targets modules. I first deciphered the role of miR167/*ARF8* in the formation of feeding cells induced in tomato and secondly the role of copper module, copper/*SPL7/MIR408-UCC2/MIR398-CSD1-BCBP* module, in the formation of nematode-induced feeding cells in *A. thaliana*.

MiR167/*ARF8* module

Functional analysis performed with the two *ARF8* in tomatoes galls induced by *M. incognita*, showed clearly that these transcription factor are involved in giant cell formation. The mechanism of action of *ARF8* in tomato galls is still unclear. *ARF8* has been described as an activator of genes responding to auxin. In order to decipher further the role of *ARF8A* and *ARF8B* in response to nematode infection, expression profile of genes which transcription is regulated by *ARF8* should analyzed in galls. We are sequencing mRNA from *arf8a* and *arf8b* and wild type galls in order to identify the targets of *ARF8* in tomato galls. The sequencing is still in progress (BGI sequencing company, China). The results of the sequencing of *arf8a* and *arf8b* tomato galls will enable to identify downstream genes and the biological processes they are involved in.

On the other hand, during my PhD I focused on the functional analysis of *ARF8* but the role of miR167 in this regulation should be further investigated. Since miR167 is downregulated in tomato galls, I have transformed tomato to overexpress miR167 using promoter 35S. One tomato transformed plant (microtom) that overexpress miR167, has the same phenotype as the double mutant CRISPR *arf8ab*: smaller plant with smaller and seedless fruits. The sterility of tomato overexpressing miR167 (Liu et al., 2014), required to backcross this plant with wild type. Infecting these lines with RKN infection will complete our understanding and test the role of miR167 in tomato giant cells.

Functional analysis of copper module in tomato galls

As showed before, our sequencing analysis identified a conserved copper module in tomato and *A. thaliana* galls with the two pairs miR408/*UCC2* and miR398/*CSD1* conserved in both plants. Functional analyses were initiated in *A. thaliana* for which the necessary biological material was already available. Functional analyzes in tomato should be performed in the future in order to investigate the conserved role of miR408/*UCC2* and miR398/*CSD1* in galls. We have started the functional analysis of miR408 in tomato. I performed stable transformation of tomatoes (WVA106) with the precursor of miR408 fused to reporter gene GUS. Since we generated the seeds of F1 that express miR408:GUS, the expression profile of this miRNA should be investigated at early and late stage of gall development, with and without copper. In the future, mutant lines of KO miR408 or *UCC2* resistant to cleavage by miR408 should be generated.

Characterization of Casparian strip and lignification in galls

Our results obtained in copper module and the role of *UCC* in CS formation suggest that RKN induce a reduction of copper level *via* *SPL7/miR408/UCC*, and this module may play a role in the formation of CS through lignification. To test hypothesis, this barrier should be characterized in the presence or the absence of copper in *A. thaliana* and tomato galls and uninfected roots. I will stain lignin in galls and roots in order to compare lignin deposition in CS. A protocol has been set up in order to stain lignin in *A. thaliana* and its compatible with chemical dyes (Ursache et al., 2018).

Characterization of ROS in galls

As we saw also most of the components of copper module (peroxidase, the lignification, SODs) are linked to ROS, a more detailed study will be needed to investigate the changes in ROS production during galls formation. We can measure H₂O₂ in galls and uninfected roots. We propose this hypothesis that the downregulation of *UCC* expression in *A. thaliana* galls, leads to a decrease in lignification in important target structures (CS) which may help the nematode infection.

Auxin and copper

In order to identify if copper affected auxin disruption in galls, we can proceed to the functional analysis of plants expressing auxin transporters (e.g. *pin1; aux1*) fused to gene reporter cultivated in medium with and without copper. Indeed, a study has showed that Cu excess affected root auxin distribution which affected the mitotic activity of the meristem. Copper induced an auxin redistribution that involves the efflux carrier of the PINFORMED1 (PIN1), which is responsible for root auxin transport (Yuan et al., 2013). Moreover, auxin have been showed to play a role in the formation of CS (Cook et al., 2021). To identify the role of auxin in CS formation in galls, galls of auxin transporters mutants could be used to characterize CS by lignin staining; treatment with auxin using mutant genes, such as *casp1-1casp3-1; esb1; sgn3*, could be also a track to explore the effect of auxin in CS formation.

New miRNAs/targets in tomato-RKN interaction

This work has also characterized the miRNAs and mRNAs differentially expressed in the galls during tomato - *M. incognita* interaction at 7 and 14 dpi. As mid-term perspectives, I propose to identify new miRNAs/targets in tomato-RKN interaction. A list of twelve pairs of miRNA/targets were identified with an anti-correlation of expression. Beyond miR167/ARF8 pair, other pairs would be interesting candidates to investigate their roles in the formation of giant cells in tomato. For example, the patatin family the target of miR7981 is upregulated while miR7981 is downregulated. The patatin genes family is well known in facilitation of pathogens host colonization in different plants (Rydel et al., 2003). miR164 family are

characterized as auxin-responsive miRNA (Guo et al., 2005). miR164 and its target *NO APICAL MERISTEM (NAC)* are also promising candidates to play a role in the regulation of gall development. A negative correlation between miR164/NAC was observed in our data at 7 and 14 dpi and this regulation was observed and confirmed by qPCR in *Kaur et al.*, at 14 dpi (Kaur et al., 2017). This highly conserved family regulate boundaries in shoot and lateral root development through the negative regulation of *NAC* family transcription factors with *CUP-SHAPED COTYLEDON 2 (CUC2)* as main targets (Peaucelle et al., 2007; Sieber et al., 2007). Since tomato CRISPR lines are available, functional analysis should be performed.

Trans kingdom RNAi

All the previous functional analysis studies and the work done during my thesis showed that plant miRNAs are involved in plant nematode interaction and in the giant cell formation. However, only few studies have investigated RKN microRNAs and no functional validation have been done so far (Wang et al., 2015b; Liu et al., 2019). Sequencing RKN miRNAs from parasitic stage is in progress in the lab in order to identify RKN miRNAs differentially expressed that may control RKN development by regulating genes in pathways associated with RKN development and/or pathogenicity.

The last 10 years pathogens were shown to manipulate the plant machinery of small RNAs by secreting small RNAs into host plant (Weiberg et al., 2013; Westwood and Kim, 2017; Cai et al., 2018b). This mechanism is known as trans kingdom RNAi and vesicles exchanges is bidirectional and it was shown that pathogen too can export sRNAs to silence gene (Dunker et al., 2020). *Botrytis cinerea* secretes fungal miRNAs which are loaded into the ARGONAUTE 1 of the plant and induce post-transcriptional repression of the genes involved in the defense of the plant host (Weiberg et al., 2013). In cotton – fungal interaction, cotton increase production of miR166 and miR159 and exports both to the fungal hyphae for specific silencing of fungal genes (Zhang et al., 2016a). Exosomes are extracellular vesicles (EV) that are involved in intracellular communication and interaction with others organisms, by transporting proteins, lipids and RNAs (Colombo et al., 2014). Exosomes from *A. thaliana* was shown to transfer small RNAs in order to silence pathogen genes *B. cinerea* (Cai et al., 2018a). We can think that similar manipulations in plant host could take place in response to RKN infection. Functional analysis of exosome markers in plant-nematode infection should be investigated.

Finally, the identification of miRNAs and their targets could be a new track to find new strategies to control nematode infection. Interestingly, new studies aim to spray small RNAs on plant surfaces as an efficient way to crop protection (Wang and Jin, 2017; Sang and Kim, 2020; Werner et al., 2020; Qiao et al., 2021). Moreover, the microRNA targets essential for giant cells and nematode development represent loss of susceptibility genes whose inactivation could lead to more resistant plant (Favery et al., 2020).

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