Cellular and molecular organization in Arabidopsis galls induced by *Meloidogyne incognita.*

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General Introduction

The root, is a dynamic interface between the plant and numerous and various microorganisms within the root rhizosphere including the nitrogen-fixing bacterial in leguminous plants, arbuscular mycorrhiza, pathogen bacteria, fungi, and plant parasitic nematodes (Horrigan, Lawrence & Walker, 2002; Koskey et al., 2017; Mani, Chowdhary & Hare, 2019; Pires et al., 2022). The soil-borne microorganisms form beneficial symbiotic, parasitic, and commensal interactions with the plant through their roots (Lan et al., 2013). Soil-borne parasitic communities primarily collaborate to cause disease, for instance, the plant parasitic nematodes-inflicted injuries form the route for secondary infection by pathogen microbes such as *Fusarium oxysporum* and *Ralstonia solanacearum* (Gildemacher et al., 2009*;* Zorrilla-Fontanesi et al., 2020). Plant parasitic nematodes are among the major players in the parasitic interaction between plants and soil-borne microbial communities and especially in below-ground ecosystems (Rich-Griffin et al., 2020).

The *Meloidogyne* genus, Root Knot Nematodes (RKNs), are perhaps among the major nematode pests in the world with their cumulative yield losses valued at 157 billion USD per annum (Abad et al., 2008). Their polyphagy nature of feeding and their ability to parasitize plants in all agroecological habitats ranging from tropical to the temperate ecosystem make them very important pests in the agricultural systems(Bertrand et al., 2001; Wesemael et al., 2011; Escobar et al., 2015; Rabello et al., 2021). The life cycle of RKNs last for an average of 28 days but may vary according to plant species and prevailing temperature. An RKN pre-parasitic second-stage juvenile (J2) spontaneously hatches from the eggs in the soil and utilizes its chemoreceptors to perceive long- and short range host-associated cues and carbon dioxide to locate a susceptible host (Turlings & Hiltpold, 2012; Kihika et al., 2017, 2020). The J2 infects the plant root at the root tip and migrates intercellularly through the vasculature where it locates several parenchyma root cells and induces their dedifferentiation into giant multinucleated and hypermetabolic feeding cells to form a functional nematode-feeding site (NFS). However, the process by which RKN selects the feeder cells remains an active subject of study. Although many studies concur on the tentative region of the target to be the root vasculature, the specific cell type targeted by RKN has been mostly hypothesized to be vascular parenchyma. Most of the cellular studies that built these hypotheses were based on ancient cellular biology techniques (Jones & Northcote, 1972; JONES, 1981; Niebel et al., 1994). The developing parasitic nematodes juvenile stages, J3 and J4, derive their nourishment from the plant and develop inside the nutrient-rich vascular tissue. The NFS's rapid growth and development collectively results in an increase in size in the infected root section. The dramatic root swelling on the infected part of the root is referred to as the root gall and is the typical symptom that characterizes RKN plant root infection (Naseer et al., 2012;

Namyslov et al., 2020; Reyt et al., 2021; Cook et al., 2021). Eventually, the nematode's sex differentiates into a vermiform free-living male or an engorged female laden with hundreds of eggs encased in a gelatinous matrix which fall out into soil (Fig. 1).

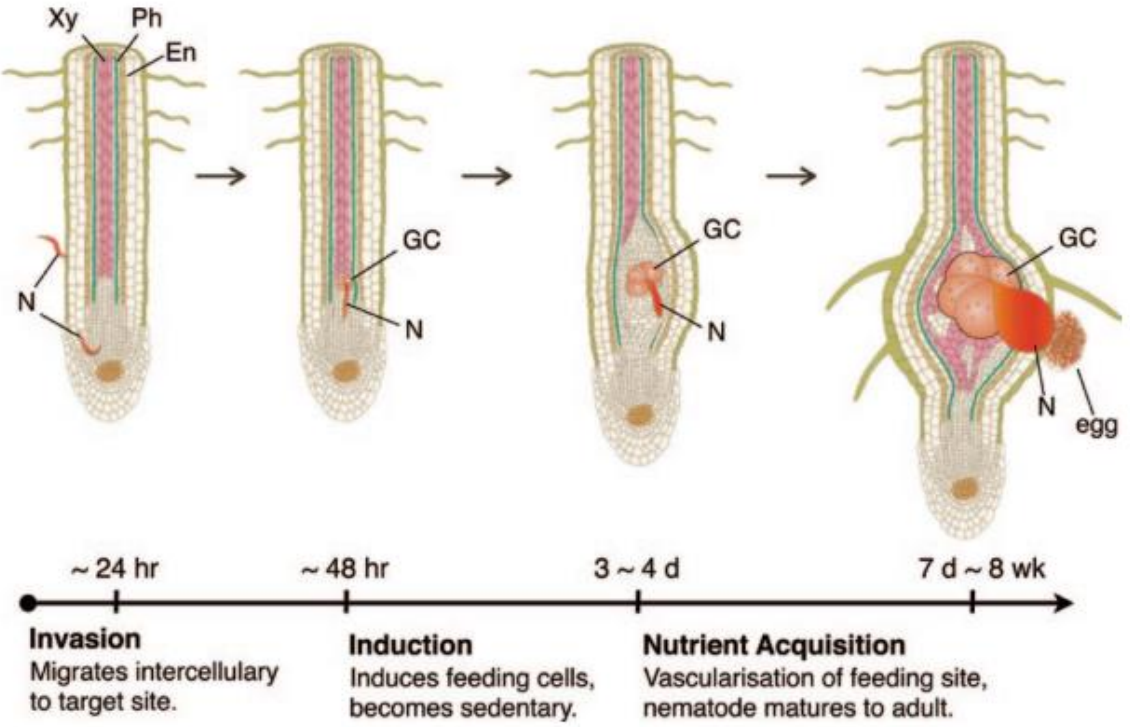

Figure 1: Schematic representation of the root-knot nematode life cycle (Bartlem et al., 2014).

The NFS is characterized by high metabolic activity to cater to high levels of karyogamy and endoreduplication. The latter increase the metabolic carbon demand within the GCs. To amply supply the GCs with this resource, the nematode extends its dedifferentiation to beyond the GCs but also other vascular cell to create cell with great ability to expand as the NFS develops but also cell that can facilitate nourishment of the GCs (Fig 2) with sufficient nutrients. These adjacent nourishing cells are referred to as the neighboring cells (NCs) (Fig 2).

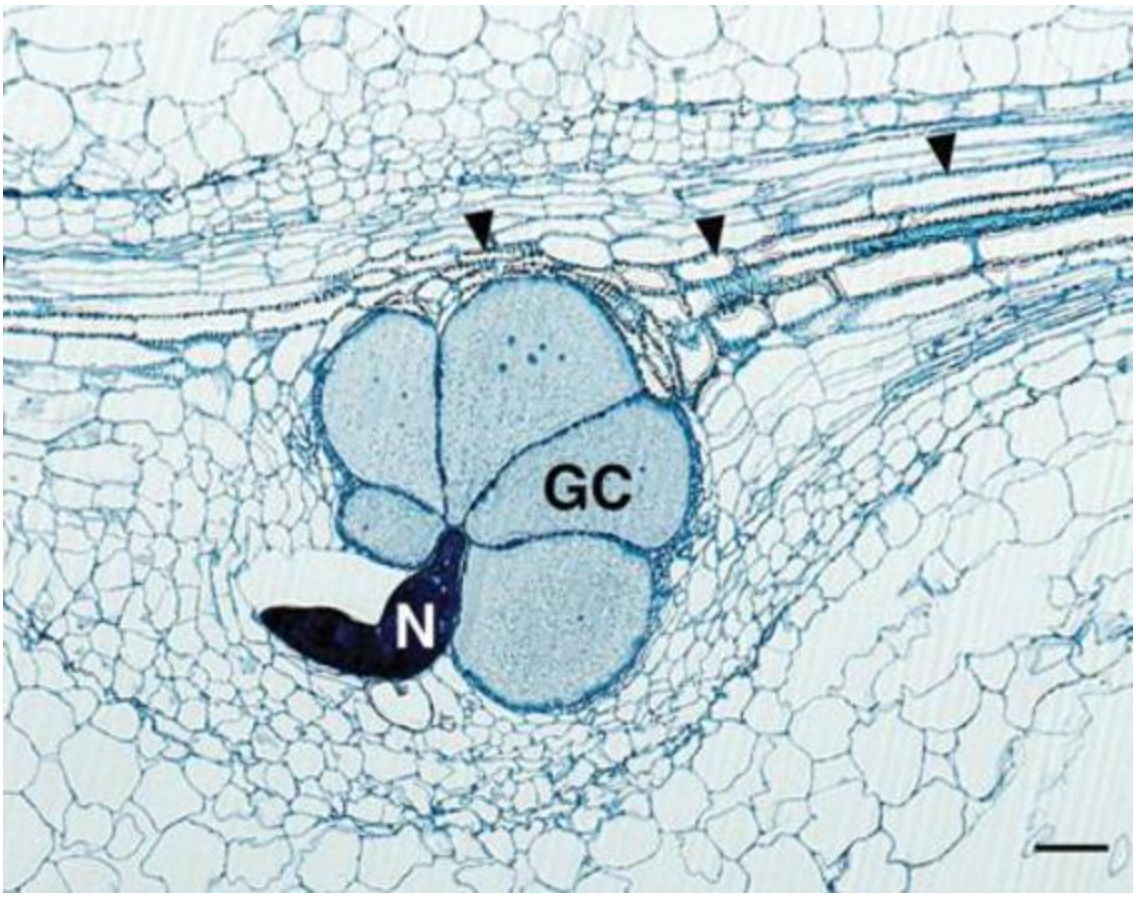

Figure 2: Giant cells (GC, one representative labelled) induced in roots of *Lotus japonicus* by *Meloidogyne hapla* root-knot nematode (N) at 2 weeks post-inoculation (Goto et al., 2013).

The GCs cells, therefore, are conveniently located at a junction of a heavily interwoven cage of *de novo* phloem and xylem network, the phenomenon is referred to as vascularization. Vascularization modifies the NFS into a terminal sink (Fig 3) to nourish the developing nematode (Hoth et al., 2008; Bartlem, Jones & Hammes, 2014). The cell wall of GCs possess a large number of plasmodesmata, they are vital in developing an optimized material exchange interface between the GCs and NCs (De Almeida Engler et al., 2004, 2010; Caillaud et al., 2008). In addition, their cell wall forms dense ingrowths and invaginations that increase the surface area for the absorption and exchange of nutrients by active transport (Yamaguchi et al., 2017).

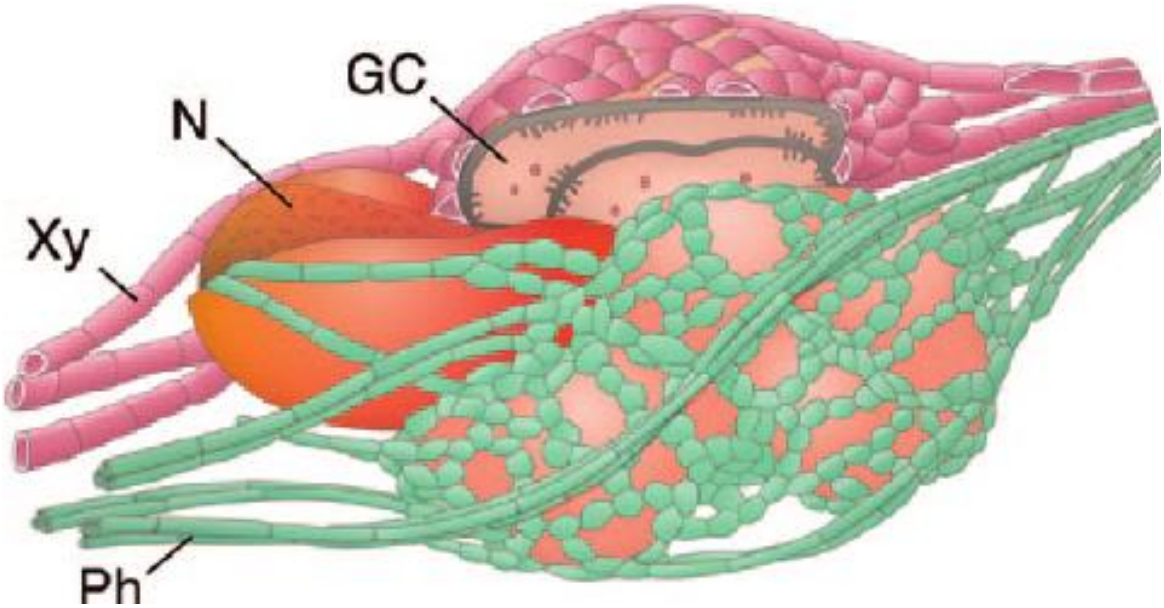

Figure 3: Modelling of the xylem (Xy) and phloem (Ph) cages surrounding the giant feeding cells (GC) and the root-knot nematode (N) (Bartlem et al., 2014).

To maintain functional feeding sites, mutual counter-defense suppression mechanisms are activated by both the nematode and host plant. The nematodes secrete effectors from the pharyngeal glands to maintain the NFS by repressing the plant defense. Plant defenses are repressed through suppression of the defense associated genes and pathways to allow the parasitic stage to develop amidst moderate plant defenses. For instance, transcriptomic analysis has demonstrated the repression of defense-associated genes, *WRKY* 45 transcription factor and PATHOGENESIS-RELATED (*PR)* both in the giant cell but also whole gall of tomato, *A. thaliana*, and *Medicago truncata* inoculated with *Meloidogyne incognita* (Jammes et al., 2005; Barcala et al., 2010; Damiani et al., 2012; Shukla et al., 2017; Kumar et al., 2019). Functional studies demonstrated the induction of PATHOGENESIS-RELATED proteins (*PR-1 and PR-2*) associated with the salicylic acid pathway on the 9th-day post-inoculation and subsequent repression at the 14th-day post-inoculation in *A. thaliana* (HAMAMOUCH et al., 2010). *WRKY* 45 gene has been reported to act downstream to a suite of plant pathways associated with the activation of plant defense (Cabrera et al., 2015). Subsequently, loss of function mutants showed an increased susceptibility evidenced by an increased infection and eventual number of galls. The latter was accompanied by an increase in Jasmonic acid and ethylene-responsive genes, suggesting their potential involvement in plant defense (Ozalvo et al., 2013). In the recent past, scientist has described possible trafficking of plant exosomes from the plant host to the fungal and viral pathogens to target there parasitic aggressiveness(Cai et al., 2018; He et al., 2021; Teng &

Fussenegger, 2021; Ruf et al., 2022). However, there is currently no evidence available to illustrate possible defense plant exosome trafficking in plant-nematode interaction.

The two objectives of my internship were; i) to identify which root cell type that are targeted by RKNs for the formation of nematode feeding sites and ii) to study the production of plant exosome as a recently reported plant defense mechanism, determine whether the plant exosomes are produced by the plant inside the GCs and if they are ingested by the developing RKN parasitic stages. To accomplish these first objective, I combined the use of confocal microscopy with i) cell type-specific promoter mcherry protein tagged Arabidopsis collection to study the specific cell targeted by RKNs for the formation of a feeding site, and to further investigate the cellular reorganization occasioned by the RKN infection in Arabidopsis galls and with ii) GFP tagged plant exosome markers Arabidopsis lines to elucidate possible ingestion of plant exosomes by the parasitic stages. I made the choice to present results on the cellular reorganization in Arabidopsis galls induced by *Meloidogyne incognita* as an article to be published in Journal of Nematology. Further, the outcomes of plant exosome production and its cross-kingdom trafficking were described in the addendum.

Cellular and molecular reorganisation in Arabidopsis galls induced by *Meloidogyne incognita.*

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Summary

Plant root infection by root-knot nematode (RKN) and the subsequent establishment of a biotrophic feeding site constitute a typical model to study the cell biology of nematode feeding sites. Pre parasitic stages understandably select the most susceptible and resource endowed root cell type for the dedifferentiation into giant- (GCs) and neighbouring cells (NCs) which make up functional feeding site (NFS). The initial NFS ontogeny results to massive reorganization of both molecular and cellular biology of the root. It is largely hypothesized that nematode target the vascular parenchyma cell during their initiation of the NFS to form the multinucleate and hypertrophied feeding cells. Additionally, earlier studies suggested that nematode development results in degradation of endodermal tissue. The degradation is extended to the Casparian Strip. Here we combine confocal in vivo imaging and GFP and mcherry tagged to cell type specific promoters to demonstrate the specific cell type targeted by RKNs for induction of the GCs and NCs. Early time point observation showed nuclear mcherry signal of *WOODENLEG* gene promoter associated with pericyclic tissue. This implied that pericycle could be the most probable target for the RKNs' dedifferentiation into GCs. Further, all the Arabidopsis line expressing vasculature associated promoter showed an mcherry signal in the NCs. On the other hand, we used *SCARECROW* AND Casparian Strip Associated Proteins 1 (CASP1) to demonstrate that the developing nematode has antagonistic effect on the Casparian Strip biosynthesis and degrades the endodermal cell layer as it develops within the root.

Key words: Pericycle, Giant cell, Neighbouring, *SCARECROW, WOODENLEG*

Plant root development is arguably one of the most crucial processes in plant growth and development. Roots anchor and facilitate plant uptake of water and mineral salts. Consequently, root cell division and differentiation are fundamental processes in transforming newly divided cells into robust specialized functional plant tissues (Kim et al., 2022). Moreover, root growth and development are influenced by a well-coordinated interaction between abiotic and biotic factors. Gravitational forces, availability of water, and mineral salts represent a section of the environmental factors that influence root development and orientation (Kobayashi, Miura & Kozaki, 2017). On the other hand, root development is driven by an ever-oscillating wave of expression of transcription factors such as *SHORTROOT* (SHR), *SCARECROW*(SCR), and *WOODENLEG* (WOL) among many other factors that regulate cell division and differentiation in the vasculature, endodermis, and pericycle root tissues respectively (Mähönen et al., 2000; Perilli, Moubayidin & Sabatini, 2010).

Terrestrial plant roots are a target for numerous plant parasitic nematodes and other pathogenic microbes found around their rhizosphere. *Meloidogyne* genus, Root-Knot nematodes (RKNs), are the most economically important nematode pests. Owing to their wide host range, they are estimated to damage more than 5,500 plants, with direct economic losses valued at about $157 billion per annum. *Meloidogyne incognita* is arguably the most studied plant parasitic nematode species, with its genome fully annotated (Abad et al., 2008). They adopt a strict endoparasitic lifestyle forming a robust biotrophic feeding site (Bird & Kaloshian, 2003). When eggs of root-knot nematode are exposed to water and optimal temperatures, they spontaneously hatch into second stage juveniles (J2s). The J2s are the infective stages for the RKN, they utilize plant cues and carbon dioxide gradient in the soil to locate and migrate to a suitable host which they infect just behind the root cap and migrates upwards within the vascular tissue (Turlings & Hiltpold, 2012; Kihika et al., 2020; Sunohara et al., 2020). Eventually, the J2 selects five to seven cells and initiates a well-coordinated dedifferentiation process to form a complex terminal sink comprising a few giant cells (GCs) and the neighbouring cells (NCs), a nourishing tissue surrounding the GCs comprising of cell that can flex to allow the expansion of the developing nematode parasitic juvenile and the GCs (M. G. K. Jones & Northcote, 1972b; de Almeida Engler et al., 2015). The GCs complex forms a nutrient sink that nourishes the developing nematode until its maturation and subsequent reproduction.

The ontogeny of the nematode-feeding site is a complex process of cell selection and active dedifferentiation into nematode-feeder sinks known as giant cells (GCs). The GCs rapidly increase in volume through coordinated repeated karyogamy with aborted cytokinesis (Jones & Payne, 1978). The latter is co-currently accompanied by rapid endoreplication in cytoplasm

resulting in a dense cytoplasm and consequently leads to a hypertrophied voluminous GC with an estimated more than 100 polyploid nuclei (Wigger et al., 1990). The NCs however, re-enter the normal cell division, they actively divide to allow for the expansion of the ever-expanding NFS (Hammes et al., 2005). A transcriptomic analysis featuring the 7-day-old micro dissected giant and the neighboring cells demonstrated 740 differentially and 498 similarly expressed genes between the GCs and neighboring cells in *Medicago truncata* inoculated with *Meloidogyne incognita* (Damiani et al., 2012)

The net effect of cumulative hyperplasia and hypertrophy around the feeding site as compared to the un-infected root tissues results in the formation of root gall, the root gall characterizes RKN root infection. The dramatic cell physiological and development underpinning occasioned by RKN infection remains an important area of study. It is an embraced fact that the nematode-secreted effectors are the primary culprits implicated in this abnormal cell physiology (Favery et al., 2004; Quentin et al., 2013; Mejias et al., 2019; Cabral et al., 2021).

Owing to the accelerated cellular activity and the metabolic activity thereof, the nutrient requirement is therefore significantly increased. Consequently, the GCs should be proximal to nutrient, water, and mineral-rich tissues. The GCs selection and the subsequent cellular reorganization process remain understudied or based on old histological studies. Owing to their level of knowledge and scarcity of information in this endeavor, conflicting reports on the cell type targeted by RKN and cyst nematodes have emerged over the years. For instance, although many scholars largely agree that the RKN target the root vasculature (Wyss & Grundler, 1992; Gheysen & Fenoll, 2002; Escobar et al., 2015; Yamaguchi et al., 2017), some literature also classify the cortical and endodermal cell as a probable target for the selection for GCs (Jones & Northcote, 1972; Jones et al., 1981) which suggested the J2s target the endodermal cell and a further description of *Meloidogyne kikuyuensis* targeting the cortical cell in the dedifferentiation into GCs (Eisenback & Dodge, 2012). Although many reports concur that the vasculature is the overall target, little is known on the specificity of cell type target by RKN in the formation the feeding sites.

To precisely deconstruct the nematode root cell-type selection and cellular reorganization occasioned by nematode infection, we combined confocal microscopy and the study of the SWELL *Arabidopsis* collection. The SWELL lines are a collection of Arabidopsis that utilizes a large variety of validated cell-type specific promoters such as *SHORTROOT* (SHR), *SCARECROW* (SCR), *WOODENLEG* (WOL), *EXPANSIN* (EXP), *ENDOPEPTIDASE* (PEP), and *GLABRA* 2 (GL2) fused to the histone H2B and the fluorescent protein mCherry (RED TIDE nuclear marker lines) resulting to red nuclear marker (Marqu S-Bueno et al., 2015). In this article,

we used the RED TIDE lines of SWELL Arabidopsis collection to investigate the root cell type targeted by *M. incognita* to induce GCs formation. Moreover, we also used *CASPI1pro:CASPI1:GFP* translational fusion Arabidopsis line that highlights the Casparian strip ring between the endodermal layers of the root.

Materials and Methods.

Nematode culture

Meloidogyne incognita culture strain 'Morelos' was multiplied on susceptible tomato (*St Pierre* cv) in plant growth chamber-maintained at 25°C and 8:16 hour light-to-darkness cycles for six consecutive weeks. Infected roots were collected, washed, and set up in the nematode hatching assays in an incubator chamber.

Arabidopsis thaliana

Soil-sand inoculation assays seedlings were pregerminated by directly sowing the seeds in potting soil and keeping them at 4 ^{0}C for 48 hours before transferring them to the germination chamber. (The list of the Arabidopsis lines used, and their respective information is found in the appendix). The pre-germinated seedlings were then transplanted into 2ml Eppendorf tubes or in pots (35mm diameter), 2 weeks after germination. The inoculation substrate was prepared by mixing sand and soil at a ratio of 1:1. The liquid fertilizer is mixed with *Bacillus thuringiensis* coated crystals (Truyens et al., 2016) and entomopathogenic nematodes *Steinernema sp.* to minimize the incidence of pests and disease build-up in the infection and growth substrate. The growth chamber conditions were maintained at 20 ^{0}C for 8-16 hours of light and darkness cycles.

Nematode inoculation

Two weeks after transplanting, the plantlets were inoculated with about 60 and 300 pre-parasitic J2s in both tubes and in pots respectively.

Confocal in-vivo imaging

Roots were collected and washed with tap water from infected plants on the 4th,7th,14th, and 21st day post-inoculation (dpi). The galls were hand dissected and then embedded in 5% Agarose in 1% PBS gel (Caillaud & Favery, 2016). Vibroslice sectioning with a thickness ranging between 30, 50, 100 and 150 µm for 4th, 7th, 14th and 21st dpi was performed from galls embedded in agarose using a Vibratome HM650V (Thermo-Fisher). The Vibroslice-sections were harvested into 1% Phosphate Buffer Saline 1X (PBS); 1,37M NaCl; 0,027M KCL; 0,080M Na_2HPO4; 0,020M KH2PO4) (Sigma Aldrich). The vibroslice was placed onto the slide and coverslip and observed by the confocal Microscope LSM880 (Zeiss). The argon laser excitation at 488 and 561nm for

GFP and mcherry fluorescence respectively. The fluorescence of the acquired images was analyzed in the Zen Blue 2.5 (Zeiss) software.

Results

Endodermis is described as the protective coat of the vasculature and therefore plays a role in regulation of what get into the vasculature (Kawa & Brady, 2022). To deconstruct the cell fate of the endodermal cell tissue, within the gall after nematode infection and subsequent establishment of a feeding site. We utilized the RED TIDE line expressing the *SCARECROW (SCR)* promoter gene, an endodermis-specific-gene encoding for a transcription factor(Fig 4.a). The plants expressing the *proSCR:H2B:mCherry* transcriptional fusion showed a clear mcherry signal during the NFS ontogenesis at 7dpi (Fig 4.b). The endodermal mcherry signal remained sparingly continuous within the developing gall up to 14dpi (Fig 4.c) and disappeared afterward (Figure 4d). As at 14dpi gall the endodermal cell-type layer was pushed outward around the nematode feeding sites (NFS). Notably, no mcherry signal was observed inside the giant nor in the neighbouring cells all along the NFS ontogeny. As a second endodermis marker, we used an Arabidopsis line that expresses a translational fusion for the gene that encodes the Casparian Strip Associated Proteins 1 (CASP1) tagged with GFP: the *CASPI1pro: CASPI1:GFP* translational fusion. CASP1 is an essential protein of the Casparian strip which is in addition to be used to follow the endodermis, the CASPI1 line was to decipher the impact of the nematode infection on the Casparian strip (CS). As observed in the *proSCR:H2B:mCherry* line, a GFP signal is observed lining the extracellular space between the endodermal cell layer at 7dpi in the *CASPI1pro: CASPI1:GFP* (Fig 3e). Strikingly, the GFP signal corresponding to the Casparian Strip seemed weaker on the gall at 7dpi (Fig 3.f) as compared to the uninfected root (Fig 3.e). As observed previously with the *AtproSCR:H2B:mCherry* line, CS and endodermal cells seem to be continuous, but pushed outward by the developing nematode feeding site (Fig 3f). Notably, the fluorescence was undetectable on the infected root at 14 and 21 dpi (Fig3. g and h). This result suggests that endodermis is not targeted by RKNs for the initiation of their feeding sites and the RKNs could have some antagonistic impact on CS biosynthesis. However, due to the absence of mcherry or GFP signal, it is in clear the fate of the endodermis as it relates to neighbouring cells.

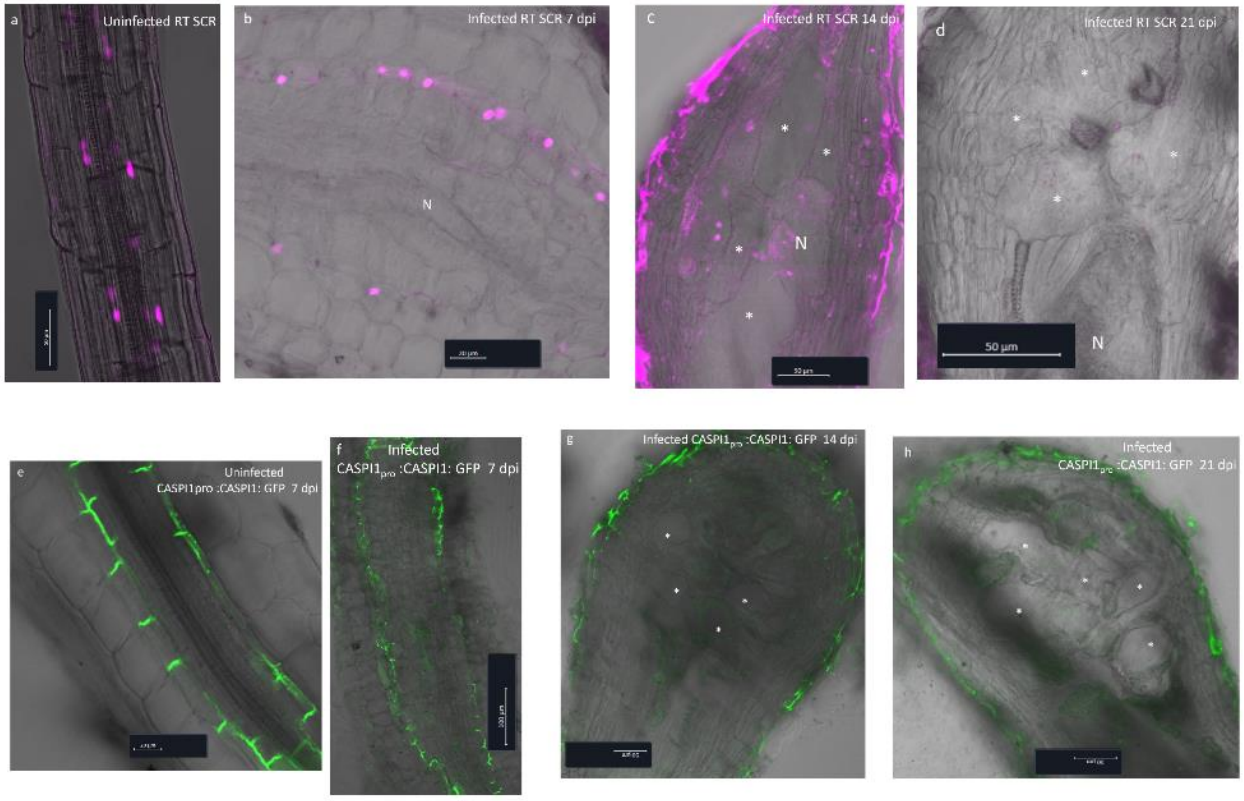

Figure 4 Promoter activity of Arabidopsis *SCARECROW* and *CASPARIAN STRIP DOMAIN PROTEIN 1* genes in uninfected roots (UR) and in *M. incognita-induced galls* at 7-21 dpi. **a:**uninfected root *Atpro*SCR*:H2B:mCherry***,** **b**) *Atpro*SCR*:H2B:mCherry* 7dpi, (**c**) *Atpro*SCR*:H2B:mCherry* 14 dpi, (**d**) *Atpro*SCR*:H2B:mCherry* 21 dpi (**e**) Uninfected root *CASPI1pro:CASPI1:GFP* (**f**) *CASPI1pro:CASPI1:GFP* 7 dpi (**g**) *CASPI1pro:CASPI1:GFP* 14 dpi (**h**) *CASPI1pro:CASPI1:GFP* 21 dpi

To further explore the specific zone targeted by the nematode we used the *SHORTROOT proSHR:H2B:*mCherry tagged Arabidopsis line, which exhibits a red nuclear fluorescence within the cells of the general vasculature or stele (Fig 4.a). The mcherry signal was scattered all over the developing giant cells (Fig5b) at 14 dpi and the fluorescent signal persisted up to 21 dpi (Fig 5.c). This observation would be a tentative lead to affirm the broadly accepted hypothesis that RKNs target the vascular tissue for the initiation and development of their feeding sites. However, there was no mcherry signal within the giant cells. Concurrently, we observed some mcherry signal in the neighboring cells which surround the giant cells (Fig5b and c).

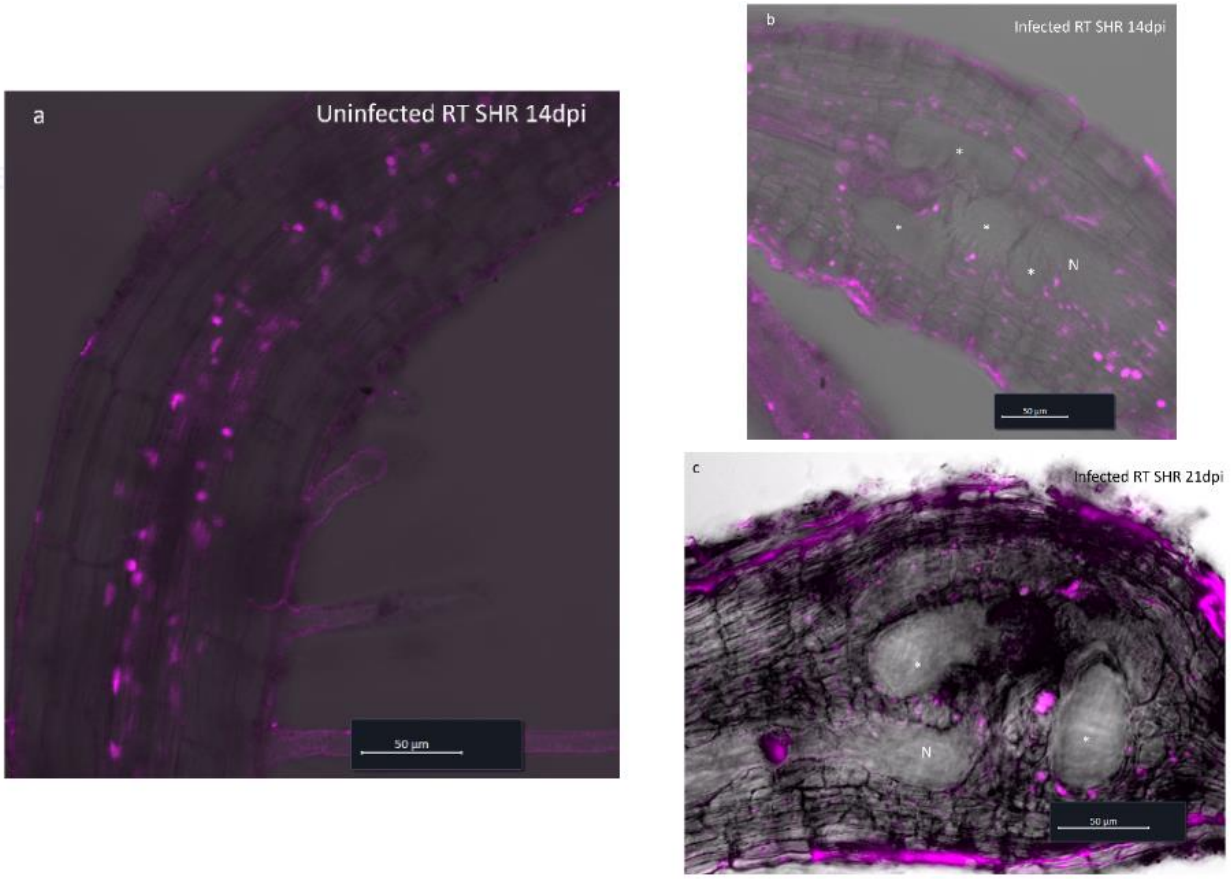

Figure 5 Promoter activity of Arabidopsis *SHORT ROOT* gene in uninfected roots (UR) and in M. incognita-induced galls at 7-21 dpi (**a)**Uninfected root **, b**) *pro*SHR*:H2B:mCherry* 14 dpi, (**c**) *pro*SHR*:H2B:mCherry 2*1 dpi

To further decipher specific cell types targeted by the root-knot nematodes within the vasculature, we used phloem-, xylem, and pericycle-specific promoter lines tagged with H2B-mCherry. We inoculated the xylem specific *INDOLE ACETIC ACID 19 (proIAA19:H2B:mCherry)* an auxin-related gene associated with pro-xylem cells (Fig 3.a). The mcherry signal rapidly increased all around nematode feeding site as the nematode developed. During the 7dpi the mcherry seems to scatter all around the gall even in non-vascular cells (Fig 3.b). At 14 and 21dpi the mcherry signal is seen to concentrate in the neighbouring cells with no mcherry signal within the giant cells (Fig 3 c and d).

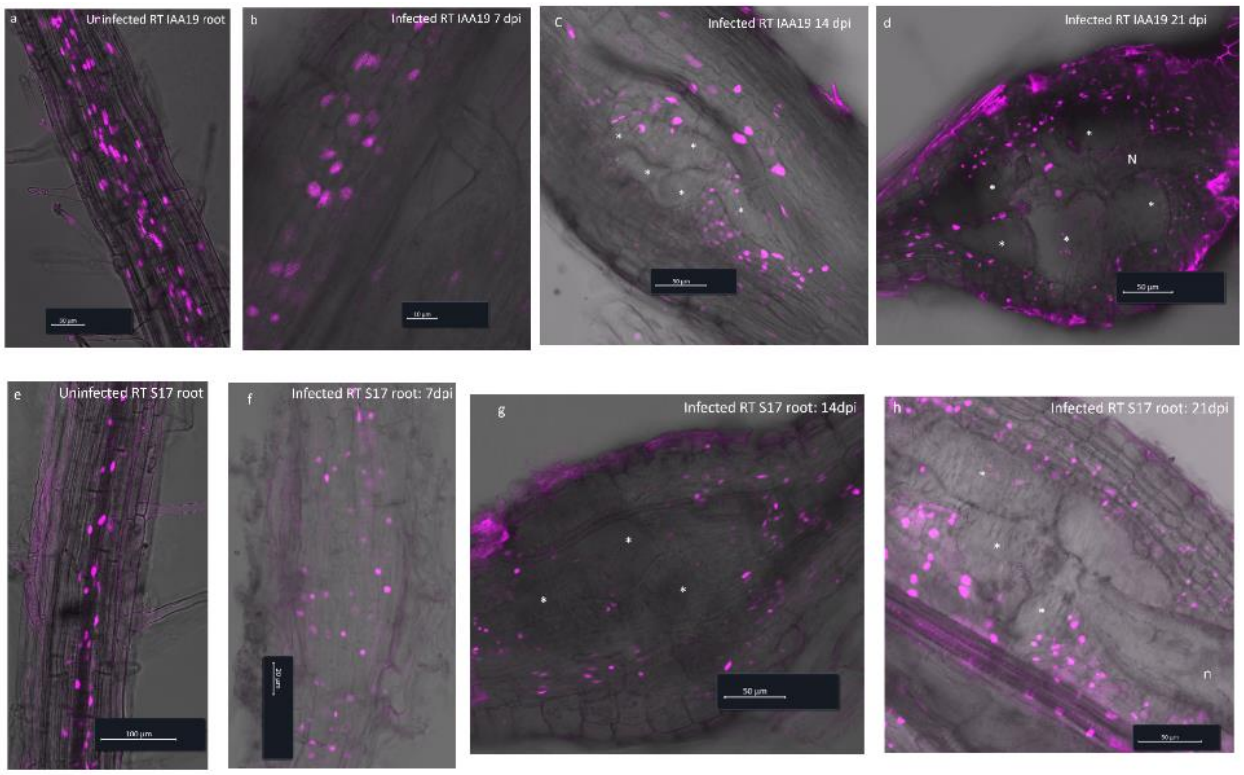

Figure 6 Promoter activity of Arabidopsis *INDOLE ACETIC ACID 19 and S17: BASIC LEUCINE ZIPPER 6* genes in uninfected roots (UR) and in M. incognita-induced galls at 7-21 dpi (**a)**uninfected root IAA19**, b**) *pro*IAA19*:H2B:mCherry* 7 dpi, (**c**) *pro*IAA19*:H2B:mCherry* 14 dpi, (**d**) *pro*IAA19*:H2B:mCherry* 21 dpi (**e**) Uninfected root *pro*S17*:H2B:mCherry* (**f**) *pro*S17*:H2B:mCherry* 7 dpi (**g**) *pro*S17*:H2B:mCherry* 14 dpi (**h**) *pro*S17*:H2B:mCherry* 21 dpi

To further probe the possibility of RKNs targeting the phloem cells we inoculated a pro-phloem specific marker line that expresses *proS17:H2B:mCherry* with RKN juveniles. S17 encodes for *BASIC LEUCINE ZIPPER 6,* a gene associated with the pro-phloem (Fig 6e). As at the 7dpi, the mcherry signal was the same as in the xylem specific line (Fig 6f) whereby nuclei signal was seen scattered all the root around the nematode. As at the 14th and 21st dpi the gall developed and the mcherry signal become more distinct in the neighbouring cell with a faint signal observed within the giant cells (Fig 6g and h), it was unclear however whether this was a sectioning technical hiccup or a true mcherry signal as it was not consistent in all the replicates at the 14 and 21 dpi. Overall, the mcherry signal in all the vasculature an associated lines spread all over the gall as at 7pi and later observed to be concentrated in the neighbouring cell after the 14dpi.There was also clear interruption of the mcherry signal lining the vascular vessels (Fig 6h).

Since it was unclear to really conclude the specific cell type that they target, we embarked on screening the possibility of the nematodes targeting the pericycle cell. To investigate the possible selection and incorporation of pericycle cells into the giant cells and neighboring cells we used the

pericycle specific SWELL line that express *WOODEN LEG (WOL) proWOL:H2B:mCherry* . WOL encodes for a gene that is associated with the pericyclic cell division and mcherry signal could be seen at the border of vasculature and endodermis (Fig 7 a). During the early stages of the infection of the nematode (4dpi), the mcherry signal could be observed in a few nuclei clustering in middle of the gall and especially around the head of the nematode at 4 and 7dpi (Fig 47b and c). Notably, the cell nuclei carry the mcherry signal were large than normal nuclei in the cell around (Fig 7d). As the nematode developed, the mCherry signal was not observed within the giant cells in 9dpi galls but the mCherry signal was observed adjacent to the giant cells but demonstrated a robust mcherry signal within the NCs surrounding the NFS (Fig 7e). This result suggests that pericycle cells are targeted by RKN(s) to initiate the giant cells development, but WOL expression is lost when the targeted pericycle cells start to dedifferentiate into GC.

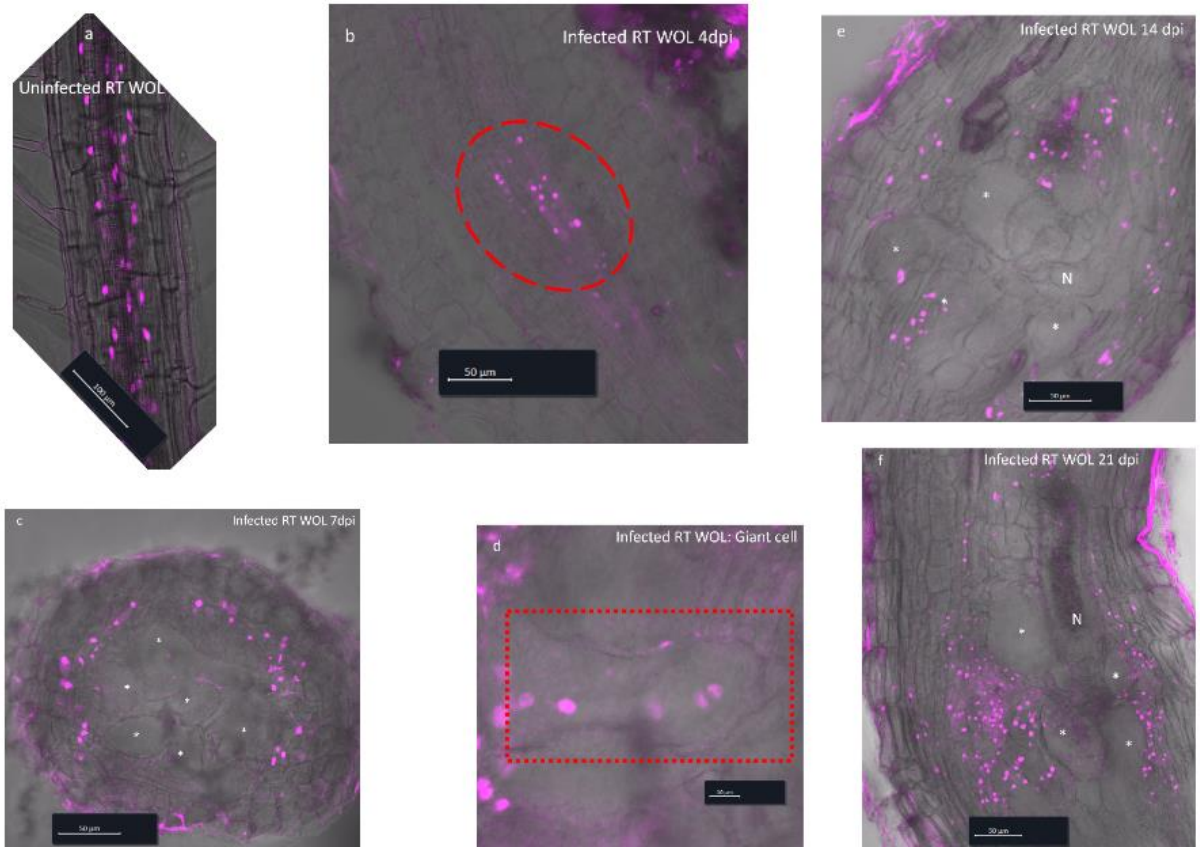

Figure 7Promoter activity of Arabidopsis WOODEN LEG genes in *AtproWOL:H2B:mCherry* uninfected roots (UR) and in M. incognita-induced galls at 4-21 dpi. (**a,** uninfected root **b**) 4 dpi, (**c, d**) 7 dpi, (**e**) 14 dpi, (**f**) *21* dpi *galls*

The mcherry signal is highly concentrated around the NFS (Fig 7f). This result implicated them in the selection processes of the GC(s)-nourishing neighboring cells. Our initial observation

demonstrated that in Arabidopsis, RKN targets the general vascular region and most probably the highly pluripotent pericycle cells.

Discussion

In this study, we showed the cell type specificity in the selection of the cells targeted for dedifferentiation into GCs and NCs making up functional RKN feeding sites in *Arabidopsis*. In particular, the pericyclic cells seemed to be the most probable target for the formation of the GCs and NCs. This was based on the early time point observation of *proWOL:H2B:mCherry* as the promoter activity is lost in the late stages of the GCs development. Since RKN infection invokes a biotrophic infection site, our study also deciphered the cell fate of the endodermal cell layer and its innate defense conferring components such as the Casparian Strip (CS) during the growth and development of the RKNs' parasitic stage in the *Arabidopsis* root. Firstly, our result strongly suggested that the developing RKN parasitic stages avoided the endodermal cell during the cell recruitment into the feeding site and clearly. While the RKN parasitic stages seem to repress and negatively impact the CS biosynthesis upon the successful initiation and development of the nematode feeding site(Fig 4.1).

The putative repression of the promoter activity in *CASPI1pro: CASPI1:GFP* and *proSCR:H2B:mCherry* transcription factor expression fluorescence as the nematode feeding develops would be a vital development insight. The latter points to a nematode's antagonistic impact on the Casparian Strip biosynthesis pathway as well as the endodermal integrity. However, we did not detect mCherry or GFP endodermis-associated fluorescence within the giant- or the neighboring cells. Based on this observation, the endodermis is not a preferred candidate for the nematode feeding site induction and ontogenesis. Occasionally, the *SCR* promoter is characterized by the inner cells of the cortex (Cui & Benfey, 2009; Koizumi, Hayashi & Gallagher, 2012). Right from the onset, the two endodermal specific markers helped to firmly establish the most popular existing hypothesis that the RKNs target the vascular bundle for the initiation of the NFS (Jones & Northcote, 1972; Wyss & Grundler, 1992; Rodiuc et al., 2014). Similar to other obligate vascular biotrophic fungal and bacterial pathogens that depend on the vasculature for survival, RKNs antagonistically repressed the development of the endodermis protective cell layer and its associated defensive components such as CS (Fester, Berg & Taylor, 2008). This finding was in line with earlier reports that described degradation of the endodermal cell layer and CS biosynthesis around RKNS' feeding sites (Holbein et al., 2019). Holbein et al further demonstrated that the degradation of the endodermis was indiscriminative to RKNs but was also observed in *Heterodera schachtii*. *H. schachtii* is an endoparasitic nematode that forms a biotrophic NFS

within the vasculature and therefore shares multiple feeding adaptations with the RKN. The CS is a hydrophobic and waxy layer that lines the surface of the endodermal layer. The repression of the CS could majorly be due to its ability to mobilize lignin and suberin compounds which build up into a barrier that regulates water and nutrient flowing into the NFS. Biotrophic pathogen would therefore exhibit an antagonistic relationship with such plant adaptions and mechanical defenses (Naseer et al., 2012).

Endodermal degradation is a widespread phenomenon among the major vascular pathogens and mutualists (Namyslov et al., 2020; Reyt et al., 2021; Kawa & Brady, 2022). Studies involving *Serendipita indica,* a mutualist colonizing the rhizodermis and cortex (Weiß et al., 2016), and the *Phytophthora parasitica,* an early temporal biotrophic phased pathogen which later assumed a dominant necrotrophic phase did not result in any significant degradation impact on the endodermis nor CS biosynthesis (Attard et al., 2010). Interestingly, *Verticillium longisporum* an obligate oomycete pathogen demonstrated severe degradation of the endodermal layer and its components during its development within the root vasculature (Ralhan et al., 2012; Fröschel, 2020). The similarity in endodermal degradation and the antagonism to CS biosynthesis for obligate vascular pathogen and mutualist would not be a mere coincidence, the results imply that obligate biotrophic pathogens such as RKNs perceives the endodermal layer as an impediment and barrier to its survival, nutritional nourishment, and development.

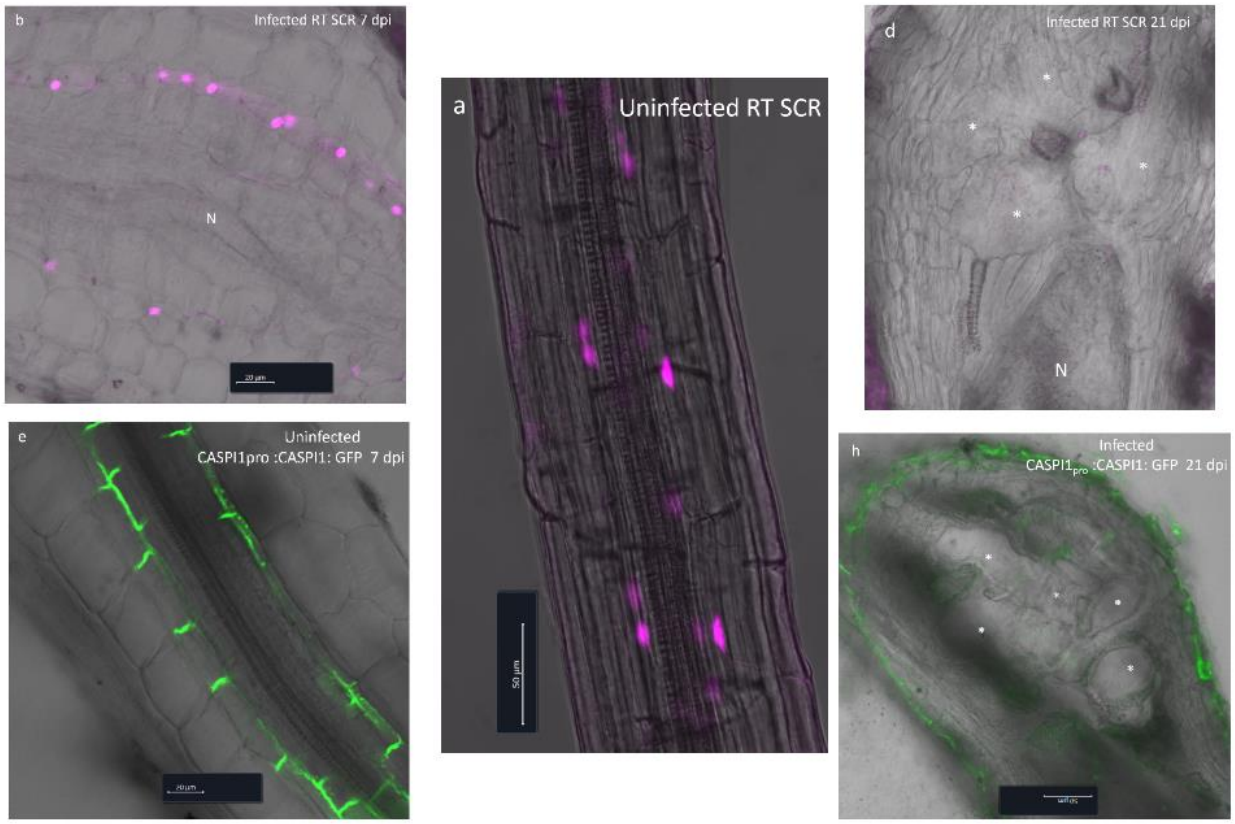

Fig 8 Promoter activity of *SCARECROW and CASPARIAN STRIP DOMAIN PROTEIN 1* genes in uninfected roots (UR) and in M. incognita-induced galls at 7-21 dpi. (**a**) uninfected root *pro*SCR*:H2B:mCherry* 7 dpi, (**b**) *pro*SCR*:H2B:mCherry* 7 dpi, (**d**) *pro*SCR*:H2B:mCherry* 21 dpi, (**c**) *CASPI1pro:CASPI1:GFP* 7 dpi (**h**) *CASPI1pro:CASPI1:GFP* 21dpi

Phloem, xylem, and general vasculature associated line expressing *proS17:H2B:mCherry, proII19:H2B:mCherry*, and *tproSHR:H2B:mCherry* cell type-specific transcription factors gall sections demonstrated a similar mcherry signal with nuclear signal scattered all over the root in the early time point and a clear signal all around the neighboring cells as the NFS develops. This observation implies that the cells of the vascular vessel could perhaps not be the probable target for dedifferentiation into GCs but could be a suitable target for differentiation into neighboring cells. The observation was very congruent to the findings of Hoth et al, who suggested that the sedentary nematode interrupts the continuous development of the vascular bundles resulting in the of the NFS into pseudo-terminal sinks, additionally, they demonstrated evidence of proliferation of de novo xylem and phloem network all around the NFS to optimize the supply of nutrients to the RKN feeding site (Hoth et al., 2008). Notably, there were faint mcherry signals in the gall section *AtproS17:H2B:mCherry (*AT2G22850*),* a pro-phloem marker (*Marqu S-Bueno et al., 2015*). This signal could be attributed to a sectioning hitch where the phloem cells were situated either below or above the GCs. Alternatively, this could be attributed to dual identity of

the pro-phloem which may be with their pericyclic founder cells (Lee et al., 2006). To distinguish between the cell dedifferentiation caused by the RKN infection and the naturally occurring plant root differentiation, early observation of *Arabidopsis* root was crucial to get the actual images of the NFS initiation phase. Early time point observation indicated that RKN could most probably target the pluripotent-pericyclic cells for the initiation of the GCs and differentiate the vascular parenchyma cells to form the neighboring cells.

On the other hand, *proWOL:H2B:mCherry* pericyclic cell type-specific mcherry signal demonstrated a concentrated nuclear signal at the center of the gall at 4dpi and observed in the GCs at 7dpi. The mcherry signal is dominant in the neighboring cell in gall sections. This result implies that either there is some pericyclic promoter that persists in the cells even after differentiating into xylem and phloem at the phloem and xylem pore (Jones & Northcote, 1972a; Böckenhoff et al., 1996) or the pericyclic cells are also a target for differentiation in neighboring cells. The presence of the mcherry signal in the giant cell is a strong indication that the pericyclic cell could be a probable target for the initiation of the GCs. This observation tallies with an earlier observation that the *Heterodera schachtii* could also be targeting the same cell layer for the initiation of the feeding site (Golinowski, Grundler & Sobczak, 1996). Old histological studies also suggested the pericycle could be a primary target for the initiation of *Meloidogyne incognita* NFS (Jones & Northcote, 1972a).

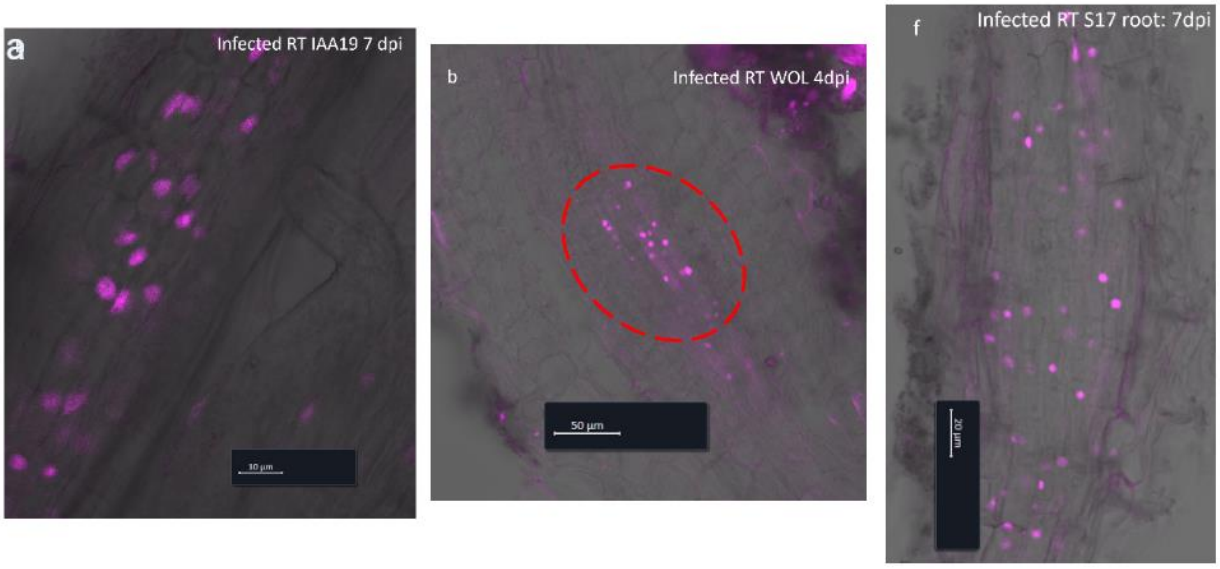

Figure 9: Promoter activity of Arabidopsis *INDOLE ACETIC ACID 19,* WOODEN LEG *and S17: BASIC LEUCINE ZIPPER 6* genes in uninfected roots (UR) and in M. incognita-induced galls at

7 dpi (**a)** *pro*IAA19*:H2B:mCherry* 7 dpi**, b**) *proWOL:H2B:mCherry* 4 dpi, (**f**) *pro*S17*:H2B:mCherry* 7dpi

The pericycle cells are highly pluripotent, which may predispose them to nematode dedifferentiation into GCs and NCs (Dembinsky et al., 2007; Rich-Griffin et al., 2020). They are the founder cell of the lateral root (Lohar et al., 2004; Dembinsky et al., 2007), whose initiation is driven by upregulation and downregulation of phytohormone signaling such as auxin and cytokinin (Hutangura et al., 1999). The latter is fundamentally important in the initiation of RKNs' giant cells and hence could potentially predispose the pericyclic cell to nematode that utilize the same pathways (Kyndt et al., 2016; Ali et al., 2018; Oosterbeek et al., 2021). Susceptibility to signaling pathways has been exploited by mutualistic nitrogen-fixing bacteria such as *Rhizobium, and Bradyrhizobium* in the initiation of plant-bacterial primordium that anchors the nodules (Liu et al., 2019; Mahmud et al., 2020). Furthermore, the pericyclic cell exhibited plasticity as the founder cell of the phloem, xylem from their phloem and xylem pores. The pericycle pores are characterized by high susceptibility to phytohormones that drive vascular development (Montiel et al., 2004; Damiani et al., 2012; Kawa & Brady, 2022). Overall, the pericycle cells are perhaps the most probable target for the initiation and development of GCs and NCs. However, to really ascertain the specific cell type targeted by RKNs the promoter activity could be combined with an early marker of giant cells such as MAP65-3 microtubule-associated protein (Caillaud, Abad & Favery, 2008). The co-localization of the protein and the nuclei mcherry signal would be undoubtedly an important insight to ascertain the specific cell type targeted by root-knot nematode for the formation of NFS.

Conclusion and Perceptive

This study elucidated three major findings that are important in comprehending the cell biology of RKN's feeding sites. To start with, we demonstrated the impact of the nematode parasitism on the endodermis and Casparian Strip development. We used GFP and mcherry fluorescent to follow the development of the endodermal layer up to 21st dpi. The result demonstrated that nematode infection led to a repression effect of the endodermis specific markers. This finding corresponds to earlier findings that the nematode degrade the endodermal layer during its development in the root (Holbein et al., 2019). Going into the future, it would be interesting to study the exact mechanism through which the nematode degrades the endodermis and CS and how breeders can use this knowledge in producing plants that are more resistant to nematode parasitism.

Our results gave a strong indication that the nematode could most probably target the pericycle cells and other vasculature cells for de-differentiation into GCs and NCs respectively. A floral dip transformation of the RED TIDE Arabidopsis lines with an early marker of giant cells such as MAP65-3 microtubule-associated protein would help in combining two robust signal localization methods(Caillaud, Abad & Favery, 2008). The combination of the promoter activity and MAP65-3 would leverage on the dynamism of the two methods. Owing to the tentative similarity in cell target by both the nitrogen fixing bacteria and RKN, it therefore crucial to jointly study the impact of each independent pathway. Plant-microbe mutualistic and parasitic relationships have different outcomes to the farmer, a big question would be whether one pathway predisposes the plant to the other or otherwise.

Addendum 1

Plants send exosomes in extracellular vesicles to root-knot nematode to silence virulence genes.

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Summary

Plant pathogens and pests inject sRNAs into plant host cells to reduce plant immunity. On the other hand, plant hosts secrete sRNAs-rich plant exosomes into the body of pests and pathogens to suppress their pathogen aggressiveness. Here, we illustrate the production of these plant exosomes within the nematode giant cells. Using TETRASPANINS (TETs), exosome markers, we demonstrated that RKN infection trigger Arabidopsis to produce numerous extracellular vesicles within the NFS. We used confocal in vivo imaging and Western blot to demonstrate that the sRNA-rich vesicles accumulated within the giant cells. However, we could not ascertain their transfer into the body of the nematode. This is a fundamental development in validating the root-knot nematode-Arabidopsis, inter-organism gene silencing.

Key words: Tetraspanin and Plant exosomes

Cross-organisms RNA interference is a recently reported phenomenon through which animal parasitic nematodes, gastrointestinal nematode *Heligmosomoides polygyrus* secreted small non coding RNAs (ncRNAs) into their mice host targeting their defense machinery (Buck et al., 2014). Similarly, plants interact with fungal pathogens by actively secreting ncRNAs into developing fungal vegetative bodies targeting their parasitic mechanisms and aggressiveness (Truong et al., 2015; Cai et al., 2018). Plant exosomes are the effective trafficking carriers of the sRNAs-rich that are produced from the plant host targeting the pest and the pathogens body to suppress the parasitic aggressiveness. Different exosome categories act as carriers of the ncRNAs from the plants into fungal bodies. Recent studies have elucidated plant exosomes secretion into pathogens, Arabidopsis-Botrytis and Arabidopsis-Mosaic viruses inter-organismal gene silencing (Truong et al., 2015; Cai et al., 2018; Kakakhel et al., 2020; Ruf et al., 2022; Zhu et al., 2022). He *et al*. used TETRASPANIN (TET) 8/9 plant exosomes marker proteins to localize fungal infection sites. The plant exosome markers were abundantly localized in *Botrytis* infection site. However, their study also highlighted that the two plant-exosome markers families did not co-localize suggesting possible varying biosynthesis pathways but associated with the similar function in plant defense. (He et al., 2021).

We embarked on a study focused on probing potential production of plant exosomes by Arabidopsis root cells during RKN infection. Interestingly, transcriptome analysis of Arabidopsis galls induced by RKN indicated a significant repression of *TET8* and *TET9* in the nematode induced galls (Yamaguchi et al., 2017). In this addendum, we investigated by use of Arabidopsis lines expressing fluorochrome-tagged TET8 or TET9s to localize plant exosomes within the galls and potential ingestion into the nematode body.

Methodology

In-vivo imaging

Arabidopsis lines expressing both the proteins as *proTET8:TET8:GFP*, *proTET9:TET9:GFP*, and *proPEN1:PEN1:mCherry* were provided by Prof Hailing Jin (University of Riverside USA). The *Arabidopsis* plants were inoculated with RKN infective J2s as described previously. Galls and uninfected roots were collected embedded agarose and hand-sectioned and the root- root- and gall sections observed at 7^{th} , 14^{th} and the 21^{st} dpi by a confocal microscope as described in the previous article.

Extraction of nematode parasitic stages

The root galls of *Arabidopsis* were collected at 14 dpi, washed in tap water, and dried by blotting the water into an absorbent paper towel. An enzymatic mixture; of pectinase and cellulase enzymes digested the root galls overnight at 170rpm and room temperature. The latter potentially disintegrated the plant cell layers and released the parasitic stages of the nematodes. The mixture of cells, root debris, and parasitic stages was separated from the filtrate using 250, 100, and 40-micrometer aperture sieves. The parasitic stages got trapped on the last and smallest sieve. The parasitic stages were resuspended in water and picked gently using a picking tool or micropipette. The nematodes were collected and gently centrifuged at 15000 for five minutes for zonal separation of residue then frozen at -80°C.

Nematode protein extraction and Western blot

The parasitic nematode stages (J3, J4) and plant root debris collected from TET8*pro*:TET8:GFP and TET9*pro*:TET9:GFP were crushed in liquid nitrogen in 1.5 ml tube by a sterile pestle and added 100µL Laemmli (SigmaS3401-10V) and 10µL protease inhibitor (Mccormack & Birnie, 2006)to break and reduce the integrity of the cell to release the transmembrane proteins through an agitation, 300rpm and at 95 ℃ for 5min.

Proteins extracted from parasitic stages collected from *Col0* galls were used as the negative control. Total nematode-proteins extraction was resolved in Polyacrylamide Gel Electrophoresis (PAGE). The blocked membrane was incubated with (PBS 1X, Tween 0.1%) buffer and anti-GFP (Invitrogen, mouse polyclonal antibody, Roche 1: 10,000) overnight to detect TET8:GFP and TET9:GFP proteins. Followed by an incubation level in secondary antibody ex. Anti-mouse HRP 1:10,000 for 2-3 hours and later stained in Coomassie blue or Ponceau red.

Results

To investigate potential plant exosomes production within the giants-cells in response to *Meloidogyne incognita* infection, three translational fusion Arabidopsis lines, TET8*pro*:TET8:GFP and TET9*pro*:TET9:GFP were used. The Arabidopsis line expressing TET-8 and 9 to follow the production of TETs associated plant exosomes. A strong GFP signal was observed in the cells surrounding the feeding site (Fig 9) and within the GC (Observation at high magnification (x63 objective lens) identified GFP tagged vesicles (Fig 9). ranging between 50-100 nm in diameter within GCs (Fig 9).

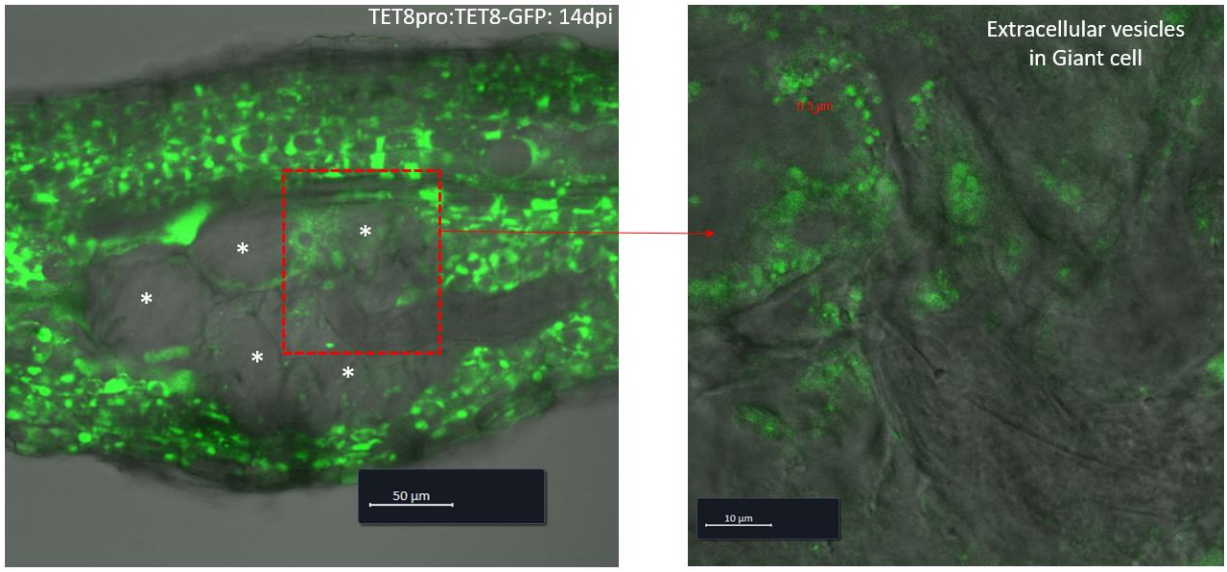

Figure 9 Translational fusion of Arabidopsis TETRASPANIN translational fusion in uninfected roots (UR) and in M. incognita-induced galls at 14 dpi: *TET8pro: TET8:GFP* 14 dpi

To ascertain possible ingestion of plant extracellular vesicles (EVs) by RKN, proteins were extracted from 100 parasitic stages of RKN bred on TETs Arabidopsis lines (TET8*pro*:TET8:GFP, TET9*pro*:TET9:GFP) and analyzed in a Western blot by using an anti GFP antibody. The Western blot results did not indicate the presence of GFP-tagged tetraspanins within the parasitic nematode protein extract (Fig10). Proteins extracted from TETs-GFP root samples were used as a positive control positive. Two bands were detected by the anti-GFP antibody, one corresponds to the TET fused to plant exosomes and the second presented free GFP were present for TET8*pro*:TET8:GFP, TET9*pro*:TET9:GFP lines (Fig 10).

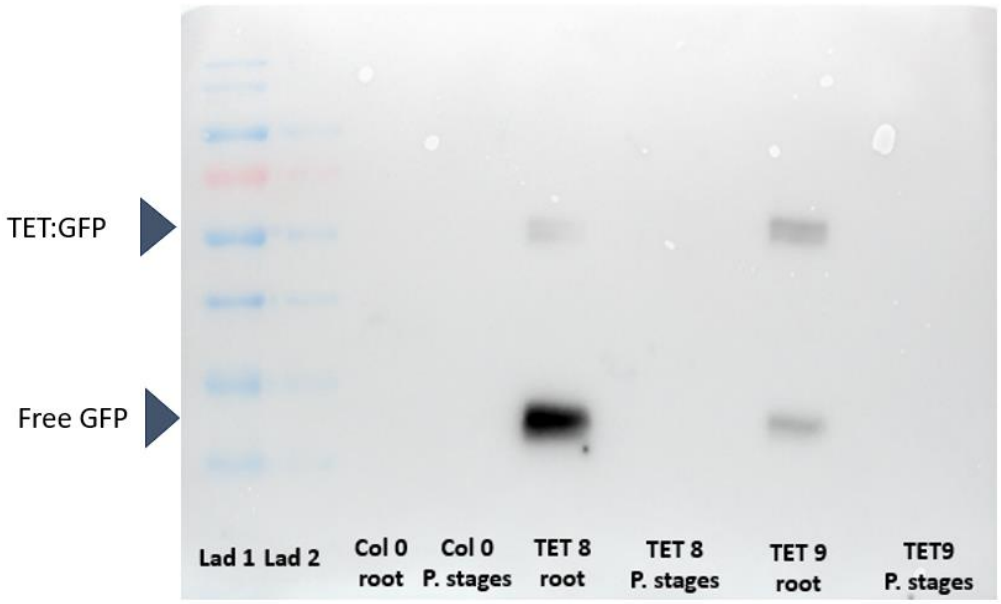

Figure 10: A Western clot image representing the protein extracted from TETRASPANINS translational fusion Arabidopsis line root galls and 100 Meloidogyne incognita parasitic stages.

Discussion and perspective

Here, we illustrated the production of the plant exosomes within the nematode giant cells using TETRASPANINS (TET8 and 9). Using plant exosome markers, we demonstrated that RKN infection trigger Arabidopsis to produce numerous extracellular vesicles within the NFS. Although we used confocal in-vivo imaging and Western blot to demonstrate that the sRNA-rich vesicles accumulated within the giant cells. It was technically difficult to get a signal of the plant exosome in the nematode body due to the primary fluorescence that made it impossible to use confocal imaging to visualize the GFP tagged *TETRASPANINS*. On the other hand, it was even more difficult to get a *TETRASPANINS* associated band in the Western blot. Even with the increased number of parasitic stages to 100, we still did not get a positive signal. The absence of the plant exosomes signal within the body of the parasitic stages could be attributed to the ultra-low concentration of the *TETRASPANINS* protein in parasitic stages' body. To counter this short fall more precise intervention could be applied to increase the chances of plant exosome detection. The other methods include immunoprecipitation, use immunogold technique coupled with transmission electron microscopy or explore mass spectrometry imaging.

Appendix

Table 1: A table representing the list of Arabidopsis line, accession numbers, phenotypes, and the respective specific cell type they characterize

No	Arabidopsis line	Accession number	Phenotype	Cell type
1.	S17	AT2G22850	BASIC LEUCINE-ZIPPER 6	Pro-phloem
2.	S18	AT5G12870	ATMYB46, MYB DOMAIN PROTEIN 46, MYB46	Pro-xylem
3.	IAA19	AT3G15540	INDOLE-3-ACETIC ACID INDUCIBLE 19, MASSUGU 2, MSG2	Xylem
4.	SCR	AT3G54220	SCARECROW, SCR, SGR1, SHOOT GRAVITROPISM 1	Endodermis
5.	WOL	AT2G01830	CRE1, CYTOKININ RESPONSE 1, WOL, WOL1, WOODEN LEG, WOODEN LEG 1	Pericycle
6.	CASPI1	AT2G27370	CASP1, CASPARIAN STRIP MEMBRANE DOMAIN PROTEIN 1	Endodermis
7.	SHR	AT4G37650	SHOOT GRAVITROPISM 7, SHORT ROOT	Vasculature

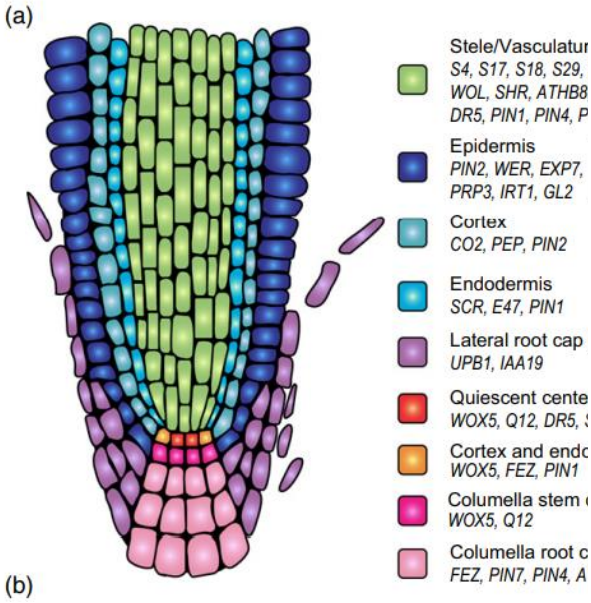

Figure 2: Strategy for generation of the SWELL promoter collection and derived transgenic lines. (a) Schematic representations of the primary root tip (left, longitudinal section, right, transversal section) with the different promoter used in this study (Bueno et al., 2015).

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My promoters' contribution was ably complemented by a team of **Scientists**; Pierre, Michaël, Laetitia, Sophie **Engineers**; Caroline, Cyril, Julia, Clare, Pauline, and **Students;** Salome, Sarah, Cliven, Vicky, Sharmistha, Joshua. I will really miss your guidance.

I heartily dedicate this piece of work to my ailing aunt Lucy, may the good LORD give fortitude and courage to surmount the most uncomfortable chemotherapy cycles, I hope to see you a few days after my graduation, See you soon.

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