DECIPHERING THE ROLE OF AUXIN AND CYTOKININ IN PLANT-NEMATODE PARASITISM

MATTHIJS P. G. OOSTERBEEK

Propositions

1. Cytokinin signalling in *Heterodera schachtii* primes cells for feeding site development and expansion.
   (this thesis)

2. Identification of the precise role of IAA in *Globodera pallida* is hampered by its possible internal as well as external functions.
   (this thesis)

3. Artificial Intelligence will never replace the need for human researchers even when a technological singularity is reached.

4. Modern-day science that builds on old literature should rigorously verify the old findings with today's methods to ensure the conclusions are still valid.

5. The Dunning-Kruger effect is a good reason for why we should not easily trust those who speak with high confidence.

6. Participating in role playing games like 'Dungeons and Dragons' makes you a more creative person.

Propositions belonging to the thesis, entitled

Deciphering the Role of Auxin and Cytokinin in Plant-Nematode Parasitism

Matthijs Oosterbeek
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Deciphering the Role of Auxin and Cytokinin in Plant-Nematode Parasitism

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Deciphering the Role of Auxin and Cytokinin in Plant-Nematode Parasitism

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Thesis

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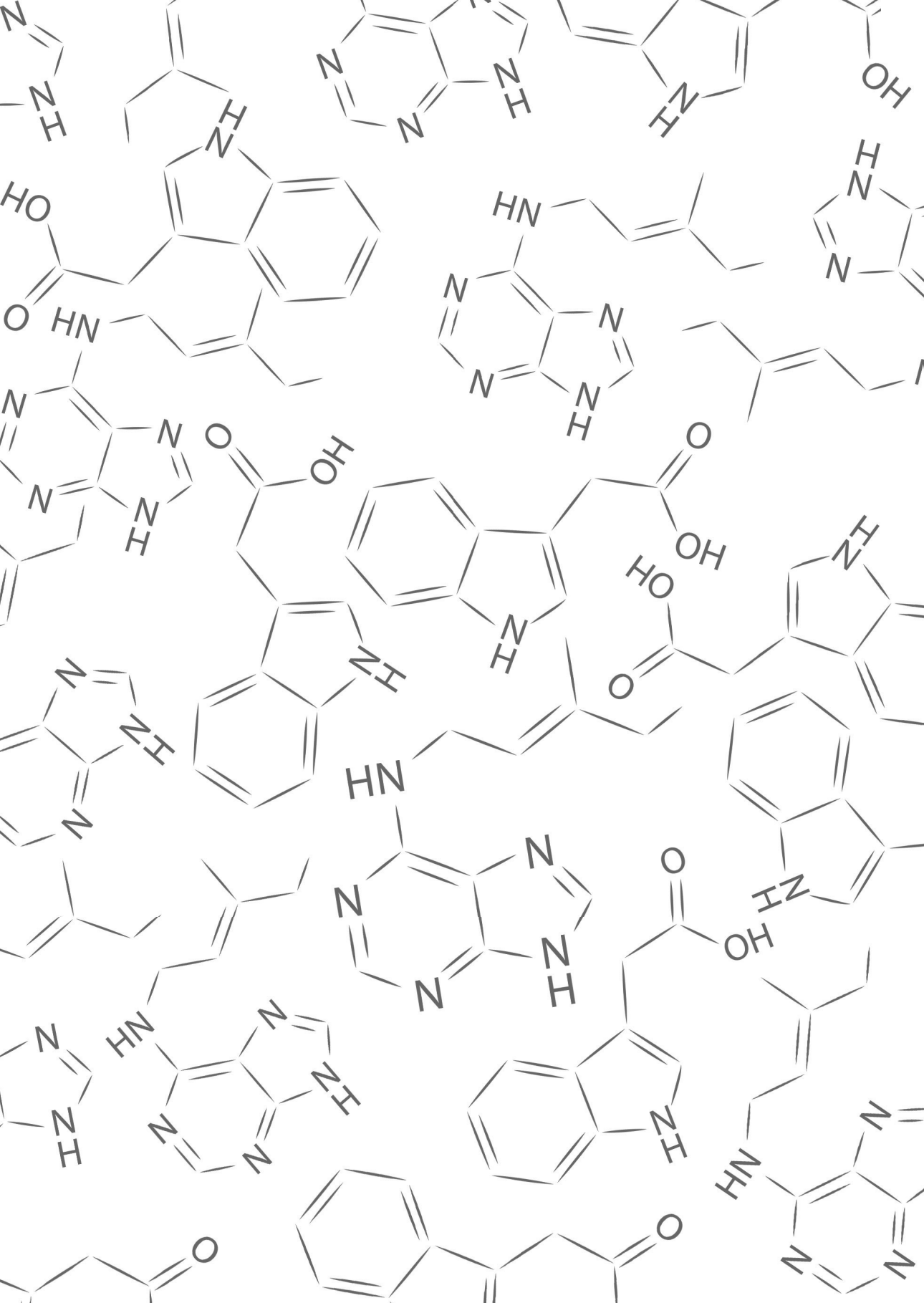

Chapter 1

General Introduction

"… if all the matter in the universe except the nematodes were swept away, our world would still be dimly recognizable, and if, as disembodied spirits, we could then investigate it, we should find its mountains, hills, vales, rivers, lakes, and oceans represented by a film of nematodes."

Nathan Cobb, 1914

Plant-parasitic nematodes

Nematodes, also known as roundworms, are the most prevalent multicellular organisms found on Earth (Decraemer and Hunt 2013). They are small, unsegmented, and vermiform creatures that can be divided into parasitic and non-parasitic species. The non-parasitic species are referred to as free-living nematodes which feed on bacteria, fungi, algae or protozoans. Free-living nematodes constitute the majority in the nematode community, up to 20 million individuals per square meter in soil making up 60-80% of the nematode community (Schratzberger et al. 2019; Neher 2010). Although parasitic nematode species are less abundant than their free-living counterparts, they are no less impactful. Parasitic nematodes can be divided into animal- or plant-parasitic and infect a variety of species from humans and cows to potatoes and rice (Jasmer, Goverse, and Smant 2003). The phylum Nematoda consists of twelve major clades that have evolved over the past 440 million years (Quist, Smant, and Helder 2015). In this time plant-parasitism has independently evolved multiple times among nematodes and can be found in four of these clades. These being clade I with Triplonchida, clade II with Dorylaimida, clade X with Panagrolaimoidea and clade XII with Tylenchida (Quist, Smant, and Helder 2015).

Among plant-parasitic nematodes (PPN) four main lifestyles can be distinguished based on sedentary or migratory behavior and endoparasitic or ectoparasitic feeding habits (Lambert and Bekal 2002). Migratory ectoparasites like *Xiphinema index*, are motile nematodes that intermittently feed from the outer cell layer of the plant root. In contrast, sedentary ectoparasites such as *Tylenchulus semipenetrans*, feed by attaching themselves to a plant root by partial penetration and subsequent induction of a feeding site. Migratory endoparasites penetrate the host's root system and migrate through the host's tissue, often leading to great damage, while feeding on multiple plant cells as is the case with *Scutellonema bradys*. Sedentary endoparasites like *Globodera pallida* penetrate and invade the host completely and after a short migration phase establish a feeding site to which they remain attached for the remainder of their life. It is amongst these sedentary endoparasitic types that some of the most harmful nematodes can be found.

Impact of sedentary endoparasitic nematodes in agriculture

Plant-parasitic nematodes annually cause an estimated yield loss between 8.8% and 14.6% worldwide with an estimated value of 157 billion dollars (Nicol et al. 2011; Singh, Singh, and Singh 2015). From these plant-parasitic nematodes the root-knot nematodes of the genus *Meloidogyne* and cyst nematodes of the genus *Heterodera* and *Globodera* are among the top 10 most harmful nematodes (J. T. Jones et al. 2013). The root-knot nematode *Meloidogyne incognita* is ranked as one of the most rapidly spreading biological threats in agricultural fields,

which can be attributed to its wide host range of more than 1000 plant species from 200 different genera (Perry, Moens, and Starr 2009). Cyst nematodes are the most damaging species for the growth of soybean and potato globally and have been listed quarantine pests in many countries around the world (Ur Rehman et al. 2021). Hence, root-knot and cyst nematodes are among the best studied species of plant-parasitic nematodes. These sedentary nematodes can feed on monocot and dicot plants. This feeding causes various symptoms, such as yellowing, stunting, and wilting which results in major crop losses. The large host range, resilience and endoparasitic nature make root-knot and cyst nematodes a persistent problem in agriculture. Once present in soils it is nearly impossible to eliminate PPN populations. Various methods to manage these parasites are employed (Duncan 1991). The most important method used to control plant-parasitic nematodes is crop rotation. By alternately growing host and non-host crops the nematode's impact can be partially mitigated. In addition, fallow can help in reducing PPN populations by removing their host plants and thereby inducing starvation. Alternatively, flooding the fields or burning crop stubble might help in decreasing the number of parasites. It is also important to note that when parasitic nematodes are present it becomes critical to prevent them from further spreading to other fields. To this end, sanitation plays an important role to prevent spread between fields through equipment and irrigation water. Another major strategy to combat PPN is the use of resistant crops. Such crops are partially resistant to the effects of infection. For example, in *Solanum peruvianum* the resistance gene *Mi-1.2* was discovered in the 1940's (Smith 1944). The Mi-1.2 gene offers resistance against tropical root-knot nematodes in tomato and is to date the most widely used resistance gene (Barbary et al. 2015). Other strategies to contain plant-parasitic nematodes include chemical and biological control options. Chemical control using compounds such as dibromochloropropane and ethylene dibromide have been applied in the past to combat PPN (Duncan 1991). Applications range from fumigation of the field that kills off parasites to erecting chemical barriers that prevent nematode migration. More recently, neurotoxins such as various organophosphates are used for PPN control (Meyer and Williams 2014). However, an increasing number of nematicides are being banned due environmental and health concerns (Hertz-Picciotto et al. 2018). Biological control might work as an alternative to nematicides and makes use of natural enemies to manage nematode populations. Examples of such enemies include nematopathogenic bacteria and fungi like *Pasteuria penetrans* and *Paecliomyces lilacinus* (Saad et al. 2022; Tian, Yang, and Zhang 2007). Although the various methods available assist in mitigating the impact of PPN, they are unable to completely solve the problem. Therefore, new control methods might offer a solution to the problem. To this end, insight into the mechanisms underlying the development of feeding sites induced by PPN might be key.

Lifecycle of cyst and root-knot nematodes

Cyst and root-knot nematodes are thought to have independently evolved their sedentary lifestyles (Baldwin, Nadler, and Adams 2004). Nevertheless, they have many features in common. For example, the lifecycle of both cyst and root-knot nematodes consists of four juvenile stages and an egg-laying adult female (Figure 1). Their life cycle generally takes 3 to 6 weeks which depends on the species and environmental conditions (Bartlem, Jones, and Hammes 2014). The pre-parasitic second-stage juvenile (J2) is the only infective stage and invades the host's roots to establish a feeding site. The specific host invasion strategies do,

however, differ between cyst and root-knot species. The infective juvenile of the cyst nematode has a preference to enter the host in the differentiation or elongation zone of the root. It enters through perforation of the epidermis by repeated stylet thrusting and enzymatic softening of the cell walls. Subsequently, the nematode migrates intracellularly through the cortex in search of a suitable feeding site and thereby causing extensive necrosis of host cells. They select a single cell to induce a feeding site. After successful establishing a feeding site the nematode goes through two other juvenile life stages before it develops into its adult form. Its sex is generally determined by environmental conditions and the frequency of males is increased in conditions of poor nutrient availability (Triantaphyllou 1985). Cyst nematodes show a high degree of sexual dimorphism with swollen females and vermiform males. The female is always sedentary, remains connected to the feeding site and lays eggs at the end of her lifecycle. In contrast, the male becomes once again vermiform and regains its motility during the third moult and subsequently leaves the root. When a female cyst nematode dies, her body wall forms a protective shell around the eggs. In addition, the nematodes inside the eggs remain dormant within, and the shell enables them to persist for up to 20 years.

Root-knot nematodes differ in the way they migrate through the host plant root and in the feeding site they initiate. Root-knot nematodes prefer to enter the root tip and move intercellularly through the root (Wyss, Grundler, and Munch 1992). Thus, in contrast to cyst nematodes, they do not leave a trail of necrotic tissue behind during migration. A combination of mechanical probing and enzymatic softening is used to separate cell tissue at the middle lamella which allows the nematode to move forwards with minimal tissue damage. The nematode moves through the cortex layer towards the root tip where it makes a U-turn around the endodermis to subsequently move acropetally through the vascular cylinder (Wyss, Grundler, and Munch 1992). Root-knot nematodes select four to ten cells to establish a feeding site. The lifecycle of root-knot nematode contains four juvenile stages and generally a male and female adult stage, similar to cyst nematodes but key differences can be found in the adult female stage. Adult females reproduce clonally through mitotic or meiotic parthenogenesis and secrete their eggs in a gelatinous matrix outside of the root.

Characteristics of nematode feeding sites

Feeding sites of cyst nematodes and root-knot nematodes are established via distinct mechanisms. Cyst nematodes select a single cell, typically in the vascular parenchyma, known as the initial syncytial cell (Abad and Williamson 2010). The nematode becomes immobile and punctures the cell with its stylet, which remains inserted for several hours before feeding begins. The initial syncytial cell is subsequently modified to become highly metabolically active and serves as a source of nutrients for the parasite. Additionally, this cell is characterised by an expanded and dense cytoplasm, an enlarged nucleus as a result of endoreduplication, various additional organelles and secondary vacuoles (Golinowski, Grundler, and Sobczak 1996). The feeding site itself expands through incorporating neighbouring cells through partial cell fusion. This process starts in the initial syncytial cell with the expansion of plasmodesmata and is followed by progressive cell wall dissolution. Initially, the feeding site develops towards the vascular bundle but afterwards expands alongside it in both directions. Cell wall protuberances are formed from the feeding site near

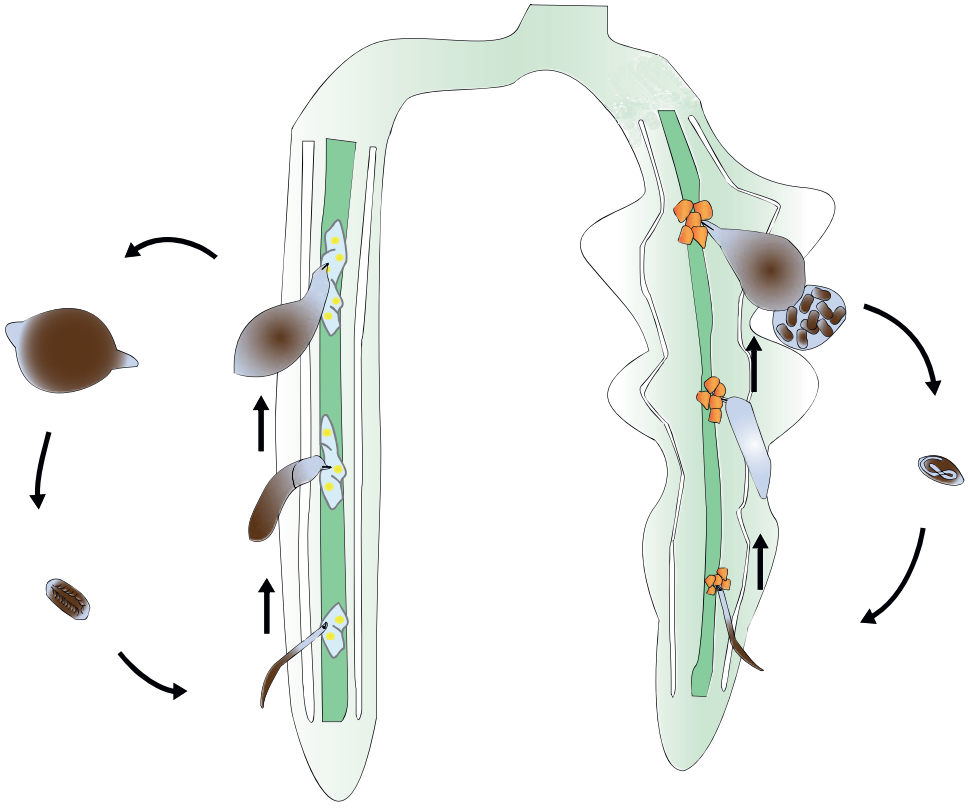

Figure 1 Schematic representation of the lifecycle the cyst nematode *Heterodera schachtii* (left) and the root-knot nematode *Meloidogyne incognita* (right). Infection of the cyst nematode starts with the eggs hatching into second stage juveniles (J2) who enter the root and establish a syncytium. The nematode progresses through the third (J3) and fourth (J4) juvenile stages within the root where each stage is accompanied by further expansion and modification of the syncytium. Subsequently, the nematode matures into an adult and afterwards the female nematode's body transforms into a cyst, serving as a protective structure that encases hundreds of eggs. Infection of the root-knot nematode starts with the eggs hatching into second stage juveniles (J2) who enter the root and induce the formation of giant cells (GCs). The nematode progresses through the third (J3) and fourth (J4) juvenile stages within the root with further expanding giant cells and the development of a gall. Subsequently, the nematode matures into an adult and afterwards the adult females lay eggs within a gelatinous matrix on the root surface.

the xylem vessels and facilitate the uptake of nutrients from the hosts (Siddique et al. 2012). It is this constant withdrawal of nutrients by the nematode that converts the feeding site into a metabolic sink for the host plant. In contrast, root-knot nematodes select four to ten cells, often located in pro-vascular regions, to establish feeding sites (M. G. K. Jones and Goto 2011). These cells then develop into so called giant cells through repeated mitosis without cytokinesis and several rounds of endoreduplication (Gheysen and Mitchum 2011). This results in a hypertrophied cell that contains multiple endopolyploidy nuclei with a high density of subcellular organelles. In addition, it contains a dense cytoplasm, a very high level of

metabolic activity and can increase in size of up to 100 times (Gheysen and Mitchum 2011). Neighbouring cells surrounding these giant cells become hypertrophic and hyperplastic and this together forms a gall. The giant cells are intermittently fed on by the nematode and serve as its nutrient source. Both types of nematodes manipulate host cells to create specialized feeding sites that support their growth and reproduction. These nematode feeding sites act as novel root organs and their formation requires significant modifications, including changes in cellular morphology, metabolism, and nutrient allocation. For example, syncytia become symplastically connected to newly formed host phloem composed of sieve elements and companion cells while giant cells are symplastically isolated (Hoth et al. 2008). Additionally, in both feeding sites, cells undergo multiple rounds of shortened cell cycles which leads to genome amplification and hypertrophy (Gheysen, de Almeida Engler, and van Montagu 1997). Moreover, large similarities are found between the transcriptomes of developing feeding sites and lateral roots (Mathesius 2003). Striking is that many of the processes in feeding sites of cyst and root-knot nematodes seem to be influenced by the phytohormones auxin and cytokinin.

The role of auxin in nematode feeding sites

Auxins are involved in numerous aspects of plant growth and development, including cell expansion, organ formation, meristem organization, vascular tissue development, and organ polarity. In root development auxin is responsible for the differentiation of various root tissues, including the vascular tissue, epidermis, cortex, and endodermis (Seo et al. 2021). It influences the pattern and differentiation of these tissues by regulating gene expression and cell fate determination. Additionally, auxin plays a crucial role in the positioning of lateral roots as lateral root primordia are initiated in response to auxin accumulation in specific regions of the root (Dubrovsky et al. 2008). The PLETHORA genes play an important role in the positioning of these auxin accumulations in specific regions (Du and Scheres 2017).

During cell expansion, auxin promotes cell elongation by influencing various processes involved in cell wall dynamics and growth. For example, auxin stimulates the activity of cell wall-loosening enzymes, such as expansins and xyloglucan endotransglucosylase/hydrolases (Catalá, Rose, and Bennett 1997). These enzymes modify the composition and structure of the cell wall, allowing it to become more flexible and extensible. Additionally, auxin influences the organization and dynamics of the cytoskeleton to enact cell expansion (Arieti and Staiger 2020). Auxin stimulates denser and more parallel, longitudinal actin filament arrays. Aside from cell expansion, auxin is also important for the development of the vasculature. One of the key roles of auxin in vascular tissue development is promoting the differentiation of procambial cells into xylem and phloem cells. Auxin stimulates the expression of specific genes involved in xylem and phloem development, leading to the differentiation of procambial cells into these specialized cell types (Simon Turner and Leslie E. Sieburth 2003; Rüscher et al. 2021). In addition to cell differentiation, auxin also influences vascular patterning (Biedroń and Banasiak 2018; Simon Turner and Leslie E. Sieburth 2003). Auxin distribution and transport patterns help establish the primary vascular strands and secondary vascular bundles where auxin gradients play a role in determining the orientation and alignment of these vascular tissues during development. Furthermore, auxin is involved in the establishment of vascular connections between different parts of the plant. Auxin acts as a

signalling molecule that guides the growth of vascular tissues towards their target destinations (Leyser 2018). By influencing the orientation and elongation of cells, it helps to establish these vascular connections. Auxin gradients and localized auxin biosynthesis contribute to the directional growth of vascular tissues, ensuring their proper alignment and connectivity (Vanneste and Friml 2009).

Both synthetic as well as natural occurring auxins are known to exist. The five types of natural occurring auxins in plants are: indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-Cl-IAA), phenylacetic acid (PAA), indole-3-butyric acid (IBA) and indole-3-propionic acid (IPA). From these five types two distinct groups can be observed: tryptophan derivative and phenylalanine derivative auxins. PAA is likely derived from phenylalanine while all other are derived from tryptophan. Four pathways are proposed for Trp-dependent IAA synthesis: (1) the indole-3-acetamide (IAM) pathway, (2) the indole-3-pyruvic acid (IPyA) pathway, (3) the tryptamine (TAM) pathway and (4) the indole-3-acetaldoxime (IAOx) pathway (Mano and Nemoto 2012). Each pathway makes use of one or more intermediate compounds to produce auxin with the IPyA pathway being the main biosynthesis pathway (Zhao 2012). The main degradation pathway known for auxins is through oxidation to 2-oxindole-3-acetic acid (oxIAA) (Pěnčík et al. 2013). Alternatively, inactivation is also known to occur through ester or amide conjugation with an amino acid, sugar or protein. These forms are thought to function as either storage or intermediates destined for degradation (WOODWARD and BARTEL 2005). Transport of IAA is generally an unregulated bulk flow through mature phloem or an actively regulated carrier-mediated cell-to-cell directional transport called polar auxin transport (PAT) (van Berkel et al. 2013). PAT is achieved through specific influx and efflux proteins. In Arabidopsis, influx is regulated by the transmembrane proteins of the AUX1/LIKE AUX1 (AUX1/LAX) family while efflux is regulated through the PIN-FORMED (PIN) proteins and the ATP-binding cassette subfamily B (ABCB)-type transporters of the multidrug resistance/phosphoglycoprotein (ABCB/MDR/PGP) protein family (Cho and Cho 2013). A family of 23 DNA-binding AUXIN RESPONSE FACTOR (ARFs) transcribe auxin-responsive genes in Arabidopsis (Li et al. 2016). These transcription factors are inhibited in the absence of IAA through dimerization with a member of the auxin/indole-3-acetic acid (Aux/IAA) family. Members of the Aux/IAA family are degraded in the presence of auxin after ubiquitination by an E3 ubiquitin ligase SKIP, CULLIN, F-BOX (SCF) complex (Salehin, Bagchi, and Estelle 2015). IAA facilitates the binding between the Aux/IAA and the F-box gene from the SCF complex. The F-box protein is part of the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOXs (TIR1/AFBs) receptor family and as such, this is called the TIR1-AFB signalling pathway (Salehin, Bagchi, and Estelle 2015).

Both cyst and root-knot nematodes have a pronounced impact on various aforementioned aspects of root growth and development, most likely involving the modulation of local IAA concentrations. The development of feeding sites is, among others, accompanied by extensive cell wall modification, alteration of cytoskeleton, enlargement of cells, reorganization of vascular tissue and adjustment of root architecture (Engler et al. 2004). These developmental changes are associated with changes in auxin signalling intensities. A transient auxin response has been detected as early as 18 hours post inoculation in both cyst and root-knot nematode infections using a DR5-GUS reporter (KARCZMAREK et al. 2004). A high level of signalling was maintained for 3-5 days after which this decreases. Auxin signalling has been observed in the galls of root-knot nematodes with the exception of the giant cells

(Absmanner, Stadler, and Hammes 2013). In contrast, auxin signalling is present in the entire feeding site of cyst nematodes including the syncytium as well as the surrounding tissue (KARCZMAREK et al. 2004; Hewezi et al. 2014). Although auxin signalling occurs in both cyst and root-knot nematodes, nuances in effect between the two can be observed when examining the expression of ARF genes. A specific set of ARF genes is expressed in the syncytium of cyst nematodes during early feeding site initiation, while a different set of ARFs is expressed in neighboring cells (Hewezi et al. 2014). The difference in auxin response hints at the possibility of multiple roles for IAA during infection and indeed, auxin has been implicated to be involved in processes such as hypertrophy, cell wall ingrowths, and cell cycle activation (de Almeida Engler et al. 1999). Auxin insensitive mutants further support a role for auxin in nematode infection. For example, the auxin-insensitive tomato mutant *diageotropica* (*dgt*) is resistant to the infection of *Globodera rostochiensis* (Goverse et al. 2000). Migration and infection seemed to occur as normal, however, the juvenile nematodes were unable to develop into their adult form. Similarly, infection is hampered for the root-knot nematode *Meloidogyne incognita* in Arabidopsis mutants of the auxin transporters AUX1 and LAX3 (Kyndt et al. 2016). In cyst nematodes, single mutants of AUX1 or LAX3 show no effect but the double *aux1/lax3* and quadruple mutant *aux1/lax1/lax2/lax3* display a marked decrease in infection (Lee et al. 2011). The relevance of Aux/IAA genes is further shown by the fact that knockout mutants of IAA16 and IAA7 result in higher susceptibility to cyst nematodes (Goverse et al. 2000; Hewezi et al. 2015). Similarly, mutants of IAA12 and IAA28 are resistant to root-knot nematode infection (Olmo et al. 2020). Alltogether the picture emerges that auxin is an important element for both cyst and root-knot nematode infection. However, the precise function of IAA remains to be elucidated in this process. In addition, it is noted that the potential relevance of other auxins (4-Cl-IAA, PAA, IBA, IPA) have hardly been addressed in plant-nematode interactions and might also be of interest for further research.

The role of cytokinin in nematode feeding sites

Similar to auxin signalling, cytokinin signalling increases during cyst and root-knot nematode infection and is known to be important for the infection of both. Cytokinins are a class of hormones with a variety of functions including the regulation of cell growth and differentiation. Amongst these functions is the vascular differentiation in the root tip. The role of AHK4 and AHP6 is thereby often given as a classic example for the function of cytokinin in vascular differentiation. The dominant-negative *wooden leg* (*wol*) allele of AHK4 shows that cytokinin signalling is required for procambial cell divisions during embryogenesis and determining the identity of vascular cells. This mutation leads to a reduced vascular system that consists solely of protoxylem and lacks both phloem and mature metaxylem (Tomáš Werner and Schmülling 2009). AHP6 is a protein that lacks phosophotransfer activity and acts as a negative regulator of cytokinin signalling during vascular development. It is thought that the inhibition of cytokinin signalling through the expression of AHP6 allows for the differentiation of protoxylem elements (Perilli, Moubayidin, and Sabatini 2010). Additionally, cytokinin is known to stimulate cell cycle progression during vascular development through CYCLIN D3;1 and AINTEGUMENTA (ANT) (Menges et al. 2007; Randall et al. 2015).

Aside from vascular development, cytokinin also plays a role in regulating the size of the root apical meristem. Cytokinins determine root meristem size by controlling the cell

differentiation rate of the vascular tissue in the transition zone of the root (Perilli, Moubayidin, and Sabatini 2010). This can be seen by the triple *ipt3ipt5ipt7* and single *ahk3*, *arr1* and *arr12* mutants that display larger root meristems, as well as CKX overexpression in the vascular tissue of the transition zone that leads to cytokinin depletion (Perilli, Moubayidin, and Sabatini 2010).

Cytokinin seems to display some inhibitory effects during the formation of lateral roots. Cytokinin is present in young primordia and creates an inhibitory field preventing the initiation of lateral roots in neighbouring cells. As such, cytokinin seems to be involved in the spacing of lateral root primordia as also *ipt3ipt5ipt7* and *log4* mutants display aberrant lateral root primordia positioning (Jing and Strader 2019). In addition to repressing lateral root initiation, cytokinin affects lateral root development. Mutants of AHP6 have shown that cytokinin regulates the pericycle founder cell divisions that initiate lateral roots (Moreira et al. 2013).

Within the class of cytokinin hormones two types can be recognised: adenine-type cytokinins such as zeatin and phenylurea-type cytokinins like thidiazuron. In plants mainly adenine-type cytokinins are created of which the following three can be found: isopentenyladenine (iP), trans-zeatin (tZ) and cis-zeatin (cZ). The first and rate-limiting step in their synthesis involves the enzyme ISOPENTENYL TRANSFERASE (IPT) (Nguyen et al. 2021). IPT exist as adenosine phosphate-IPTs or tRNA-IPTs where the former is responsible for the biosynthesis of iP and tZ while the latter is used for biosynthesis of cZ. All IPTs use dimethylallyl pyrophosphate (DMAPP) for the synthesis of cytokinins in their nucleotide precursor form. The release of cytokinin to its active form is known to be directly catalysed by 5'-monophosphate phosphoribohydrolase, called LONELY GUY (LOG) (Šmeringai, Schrumpfová, and Pernisová 2023). Degradation of cytokinin is facilitated by cytokinin oxidase/dehydrogenase (CKX) enzymes. CKXs cleave the unsaturated isoprenoid side chains from cytokinins which results in the formation of adenine/adenosine and the corresponding side chain aldehyde (T. Werner et al. 2006). Three types of membrane transport proteins are thought to facilitate the cytokinin import and export. A subset of the purine permeases (PUPs) and equilibrative nucleoside transporters (ENTs) are thought to act as influx carriers for cytokinin (Liu, Zhao, and Zhang 2019). Less is known about cytokinin exporters but in Arabidopsis the ATP-binding cassette transporter G subfamily member ABCG14 acts as an efflux pump for the long-distance acropetal transport of the root-born cytokinins. Cytokinins are perceived by the ARABIDOPSIS HISTIDINE KINASE (AHK) receptors which initiate a phosphorylation signalling cascade (ref). Upon cytokinin perception the AHK receptors are autophosphorylated which leads to the phosphorylation and activation of the B-type ARABIDOPSIS RESPONSE REGULATORS (ARRs) through the ARABIDOPSIS HISTIDINE PROTEINS (AHPs). The ARRs in turn activate the transcription of cytokinin-responsive genes. Among these cytokinin responsive genes are the A-type ARRs and CYTOKININ RESPONSE FACTORS (CRFs) which inhibit the activation of B-type ARRs leading to a negative feedback loop.

Cytokinin is thought to play an important role in nematode feeding site development. For example, induction of the cytokinin-responsive ARR5 reporter gene was observed in the vascular bundle prior to feeding site formation by *M. incognita* in *Lotus japonicus* (Lohar et al. 2004). The signal persisted throughout gall development and remained in mature galls. ARR5 signal was mainly found in small rapidly dividing cells in the mature gall but not in giant cells.

Comparable patterns were observed for *Oryza sativa* where infection with *M. graminocola* resulted in altered expression of genes involved in cytokinin signalling at 3- and 7-days post inoculation (dpi) (Kyndt et al. 2012). Overexpression of a CKX gene in *L. japonicus* resulted in a decrease in the number of galls when infected with *M. incognita* indicating that cytokinin is involved in infection (Lohar et al. 2004). Similar to root-knot nematodes, cytokinin signalling is also found in the feeding sites of cyst nematodes and is present at 2 to 3 dpi in the root tissues surrounding the nematode and at the site of the developing syncytium (Shanks et al. 2016). Signalling increases in developing syncytia from 5 to 10 dpi and subsequently declines in mature syncytia around 14 dpi. RNA-seq data shows that various cytokinin related genes are differentially expressed during cyst nematode infection and that various cytokinin insensitive mutants show a decrease in formed syncytia. Similarly, cytokinin hypersensitive mutants show a decrease in the number of infections. This disparity might be explained by the idea that cytokinin is not only needed for proper feeding site formation but has an additional function at elevated levels as a signalling molecule to trigger defence responses to invading parasites and pathogens (Shanks et al. 2016). Cytokinin deficiency not only leads to a decrease in quantity but also in size as syncytia induced in PYK10:CKX3 and *ahk3/4* lines appear to be smaller (Siddique et al. 2015). Both *H. schachtii* and *M. incognita* are reported to produce cytokinins themselves. Predominantly benzyladenine and zeatin-type varieties were found (Bird and Loveys 1980; De Meutter et al. 2003; Dimalla and van Staden 1977). However, the iP type cytokinin was also found in *H. schachtii* together with an associated IPT biosynthesis gene in the nematode's genome (Siddique et al. 2015). It was shown that this HsIPT was responsible for some of the cytokinin in the nematode and that silencing of this gene attenuated the infection process. No cytokinin biosynthesis genes in root-knot nematodes are reported to date. However, considering the higher levels of cytokinin reported in root-knot nematodes, it is assumed that cytokinin-synthesizing genes are also present in root-knot nematodes (Siddique and Grundler 2018). Although cytokinin signalling is required for proper feeding site formation in both root-knot nematodes as well as cyst nematodes, there is a difference in the response of cytokinin related genes. Differences in the expression of cytokinin biosynthesis, catabolism and signalling genes were found, suggesting that the manipulation of cytokinin differs between cyst and root-knot nematodes (Dowd et al. 2017).

Auxin and Cytokinin interplay

Auxin and cytokinin are key regulators of plant growth as well as developmental processes and interplay between the two is known to control plant development and differentiation. Various data demonstrate that the ratio between auxin and cytokinin levels plays a central role in organogenesis. For example, classical experiments have shown that exposing callus to a high auxin-to-cytokinin ratio results in root formation, whereas a low ratio of these hormones leads to shoot development (SKOOG and MILLER 1957). Also during root development auxin is known to interact with cytokinin at various levels. It was shown that auxin induces the expression of the cytokinin biosynthesis gene IPT5 likely mediated through IAA3 (SKOOG and MILLER 1957). In a similar manner cytokinin can induce the expression of auxin as a reduction in endogenous cytokinin levels can lead to a reduction in auxin biosynthesis (B. Jones et al. 2010). On the other hand, cytokinin can also negatively regulate auxin through ARR1 by inducing the expression of IAA3 which functions as an auxin transcription inhibitor (Ioio et al. 2008). Such interplay between auxin and cytokinin is not

only seen during biosynthesis but also catabolism is affected. Auxin downregulates the CKX2, CKX4, and CKX7 cytokinin degradation genes and upregulating CKX1 and CKX6 (T. Werner et al. 2006).

Another clear example is found during the vascularisation of the root meristem where cytokinin allows for the differentiation of protoxylem elements through AHP6. It was shown that the auxin induced TARGET OF MONOPTEROS5/LONESOME HIGHWAY (TMO5/LHW) basic helix-loop-helix (bHLH) transcription factor dimer regulates periclinal cell divisions (De Rybel et al. 2013). Mutants of TMO5/LHW show a reduction in vascular tissue size as a result of a reduced number of periclinal cell divisions. The key interplay is found when the auxin induced TMO5/LHW complex induces the expression of the LOG4 cytokinin biosynthesis gene (De Rybel et al. 2014). The induction of LOG4 by TMO5/LHW does lead to the production of cytokinin as well as a transcriptional response where AHP6 is induced. It is this interaction that is thought to affect vascular tissue patterning.

On the other hand, auxin induces the expression of ARR7 and ARR15 (Müller and Sheen 2008). These A-type ARRs inhibit cytokinin signalling for the root stem-cell specification during early embryogenesis. Mutants of ARR7 and ARR15 result in a defective root stem-cell system showing that auxin limits cytokinin signalling for specifying the first root stem-cell niche. Conversely, cytokinin regulates root meristem activity through modulation of auxin transport (Růžička et al. 2009). It was shown that cytokinin inhibits the expression of various PIN proteins including PIN1, PIN3 and PIN7 in root tips. Changes in cytokinin levels lead to changes in transcription of several PIN proteins and had a direct impact on auxin efflux in cultured cells and on auxin distribution in the root apex. Overall, it can be seen that the auxin cytokinin interplay takes place on many levels including transport, signalling, biosynthesis and catabolism. Additionally, this interaction can take many forms ranging from of synergistic, antagonistic, and additive. This complex web of interactions is yet to be fully uncovered.
Independent studies on auxin and cytokinin signalling have demonstrated the presence of these hormones during infection by both cyst and root-knot nematodes. It has been shown that auxin signalling is present as early as 18 hours post inoculation and remains present to 3-5 days post inoculation (KARCZMAREK et al. 2004). Similarly, cytokinin signalling has been reported in both cyst and root-knot nematodes at 3 days post inoculation (Kyndt et al. 2012; Shanks et al. 2016). These studies suggest that auxin and cytokinin signalling occur simultaneously and at the same location during nematode feeding site development. However, little is known about the interplay between auxin and cytokinin during nematode infection and what effect this might have on feeding site formation and functioning. So far, the effects of auxin and cytokinin signalling on feeding site development have predominantly been addressed in separate studies and there is remarkably little research reported on their combined effect.

Scope of the thesis

In this thesis, I explore strategies of plant-parasitic nematodes to manipulate the local hormone balance to establish a successful feeding site for further development and reproduction. The main objective of this thesis is to obtain deeper insight into how nematodes manipulate auxin levels in plant roots upon infection, either directly or indirectly, and how

this affects downstream signalling events. In addition, my aim is to compare the spatiotemporal dynamics of auxin and cytokinin signalling during infection as a first step towards understanding the interaction between both hormones during the various stages of the development of syncytia induced by the beet cyst nematode *H. schachtii*.

Chapter 2 presents a systematic analysis of past and current knowledge on the role of auxin in plant-nematode interactions providing a comprehensive report on auxin homeostasis in plants, encompassing its synthesis, breakdown, transport, and signalling during infection. It highlights the ability of plant-parasitic nematodes to manipulate auxin levels in various ways as part of a successful parasitic relationship with their host plants.

The phytohormone cytokinin is also known to play an important role in the plant-nematode interaction, but hardly been studied simultaneously with auxin. In Chapter 3, the spatiotemporal dynamics of auxin and cytokinin signalling during infection by the cyst nematode *Heterodera schachtii* are investigated using live-cell confocal imaging. Live-cell imaging uses the idea of following a single sample over time on a cellular level with time-lapse microscopy. To this end we established a method to adapt live-cell imaging for studying plant nematode interactions. With most research focused on using single auxin or cytokinin reporter constructs, here, an auxin-cytokinin double reporter was used to allow for simultaneous visualisation of both hormone signals. This resulted in time-lapse movies of infection from which these data gave insight into the induction and overlap of auxin and cytokinin signalling domains. Additionally, a semi-quantitative method was employed to estimate auxin and cytokinin signalling levels in syncytia compared to root tips.

Previous studies have shown that the manipulation of auxin transporters result in a local transient auxin accumulation during nematode infection. However, a local increase in auxin could be attributed as well to nematode-derived auxin. This prompted us to test the hypothesis in Chapter 4 that the cyst nematode *Globodera pallida* is able to produce and release auxin. First, it was assessed with the use of bioinformatic tools, whether *G. pallida* possessed potential auxin biosynthesis genes. Next, it was determined whether IAA could be detected inside the nematode or its secretions with mass spectrometry. Subsequently published transcriptomic data was utilised to find support for the expression of these auxin biosynthesis genes during nematode infection of plant roots. Lastly, the presence of auxin biosynthesis genes in other parasitic and non-parasitic nematodes was investigated to reflect on the broader role of auxin in the interaction between nematodes and their environment.

The production and release of auxin as well the local manipulation of the auxin level in plant roots by plant-parasitic nematodes is expected to induce the activation of downstream signalling events. In Chapter 5, the role of auxin-induced transcription factors PLETHORA (PLT)3, PLT5, and PLT7 was investigated in root-knot nematode (*M. incognita*) infected Arabidopsis roots. The role of these transcription factors in nematode feeding site formation is unknown, Therefore, the hypothesis was tested whether they contribute to the development of feeding sites and if so, whether they may regulate PIN expression during feeding site formation. The effect of PLTs on the development of root-knot nematode galls was assessed using an infection assay on mutant lines of Arabidopsis. Because of their reported redundancy, single, double and triple knockout mutants of these genes were tested.

To study their influence on polar auxin transport during gall formation, both the expression of PIN1 and PIN3 was studied with fluorescent microscopy in the absence of PLT genes.

Finally, in Chapter 6 mechanisms by which plant-parasitic nematodes can manipulate the balance between auxin and cytokinin levels are discussed. The main findings are integrated and evaluated in the context of basal plant developmental pathways that are recruited by plant-parasitic nematodes to induce feeding sites.

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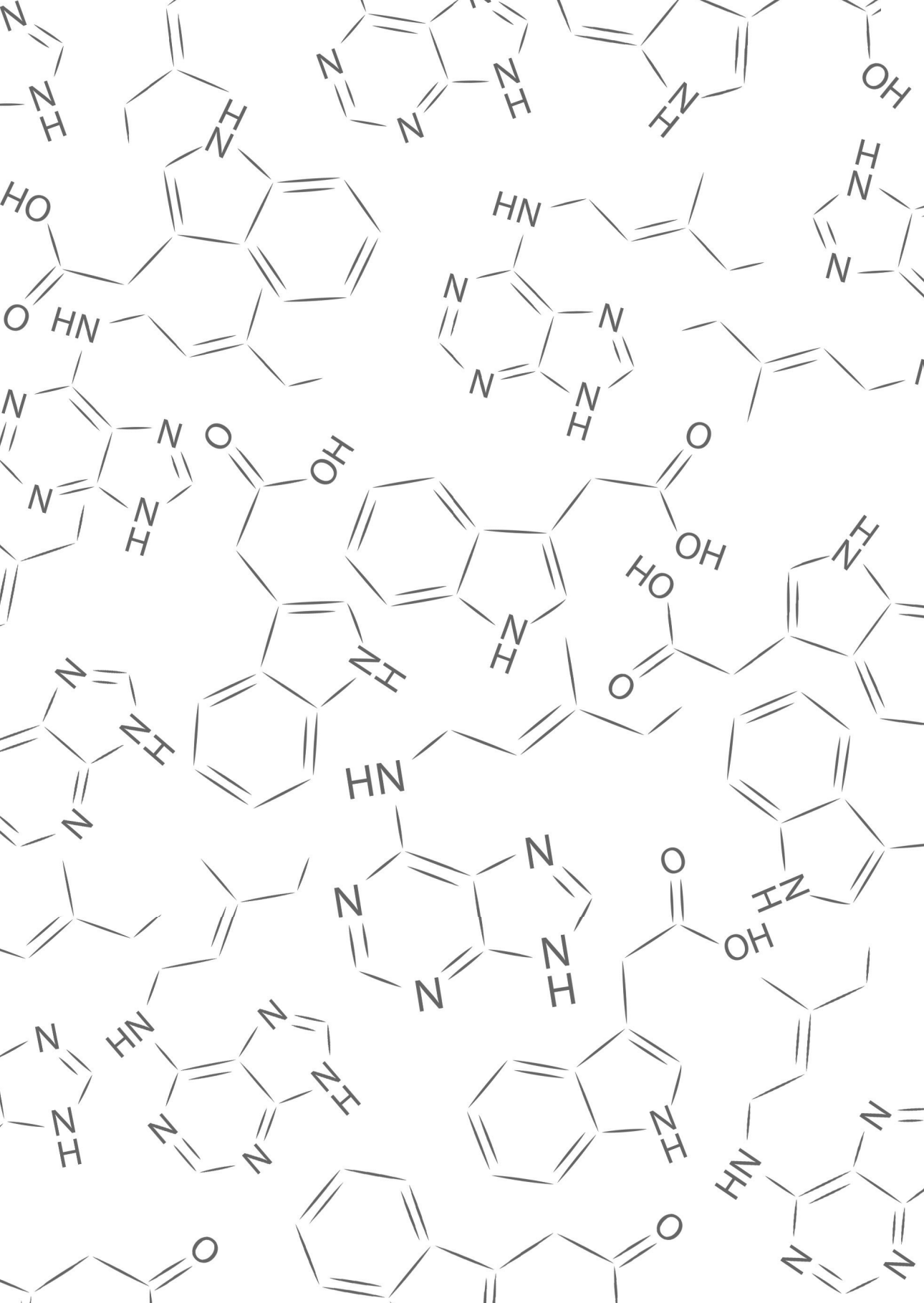

Chapter 2

Sedentary plant-parasitic nematodes alter auxin homeostasis via multiple strategies

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Abstract

Sedentary endoparasites such as cyst and root-knot nematodes infect many important food crops and are major agro-economical pests worldwide. These plant-parasitic nematodes exploit endogenous molecular and physiological pathways in the roots of their host to establish unique feeding structures. These structures function as highly active transfer cells and metabolic sinks and are essential for the parasites growth and reproduction. Plant hormones like IAA are a fundamental component in the formation of these feeding complexes. However, their underlying molecular and biochemical mechanisms are still elusive despite recent advances in the field. This review presents a comprehensive overview of known and potential functions of various auxins in plant-parasitic nematode infection sites, based on a systematic analysis of current literature. We evaluate multiple aspects involved in auxin homeostasis in plants, including anabolism, catabolism, transport, and signalling. From this analysis, we conclude that plant-parasitic nematodes have evolved multiple strategies to manipulate auxin homeostasis to establish a successful parasitic relationship with their host. As such, this review provides novel insights in the complex role of auxin in the nematode parasitism of plants.

Introduction

The establishment of a long-term relationship with a plant can result in the formation of unique specialized organs. For rhizobia, these are nodules on the roots of legumes that encapsulate the nitrogen-fixing bacteria (Oldroyd, Murray et al. 2011). For *Agrobacterium tumefaciens*, these are crown galls in plant stems that form a complex network to facilitate nutrient assimilation within the host (McCullen and Binns 2006). The plant-parasitic cyst and root-knot nematodes follow a similar strategy to form a feeding structure inside the plant root to serve as a nutrient source (Abad and Williamson 2010). These nematodes are obligate biotrophic endoparasites that infect a plant and subsequently manipulate the host machinery to facilitate pathogen survival. Plant-parasitic nematodes (PPN) are a major agro-economical pest partly due to the lack of available effective countermeasures and their complex interaction with their host. Infections by PPN result on average in a yield loss of 12.3% and cause yearly a financial harm of 157 billion dollars (Singh, Singh et al. 2015). The root-knot nematodes *Meloidogyne incognita* and *M. javanica* are considered to be the most rapidly spreading pests among all pests and pathogens worldwide (Bebber, Holmes et al. 2014). In addition, root-knot nematodes of the genus *Meloidogyne* and cyst nematodes of the genus *Heterodera* and *Globodera* are among the top 10 most harmful nematodes (Jones, Haegeman et al. 2013).

Cyst nematodes (CN) hatch from their eggs as second stage juveniles (J2). They subsequently locate a suitable host and penetrate their root system. They move intracellularly through the root cortex and select an initial feeding cell near the vascular bundle by injecting a suite of secretory compounds. These compounds modify host cell metabolism, leading to a partial breakdown of plant cell walls and subsequent merging of neighbouring cells (Sobczak and Golinowski 2011). This results in a so-called syncytium that acts as a nutrient source for the nematode. Root-knot nematodes (RKN) in the J2 stage enter the root near the root tip and move intercellularly towards the apical meristematic region. There RKN turn around and migrate away from the root tip until they reach the differentiating vascular tissue. RKN select several cells in their vicinity, usually pro-vascular, to establish a feeding site upon injection of stylet secretions (Jones and Goto 2011). Subsequently, these cells undergo several rounds of nuclear division and DNA replication that result in large multinuclear and hypertrophied cells. These are known as Giant Cells (GC) and serve as a nutrient source for the nematode. Typically, the RKN creates five to seven giant cells which can increase up to 100 times in size.

While the initiation and development of these feeding sites differ between RKN and CN, there are several striking similarities. Most notably, the accumulation of auxin at the site of infection at the onset of feeding site initiation. Auxin has long been suspected to play a crucial role during nematode infection (Sandstedt and Schuster 1966). However, this had been difficult to study due to the absence of proper molecular methods. Due to the emergence of new techniques over the years, such as confocal microscopy and mass spectrometry more methods have become available to study auxin during nematode infections. For example, reporter studies for both RKN and CN have shown the presence of auxin early on during feeding site initiation and for some time thereafter (Absmanner, Stadler et al. 2013). In addition, preventing auxin accumulation from occurring in Arabidopsis mutants is harmful and compromises nematode infection (Goverse, Overmars et al. 2000). Nevertheless, despite technological advances no clear picture of the role of auxin in nematode infection is available.

Auxin is arguably the most important signalling molecule in plants and plays a key role in nearly all growth and developmental processes (Weijers and Wagner 2016). In turn a lot is known about how auxin synthesis, transport, and perception work in addition to the genes that are transcriptional activated by this hormone (Zhao 2010, Leyser 2018). Auxin is often synonymously used with indole-3-acetic acid (IAA) but it is actually a group of several hormones with similar functions that can where several can also activate classical reporter genes such as DR5-GUS (Sugawara, Mashiguchi et al. 2015). IAA is a major player in many developmental processes such as cell division, root development and organogenesis, but despite the knowledge we have on these processes a complete overall picture of its role during nematode infection remains absent.

In this review, we bring together the scattered information on the many aspects of auxin metabolism in feeding site initiation and development, from biosynthesis, catabolism and transport to perception and signalling. In addition, we will discuss how plant-parasitic nematodes may enact these changes in auxin homeostasis. We first describe the current knowledge on auxin-related processes in plants and afterwards explore how PPN affect these processes upon infection. This is not only done for the auxin known as IAA but also for all other naturally occurring plant auxins to assess what role they may have during parasitic infection. Here, we aim to provide novel insights in the molecular and physiological mechanisms underlying the role of auxin in feeding site formation in plant roots and to highlight new avenues to explore in future research.

Auxins in Nematode feeding sites

In plants, five types of endogenous auxins are present: indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-Cl-IAA), phenylacetic acid (PAA), indole-3-butyric acid (IBA) and indole-3-propionic acid (IPA). IAA has been widely recognised as the most influential auxin in regulating plant growth and development, but other endogenous auxins have been acknowledged to play various important roles. The auxin 4-Cl-IAA is synthesised by several plant species in the phylogenetic clades of the Fabeae and Trifoleae from the Fabaceae family and appears to affect fruit and seed development (Ernstsen and Sandberg 1986, Ozga, Reinecke et al. 2009, Lam, McAdam et al. 2015, McAdam, Meitzel et al. 2017). PAA is widespread among plants, occurs at higher concentrations than IAA, and is capable of regulating the same set of auxin-responsive genes through the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) pathway (Wightman and Lighty 1982, Sugawara, Mashiguchi et al. 2015). IBA was originally thought to be a synthetic auxin until it was detected as an endogenously produced auxin. Although the underlying mechanism remains unknown, IBA strongly induces adventitious root formation at a higher rate than IAA (Epstein and Ludwig-Müller 1993, Ludwig-Müller 2000). IPA is speculated to be present in soybean and has also been found in *Cucurbita pepo* and *Pisum sativum* (L.M. and F. 1982, Schneider, Kazakoff et al. 1985, Jiang, Liu et al. 2020). Additionally, IPA has been detected in Rhizobium root nodules of pea where it binds to horseradish peroxidase C (Badenoch-Jones, Summons et al. 1984, Veitch and Williams 1990). While IAA undoubtedly will stay a focal point of research, the potential roles and functions of other endogenous plant auxins have gained increasing interest of developmental biologists (Ludwig-Müller 2011, Simon and Petrášek 2011, Frick and Strader 2018, Cook 2019).

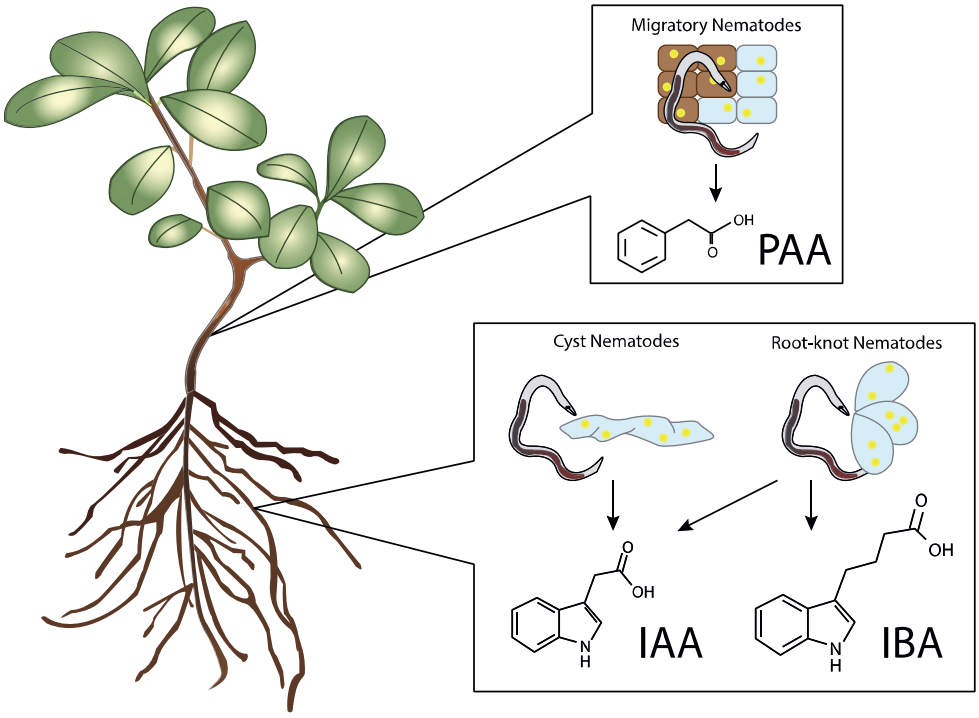

Figure 1 Auxins detected in nematode infected plant tissues. PAA was detected in callus upon infection by the necrotrophic plant-parasitic nematode *Bursaphelenchus xylophilus* (Kawazu, Zhang et al. 1996, Zhang, Kanzaki et al. 1997). Increased levels of IAA have been found in the infection sites of the cyst nematodes *Heterodera schachtii*, *H. cruciferae*, *H. trifolii* and *Globodera rostochiensis*, and in the galls of the root-knot nematodes *Meloidogyne incognita*, *M. hapla* and *M. javanica* (Yu and Viglierchio 1964, Viglierchio and Yu 1968, Hanschke, Borriss et al. 1975, Agarwal, Goel et al. 1985, Ganguly and Dasgupta 1987). Elevated levels of IBA were only found in the infection sites of the root-knot nematode *M. incognita* (Yu and Viglierchio 1964).

Elevated levels of IAA and other auxins have been detected in nematode infection sites of various plant species and in some cases even in egg masses of nematodes (Yu and Viglierchio 1964, Viglierchio and Yu 1968, Hanschke, Borriss et al. 1975, Agarwal, Goel et al. 1985, Ganguly and Dasgupta 1987). These auxins were detected during infections by the root-knot nematodes *Meloidogyne incognita*, *M. hapla*, *M. javanica*, and the cyst nematodes *Heterodera schachtii*, *H. cruciferae*, *H. trifolii*, and Globodera r*ostochiensis* (Figure 1). These auxins were measured using chromatography combined with the Avena first internode test, as well as Ehrlich and Salkowski chemical tests (Yu and Viglierchio 1964). Thus, already in the early 60s a prominent role for auxins in nematode feeding site development was recognized. Besides IAA, IBA was exclusively be detected in the galls of *M. incognita* (Viglierchio and Yu 1968). However, the absence in other species might be attributed to the difficulty in measurement because of the transient nature of this hormone due to its conversion to IAA. IBA is thought to mainly act through conversion to IAA and contributes to root hair expansion, secondary root formation, and the auxin pool in the root apical meristem (Frick and Strader 2018). As such, there is a potential role for IBA in feeding cell development, because lateral root formation and feeding cell development share similar developmental processes (Goverse

and Bird 2011). Infection of *M. incognita* as well as other RKNs and CNs lead to spontaneous secondary root formation originating from the feeding site to which auxins seems to contribute (Olmo, Cabrera et al. 2017, Levin, Tucker et al. 2020). Whereas a role of auxin in cyst and root-knot nematode parasitism has been widely accepted, this is not the case for plant-parasitic nematodes with a migratory lifestyle. Therefore, it is interesting to note that PAA has been detected in callus tissue infected with *Bursaphelenchus xylophilus* (Kawazu, Zhang et al. 1996, Zhang, Kanzaki et al. 1997). This nematode inflicts the pine wilt disease and it has been suggested that PAA induces benzoic acid accumulation which results in the wilting of plant tissue (Kawazu, Zhang et al. 1996). However, not all auxin types have been studied during PPN infection. To the best of our knowledge, no records have been published describing measurements of the auxins 4-Cl-IAA and IPA in infection sites of plant-parasitic nematodes.

Manipulation of *de novo* auxin biosynthesis in nematode feeding sites

Elevated levels of auxins during nematode infection can be the result of various mechanisms, one of which is increased auxin biosynthesis. Indole moiety containing auxins (IAA, 4-Cl-IAA, IBA, IPA) are derived from tryptophan (Trp) while non-indole auxins such as PAA are presumably synthesized from phenylalanine (Cook, Nichols et al. 2016). Both tryptophan and phenylalanine derive from the shikimate metabolic pathway, which is not present in animals, making these compounds essential amino acids. *De novo* IAA is synthesized from amino acids in the aerial part of the plant as well as specific regions in the root, such as the meristematic zone (Ljung, Hull et al. 2005). Several biosynthesis routes have been postulated for the synthesis of indole-3-acetic-acid. For Trp-dependent IAA synthesis four pathways are proposed: (1) the indole-3-acetamide (IAM) pathway, (2) the indole-3-pyruvic acid (IPyA) pathway, (3) the tryptamine (TAM) pathway and (4) the indole-3-acetaldoxime (IAOx) pathway (Mano and Nemoto 2012). Here we discuss which auxin biosynthesis routes may be relevant for successful nematode infections.

All known auxin biosynthesis routes of IAA start with tryptophan although it is speculated that an non-Trp dependant pathway exists. Tryptophan concentrations increase in the feeding sites of *M. incognita* (Setty and Wheeler 1968). In addition, the intermediate compounds indole-3-acetonitrile (IAN) can be found in galls of this nematode as well (Yu and Viglierchio 1964). IAN is an intermediate compound of the IAOx biosynthesis pathway. The presence of this compounds suggests that local auxin biosynthesis occurs in feeding sites (Figure 2). This is supported by transcriptome analyses of cyst and root-knot nematode infections showing that IAA biosynthesis genes are differentially expressed, such as *AMI*, *CYP79B2*, *CYP79B3* and *YUCCA* (Supplementary Table S1 at *JXB* online). Most differentially expressed auxin biosynthesis genes are upregulated upon nematode infection. Additionally, some of these genes support a role for the IAM and the IPyA pathway in local auxin biosynthesis. Although no intermediary compounds for these pathways are reported, modern day techniques may shed light on this. The transcriptome data together with the increased amount of tryptophan and IAN indicate that local auxin biosynthesis occurs during infection of plant roots by CN and RKN.

Aside from the IAOx, IAN, and IPyA pathway, the TAM biosynthesis route is affected as well during parasitism. The first step in the TAM biosynthesis pathway is the conversion of Trp to

TAM and is catalysed by the enzyme tryptophan decarboxylase (TDC). TDC has been shown to be transcriptionally upregulated in feeding sites of the cyst nematodes *H. avenae* and showed increased enzyme activity in that of *G. rostochiensis* (Giebel, Jackowiak et al. 1979, Huang, Li et al. 2018). TDC loss-of-function or overexpression mutants in *Aegilops variabilis*, a close relative to wheat, do not seem to affect IAA content during *H. avenae* infection despite its role in auxin biosynthesis (Huang, Li et al. 2018). This is in accordance with experimental evidence showing a low contribution of the TAM pathway to the overall IAA pool in plants (Facchini, Huber-Allanach et al. 2000). Furtheremore, TDC regulates secondary metabolites rather than auxin biosynthesis suggesting that TDC has a secondary role in plant defence (Huang, Li et al. 2018).

Aforementioned, changes in local auxin biosynthesis seem to occur during nematode infection. The nematode effector chorismate mutase (CM) is suspected to contribute to these changes. Although the nematodes inject a suite of effectors into host plants, little is known about effectors directly targeting auxin biosynthesis, with the exception of CM. CM was first discovered in *M. javanica*, was later found to be widely distributed in both cyst and root-knot nematodes, and is currently used as a diagnostic marker for nematode parasitism (Lambert, Allen et al. 1999, Bekal, Niblack et al. 2003, Doyle and Lambert 2003, Jones, Furlanetto et al. 2003, Huang, Dong et al. 2005, Long, Wang et al. 2006, Vanholme, Kast et al. 2009, Yu, Chronis et al. 2011, Chronis, Chen et al. 2014). Upon discovery, it was suggested that CM alters plant cell development by negatively affecting auxin biosynthesis (Doyle and Lambert 2003). Endogenous CM converts chorismate to prephenate in the shikimate pathway which occurs in plastids (Tohge, Watanabe et al. 2013). Coincidently, chorismate is also the branching point in the shikimate pathway from the biosynthesis of indole to the biosynthesis phenylalanine and tyrosine. As such, the release of the effector chorismate mutase could lead to resource competition over chorismate and as a result shift more to the synthesis of phenylalanine and tyrosine than to indole and by extension IAA (Doyle and Lambert 2003). As phenylalanine and tyrosine are also amino acids it is possible that the nematode uses chorismate mutase to increase nutrient production. Alternatively, the increase in phenylalanine via the shikimate pathway may enhance the production of the non-indole auxin PAA although its presence in feeding sites of cyst and root-knot nematodes remains to be elucidated. In addition, it is worthwhile noting that the plant defence hormone salicylic acid (SA) is also produced from phenylalanine. However, various data suggest that the secreted CM actually reduces the synthesis of the plant defence hormone salicylic acid (SA) (Djamei, Schipper et al. 2011, Wang, Xue et al. 2018). Biosynthesis of SA occurs in plastids and uses the plastid fraction of chorismate, while a secreted CM would act on the cytoplasmic fraction of the chorismate pool. Likely, the secreted CM likely reduces the cytoplasmic fraction of chorismate leading to an increased flow of chorismate from the plastids to the cytosol resulting in a depletion of the chorismate in the plastid fraction and therefore a decrease in SA biosynthesis.

It can be concluded that nematodes seem to locally increase auxin biosynthesis through increasing precursor metabolites and increasing biosynthesis gene expression, thereby stimulating production of IAA most likely through the IAOx pathway. In addition, transcriptional analysis revealed that nematodes affect the IAN and IPyA pathway as well as the TAM pathway despite its low contribution to the overall IAA pool. Lastly, cyst and root-knot nematodes inject effectors e.g. chorismate mutase to reduce SA biosynthesis, which may

have hypothetically an inhibitory effect on local IAA synthesis and a positive effect on PAA production (Figure 2).

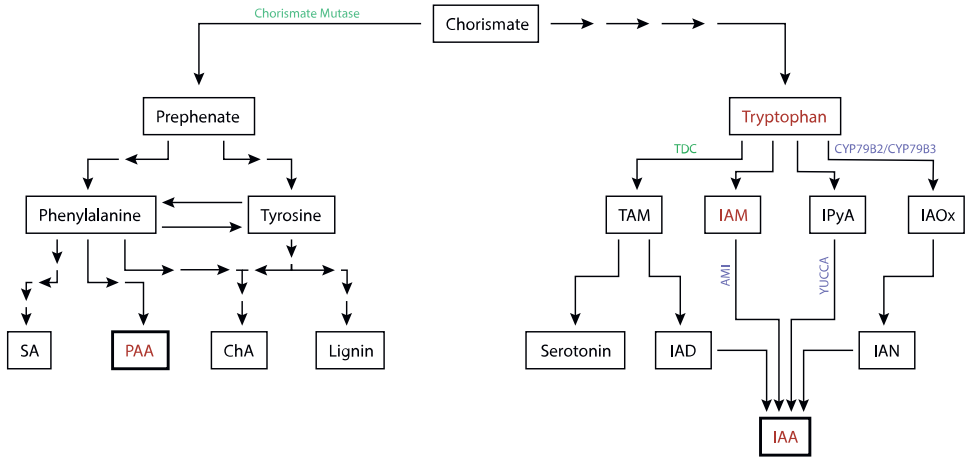

Figure 2 Simplified biosynthesis pathway of the auxins IAA and PAA in plants from the common precursor chorismate. Auxins and auxin precursors for which upregulation is shown are indicated (red). Enzymes involved in the biosynthesis pathway shown to be differentially regulated either at the protein level (green) or at the transcriptome level (purple) are depicted (Supplementary Table S1). The number of arrows indicate the number of conversion steps with four indicating four or more intermediate steps. Converging arrows show a shared intermediary compound.

Abbreviations: SA - Salicylic acid, PAA – Phenylacetic acid, ChA – Chlorogenic acid, TAM – Tryptamine, IAM – Indole-3-acetamide, IPyA – Indole-3-pyruvic acid, IAOx – Indole-3-acetaldoxime, IAD – Indole-3-acetaldehyde, IAN – Indole-3-acetonitrile, IAA – Indole-3-acetic acid

Manipulation of auxin catabolism in nematode feeding sites

An alternative strategy for nematodes to elevate auxin levels is to interfere with auxin breakdown. The main inactivation pathway known for IAA is oxidation to 2-oxindole-3-acetic acid (oxIAA) (Pěnčík, Simonovik et al. 2013). The mechanisms of IAA oxidation have long been considered to be enacted through peroxidases. In addition, it was believed that IAA levels were affected by phenolic compounds via inhibition of peroxidase activity (Sági and Garay 1961). This has been the prevailing view in plant nematology for many years as well. Various studies demonstrated that peroxidase activity is downregulated upon infection with cyst and root-knot nematodes (Giebel 1970, Szczygiel and Giebel 1970, Wilski and Giebel 1971, Janas 1976, Ganguly and Dasgupta 1987, Molinari 1991, Leela, Khan et al. 1993). A commonly used method to assess IAA oxidase activity upon nematode infection was to add horseradish peroxidases to root homogenates and measure O_2 production (Giebel 1970, Giebel 1974, Giebel and Lamberti 1977). These measurements revealed that phenolic compounds are key in regulating oxidase activity upon nematode infection (Figure 3A). Later on, it was shown that peroxidases do not play a significant physiological role as IAA oxidases in plants (Normanly, Slovin et al. 1995, Ljung, Hull et al. 2002, Stepanova and Alonso 2016).

Considering that peroxidases have no significant IAA oxidase activity *in planta*, the relation between phenols and nematode infection needs to be re-examined and therefore we propose a new relation (Figure 3). Early studies have shown that phenol content is often correlated with susceptibility to PPN (Szczygiel and Giebel 1970, Knypl and Chylinska 1975, Giebel and Dopierala 1982), where the polyphenolic fraction has an inhibiting effect on IAA oxidation and the monophenolic fraction a stimulating one. It was shown for *M. incognita* and *G. rostochiensis* that the ratio between mono- and poly-phenols correlates to susceptibility (Giebel 1970, Yuan, Cappellini et al. 1996). A plausible function of peroxidases in mediating resistance (Simonetti, Alba et al. 2010, Jin, Hewezi et al. 2011, Sung, Lee et al. 2019) is the generation of reactive oxygen species (ROS). ROS stimulate cell wall reinforcement through lignification and extensin cross-linking and incite additional defence mechanisms (Almagro, Gómez Ros et al. 2009, Marhavý, Kurenda et al. 2019). Thus, peroxidases remain related to nematode infection, albeit through a different function than originally thought (Figure 3B).

In 2013, the characterization of a male-sterile mutant in rice resulted in the identification of the candidate enzyme DIOXYGENASE FOR AUXIN OXIDATION (DAO) as a player in this oxidation mechanism (Zhao, Zhang et al. 2013). Orthologs of this gene have subsequently been identified in Arabidopsis demonstrating the conserved nature of this gene between monocots and dicots (Mellor, Band et al. 2016, Porco, Pěnčík et al. 2016). Mutant and expression studies of DAO showed little impact on the general oxIAA pool, making it unlikely that DAO is the main component in this process (Stepanova and Alonso 2016). Various transcriptomic datasets suggest that *AtDAO1* and *AtDAO2* in *A. thaliana* are upregulated upon cyst and root-knot nematode infection (Supplementary Table S1). We therefore hypothesise that feeding sites may contain several redundant oxidation pathways that have yet to be identified.

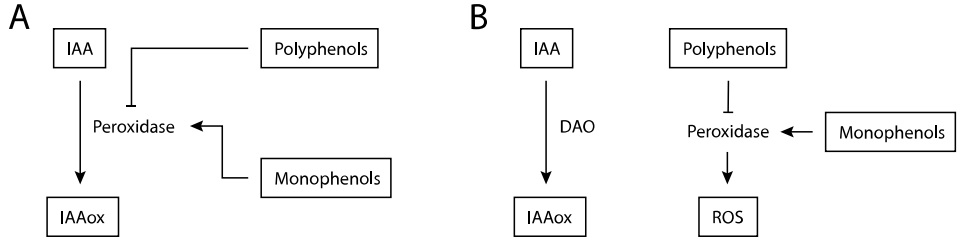

Figure 3 Proposed mechanisms involved in IAA inactivation during nematode infection of plants. (**A**) Previously, a mechanism was suggested for IAA inactivation during nematode infection based on Giebel 1970, Giebel 1974, Giebel and Lamberti 1977. Peroxidase is assumed to facilitate the conversion of IAA to IAAox, which is stimulated by increased monophenols levels and inhibited by polyphenols. (**B**) Based on new insights (Normanly, Slovin et al. 1995), an alternative model for IAA inactivation is proposed. DAO facilitates IAA oxidation, but it is anticipated that additional inactivation mechanisms are involved. In this model, peroxidases remain regulated by phenols but now enhance ROS production as reported by Simonetti, Alba et al. 2010, Jin, Hewezi et al. 2011, Sung, Lee et al. 2019.

Abbreviations: IAA – Indole-3-acetic acid, IAAox – oxidized indole-3-acetic acid, DAO – DIOXYGENASE FOR AUXIN OXIDATION, ROS – Reactive Oxygen Species

Conjugation of auxins in nematode feeding sites

An alternative method for inactivating auxins is through ester or amide conjugation with an amino acid, sugar or protein. The conjugated forms are presumed to function as either storage or intermediates destined for degradation (Woodward and Bartel 2005). The conversion of an auxin to an ester conjugate with sugar involves UDP-glucose transferases, whereas conversion to an amide conjugate with amino acids transpires through IAA-amino acid conjugate synthetases (Ludwig-Müller 2011). Conversion to an amide protein conjugate occurs through a still unknown mechanism. Beside IAA, all other auxins can be conjugated in this manner (Bajguz and Piotrowska 2009). Although auxins can be conjugated to nearly all amino acids, their functions differ. IAA conjugates IAA–Asp and IAA–Glu are considered precursors for a degradation pathway while IAA-Trp acts as an inhibitor of auxin biosynthesis (Staswick 2009). Additionally, a small fraction of auxin amino acid conjugates acts as storage forms and can be hydrolysed back to their unbound form via auxin amino acid conjugate hydrolases (LeClere, Tellez et al. 2002).

These processes seem to be affected during nematode infection at various time points. The GRETCHEN HAGEN 3 (GH3) family is such an example that is active in white clover (*Trifolium repens* cv. Haifa) during the initiation of root galls by *M. javanica* (Hutangura, Mathesius et al. 1999). The GH3 family is co-responsible for regulating the activity of IAA, SA and JA and consists of three phylogenetic groups. The second group consists of IAA-amino acid conjugate synthetases and is one of the primary auxin-response genes (Zhang, Zhang et al. 2018). As such, these GH3 genes are responsible for creating IAA-amino acid conjugates for either storage or degradation purposes. Transcriptomic data suggest that several GH3 genes are expressed in the feeding sites of cyst and root-knot nematodes (Beneventi, da Silva et al. 2013). They are predominantly upregulated, indicating a positive role for GH3 genes in feeding site development (Supplementary Supplementary Table S1). While upregulated at early stages of infection, a reporter study in white clover (*Trifolium repens* cv. Haifa) with *M. javanica* shows that GH3 disappears after 72 hours post inoculation (Hutangura, Mathesius et al. 1999). This suggests that GH3 genes may play a role in regulating hormone levels during early feeding site initiation and afterwards might be downregulated to maintain high levels of free active IAA. The biological significance of the downregulation after 72H is supported by the overexpression mutant of *GH3.1* in *Oryza sativa*, which decreases the infection by *M. graminicola* (Yimer, Nahar et al. 2018).

In conclusion, all natural auxins can be conjugated with an amino acid, sugar or protein and this process seems to contribute to fine tuning IAA levels during nematode infection. The temporal variation in GH3 expression levels seems to be required for successful infections. However, it remains unknown which IAA conjugates are created in the feeding site and if other auxins see a similar fate.

Auxin transport in nematode feeding sites

Next to auxin anabolism and catabolism, transport processes are key players in the accumulation of auxins at specific locations. IAA is transported as either a general unregulated bulk flow of auxin through mature phloem or through an actively regulated carrier-mediated

cell-to-cell directional transport called active polar auxin transport (PAT) (van Berkel, de Boer et al. 2013). PAT is mediated by a set of specific efflux and influx carriers. In Arabidopsis, the transmembrane proteins of the AUX1/LIKE AUX1 (AUX1/LAX) family act as influx carriers. Efflux is enacted by the PIN-FORMED (PIN) proteins and the ATP-binding cassette subfamily B (ABCB)-type transporters of the multidrug resistance/phosphoglycoprotein (ABCB/MDR/PGP) protein family (Cho and Cho 2013, Adamowski and Friml 2015). Of the PIN protein family PIN5, PIN6, and PIN8 are presumed to regulate IAA transport between the endoplasmic reticulum and the cytosol and are suspected to regulate free IAA levels in the cytoplasm. The other PIN proteins (PIN1, PIN2, PIN3, PIN4, PIN7) are localised at the plasma membrane and facilitate cellular auxin efflux (Krecek, Skupa et al. 2009). Five members of the ABCB subfamily have so far been associated with auxin transport: ABCB1, ABCB4, ABCB14, ABCB15 and ABCB19. The ABCB proteins localise to the plasma membrane of the cell in a non-polar manner and maintain auxin homeostasis providing a stable and uniform auxin distribution among cells (Cho and Cho 2013).

Transport proteins for the auxins IPA and PAA have yet to be discovered. However, PAA forms concentration gradients in maize coleoptile similar to IAA (Sugawara, Mashiguchi et al. 2015). These PAA gradients are unaffected by the PIN transport inhibitor NPA and do not change when subjected to gravitropism stimuli. From this it was concluded that PAA is not transported actively and directionally in plants. As such PAA has distinct transport characteristics compared to IAA and it was speculated that the formation of the PAA gradient might be attributed to regulation of local biosynthesis. The exact transport proteins of 4-Cl-IAA are unknown, but an auxin transport competition assay suggests that IAA influx and efflux proteins transport 4-Cl-IAA in addition to IAA (Simon, Kubeš et al. 2013). In the case of IBA some transporters have been identified. Its efflux is promoted by ATP-BINDING CASSETTE G36/PLEIOTROPIC DRUG RESISTANCE 8/PENETRATION 3 (ABCG36/PDR8/PEN3) and ABCG37/PDR9/PIS1 (Strader and Bartel 2009, Růžička, Strader et al. 2010). Although IBA uptake is a saturable process it is suspected to be transported across long distances in the plant mainly in a conjugated form (Rashotte, Poupart et al. 2003).

PAT is important for induction and development of nematode feeding sites and contributes to the local accumulation of auxin upon infection. This was already shown in 1972, where removal of the shoot tip from *Lycopersicon esculentum* resulted in a sharp decrease in infections of *G. rostochiensis* (Steinbach 1972). Additionally, the disruption of PAT through inhibition with N-(1-naphthyl)phtalamic acid (NPA) during *G. rostochiensis* infection resulted in abnormal syncytium development (Goverse, Overmars et al. 2000). PAT is significantly altered during feeding site development by both cyst and root-knot nematodes. Reporter studies have shown that during infection of *H. schachtii* PIN1 and PIN7 are absent from the feeding site suggesting a strong decrease in downward auxin flow (Grunewald, Cannoot et al. 2009). Interestingly, PIN3 and PIN4 seem to be highly expressed and specifically localise towards the lateral sites of the feeding site. During RKN infection PIN3 seems to be active during early infection in neighbouring cells at the basipetal side of the gall but not inside the feeding site. However, PIN3 expression changes at later stages where it also becomes active in giant cells. PIN1 seems to be slightly higher expressed at the basal site of young galls whereas PIN7 expression seems to be suppressed. This suggests that in giant cells the downward auxin flow is stemmed and laterally redistributed.

PIN localisation is tightly controlled and influenced by multiple factors. An intriguing example of this is the cyclophilin A ortholog *DIAGEOTROPICA* (*DGT*) gene in *Solanum lycopersicum* (Goverse, Overmars et al. 2000). The *DGT* mutant abolishes the organogenesis of lateral roots and affects PIN expression and localization to the plasma membrane at the root tip (Ivanchenko, Zhu et al. 2015). This has been shown in an expression study where *PIN2* transcripts decrease in the *dgt* mutant and the localisation of PIN1 and PIN2 is affected when *DGT* is expressed. When infecting a tomato *dgt* mutant with *G. rostochiensis* inhibition of nematode development was observed, suggesting that the regulation of *PIN1* and *PIN2* through *DGT* is necessary for the development of nematode feeding sites (Goverse, Overmars et al. 2000). The auxin efflux protein PIN2 is not only regulated by DGT, but additionally seems to be the target of several hormonal signalling pathways that control its localisation during nematode infection. PIN2 is also known as ETHYLENE INSENSITIVE ROOT 1 (EIR1) and its gene was first identified as a knockout plant insensitive to ethylene which later was recognised as PIN2. This mutant showed decreased susceptibility to *H. schachtii* in Arabidopsis (Goverse, Overmars et al. 2000, Wubben, Su et al. 2001). Additionally, PIN2 localisation is affected by a novel hormone class known as strigolactones (SL) that function as branching inhibitors. It has been shown that the SL deficient mutant *max4-1* and the SL signalling mutant *max2-1* have enhanced nematode infection due to the formation of enlarged feeding cells possibly through interaction with PIN2 (Escudero Martinez, Guarneri et al. 2019). In addition to ethylene and strigolactones, the cytokinin hormones could affect PIN localisation during nematode infection as well. Cytokinins accumulate in feeding sites and are essential for proper nematode development (Lohar, Schaff et al. 2004, Siddique, Radakovic et al. 2015). Interestingly, it has been shown that *H. schachtii* can produce cytokinins that affect infection (Siddique, Radakovic et al. 2015). Cytokinins are known to affect the localisation of PIN1, PIN3 and PIN7 but whether this occurs during nematode infection remains unknown (Bishopp, Help et al. 2011, Marhavý, Duclercq et al. 2014, Šimášková, O'Brien et al. 2015, Waldie and Leyser 2018). Interestingly, PIN3, but not other PIN proteins, can also be induced by the hormone PAA, suggesting that PAA might also be a regulator of PIN expression (Sugawara, Mashiguchi et al. 2015).

The CN infection process goes along with an increased expression of the AUX1 importer in young feeding sites, potentially resulting in a greater auxin influx (Mazarei, Lennon et al. 2003). LAX genes together with AUX1 are important during nematode feeding sites initiation as single mutants show no effect on infection but the double *aux1/lax3* and quadruple mutant *aux1/lax1/lax2/lax3* display a marked decrease in infection. Additionally, the effector protein 19C07 of *H. schachtii* interacts with LAX3 and is thought to assist in increasing auxin influx and induce numerous cell wall–remodelling enzymes (Lee, Chronis et al. 2011). During RKN infection the expression of AUX1 and LAX3 is increased and likely, also leads to an increase of auxin similar to cyst nematode infections (Kyndt, Goverse et al. 2016). However, no responsible effector has been identified yet.

The role of ABCB efflux carriers remain elusive in the infection process, but interestingly transcriptomic data suggest that these transporters are generally downregulated upon nematode infection by *H. schachtii*, *H. glycines* and *M. incognita* (Supplementary Table S1). This suggests that these efflux carriers are downregulated to prevent the establishment of a uniform auxin distribution. This in turn would keep the auxin maxima localised at the feeding site and prevents it from dissipating.

Overall, it seems that cyst and root-knot nematodes manipulate both influx and efflux carriers to induce an auxin accumulation in feeding sites. PIN proteins show complex alterations in expression and localisation patterns to facilitate auxin accumulation, whereas auxin importers are upregulated upon infection ABCB transporters are downregulated to prevent auxin leakage. The precise mechanisms by which efflux and influx carriers can be manipulated by nematodes remain unknown, but both nematode effectors and changes in hormonal pathways seem to be relevant.

Auxin perception in nematode feeding sites through the TIR1-AFB signalling cascade

The nematode employs various means to generate and maintain an auxin maximum in its feeding site. These elevated levels of auxin result in the transcription of a large set of genes that enact their function and contribute to the infection. In Arabidopsis, a family of 23 DNA-binding AUXIN RESPONSE FACTOR (ARFs) transcribe auxin-responsive genes (Li, Xie et al. 2016). In the absence of IAA, these transcription factors are inhibited through dimerization with a member of the auxin/indole-3-acetic acid (Aux/IAA) family. In the presence of IAA the inhibitor is ubiquitinated by an E3 ubiquitin ligase SKIP, CULLIN, F-BOX (SCF) complex and subsequently degraded (Salehin, Bagchi et al. 2015). IAA facilitates the binding between the Aux/IAA and the F-box gene from the SCF complex that is part of the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOXs (TIR1/AFBs) receptor family (Salehin, Bagchi et al. 2015). As such, the subsequent activation of the ARF transcription factors is called the TIR1-AFB signalling pathway.

Other auxins can activate this TIR1-AFB signalling pathway in varying degrees. The auxin IPA does not significantly interact with the TIR1-AFB pathway with only a very weak binding and with a noticeably more rapid dissociation rates (Uzunova, Quareshy et al. 2016). IBA does not activate the IAA signalling cascade as the molecule possesses a lengthened side chain that makes it unable to adopt a conformation for binding into the TIR1–Aux/IAA co-receptor pocket (Uzunova, Quareshy et al. 2016).The auxin 4-Cl-IAA binds to the TIR1 auxin receptor (Jayasinghege, Ozga et al. 2019) and is able to activate the signalling cascade with at least partial overlap in gene activation (Johnstone, Reinecke et al. 2005). As 4-Cl-IAA can activate the same signalling cascade as IAA it was expected that the response to the former would barely differentiate from that of IAA. However, 4-Cl-IAA stimulates gibberellin biosynthesis and regulates the ethylene response in a manner that IAA cannot (Jayasinghege, Ozga et al. 2017). Similarly, PAA activates the TIR1-AFB signalling pathway and has many overlapping primary response genes with IAA (Sugawara, Mashiguchi et al. 2015). However, all major processes regulated by PAA can also be regulated by IAA and its functional difference from IAA is attributed to its difference in transport. Activation of the TIR1-AFB signalling complex by any auxin results in the transcription of primary auxin response genes.

The primary auxin response genes can be categorised into three major groups: Aux/IAA, GRETCHEN HAGEN 3 (GH3), and small auxin up RNA (SAUR) (Abel and Theologis 1996). The Aux/IAA family functions as transcriptional repressors of auxin-response genes but are also one of the first major groups to be upregulated upon auxin signalling. This negative feedback

loop results in a complex regulatory mechanism of IAA signalling given the flexibility in the binding specificity of Aux/IAA to a particular ARF (Luo, Zhou et al. 2018). The GH3 family of acyl-acid-amido synthetases catalyse the conjugation of amino acids to various endogenous hormones among which is auxin (Chen, Westfall et al. 2010). IAA-amino acid conjugates are essential for maintaining auxin homeostasis as they can function as either degradation intermediates or storage forms (Woodward and Bartel 2005). The SAUR family is the largest group of early response genes and was originally identified in a differential hybridization screen for genes that were rapidly induced by auxin in elongating soybean hypocotyl sections (McClure and Guilfoyle 1987). SAUR genes contain no known biochemical motif to indicate their function but have a variety of different roles in cellular, physiological, and developmental processes (Ren and Gray 2015). Of note here is the capability of some SAURs to regulate auxin transport, which is suspected to occur through either elevated plasma membrane H^+-ATPase activity or Ca^{2+} regulation due to the CaM-binding activity of those SAURS (Ren and Gray 2015).

For *H. schachtii* it has been shown that nearly all ARFs are active in syncytia and their expression patterns vary among infection stages (Hewezi, Piya et al. 2014). A select group of ARFs (3, 6, 10-12, 14, 15, 20-22) seems to be expressed in the syncytium during early feeding site initiation, while a different set of ARFs (1, 2, 4, 5, 7, 9, 17-19) is expressed in neighboring cells. Transcriptomic databases reveal that several ARF genes are also expressed during *M. incognita* infection, but the precise timeframe and localisation remain unclear (Supplementary Table S1). LATERAL ORGAN BOUNDERIES-DOMAIN (LBD) genes are a family of transcription factors transcribed by various ARF proteins and play a crucial role in the growth and development of plants (Lee, Cho et al. 2015). *LBD16* is an example of a downstream signalling gene that is expressed in RKN feeding sites and is coincidentally induced through both IAA and PAA (Cabrera, Diaz-Manzano et al. 2014, Sugawara, Mashiguchi et al. 2015, Olmo, Cabrera et al. 2017). *LBD16* is induced by either ARF7 or ARF19 and functions as a transcription factor leading to the divisions in the xylem pole pericycle for lateral root formation. Its importance in root-knot nematode feeding site formation is demonstrated by the *35S::LBD16-SRDX* overexpression line in which no feeding sites could be established and by the *lbd16* knockout mutant which shows a lower infection rate (Cabrera, Diaz-Manzano et al. 2014, Olmo, Cabrera et al. 2017). The result of LBD16 repression by the SRDX protein is more severe than that of the *lbd16* knockout but this is potentially explained by the fact that the *35S::LBD16-SRDX* transgene might also repress the transcription of other LBD16 homologues, thereby exacerbating the effect. In contrast to root-knot nematodes, *LBD16* and its co-regulated genes seem to be selectively repressed in feeding sites of the cyst nematode *Heterodera schachtii* (Cabrera, Fenoll et al. 2015). In addition, neither an overexpression mutant or a SRDX repression line seems to have any effect on the infection rate (Cabrera, Diaz-Manzano et al. 2014).

Regulation of ARF expression is required in nematode feeding sites, given their distinct expression profiles. The major functional regulator of ARF activity is the Aux/IAA repressor family. As a primary auxin response group several Aux/IAA genes are upregulated in nematode feeding sites (Supplementary Table S1). It has been shown that the effector protein 10A07 of *H. schachtii* physically interacts with Aux/IAA16 (IAA16) and presumably inhibits its function to promote auxin signalling (Hewezi, Juvale et al. 2015). This hypothesis is supported by the fact that knockout mutants of IAA16 and IAA7, a protein from the same clade, result

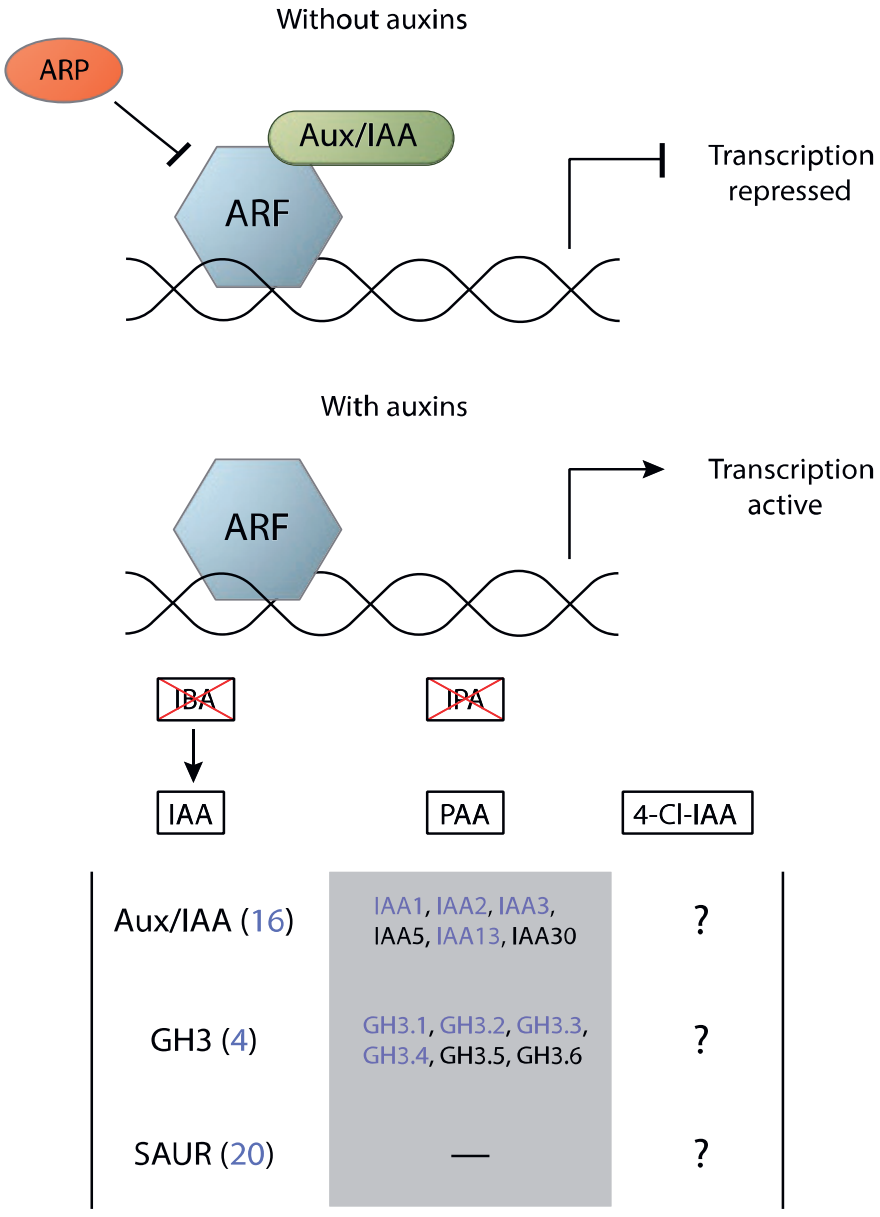

Figure 4 Simplified mechanism of auxin perception through the TIR1-AFB signalling cascade leading to the activation of primary auxin response genes. Transcription of auxin response genes is repressed by the Aux/IAA and ARP proteins (top). The presence of the auxins PAA, IAA, and 4-Cl-IAA degrade Aux/IAA and allow for gene transcription by ARF (bottom). The effect of the auxin IBA on transcription by ARF is mediated via conversion into IAA and the auxin IPA does not significantly activate the signalling pathway. Members of three primary response gene families can be activated by this system, of which various members are differentially regulated in feeding sites of cyst and root-knot nematodes (indicated in purple) (Supplementary Table S1). There is partial overlap between the genes that can be activated by PAA and those that are activated by IAA. On the other hand, it is unknown which primary response genes 4-Cl-IAA can activate.

in higher susceptibility to cyst nematodes (Goverse, Overmars et al. 2000, Hewezi, Juvale et al. 2015). Interestingly, a mutant of another closely related protein, *IAA14* results in a more resistant plant (Grunewald, Karimi et al. 2008). Mutants of the Aux/IAA genes *IAA12* and *IAA28* additionally show a root-knot nematode resistant phenotype (Olmo, Cabrera et al. 2020). Another regulator of ARF is AUXIN REPRESSED PROTEIN (ARP). It has been shown that ARP1 can act as a repressor of plant growth and an activator of disease resistance by regulating the expression of ARF8 (Zhao, Li et al. 2014). The *ARP* gene in a wild peanut relative, *Arachis stenosperma* is upregulated during infection by *M. arenaria* (Guimaraes, Brasileiro et al. 2010). There it likely functions as a regulator of ARF expression during infection.

Activity of the primary auxin response group GH3 can be detected in feeding sites early on in the infection of white clover (*Trifolium repens* cv. Haifa) by *M. javanica* (Hutangura, Mathesius et al. 1999). GH3 genes are mainly involved in hormone storage and degradation to maintain auxin homeostasis. They are downregulated 72H post inoculation suggesting that they play a role in regulating hormone levels during early feeding site initiation. Afterwards, GH3 genes might be downregulated to maintain high levels of free active IAA. Coincidentally, PAA induces, similarly to IAA, the expression of several GH3 (*GH3.2*, *GH3.3*, *GH3.4* and *GH3.5*) and Aux/IAA (*IAA1*, *IAA2*, *IAA5* and *IAA13*) genes (Sugawara, Mashiguchi et al. 2015).

The last primary auxin response group, SAUR, is likewise differentially expressed in nematode feeding sites and generally seems to be downregulated during infection (Supplementary Table S1). Little is known about the role of individual SAURs during nematode infection, however, given their potential to regulate auxin transport a better understanding of SAURs might give insight in how nematodes affect PIN localisation during infection. The relation between SAURs and PINS is still unclear, but it has been shown in *A. thaliana* that PIN2 is crucial for the proper functioning of SAUR19-mediated root waving (Spartz, Lee et al. 2012).

Overall, during nematode infection the perception of auxin changes and all major primary response groups are involved (Figure 4). A large set of ARF genes becomes active and exhibit distinct spatial expression patterns in the infection site. ARF activation leads to the transcription of all three primary response groups. The transcription of the Aux/IAA group gives in turn another layer of complexity to the regulation of auxin-mediated ARF signalling. In addition, GH3 genes become active likely to regulate hormone levels and the SAUR family, although active, remains an unknown factor in the infection process.

Auxin signalling in nematode feeding sites through non TIR1-AFB pathways

Perception of IAA above a certain threshold activates the TIR1-AFB signalling cascade, which in turn expresses a certain set of auxin-responsive genes. However, not all auxins activate this signalling pathway. Independent from or in addition to the TIR1-AFB signalling cascade some auxins affect gene transcription through not yet identified signalling pathways. This section will discuss the various effects of natural-occurring auxins upon nematode infections through these unknown signalling mechanisms.

Of the five natural occurring auxins the ones that are indicated to act outside the classical signalling cascade are 4-Cl-IAA, IPA, and IBA. Although 4-Cl-IAA can activate the TIR1-AFB

signalling pathway, it stimulates gibberellin biosynthesis and regulates ethylene responses independent from this pathway (Jayasinghege, Ozga et al. 2017). In pea, the ethylene response is regulated by 4-Cl-IAA through modulating the expression of ethylene biosynthesis genes such as *1-aminocyclopropane-1-carboxylate oxidase* (*ACO) 1*, *ACO2* and *ACO3*. These genes catalyse the final step in the biosynthesis pathway and convert 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene. The notion that IAA induces ethylene in feeding sites has long been proposed and 4-Cl-IAA could play a role here as well. This holds true specifically for the plant species in the phylogenetic clades of the Fabeae and Trifoleae from the Fabaceae family where 4-Cl-IAA is found.

In contrast to 4-Cl-IAA, the hormone IPA is unable to properly activate the TIR1-AFB signalling pathway and its molecular mechanisms in plants remain unknown (Simon, Kubeš et al. 2013). However, IPA inhibits primary root elongation and induces secondary root formation at high concentration in *A. thaliana*. In addition IPA failed to significantly activate the *DR5rev:GFP* reporter line showing its biological irrelevance to activate the TIR1-AFB signalling pathway. Alternatively, IPA functions as a potent antioxidant by reacting with hydroxyl radicals at a controlled diffusion rate (Poeggeler, Pappolla et al. 1999). Hydroxyl radicals are members of ROS and act as core regulators in a sophisticated network of signalling pathways in plants (Bhattacharjee 2012). Additionally, ROS plays an important role in plant defence against pathogens, such as nematodes, through the induction of a hypersensitive response, followed by programmed cell death (O'Brien, Daudi et al. 2012). *M. javanica* prevents this response using the effector TTL5 which interacts with a ferredoxin-thioredoxin reductase catalytic subunit (AtFTRc) in Arabidopsis. This interaction increases the hosts ROS scavenging activity and suppresses host resistance (Lin, Zhuo et al. 2016). Similarly, the nematode could suppress programmed cell death through localized accumulation of IPA. Whether IPA is present in nematode feeding sites and acts there as a ROS scavenger remains unknown.

IBA seems to be widespread in plants and has been detected in Arabidopsis, tobacco, pea, maize and potato (Korasick, Enders et al. 2013). IBA is unable to use the TIR1-AFB signalling cascade and a role outside of IBA to IAA conversion has also been speculated. IBA seems to be more effective in plant rooting and propagation than IAA. Furthermore, IBA is capable of restoring both lateral rooting and gravitropism in the *lrt1* rice mutant, while IAA only seems to rescue the former (Chhun, Taketa et al. 2003). Therefore, it has been speculated that IBA can function as a signalling molecule independent of IAA (Ludwig-Müller 2000) but no concrete signalling pathway has been identified.

Overall, the hormones 4-Cl-IAA, IPA and IBA might affect nematode infection through non TIR1-AFB signalling pathways. For IPA this might be in the form of an antioxidant, while for 4-Cl-IAA this might be through activating ethylene biosynthesis. IBA is involved in secondary root formation and its presence in the feeding sites of *M. incognita* suggests a role in the formation of secondary roots during infection.

Conclusions and Perspectives

In this review we evaluated how different auxins could play a role during nematode infection and how auxin related processes are changed upon infection (Figure 5). Bioassays and

chemical tests indicated already in the early 60s that IAA is of importance in feeding cell development induced by root-knot and cyst nematodes. However, other auxins, such as IBA (Kawazu, Zhang et al. 1996, Zhang, Kanzaki et al. 1997), may also accumulate during nematode infection. Clearly, examining the presence of IBA, PAA, 4-Cl-IAA and IPA in a range of plant species with state-of-the-art metabolomics is worthwhile to clarify the role of auxins in feeding sites. Also studying the precursors of auxins with novel technologies is essential to uncover the pathways. In feeding sites, the IAOx pathway has the most support for *de novo* IAA synthesis to occur through (Figure 2). Surprisingly, no intermediate metabolites have been reported for the IPyA pathway which is the main auxin biosynthesis route in plants. The IAOx pathway is mainly found in members of the Brassicaceae family and as such manipulation of that pathway would only be relevant in that family. Given the broad host range of some nematodes one would expect them to manipulate an auxin biosynthesis pathway that is common in all their host plants. Therefore, it would be interesting to study which auxin biosynthesis pathways are induced by nematodes in non-Brassicaceae species.

Expression data and overexpression studies indicate that degradation and conjugation are involved in controlling and containing auxin levels in infected tissues (Supplementary Table S1). The old concept that peroxidases inactivate auxin and IAA oxidases are regulated by phenolic compounds seems invalid and, it is more likely that enzymes such as DAO tune auxin levels. The early upregulation of conjugation enzymes seems contradictory as elevated auxin levels are required for proper feeding site initiation. However, the gene family responsible for auxin conjugation is also involved in the catabolism of other plant defence related hormones such as JA and SA (Chen, Westfall et al. 2010). An alternative explanation is that the early upregulation of conjugation enzymes is required to dampen auxin signalling in neighbouring cells, while a high level of auxin is maintained in the actual feeding cell. It is noted that the interpretation of expression data is often hampered by the absence of data at the cellular level because usually data are collected at the level of tissues i.e. feeding cells surrounded by a variety of neighbouring cells.

Influx and efflux dynamics of IAA at the cellular level changes drastically upon nematode infection, but the underlying regulatory mechanisms remain actually unknown (Grunewald, Cannoot et al. 2009, Kyndt, Goverse et al. 2016). In the case that other natural auxins are also present in feeding sites, this may add an additional regulatory layer to reprogram plant cells into feeding sites because the intercellular transport mechanisms may differ. While this review gives some examples of possible regulators of intercellular IAA transport via PINs it might be worthwhile to investigate the expression and changes in vesicle transport and PIN internalisation in feeding sites in more detail. The finding that sedentary nematodes secrete the plant hormone cytokinin might provide a clue in this process, since this plant hormone is shown to regulate both PIN expression and localisation during lateral root formation.

Local accumulation of auxin in both cyst and root-knot nematode feeding sites leads to TIR1-AFB-mediated signalling. This perception of auxin has been shown mainly using transcriptomic data and via the activation of the DR5 promotor in localisation studies. However, this does not prove that solely IAA is present as both PAA and 4-Cl-IAA can activate the same signalling pathway. Data on the role of any other auxin during nematode infection beside IAA is grossly underrepresented, while it might be able to shed light on important infection events such as nematode feeding site initiation and development. Another relatively

unexplored research area is the biological role and significance of the various components of the TIR1-AFB signalling cascades in nematode infection of which only few have been studied in detail.

Overall, sedentary nematodes use various means to accumulate IAA and potentially other auxins at their feeding site during infection. These nematodes stimulate local IAA biosynthesis, regulate auxin catabolism, and modify IAA transport. Achieving this auxin accumulation is an import step in the life cycle of the nematode and having multiple methods to this goal provides a robust infection mechanism. CN and RKN both possess tools to modify the various routes leading to auxin accumulation, albeit in a different manner, as is clearly demonstrated by their difference in regulation of IAA transport through PIN. Identifying what role other auxins play in the infection process and identifying the tools each nematode uses to manipulate auxin homeostasis will bring us closer to understanding how nematodes develop their feeding sites.

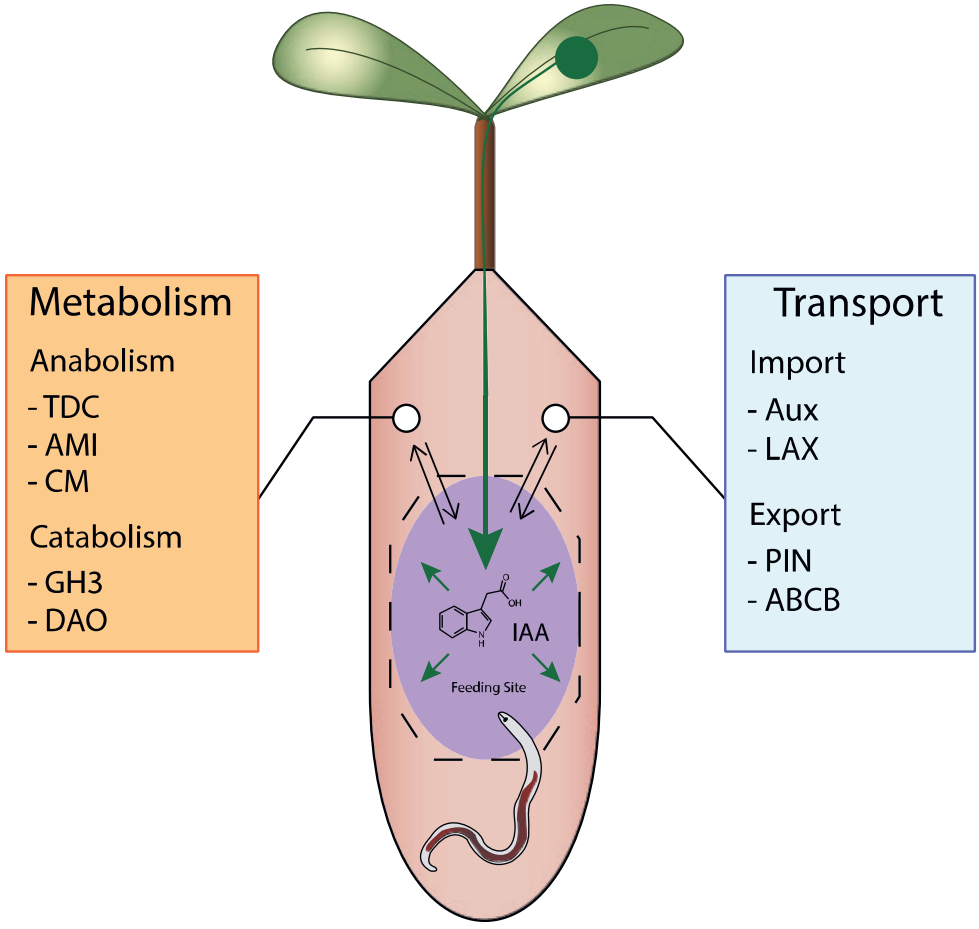

Figure 5 Overview of potential mechanisms leading to IAA accumulation during nematode feeding sites formation. A schematic feeding site is depicted in purple with auxin flows shown in green. IAA accumulates due to regulation of auxin transport redirecting IAA to the feeding site from the vascular bundle. In addition, local IAA synthesis occurs in and/or around the feeding site while auxin catabolism is repressed.

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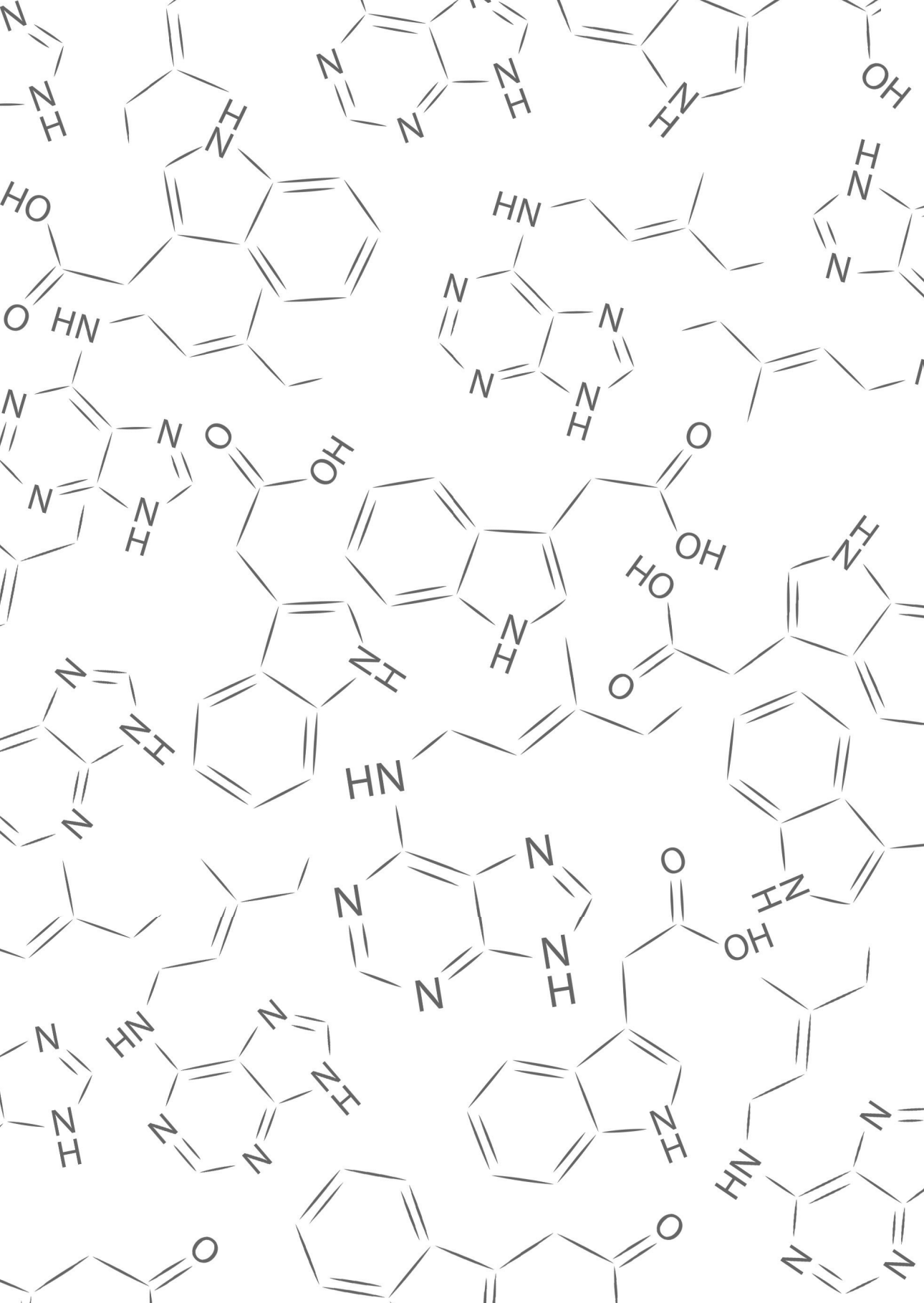
Supplementary Data

Supplementary Table available at:
https://www.frontiersin.org/articles/10.3389/fpls.2021.668548/full#supplementary-material



Chapter 3

Simultaneous and quantitative live-cell imaging of auxin and cytokinin signalling in developing feeding sites of *Heterodera schachtii*

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Abstract

Cyst nematodes are sedentary endoparasites that establish unique feeding structures (syncytia) inside host roots from which the nematode withdraws nutrients. Formation of the syncytium is essential for the completion of the nematode's life cycle and starts with the redifferentiation of a normal root cell into an initial syncytial cell that subsequently expands through fusion with neighbouring cells. The cross-talk between the phytohormones auxin and cytokinin is thought to play a crucial role at the onset of this process as both seem to accumulate in the early stages of syncytium formation. However, the combined spatial and temporal changes of auxin and cytokinin signalling during nematode infection are unknown. Here, we used an auxin-cytokinin double reporter line for simultaneous analysis of the spatio-temporal dynamics of auxin and cytokinin signalling by live-cell confocal imaging during the initiation and expansion phase of syncytium development in *Arabidopsis thaliana* roots infected by the beet cyst nematode *Heterodera schachtii*. Three movies consisting of time lapse recordings for up to 140 hours were obtained capturing images of the redifferentiation process of root cells into a nematode feeding structure every 30 minutes. Quantitative analysis of reporter gene expression in this dataset shows that upon nematode root infection cytokinin signalling occurs in nearly all cell types, also outside the syncytial area. Cytokinin signalling increases rapidly and precedes auxin signalling, which is – in contrast - restricted to the syncytial area. Auxin signalling starts with a lag phase and is induced after the first contours of the syncytial area have become visible. Within the syncytial area the signalling domains overlap and elevated auxin signalling intensities in nuclei are associated with high cytokinin responses. In addition, cytokinin signalling is relatively high in nematode infection sites when compared to signalling maxima in root tips, while auxin signalling is relatively weak when compared to signalling maxima in root tips. Together, these data show in a qualitative and quantitative manner the spatio-temporal dynamics of auxin and cytokinin signalling during the onset of syncytium formation by cyst nematodes and how they overlap specifically in the syncytial area. Our findings are discussed in the context of previous reports on the role of auxin and cytokinin signalling in the induction of nematode feeding structures upon infection of plants roots.

Introduction

Plant-parasitic nematodes cause an estimated annual crop yield loss between 8.8% and 14.6% worldwide equalling 157 billion of dollars (Nicol et al. 2011; Singh, Singh, and Singh 2015). Of these plant-parasitic nematodes the root-knot nematodes of the genus *Meloidogyne* and cyst nematodes of the genus *Heterodera* and G*lobodera* are among the top 10 most harmful nematodes (J. T. Jones et al. 2013). Due to this, the best studied species of plant-parasitic nematodes are among the root-knot nematodes and cyst nematodes. These sedentary nematodes can feed on monocot and dicot plants where they establish a unique feeding structure inside the plant root through a complex interaction at cellular and molecular level (Perry and Moens 2011). Cyst and root-knot nematodes hatch from their eggs and second stage juveniles search for a host to invade the roots using distinct modes of action. Cyst nematodes move intracellularly through the plant's cortex where they select an initial feeding cell in or near the vascular bundle (Bohlmann and Sobczak 2014). Subsequently, they inject this cell with a suite of secretory compounds known as effectors that start to alter the hosts metabolism leading to the formation of a syncytium (Haegeman et al. 2012). This syncytium is formed through partial breakdown of the neighbouring cell walls and followed by fusion of their cell membranes (Sobczak and Golinowski 2011). Second stage juveniles of root-knot nematodes, however, enter the root near the meristematic zone and move intercellularly through the vascular bundle. There they select up to 10 individual cells and transform these into giant cells by injecting effector compounds that induce cell division without cytokinesis (Caillaud, Abad, and Favery 2008). Although cyst and root-knot nematodes develop distinct feeding structures they do share common characteristics as both form large multinucleate cells with proliferated endoplasmic reticulum through reprogramming of normal root cells (Eves-van den Akker et al. 2014). Feeding site formation in general is paired with aberrant cell cycle activation and drastic changes in gene transcription (Gheysen, de Almeida Engler, and van Montagu 1997). Feeding sites deplete the host of resources and function as the sole nutrient source for the parasite. Interruption or prevention of the formation of these feeding sites lead to the demise of the nematode (de Almeida Engler et al. 1999).

The infection process by cyst and root-knot nematodes is irrevocably linked to changes in hormone balance of the plant. Auxins together with cytokinins are the key plant hormones involved in cell division and differentiation, which also play a significant role in feeding site initiation and development. The phytohormone auxin, indole-3-acetic acid (IAA), is considered to play a pivotal role in feeding site formation of both cyst and root-knot nematodes and is thought to be responsible for many of these processes such as hypertrophy, cell wall ingrowths, and cell cycle activation (Gheysen and Mitchum 2019). Several auxin mutants in *Arabidopsis thaliana* such as *dgt*, *aux1*, and *arf7/arf19* are significantly less susceptible to either root-knot or cyst nematode infection (Olmo et al. 2017; Goverse et al. 2000; Kyndt et al. 2016) Moreover, the auxin responsive DR5 reporter revealed that nematode induced feeding site formation is accompanied by a local and transient auxin accumulation as early as 18 hours post infection (Goverse et al. 2000; Karczmarek et al. 2004). IAA accumulation seems to occur in expanding syncytia and in cells surrounding the syncytia where the majority of these neighboring cells will be incorporated in the syncytium at a later stage of development. In addition, auxin is suspected to trigger distinct developmental processes during cyst and root-knot nematode infection. For example, auxin induced LATERAL ORGAN BOUNDARIES-DOMAIN (LBD) 16 expression is important in the formation of

secondary roots and is upregulated in giant cells of root-knot nematodes but it is downregulated during cyst nematode infection (Cabrera et al. 2014; Cabrera, Fenoll, and Escobar 2015). Reporter and transcriptome studies have investigated the expression of auxin related genes at various days after infection and these snapshots suggest a dynamic and complex spatial as well as temporal expression pattern of auxin related genes such as PINs, ARFs, and GH3 (Kyndt et al. 2016; Hewezi et al. 2014; Hutangura et al. 1999). In these studies, auxin biosynthesis and auxin-response genes such as tryptophan decarboxylase (TDC) and GRETCHEN HAGEN 3 (GH3) are predominantly up-regulated at the early stages of nematode infection, where in contrast auxin repressors like the IAA family are down regulated. From this it can be inferred that the accumulation itself likely arises, at least partially, through changes in local auxin biosynthesis and catabolism as well as auxin transport (Oosterbeek et al. 2021).

The phytohormone cytokinin is, similar to auxin, known to play an important role in nematode feeding site development. In plants, cytokinins exert cell cycle control, are able to delay senescence and convert tissues into sinks by modulating nutrient translocation (W. Wu et al. 2021). Due to these properties, they have long been thought to play a role during nematode infection. Several cytokinin insensitive mutants are less susceptible to cyst or root-knot nematode infection like *ahp1/2/3*, *ahk4*, and *arr1/12* as well as those with reduced cytokinin levels such as CKX3, and CKX4 (Lohar et al. 2004; Dowd et al. 2017; Siddique et al. 2015; Shanks et al. 2016). Cytokinin has been shown to accumulate in the feeding site during the infection cycle of both cyst and root-knot nematodes using the cytokinin reporters TCSn::GFP and ARR5::GUS (Dowd et al. 2017; Shanks et al. 2016). These studies show that cytokinin expression occurs in the same timeframe compared to auxin but is induced later from at least 4 dpi up to 14 dpi (Dowd et al. 2017; Lohar et al. 2004; Siddique et al. 2015). Moreover, the cytokinin reporter signals are also present in the surrounding tissue of both cyst and root-knot nematode feeding cells. In addition, they can be found in the syncytium of cyst nematodes, but curiously enough not inside the giant cells of root-knot nematodes (Absmanner, Stadler, and Hammes 2013). These differences are also observed in transcriptomic studies for the expression of cytokinin biosynthesis, catabolism, and signalling genes, which is attributed to the underlying difference in cell cycle progression during syncytium and giant cell formation (Dowd et al. 2017). This was demonstrated by the importance of the cytokinin signalling gene *AhK4* in syncytium development, while *AhK2* and *AhK3* were shown to be crucial for giant cells development (Siddique et al. 2015; Dowd et al. 2017).

Although auxin and cytokinin signalling have until now been studied separately, it can be inferred from previous studies that auxin and cytokinin responses occur simultaneously in feeding sites of both cyst and root-knot nematodes. This is remarkable given the antagonistic nature of auxin and cytokinin signalling in normal plant root development (Coenen and Lomax 1997). For example, auxin decreases the expression of the cytokinin biosynthesis gene CYP735A in *Arabidopsis* but also directly activates two type A ARRs, ARR7 and ARR15, thereby inhibiting cytokinin signalling (Müller and Sheen 2008; Takei, Yamaya, and Sakakibara 2004). Conversely, cytokinin modulates auxin metabolism and transport by, for example, affecting IAA17 which is important in maintaining auxin levels and regulating the expression of the PIN auxin export family (Laplaze et al. 2007; B. Jones et al. 2010). However, auxin and cytokinin interaction is not always antagonistic as additive and synergistic effects also occur, like auxin-

mediated regulation of cytokinin biosynthesis and their collaborative role in cell divisions in vascular tissues. In this case, the cytokinin biosynthesis genes IPT5 and IPT7 are upregulated in Arabidopsis roots in response to auxin treatment through *SHY2/IAA3* expression (Ioio et al. 2008; Jing and Strader 2019). In addition, auxin decreases the expression of the cytokinin catabolism genes CKX2, CKX4, CKX7, and allows cytokinin to promote vascular differentiation and regeneration (ALONI et al. 2006; Werner et al. 2006). Interestingly, vascularisation is also associated with cyst and root-knot nematode feeding site formation and may be the result of auxin-cytokinin cross talk (Hoth et al. 2008; Bartlem, Jones, and Hammes 2014). However, current auxin and cytokinin data are based on independent studies and it is *de facto* unknown if and how auxin and cytokinin signalling events merge during feeding site initiation and development. Moreover, as they have never been investigated simultaneously in this context, it is also unknown whether within feeding sites auxin and cytokinin signalling occurs simultaneously or consecutively. Additionally, previous analytical approaches have favoured qualitative assessments over quantifiable numerical data by evaluating results as either increased, elevated or high but not quantifying these (Goverse et al. 2000; Dowd et al. 2017; Shanks et al. 2016). The spatial and temporal dynamics are further obfuscated by examining non-synchronous infection sites based on daily observations thereby only observing general trends.

Therefore, the aim of this study was to monitor the spatio-temporal dynamics of auxin and cytokinin signalling simultaneously in individual feeding sites of the cyst nematode *H. schachtii* using quantitative live-cell confocal imaging. For this, roots of an Arabidopsis reporter line were infected in vitro containing a double reporter construct consisting of the auxin-responsive DR5revV2 promoter driving the expression of nuclear targeted green fluorescent protein (n3GFP) and the cytokinin responsive promoter TCSn driving nuclear targeted ntdTomato expression. Upon infection, single juveniles were observed and selected when in the sedentary phase 18 hours after inoculation. Next, they were transferred to a mini-growth chamber for continuous growth and time lapse recording of dual reporter gene expression up to 140 hours using confocal laser scanning microscopy (CLSM). Every 30 minutes 7 to 10 optical slices were collected, capturing the induction and development of a syncytium, including changes in its surrounding root tissues. In total, a collection of more than 6000 optical slices were obtained to monitor the spatio-temporal dynamics in auxin and cytokinin signalling during feeding site formation. Quantitative and comparative analyses of these data show that auxin is induced locally and specifically upon infection in the syncytium, whereas cytokinin signalling occurs throughout the root though slightly enhanced in local feeding sites. When these data from individual samples are combined, it is clear that high auxin responses are associated with high cytokinin responses in contrast to what is observed in root tips. Interestingly, the signalling dynamics of auxin and cytokinin show distinct patterns in time and space. The earliest events of the infection process are associated with a steep increase in cytokinin signalling reaching a plateau likely reflecting a cytokinin signalling maximum when feeding site formation progresses. Moreover, a strong and heterogeneous cytokinin response was observed in various cell types, including cells that are not incorporated into the syncytium. The increased auxin signalling, however, was restricted predominantly to the syncytial area and observed after a lag phase during which the first contours of the syncytial area became visible with bright-field microscopy. This indicates that the cytokinin signal precedes the auxin signal which is induced later after nematode feeding site initiation has already started. So, simultaneous monitoring of auxin and cytokinin

signalling using live-cell imaging not only uncovers overlap of auxin and cytokinin signalling domains within the syncytial area, but also reveals that elevated auxin signalling is associated with high cytokinin responses in the same cells during feeding site formation. In addition, our data also suggests that the initial phase of syncytia development may be independent of auxin signalling. The possible implications of cross-talk between auxin and cytokinin as well as their independent roles in feeding site formation by cyst nematodes are discussed.

Results

A fluorescent double reporter reveals overlapping auxin and cytokinin signalling domains in young feeding sites induced by *H. schachtii*

Prior to the establishment of a live-cell imaging system to monitor the spatio-temporal dynamics in auxin and cytokinin signalling domains during feeding site development, a classical approach was used to investigate the expression patterns of the auxin-cytokinin double reporter DR5revV2::n3GFP-TCSn::ntdTomato (Smet et al. 2019) in roots of Arabidopsis seedlings infected with *H. schachtii* juveniles. As a reference, roots of uninfected Arabidopsis seedlings were studied to validate the expression pattern of this dual reporter system. In eleven-day-old uninfected seedlings cytokinin signalling was observed in the vascular cylinder and cortex of the mature root (Figure 1G). In a number of seedlings a cytokinin response was also observed in the epidermis of the mature root. For the root tip, expression of TCSn::ntdTomato has been reported for procambium, columella, epidermis and lateral root cap cells (Smet et al. 2019), which is in concordance with the observed fluorescence produced by the double reporter in our study (Figure 1C). Little to no auxin signal was observed in the root above the maturation zone (Figure 1F). Expression of DR5revV2::n3GFP was evident in the root tip (Figure 1B), and as reported (Liao et al. 2015) most prominent in the quiescent centre (QC) and neighbouring stem cells, and protoxylem, and relatively low in metaxylem, pericycle, lateral root cap and epidermal cells. Visual inspection of the photographs (Figure 1B, 1C and 1D) reveals that high auxin and high cytokinin signalling occurs in distinct nuclei. Altogether it is concluded that the expression patterns of auxin-cytokinin double reporter DR5revV2::n3GFP-TCSn::ntdTomato are consistent with previous data and show that domains with high auxin and high cytokinin signalling do not overlap.

For *H. schachtii* infected root samples, a profound ntdTomato fluorescent signal was observed as compared to similar regions of uninfected roots indicating enhanced cytokinin signalling within and around the 'syncytial area' at 4 days post-infection (DPI) (Figure 1K). Measurements were performed at 4 DPI as this timepoint is indicative of both the auxin and cytokinin signalling window during early feeding site development (Hewezi et al. 2014; Shanks et al. 2016). The term 'syncytial area' is used, since with our microscope setup syncytial cells cannot be distinguished from neighbouring cells that are not connected to the syncytium. As described in previous studies (Golinowski, Grundler, and Sobczak 1996), syncytial elements are surrounded by cambial and peridermal cells. Also root cells preconditioned to become part of the syncytium by progressive cell wall dissolution during feeding site expansion cannot be recognized. Hence, we refer in this study to the syncytium and these adjacent cell layers as 'syncytial area'. The fluorescence signal of ntdTomato was not specific to any type of tissue as it was observed in the syncytial area as well as in surrounding tissues i.e., in the epidermis,

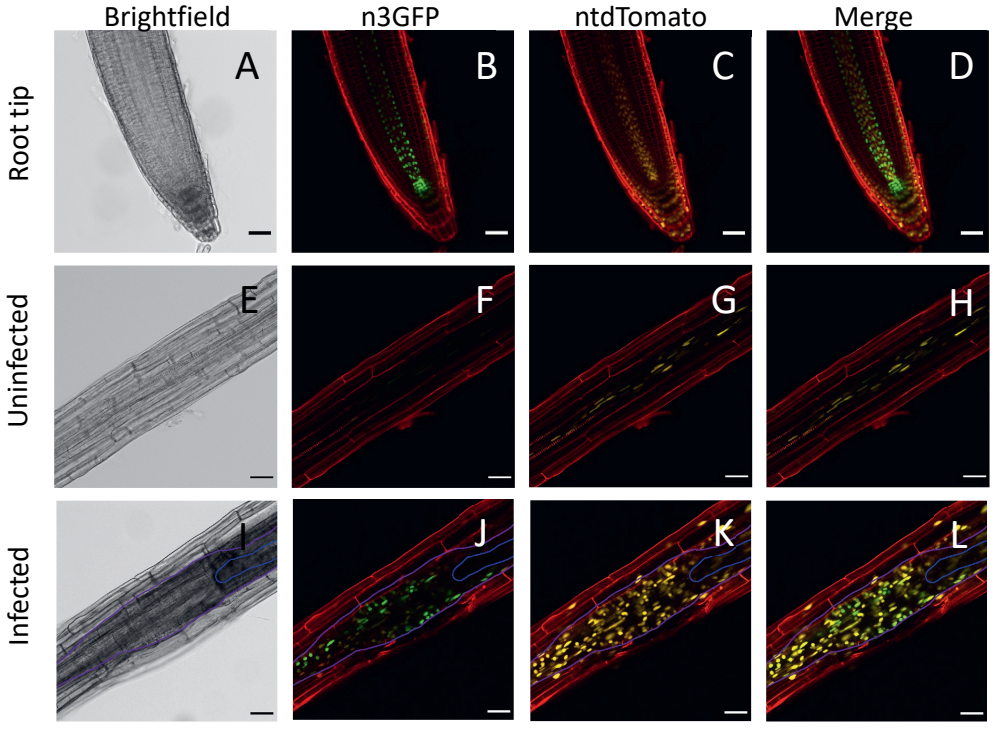


Figure 1 Visualisation of cytokinin and auxin signalling through the use of the double reporter DR5revV2::n3GFP-TCSn::ntdTomato in uninfected roots and Arabidopsis roots infected with *H. schachtii* at 4 DPI. (**A-D**) Auxin (green) and cytokinin (yellow) signalling in uninfected *Arabidopsis* root tip. Scale bar represents 20 µm. (**E-H**) Auxin and cytokinin signalling in the mature zone of uninfected *Arabidopsis* root. Scale bar represents 40 µm. (**I-L**) Root infected with *H. schachtii* at 4 DPI. Blue shows the contour of the nematode and pink outlines the syncytial area. Scale bar represents 40 µm. Cell walls are stained with propidium iodide (red). Data displayed is a representative image of over 25 observations from three independent replicates with similar results using a confocal laser scanning microscope (CLSM).

cortex and vascular bundle outside the syncytial area (Figure 1K). However, a local increase of ntdTomato fluorescence was observed in the syncytial area as compared to the ntdTomato background signal in normal root tissue.

In contrast to cytokinin, auxin signalling was clearly observed at 4 DPI in the syncytial area but not in surrounding cells (Figure 1J). No auxin signal was observed in the epidermis and cortex, and vascular cylinder outside the syncytial area indicating a specific local increase in auxin signalling during the onset of feeding site formation. Interestingly, the observed auxin signalling domain seems to overlap with that of the cytokinin signalling domain inside the syncytial area when merging images of the same sample (Figure 1L). No differences were observed between root tips of infected and uninfected seedlings, both showing a typical expression pattern for auxin and cytokinin as reported (Smet et al. 2019). Overall, in infected samples the double reporter reveals ubiquitous cytokinin signalling throughout the root,

while auxin signalling is restricted to the syncytial area. When combining these data from individual samples, it seems that high auxin and high cytokinin signalling domains overlap in the syncytial area in contrast to what is observed in root tips.

Mini-growth chamber design enables continuous live-cell imaging of auxin-cytokinin double reporter activity in syncytial areas

Before starting live-cell imaging of auxin and cytokinin signalling in developing feeding cells, experimental conditions were established to allow proper growth of uninfected and infected plants, including the support of nematode feeding and development over longer time periods. To enable monitoring of feeding site formation from the onset of nematode parasitism in real-time, *in vitro* root samples were collected with parasitic 2nd stage juveniles that had just ceased migration while visibly probing root cells to select an initial syncytial cell. Hereto, primary roots of around 200 seven-day old Arabidopsis seedlings were inoculated in vitro and after 18 hours roots were screened using a binocular to select seedlings with single 2nd stage parasitic juveniles in this specific stage of parasitism. Next, individual samples were placed in sterile chambered cover glasses supplemented with both solid and liquid nutrients to allow

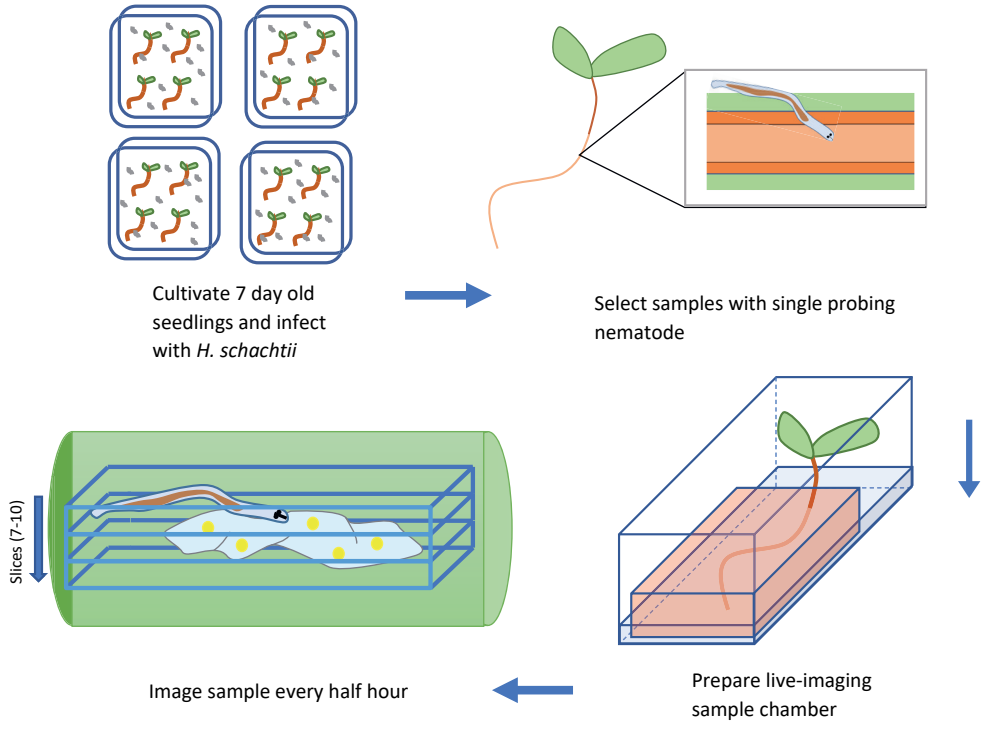


Figure 2 Schematic outline of all steps involved in the selection of sedentary second stage juveniles from *H. schachtii* for live-cell imaging using a mini-chamber set up as described (see M&M). Seven-days old infected samples were observed and those with a single nematode at the onset of feeding site initiation were selected. Three to five samples are prepared in a live-imaging chamber and one is imaged. Using optical sectioning with a CLSM, a major part of the syncytium can be captured using ~7 optical slices (z-stack).

further growth and development in vitro (Figure 2). Prolonged culturing of plant samples in these mini-growth chambers showed that root growth and the formation of feeding cells continued normally and that juveniles were able to feed resulting in normal moulting into subsequent juvenile life stages. From this, it was concluded that the method established enables normal root parasitism by the cyst nematode H. schachtii and could be used for continuous monitoring of the expression of the reporter genes during feeding cell formation. To visualise cell boundaries and facilitate the detection of syncytium formation during imaging, infected roots were incubated with propidium iodide or calcofluor white. A suitable concentration was determined for each dye by performing a toxicity assay. Inhibition of plant growth was tested by supplementing growth media with either 10, 50 or 100 µm propidium iodide (PI) or 0.1, 1, or 10% (w/v) calcofluor white. All concentrations of calcofluor white and concentrations of PI at or above 50 µm visibly hampered root growth as observed after one week incubation. These results show that PI is the most suitable cell stain in this set up for Arabidopsis plants when used at concentrations of 10 µm or below. At 10 µm PI no visible effects on nematode growth were observed while still providing proper counterstaining of the plant root cells. Hence, it is concluded that the experimental conditions within the mini-growth chamber as established in this study allows nematode growth and feeding cell development for prolonged periods of time while facilitating conditions suitable for monitoring gene expression using CLSM (for details see M&M). To mitigate photobleaching, the microscopic settings were tuned to the cytokinin (stele and columella) and auxin (quiescent centre and surrounding cells) signalling maxima in the root tip. The intensity of the laser power was relatively low and set in such a way that these maxima were clearly visible. To minimize loss of resolution in cells with lower expression levels of the double reporter, this was compensated for using the gain settings during image acquisition to make full use of the detector range.

Live-cell imaging reveals a steep and wide-spread increase in cytokinin signalling upon infection

To study the auxin and cytokinin signalling dynamics during early stages of feeding site formation, GFP and ndtTomato fluorescence was monitored with an interval of 30 minutes for a time period of at least 80 hours in three independent *H. schachtii* infection sites as well as an uninfected control root segment obtained from the Arabidopsis double reporter line. The resulting timelapse series were compiled into four movies to visualize the spatio-temporal changes in reporter gene expression as an indicator for auxin and cytokinin signalling (Supplementary Data). In these movies the infection process can be observed real-time from the onset of parasitism, including other nematode-induced changes like the asymmetric emergence of multiple secondary roots in close proximity of the syncytial area (Guarneri et al. 2023). In two samples, the nematodes successfully developed into the fourth juvenile stage and emerged from the root due to an increase in body size, indicating that continuously monitoring with a CLSM laser beam for up to 140 hours had no pronounced effects on the feeding process and nematode growth. In one sample, however, the formation of the syncytial area progressed normally until 80 hours after which the host plant died as

inferred by a sudden arrest of nuclear movement and cell permeation by propidium iodide (MovieS1_Rep1). Therefore, data obtained after 80 hr were excluded from further analyses for this sample. Additionally, it should be noted that for one replicate no cell wall counterstaining with PI was used (MovieS3_Rep3).

Over the course of the infection process, the main root thickened and in two cases the nematode gradually swelled and developed into a J4, bursting from the root (MovieS2_Rep2 and MovieS3_Rep3). Additionally, in all samples 3-6 secondary roots developed in close proximity to each other. Furthermore, a strong increase in ndtTomato fluorescence was noted across the entire sample (Figure 3A-D). The increase of ndtTomato fluorescence was not limited to the stele but was also observed in the cortex and epidermis (Figure 3A-D). This increase is in agreement with the observations made previously from the independent snapshot experiment (Figure 1). During the imaging process 7 to 10 optical slices were made every 30 minutes over a depth of 64 microns capturing a major part of the syncytium. To compare between samples, the head of the nematode was taken as reference for the starting point of feeding site induction and subsequent development. In this way, a similar part of the syncytial area was compared for all samples; thus, the optical slice containing the head of the nematode was used as starting point (Figure 3A-D). In case the head of the nematode gradually changed position from optical slice during the measurements as a result of sample drift, a switch was made to the desired optical slice. At later stages of infection, the precise position of the nematode's head became invisible by bright-field microscopy due to local root thickening. At these later stages, sample drift was monitored by analysing the signalling intensities of the entire imaged field between subsequent time points. Between most time points the intensity values changed gradually and in case of unexpected shifts neighbouring optical slices were analysed to evaluate whether the gradual trend could be restored by switching to another optical slice.

To quantify the observed changes in fluorescence over time in cytokinin signalling, nuclei showing ntdTomato fluorescence signals were converted into the Relative Mean Intensity (RMI_{tomato}) and Fluorescent Area (FA_{tomato}). The RMI_{tomato} is the average of the fluorescence intensities of all fluorescent pixels as measured in a single optical slice at a certain time point relative to the same measurement of the first timepoint (Figure 3E). Thus, the RMI_{tomato} reflects the fluorescence intensities in cytokinin signalling as a ratio compared to the first time point (Figure 3E). The FA_{tomato} is defined as the sum of all fluorescent pixels as detected in fluorescent nuclei at a given timepoint for the observed optical slice (Figure 3F). The FA_{tomato} reflects as such the number and size of the fluorescent nuclei which serves as another indicator for cytokinin signalling. Thus, the RMI_{tomato} is a measure for the relative strength of the cytokinin signal within an area compared to the start of the first measurement, whereas the FA_{tomato} is a measure for the total number cytokinin signalling pixels within an area. The nuclei were selected for pixel measurements by using a mask based on the signalling intensity and a noise threshold cut-off (see Material and Methods). Measuring the cytokinin response in the entire main root in the optical slice with the head of the nematode reveals in all three replicates a steep increase of the RMI during the first 40 hours of real-time live-cell imaging (Figure 3E). In this timeframe the rise in signalling intensities varies between the replicates and ranges between a 2 to 4-fold increase (Figure 3E). After 40 hours the intensity of cytokinin signalling in the nuclei remains relatively stable in two of the three replicates (Figure 3E). For one sample (Rep1), stabilization was already visible after 20 hours. This early arrest in

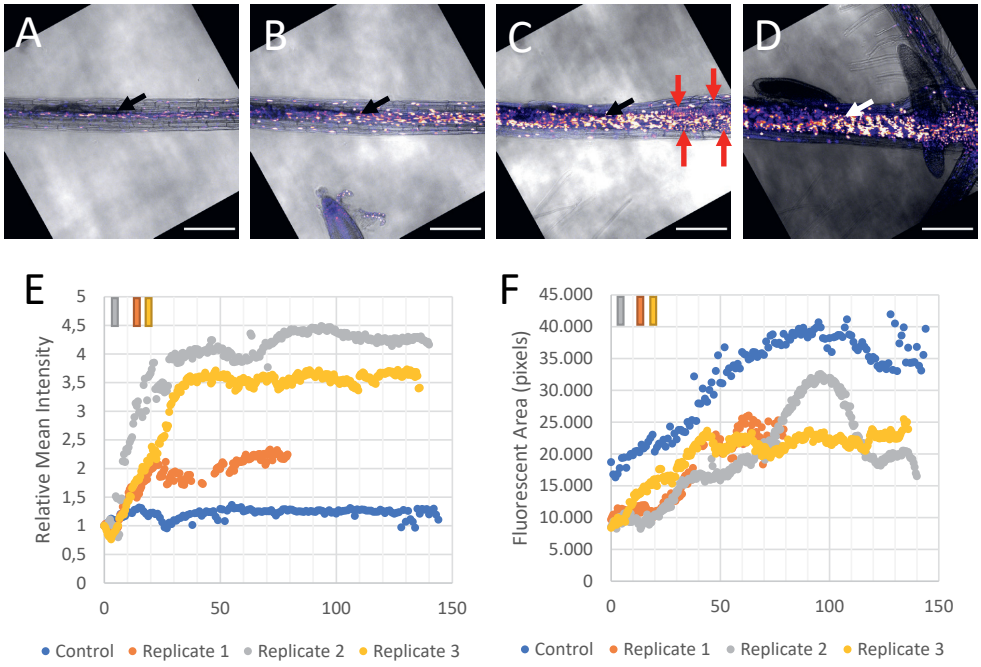


Figure 3 Cytokinin signalling revealed by the double reporter DR5revV2::n3GFP-TCSn::ntdTomato with continuous live-cell imaging in Arabidopsis roots infected with *H. schachtii*. Snapshots from the movie of the 3rd replicate (Rep3) at (**A**) the start of live-cell imaging (t=0), (**B**) at the first appearance of the syncytial area (t=21.5h), (**C**) during expansion of the syncytial area (t=68h) and (**D**) at the end of the measurements with fourth stage juvenile and outgrowing secondary roots (t=130h). Black and white arrows indicate the head of the nematode and red arrows show the developing root primordia. The scale bar represents 200 µm. Fluorescent intensity is displayed as a blue<orange<white (fire) gradient. (**E**) The average intensity per pixel of fluorescent cytokinin signalling nuclei in one optical slice per timepoint relative to the first measurement (t=0) (RMI) of the entire imaged primary root for each replicate. Bars on the upper left indicate the time at which the syncytial area was first observed with bright-field microscopy. (**F**) The number of fluorescent pixels (FA) in the main root for each replicate in one optical slice per timepoint. Bars on the upper left indicate the time at which the syncytial area was first observed.

cytokinin signalling may be caused by the premature starvation of the corresponding plant after 80 hours as described (MovieS1-Rep1). In contrast to the infected samples, the cytokinin signalling (RMI_{tomato}) in the control remains constant and is hardly affected during 140 hours of live-cell imaging (Figure 3E), indicating that the contribution of stress related induction of reporter activity due to the growth conditions in the mini-growth chamber can be neglected.

During live-cell imaging, the number of signalling nuclei increased significantly (Figure 3A-D) most likely due to multiple cell divisions, but potentially also by enlargement of existing nuclei caused by endoreduplication (de Almeida Engler and Gheysen 2013). Both phenomena are reflected in the gradual increase in the total number of fluorescent pixels (FA_{tomato}) (Figure 3F). The more than two-fold increase of the FA_{tomato} in the uninfected root can be explained by periclinal and anticlinal cell divisions responsible for secondary growth, as can be viewed by a gradual thickening of the vascular bundle (movieS4_Uninf). In the infected samples the

rise of the FA_{tomato} ranges from 2 to 3-fold. As described in previous studies, syncytial elements are surrounded by cambial and peridermal cells showing extensive proliferation, which may result in a ten-fold increase in cell numbers when compared with neighbouring regions outside the syncytial area (Golinowski, Grundler, and Sobczak 1996). In addition, the FA_{tomato} is also influenced by multiple cell division giving rise to the development of secondary root primordia in proximity of the expanding syncytial area (Figure 3C and 3D, see also movieS3_Rep3). Measuring the signalling area per nucleus demonstrated that also the fluorescent area per nucleus increased during the measurements. Quantification of the fluorescent area per nucleus at the start of live-imaging and at 100 hours revealed an average increase of 45% as established by a random selection of 30 nuclei across the infected root at both time points (Supplementary Figure 1).

Overall, our data demonstrate that in the control as well as in the infected root the number of fluorescent pixels (FA_{tomato}) gradually rise over time while only in the latter the intensity of the fluorescence (RMI_{tomato}) increases. Since ndtTomato fluorescence is an indicator for cytokinin signalling we can conclude that early events of the infection process are associated with a steep increase in cytokinin signalling reaching a plateau, likely reflecting a cytokinin signalling maximum.

Strong and heterogeneous increase in cytokinin signalling intensity within syncytial area

The next step was to analyse the spatio-temporal dynamics of cytokinin signalling within the syncytial area (Figure 4). To examine the signalling dynamics solely within the syncytial area and to exclude the cytokinin signal in the surrounding tissues (e.g., the epidermis, cortex and secondary root primordia), its size and growth were firstly approximated by manually marking the borders of the syncytial area at four (Rep1 and Rep3) or six (Rep2) timepoints using the bright field channel of the movie. Subsequently, the borders of the syncytial area were interpolated between these time points. This resulted in a linear approximation of the growth and shape of the syncytial area, thereby providing a simulated shape of the syncytial area for each time point in the movie (Figure 4A-D). The relative intensities of the cytokinin signal (RMI_{tomato}) were then measured inside the borders of the syncytial area (Figure 4E). The RMI_{tomato} within the syncytial area shows in all three replicates a pronounced increase, especially during the initial phases of the expanding syncytial area (Figure 4E). The magnitude of the increase varies between the replicates and ranges from 1.5 to about 5-fold, which may reflect the differences in the rate of development of the three individual nematodes and their expanding syncytia. It is noted that the time points at which the first contours of the syncytial area are observed differ between the three replicates (Figure 3E), and hence also the start of the RMI_{tomato} measurements within the syncytial areas varies between replicates (Figure 4E).

In order to gain an indication of the strength of the cytokinin response, the mean signal intensity of nuclei inside the syncytial area was compared to the mean signal intensity of nuclei in the root tips of secondary roots that could be observed within the same sample for all three replicates (Figure 4F). To this end, the mean intensity inside the syncytial area was taken at the point in time at which the highest RMI_{tomato} (Figure 4E) was measured (t=71,5h (Rep1); 97,5h (Rep2); 130,5h (Rep3)). Then, in the same replicate, the mean intensity of cytokinin signallig was measured in secondary root tips by selecting an optical slice with the

highest intensity (see examples in Supplementary Figure S9). As such the highest measured mean intensity inside the syncytial area was compared to the highest measured mean intensity in secondary root tips. This comparison revealed that the average cytokinin response in the feeding site is up to five times stronger than in the root tips of secondary roots.

To study the spatio-temporal dynamics within the syncytial area, the optical slice of the infected primary root sample was divided into 10 optical cross sections (segments) of 90 µm along the length of the syncytial area. Next, the mean signal intensities of the mask-selected nuclei were calculated for each segment only within the boundaries of the syncytial area (Figure 4A-D). The values obtained from the optical slices (that were made every 30 minutes) were subsequently averaged over 5 hours of measurement and plotted as a heatmap (Figure 4G). Noticably, the mean intensities of the cytokinin response are not homogeneously distributed within the syncytial area and show large variations between segments and may differ at certain timepoints more than 7-fold (Figure 4G). A similar pattern is observed in the other two replicates (Supplementary Figures S2 and S3). Also within segments large differences in signalling intensities between individual nuclei are observed (Figure 4A-D), indicating that the syncytial area is composed of a heterogeneous population of (fused) cell types with inherent differences in cytokinin signalling. Also the numerous cell divisions may contribute to the heterogeneity of the signal ('dilution effect'). It is noted that the microscopic resolution is too low to reveal the identity of the various cell types within the stele and to follow the incorporation of individual cells into the syncytium.

The segments that include the head of the juveniles show in all three replicates (Figure 4G, Supplementary Figures S2 and S3) relatively high signalling intensities. However, high signalling levels are also observed in neighbouring segments, which often exceed the signalling level of the segment with the nematode's head. Similarly, the first observed rise of the signal occurs simultaneously in two or more neigbouring segments, and not exclusevily in the segment with the nematode's head (Figure 4G, Supplementary Figures S2 and S3). Despite the irregular spatial signalling pattern, nearly all segments show a gradual increase in cytokinin response over time, with an increase up to 7 fold (Figure 4G). It is noted that also outside the syncytial area, a strong increase in signalling intensities is observed in the vascular tissue (Figure 4A-D). In addition, nuclei in the cortex and epidermis also show a strong increase (Figure 4A-D).

It can be concluded that the rise in cytokinin signalling is heterogeneously distributed within the syncytial area and that this heterogeneous increase is also observed in surrounding tissues. The signal intensities within the syncytial areas show in most segments a steady increase during the first 70 to 90 hours after which the signal reaches a maximum level. Altogether, our data indicate that the induction and expansion of a syncytial area is accompanied by a strong cytokinin response in various cell types, including cells that are not incorporated into the syncytial area.

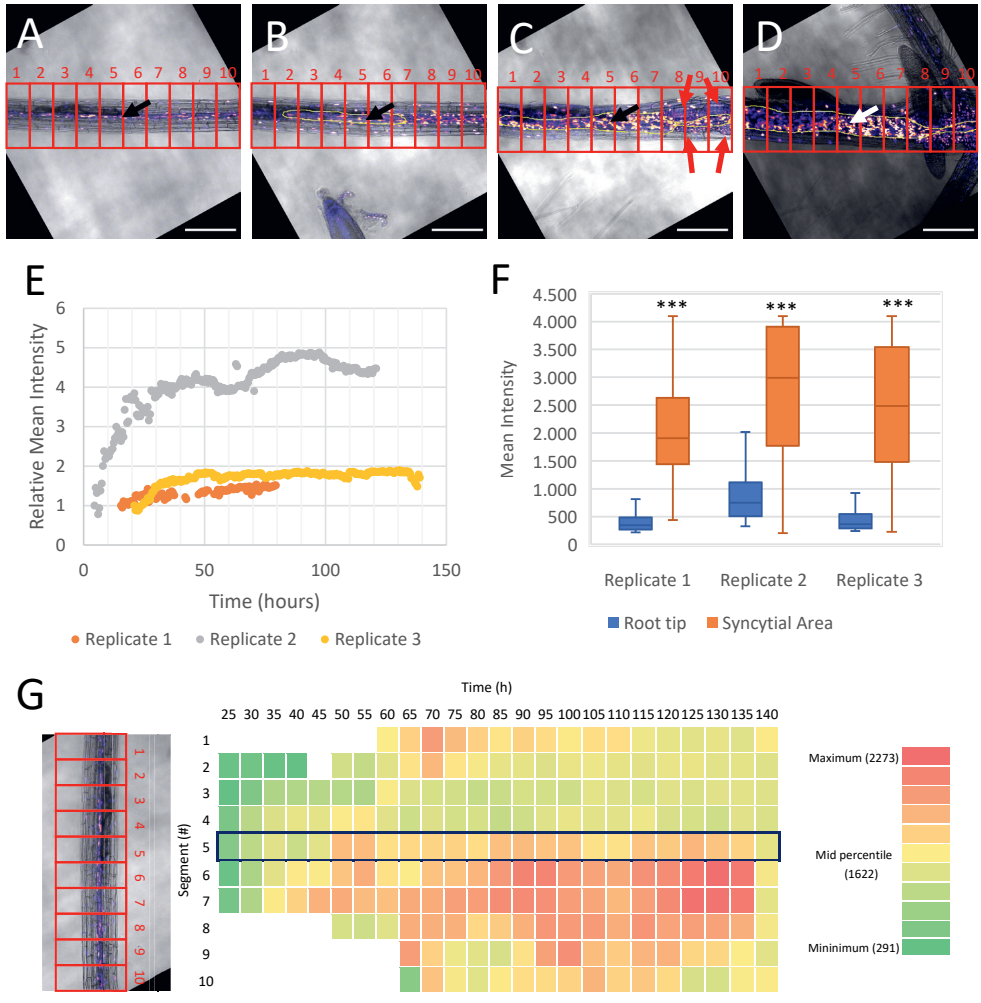


Figure 4 Analysis of cytokinin signalling dynamics within the syncytial area induced by *H. schachtii* in a root of Arabidopsis revealed by the double reporter DR5revV2::n3GFP-TCSn::ntdTomato with continuous live-cell imaging. Snapshots from the movie of the 3rd replicate (Rep3) at (**A**) the start of live-cell imaging (t=0), (**B**) at the first appearance of the syncytial area (t=21.5h), (**C**) during expansion of the syncytial area (t=68h) and (**D**) at the end of the measurement (t=130h) showing the emergence of a fourth stage juvenile and secondary roots. The border of the syncytial area is indicated in yellow and the root is divided into 10 optical segments indicated in red. Black and white arrows indicate the head of the nematode and red arrows show the developing root primordia. Fluorescent intensity is displayed as a blue<orange<white (fire) gradient. The scale bar represents 200µm. (**E**) Relative Mean Intensity of the TCSn::ntdTomato signal (RMI_{tomato}) measured inside the marked syncytial area for all three biological replicates. Bars on the upper left indicate the time at which the syncytial area was first observed. (**F**) Mean intensity of cytokinin signalling in nuclei in the syncytial area compared to nuclei in root tips of secondary roots (t=71,5h (Rep1); 97,5h (Rep2); 130,5h (Rep3)). Significance was determined using a two tailed homoscedastic T-test. ***p<0,001 (**G**) Heatmap of the average fluorescent intensity within the syncytial area per root segment over time for the 3rd replicate (Rep3). Red indicates a higher intensity and optical segments are 90 µm long. The segment with the head of the nematode (#5) is marked by a blue border.

Auxin signalling starts with a lag phase followed by a moderate and transient rise

Snapshot images of the fluorescent auxin reporter (3nGFP driven by the DR5revV2 promoter) show that GFP fluorescence was only visible in root tips and secondary root primordia in the uninfected control sample, and little to no signal was perceived from any other cell types (Figure 1B-F). A similar situation was seen in the movie of the infected samples at the start of the measurement where no auxin response was detectable (Figure 5A). During the course of the measurement, auxin signalling was induced in the infection site as well as in the developing secondary root primordia (Figure 5A-D). It is noted that, apart from secondary root primordia, little auxin signalling is observed outside the syncytial area. To gain insight into the dynamics of this induction, the RMI_{GFP} and FA_{GFP} were measured in all three samples using the same procedure as has been followed for quantifying the cytokinin signal. Analysis of the RMI_{GFP} in the optical slice of the main root that includes the head of the nematode (Figure 5E) and emerging secondary root primordia, shows that the auxin response in the three replicates rises to a maximum RMI_{GFP} value between 30 and 70 hours depending on the sample and varies between 1.5 and 3. Thereafter, the RMI_{GFP} drops to a slightly lower level in all replicates which is maintained until the end of the measurements. Notably, during the first hours of the sedentary phase of the juveniles no auxin signal could be detected. Apparently, the observed rise in auxin signalling is preceded by a lag phase of about 20 to 30 hours after the start of the measurements (Figure 5E). As expected, the number of pixels showing auxin signalling (FA_{GFP}) rises after the lag phase (Figure 5F) due to an increase in nuclei showing auxin signalling (Figure 1B-F). Similar to cytokinin signalling, the number of auxin signalling pixels (FA_{GFP}) increases during live-cell imaging in all three infected replicates although to different levels depending on the sample (Figure 5F). No auxin signal was observed in the uninfected control sample (Figure 5F), showing the specificity of the local auxin response upon nematode infection of the roots.

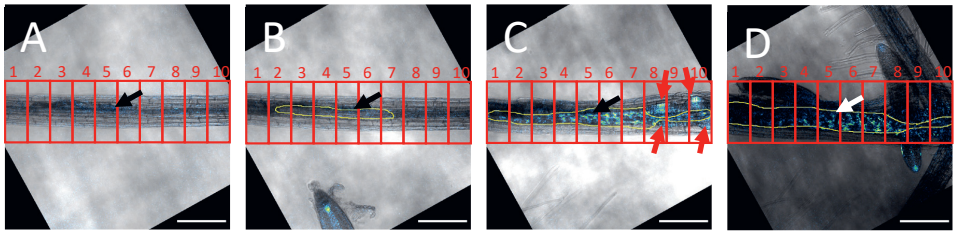
It is noted that the fluctuations observed in the RMI_{GFP} measurements of the 3rd replicate (Figure 5E) are caused by the additive effect of auxin signalling during the development of secondary root primordia. First, emerging secondary roots contribute to a pronounced rise in auxin signalling when still inside the main root, but a decrease in auxin signalling occurs when the secondary roots grow out from the analysed area (main root). To exclude the influence of auxin signalling in secondary root primordia, the RMI_{GFP} was also measured only within the syncytial area (Figure 5H). Similar to the cytokinin analysis, the syncytial area was manually marked at various time points and the growth of the syncytial area was interpolated between these marked borders as described. Next, the RMI_{GFP} was determined in the syncytial area, which revealed a pronounced increase in auxin response reaching maximum RMI_{GFP} values between 2.5 and 4.5 (Figure 5H). After a lag phase, the RMI_{GFP} values of the entire syncytial areas reach their maximum around 40 (1st and 2nd replicate) or 70 hours (3rd replicate) followed by a decline in auxin signalling to an intermediate steady state level (Figure 5H).

In order to gain an indication of the relative strength of the auxin response, the mean intensities of nuclei inside the syncytial area were compared to the mean intensities of auxin signalling nuclei in secondary root tips. To this end the mean intensity inside the syncytial area was taken at the point in time at which the highest RMI_{GFP} was measured (t=42,5h (Rep1); 46,5h (Rep2); 76,5h (Rep3)) (Figure 5G). Then, the mean intensity was measured within well-developed secondary root tips for comparison (Supplementary Figure S9). Secondary root tips

were selected from each replicate and optical slices with highest mean intensity were used for comparison. In contrast to the cytokinin analysis (Figure 4F), this comparison reveals that the auxin response within the syncytial area is significantly weaker, up to a factor of five, compared to the auxin response in secondary root tips.

To study the spatio-temporal dynamics of auxin signalling within the syncytial area, the infected root samples were divided in optical cross sections of 90 μm for each time point as described for cytokinin. Although the boundaries of the syncytial area became visible with bright field microscopy at 5h (2nd replicate), 16h (1st replicate) and 22h (3rd replicate) after the start of the measurements, no auxin signal could be detected yet. After this lag phase, the auxin signalling intensities showed an increase in all three replicates and is observed in nearly all segments (Figure 5I). The time points at which the auxin response maxima are reached vary between replicates. Also, within a single syncytial area the auxin signalling intensities are heterogeneously distributed and the height of the auxin maxima differ between segments. These variations seem to reflect the fluctuations in expansion rate and also the direction of expansion of the individual feeding cells. For example, between 21 and 65 hours the largest expansion in the 3rd replicate is observed in segments 5, 6 and 7 in front of the nematode's head and later on the syncytial area fully expands in segments 1, 2, 3 and 4 below the main body of the nematode (Figure 5 B-D). This differential expansion is in part also reflected in the heatmap of the segment analysis (Figure 5I). It is noted that this pattern is also observed for the cytokinin signal (Figure 4G). No evidence is obtained for consistently stronger fluorescence signals around the head of the nematode compared to other areas. In all three replicates, elevated levels of auxin signalling are observed in the segment containing the head of the nematode (Supplementary Figures S4 and S5). However, high auxin signal intensities are also observed in two or more neighbouring segments, often exceeding the segment harbouring the nematode's head. In addition, the first increase in auxin signalling intensities does not originate from a single segment and is also observed in two or more, often neighbouring segments. Also, the decline in auxin signalling occurs nearly synchronously in neighbouring segments.

Overall, it can be concluded that the transient rise in auxin signalling is restricted to the syncytial area and is preceded by a lag phase during which the first contours of the syncytial area become visible with bright-field microscopy. From this, we infer that the initial phase of syncytia development is independent of auxin signalling or accompanied by very low auxin signalling levels below the GFP detection threshold of the CLSM used. In contrast to cytokinin signalling, the auxin signalling intensities are relatively low in the syncytial area. A comparative analysis showed that the auxin signalling intensities were significantly lower in the syncytial area than in secondary root tips, while for the cytokinin response the opposite was observed.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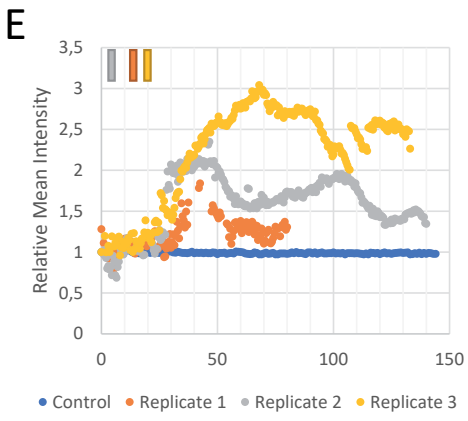
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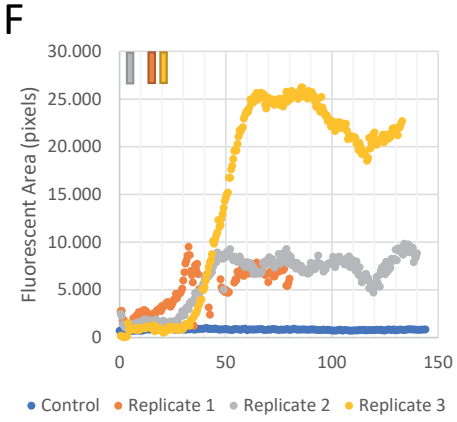
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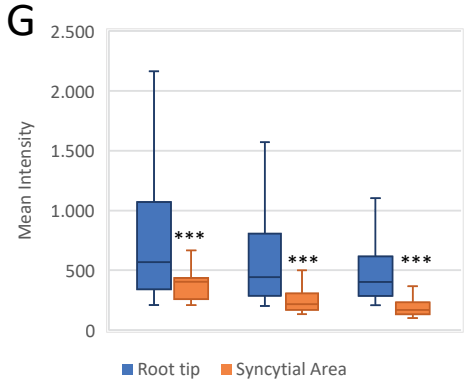
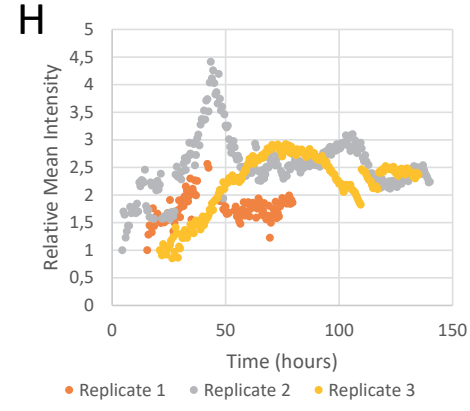

G
Mean Intensity
2.500
2.000
1.500
1.000
500
0
***
***
***
Root tip
Syncytial Area


H
Relative Mean Intensity
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
0
50
100
150
Time (hours)
Replicate 1
Replicate 2
Replicate 3

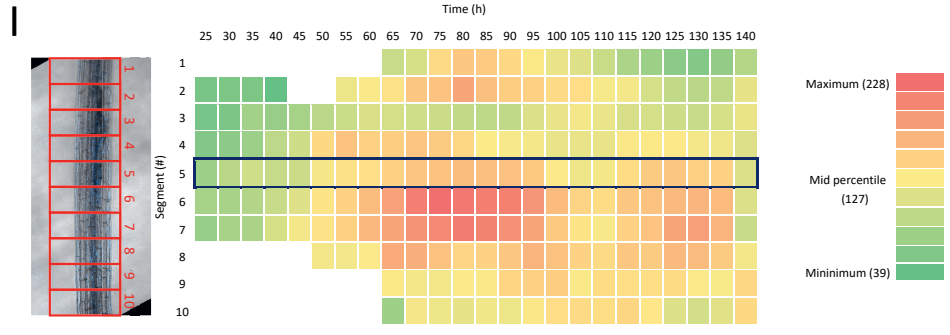

I
Time (h)
25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140
Segment (#)
1 2 3 4 5 6 7 8 9 10
Maximum (228)
Mid percentile (127)
Mininimum (39)

Figure 5 Analysis of auxin signalling dynamics within syncytial area induced by *H. schachtii* revealed by the double reporter DR5revV2::n3GFP-TCSn::ntdTomato with continuous live-cell imaging. Snapshots from the movie of the 3rd replicate (Rep3) at (**A**) the start of live-cell imaging (t=0), (**B**) at the first appearance of the syncytial area (t=21.5h), (**C**) during expansion of the syncytial area (t=68h) and (**D**) at the end of the measurement (t=130h) showing the emergence of a fourth stage juvenile and secondary roots. The border of the syncytial area is indicated in yellow and the root is divided into 10 optical segments indicated in red. Black and white arrows indicate the head of the nematode and red arrows show the developing root primordia. Fluorescent intensity is displayed as a blue<green<white (green fire blue) gradient. The scale bar represents 200 µm. (**E**) The average intensity per pixel of fluorescent auxin signalling nuclei in one optical slice per timepoint relative to the first measurement (t=0) (RMI) of the entire imaged primary root for each replicate. Bars on the upper left indicate the time at which the syncytial area was first observed with bright-field microscopy. (**F**) The number of fluorescent pixels (FA) in the main root for each replicate in one optical slice per timepoint. Bars on the upper left indicate the timepoint at which the syncytial area was first observed. (**G**) Mean intensity of auxin signalling in nuclei of the syncytial area compared to nuclei in secondary root tips (t=42,5h (Rep 1); 46,5h (Rep2); 76,5h (Rep3)). Significance was determined using a two tailed homoscedastic T-test. ***p<0,001 (**H**) Relative Mean Intensity (RMI) of the DR5revV2::n3GFP signal measured inside the marked syncytial area for all three biological replicates. (**I**) Heatmap of the average fluorescent intensity within the syncytial area per optical root segment (90 µm) over time for the 3rd replicate. Red indicates a higher fluorescent intensity and the segment that includes the head of the nematode is marked by a blue border.

Rise in cytokinin signalling within syncytial area precedes increase in auxin signalling

To understand the differences in cytokinin and auxin signalling in more detail, the nuclei were divided in three types that showed either auxin signalling, cytokinin signalling or both (Figure 6 A-D). Next the number of pixels (FA) displaying auxin signalling, cytokinin signalling or both was quantified in the entire imaged main root. At the end of the migratory phase, at the start of the measurements, all nuclei show only a cytokinin response (Figure 6E). Moreover, during the steep increase in cytokinin signalling, no auxin signalling nuclei are observed in the primary root. After 30 hours a rise in the number of nuclei displaying both auxin and cytokinin responses are observed (Figure 6E). Cells that show an auxin response but no cytokinin signal are only observed in developing secondary root primordia (Figure 6E, Supplementary Figure S6). It is noted that the observed decrease of the FA_{GFP} values of these auxin signalling nuclei after 70 hours (Figure 6E) is due to the outgrowth of the secondary roots (Figure 6C and 6D) and as a consequence, their root tips showing auxin signalling moved outside the analysed microscopic field.

To compare the differences in auxin and cytokinin signalling dynamics, the FA_{GFP} (Figure 5F) and FA_{tomato} (Figure 3F) were combined in a single figure (Figure 6F). Integration of data for each time point was possible given the simultaneous monitoring of both auxin and cytokinin signalling in the same infected root using a double reporter line. This comparison confirms that the relative increase in cytokinin signalling starts earlier than the increase in auxin response. The cytokinin response is continuously rising from the start of the first measurements (t=0) in all three replicates, while the auxin signal is observed later. For example, upon the first appearance of the syncytial area (t= 21.5h; Rep3), the level of cytokinin signalling is already high with a fluorescent area near 15.000 pixels, while the auxin signal is still absent. The rise in auxin signalling starts at 30 hours in the 1st replicate, 25 hours in the 2nd replicate and at 35 hours in the 3rd replicate, after which the auxin response increases in all three replicates ((Figure 6F, Supplementary Figure S7). These findings indicate

that the cytokinin signal precedes the auxin signal, after the first contours of the syncytial area becomes visible with bright field microscopy.

In order to determine the correlation between auxin and cytokinin signalling during infection, the Mean Intensity (MI) of both auxin and cytokinin signalling was determined in the main root for each timepoint (Figure 6G). It was noticed that high auxin signalling intensities are associated with high cytokinin signalling intensities. However, the inverse is not always true as high cytokinin intensities do not necessarily always correlate with high auxin signalling intensities. For example, cortical and epidermal cells with a high cytokinin response do not show auxin signalling. Because auxin signalling is contained to the syncytial area this mainly reflects the relation between auxin and cytokinin signalling within the syncytial area. As such, this suggests that the induction of auxin signalling co-occurs with an increase in cytokinin signalling within the syncytial area. Moreover, our imaging data (Figure 6A-D) indicate that within the syncytial area auxin and cytokinin signalling occurs simultaneously in the same nuclei. To further elucidate the ratio between auxin and cytokinin signalling, their mean intensities were measured in individual nuclei (Figure 6H). Nuclei with high auxin and high cytokinin signalling intensities within the syncytial area were selected and the mean fluorescence intensities within these nuclei were compared to those of nuclei with high auxin or cytokinin signalling in secondary root tips within the same replicate (Supplementary Figure S9A and 9B). Selection of high auxin or high cytokinin signalling nuclei within the syncytial area and secondary root tips was done by selecting nuclei having the majority of pixels with signalling intensities representing the highest 10% of measured intensities. The selection of nuclei was based on using cytokinin signalling intensities (Supplementary Figure S9A) as well as auxin signalling intensities (Supplementary Figure S9B). From the comparison between the different groups of nuclei it can be concluded that within the syncytial area auxin signalling maxima are associated with a high cytokinin response and vice versa, in contrast to what is seen in root tips (Figure 6H). In root tips, nuclei in the root cap and developing vascular tissue with a high level of cytokinin signalling are accompanied with relatively low levels of auxin signalling, while the reverse is the case for nuclei in the quiescent centre and neighbouring cells with a high auxin response. Overall, it can be concluded that selection of nuclei within the syncytial area with high auxin or high cytokinin signals results in cells with similar auxin-cytokinin ratios, which are distinct from the auxin-cytokinin ratios observed in signalling maxima of root tips (Figure 6H).

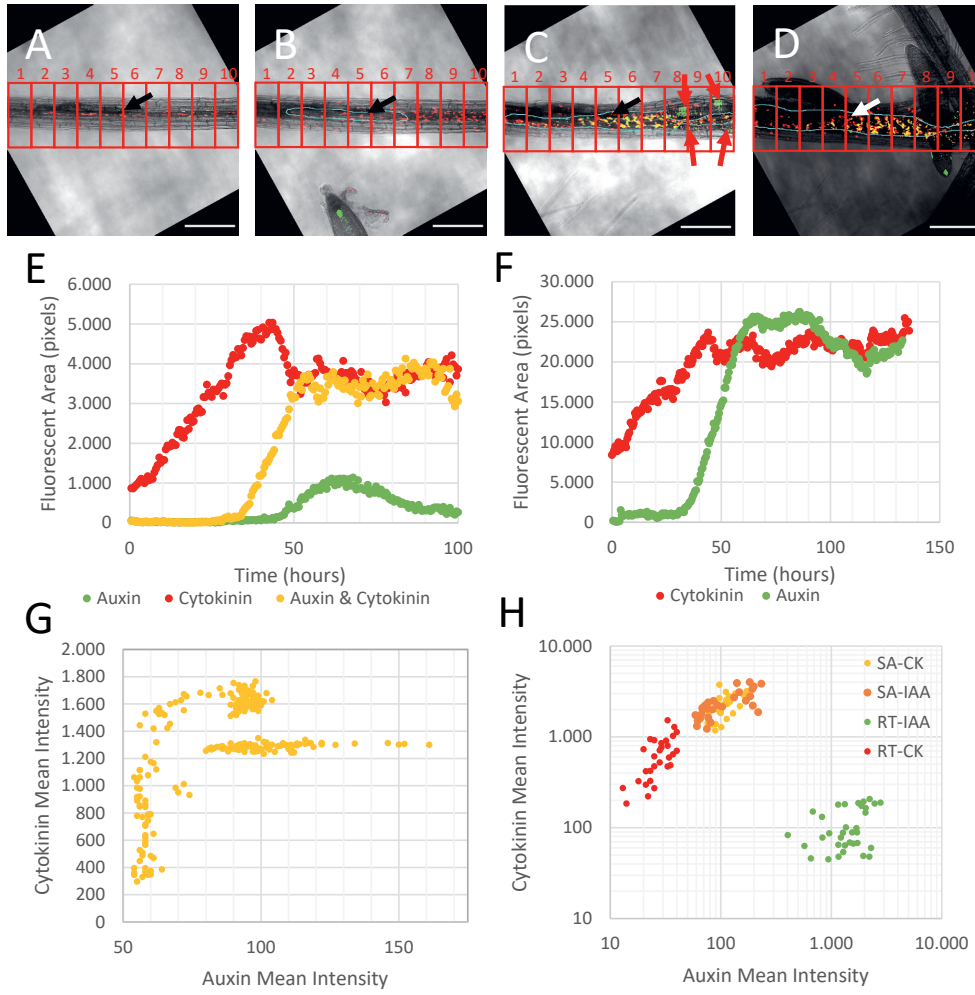


Figure 6 Comparative analyses of auxin and cytokinin signalling dynamics during development of syncytia induced by *H. schachtii* using the Arabidopsis DR5revV2::n3GFP-TCSn::ntdTomato double reporter line. Auxin (green) and cytokinin (red) signals were merged, resulting in a yellow signal when they overlap in the syncytial area of a single replicate (Rep3) at (**A**) the start of live-cell imaging (t=0), (**B**) at the first appearance of the syncytial area (t=21.5h), (**C**) during expansion of the syncytial area (t=68h), and (**D**) at the end of the measurement (t=130h) showing the emergence of a fourth stage juvenile and secondary roots. Black and white arrows indicate the head of the nematode and red arrows show the developing root primordia. The scale bar represents 200µm. (**E**) Fluorescent area (FA) as an indication of the number of nuclei that show auxin signalling, cytokinin signalling or both as measured over time in the entire image of the main root infected with *H. schachtii* (Rep3) using a binary masking method. (**F**) Fluorescent area (FA) of auxin and cytokinin signalling in main root infected with *H. schachtii* (Rep3) showing a delay for auxin signalling. (**G**) The mean intensity of the cytokinin signal plotted against the mean intensity of the auxin signal in the entire main root for all measured time points of the 3rd replicate (Rep3), showing the auxin and cytokinin ratios measured at intervals of 30 min during a period of 140h (**H**) The mean intensity of auxin and cytokinin signals found at a single time point (t=113h) in the 3rd replicate in single nuclei. At least thirty nuclei were selected from the top 10% highest auxin and cytokinin signalling nuclei located in the syncytial area (NFS-IAA and NFS-CK) as well as nuclei present in secondary root tips (RT-IAA and RT-CK) resulting in three groups of cell types based on their auxin-cytokinin ratios. Further explanation see main text. Abbreviations: IAA – Indole-3-acetic acid, CK – Cytokinin, SA – Syncytial Area, RT – Root Tip

Discussion

Here, we developed and tested a novel live-cell imaging method to study simultaneously the spatio-temporal dynamics of auxin and cytokinin signalling in plant roots during the initiation of syncytia induced by the cyst nematode *H. schachtii*. Upon infection of a transgenic *Arabidopsis thaliana* line harbouring a nuclear-targeted dual reporter, the auxin and cytokinin signalling responses could be monitored continuously in a single specimen by confocal laser scanning microscopy (CLSM) and images were obtained at 30 minutes intervals. Quantitative analyses of the optical slices revealed an early wide-spread increase of the cytokinin response during infection, while auxin signalling showed a lag phase and was restricted to the syncytial area. Auxin signalling is observed after the first contours of the syncytial area become visible and reaches its maximum within 70 hours and subsequent decreases to a relatively constant level. In contrast, the cytokinin signalling intensities rise early during infection and stay at a constant level afterwards. Interestingly, a comparative analysis shows that the cytokinin signals in the syncytial area are higher than the cytokinin signalling maxima in secondary root tips, while for auxin the response is lower. In addition, we show that within the syncytial area auxin signalling maxima are paired with high cytokinin signalling intensities, which contrasts the auxin and cytokinin ratios seen in the root tip. Altogether our data indicate that auxin signalling increases after the first contours of syncytial area become visible suggesting that auxin plays a role during expansion of the syncytium rather than initiation. The widespread occurrence of cytokinin signalling, also outside the vascular tissue, may point at multiple roles during infection, which may include initiating and enlarging of the syncytial area as well as regulating early defence responses. Overall, our data suggest that cytokinin signalling plays a role in syncytium formation before auxin does. This necessitates a shift in our current view on the role of cytokinin and auxin in syncytium induction and development.

In this study, an increase in cytokinin signalling was observed overtime throughout the entire feeding site within the first day of measurement (Figure 3E). These findings are in accordance with prior studies employing specific reporter genes, such as TCSn::GFP and ARR5::GUS, which have demonstrated an upregulation of cytokinin signalling within the syncytium of *H. schachtii* in *Arabidopsis thaliana* as early as 2 and 3 days post inoculation (dpi), respectively (Shanks et al. 2016; Siddique et al. 2015). This is roughly the same timeframe used in our study, as we introduced the nematodes into the sample approximately 18 hours prior to live-cell imaging (t=0). Thus, a day after the start of live-cell imaging in fact corresponds to a time point of around 2 days post inoculation. With regard to the spatial distribution of cytokinin signalling, we observed a fluorescent signal in the cortical and epidermal cells during live-cell imaging (Figure 3A-D). In contrast, previous reports on cyst nematodes did not show the presence of cytokinin signalling in the cortical and epidermal cells despite the use of the same nematode and plant species (Dowd et al. 2017; Shanks et al. 2016). However, the key difference might be the use of different promotors or fluorophores in the reporter gene constructs. In the case of the ARR5::GUS reporter, the TCSn promotor was shown to be more sensitive to cytokinin signalling compared to several ARR promotors (Zürcher et al. 2013). Furthermore, the ntdTomato fluorophore is up to six times brighter than EGFP and, as such, it is likely that we could visualise responses to lower cytokinin concentrations as compared to the use of TCSn::GFP (Shaner et al. 2004). Alternatively, the difference might be explained by the fact that the TCSn promoter also responds to plant stress, which results in expression in the epidermis and cortex. The epidermal and cortical cytokinin signal might thus be explained

by an abiotic stress response induced by the measurement setup as this is seen in both the infected as well as the uninfected samples. A possible source of stress within the measurement setup could be the regular exposure to laser light in addition to growth under *in vitro* conditions. Additionally, light might affect root development mediated by cytokinin via the expression of the Cytokinin Independent-1 (CKI1), which in turn is regulated by light through PhysA (Pavlů et al. 2018).

Cytokinin signalling induction during parasitism is not exclusive to *H. schachtii*, and is also observed during the formation of giant cells induced by root-knot nematodes (Dowd et al. 2017). Moreover, an early and rapid induction of cytokinin signalling is also seen in other biotic interactions. For example, early activation of cytokinin signalling is observed in plants invaded by parasitic plants like *Phelipanche ramose*, where cytokinins play a pivotal role in prehaustorium induction (Goyet et al. 2019). Cytokinin levels and signalling are also induced upon wounding leading to a more widespread cytokinin response during herbivory by larvae of the moth *Manduca sexta* (Schäfer et al. 2015). In the case of *Lotus japonicus*, the TCSn:YFPnls cytokinin reporter showed a progressive fluorescent signal in response to Rhizobium bacterial colonization from 2 dpi onwards. The signal was located in foci of dividing cortical cells, which correspond to the development of nodule primordia (Reid et al. 2017). Previously, it has been suggested that the induction of cytokinin signalling during cyst nematode infection contributes both to developmental processes and defence (Shanks et al. 2016). Thus, the wide-spread cytokinin accumulation within and around syncytia as observed in our study might reflect a dual role for cytokinin in syncytial development and defence responses induced by the invading nematode. However, the use of the auxin/cytokinin double reporter in this study does not allow to untangle defense and developmental responses upon nematode root infection as they are expected to partially overlap in space and time.

Our data show that the cytokinin signalling intensity decreases when further removed from the nematode's head and nearing the edges of the syncytial area (Figure 4G). As such, the decrease near the edge of the syncytial area might be the result of the injection of cytokinin into the feeding site as this pattern is in line with what could be expected upon the release of cytokinin through the nematode's stylet into the initial syncytial cells. For *H. schachtii*, the biosynthesis and release of cytokinin was reported, probably to control feeding site formation (Siddique et al. 2015). Cytokinins have been measured in *H. schachtii* and a cytokinin-synthesizing isopentenyltransferase gene (HsIPT) has been found. Silencing of this gene lowers the level of free base *iP*, an active form of cytokinin, within juveniles and attenuates the infection process, consistent with a possible role for cytokinin secretions in the induction of cytokinin signalling during the onset of parasitism as observed in our study. Alternatively, local plant-based biosynthesis of host cytokinin might explain the distribution of the observed cytokinin signal. Infection by *H. schachtii* was shown to upregulate cytokinin biosynthesis (IPT) gene expression in *Arabidopsis thaliana* (Dowd et al. 2017). As such, the increased cytokinin signalling near the nematode's head might be the result of increased local cytokinin biosynthesis or the release of cytokinin by the nematode, or even a combination of the two.

Similar to cytokinin, the auxin signalling dynamics could be studied in time and space using the double reporter. The live-cell imaging data shows the induction of auxin signalling within the syncytium of *H. schachtii* during the infection at 2 to 3 dpi (Figure 5E). However, auxin accumulations have been reported as early as 18 hours post inoculation while our live-cell

data shows an accumulation between 2-3 dpi (Karczmarek et al. 2004). This difference might be explained by variations in the migration process as this study only included parasitic juveniles near the end of the migratory phase at 18 hpi while nematodes in other studies might not have had this delay and started earlier with the induction of syncytia. Alternatively, the early auxin induction (Karczmarek et al. 2004) might perhaps be attributed to the GUS reporter as the expression of this enzyme results in a signal amplification due to the conversion of substrate into a stable product in contrast to the more gradual accumulation of a fluorophore that may need more time to reach the threshold for detection. In addition to plant-nematode interactions, the induction of auxin signalling is observed in other biological systems as well. For instance, an initiation of auxin signalling was observed during nodule formation by Rhizobium (Kohlen et al. 2018). Similarly, in the case of the parasitic plant *Striga hermonthica*, the induction of auxin signalling is a fundamental aspect for the switch of *S. hermonthica* roots to form the invasive prehaustoria (Xiao et al. 2022). Together, these examples including our findings underscore the role of auxin signalling in the establishment of symbiotic and parasitic interactions on plant roots by contributing to the development of specialized structures required for nutrient exchange.

Comparable to cytokinin, although later in time, a relatively strong auxin signal is observed in segments close to the head of the nematode (Figure 5I). This might be due to the potential injection of auxin by the nematode into the plant tissue as suggested by the report on auxin in stylet secretions in previous studies (Meutter et al. 2005, chapter 4). Alternatively, auxin accumulations might be the result of plant-based processes including the re-localisation of PIN proteins which act in polar auxin transport. The role of auxin export proteins during nematode infection have been well-documented (Grunewald et al. 2009). For example, during cyst nematode infection it is thought that PIN1 is responsible for the flow of auxin into the syncytium, while PIN3 and PIN4 may distribute auxin throughout the feeding site. However, the accumulation of auxin can also be the result of both nematode and plant-based activities. For example, auxin transport is affected by the nematode effector 19C07 from *H. schachtii* that was reported to interact with the auxin influx transporter LAX3 (Lee et al. 2011). Finally, increased local auxin biosynthesis might play a role as well. For example, upregulation of IAM and YUCCA auxin biosynthesis genes is observed during infection of *H. schachtii* or *M. incognita* (Oosterbeek et al. 2021; Chapter 2). Additionally, the Ethylene Response Factor 9 (ERF109) promotes auxin biosynthesis by binding to the promoters of YUC2 and ASA1 and has been suggested to regulate local auxin biosynthesis at the nematode infection site as DR5::GUS expression was significantly lower in *erf109* mutants (Guarneri et al. 2023). This suggests that the auxin accumulation in syncytia is at least partially the result of local auxin biosynthesis. As such, the relatively strong auxin signal in segments near the head of the nematode as observed in this study might be the result of hormone injection, increased local biosynthesis or transport manipulation.

Aside from the analysis of signalling dynamics of individual hormones, the use of the double reporter has allowed us to study auxin and cytokinin signalling simultaneously in a single nematode infection site. Using this reporter, we observed an increase in auxin signalling during infection, but its intensity is significantly weaker compared to that in secondary root tips (Figure 5G). In contrast, cytokinin signalling inside the syncytial area seems to exceed the levels detected inside root tips (Figure 4F). This might be an indication that cytokinin signalling is more prevalent during nematode infection compared to auxin. The differences in auxin and

cytokinin signalling in the syncytial area seems consistent with earlier data based on the transcriptional analysis of nematode feeding sites. Comparative analysis of feeding sites induced by root-knot nematodes and feeding sites induced by cyst nematodes revealed that cytokinin related genes are enriched compared to auxin related genes. For example, LBD16 and its positively co-regulated genes were repressed in syncytia while in contrast cytokinin-induced genes were enriched (Cabrera, Fenoll, and Escobar 2015). This supports our data that cytokinin plays a more dominant role in syncytium development. Interestingly, for gall formation by the root-knot nematode the opposite was shown indicating that auxin might be a more dominant factor in giant cell formation (Cabrera, Fenoll, and Escobar 2015). While the strength, tissue and timing of the hormone signals seems to differ in infected root segments, a substantial part of the auxin and cytokinin signalling domains overlap in time and space. We could even demonstrate that auxin and cytokinin signalling occurs simultaneously in single nuclei located inside the syncytial area during the onset of feeding site development (Figure 6A-D). Interestingly, nearly all of the auxin signalling that is induced due to nematode infection seems to be co-localised with cytokinin signalling on a cellular level (Figure 6E). The absence of nuclei inside the syncytium in which only auxin signalling occurs and that the auxin signal is always paired with a high cytokinin signal on an individual cell level, suggests that the role of auxin during nematode development is through the interplay with cytokinin.

Previous reports have shown that in developing vascular tissues of root tips, auxin and cytokinin signalling maxima do not overlap, in contrast to what is observed within the syncytial area (Bishopp et al. 2011; Wybouw and De Rybel 2019; De Rybel et al. 2014). In the developing vascular tissue of the root tip auxin signalling is confined to metaxylem and protoxylem cells, while neighbouring procambium cells and phloem cells display cytokinin signalling (Bishopp et al. 2011; Wybouw and De Rybel 2019). Similar patterns are observed in the root cap, quiescent centre and neighbouring stem cells, though the precise position of the signalling maxima may vary depending on the type of reporter (Zürcher et al. 2013; Petersson et al. 2009; L. Wu et al. 2021; Grieneisen et al. 2007; Liao et al. 2015; Fisher et al. 2018). For example, the DR5 reporter has the highest response in the quiescence centre, while the DR5revV2 reporter has its maxima in subtending columella cells (Liao et al. 2015). Also direct measurements and predictive modelling of auxin and cytokinin concentrations may lead to deviations from reporter gene expression studies (Moore et al. 2024). A well-known example is the quiescence centre where high cytokinin concentrations have been measured, while the cytokinin response is relatively low, and is presumable repressed by high auxin concentrations via AHP6 signalling (Moore et al. 2024). Nevertheless, expression profiles of reporters are widely accepted to study the interaction between auxin and cytokinin, but should be interpreted in the appropriate context and in a comparative way without referring to actual hormone concentrations. Therefore, it is feasible to conclude that the overlapping auxin and cytokinin signalling maxima revealed by the double reporter indicate that, in contrast to root tips, signlling modules leading to the separation these maxima are not operating within the syncytial area. Altogether, the pairing of auxin signalling maxima with high cytokinin responses observed in this study underlines the unique features of feeding cell development.

Despite their divergent functions, the interplay between auxin and cytokinin is known to be responsible for developmental processes such as lateral root formation, vascular patterning, and shoot and root meristem development (Bishopp et al. 2011; Jing and Strader 2019; Su, Liu, and Zhang 2011). As such, not the individual actions of auxin and cytokinin but the

interplay between them might explain various processes that occur during feeding site formation like the formation of phloem. It has been speculated that the difference in ratio of auxin and cytokinin between galls and syncytia affect the formation of phloem, but not the feeding site itself (Absmanner, Stadler, and Hammes 2013). Phloem is induced around syncytia and giant cells to support feeding site and nematode development. Giant cells are symplastically isolated and obtain nutrients through transporter-mediated processes while, in contrast, syncytia are connected to the phloem by plasmodesmata. It was shown that the phloem around giant cells does not respond to cytokinin while the phloem around syncytia does (Absmanner, Stadler, and Hammes 2013). As such, the process of vascularization around feeding sites for cyst and root-knot nematodes could potentially arise from the fine-tuned interplay between auxin and cytokinin signalling pathways.

Finally, as shown in this study the use of an auxin-cytokinin double reporter represents a potent method for investigating hormonal signalling dynamics in plant nematode interactions. However, it is vital to acknowledge and address certain inherent pitfalls in this approach and how these were overcome to ensure the precision and reliability of the acquired data. One primary concern in employing the auxin-cytokinin double reporter system is the potential impact of laser-induced photobleaching. Photobleaching, the loss of fluorescence due to laser exposure, can compromise the accuracy of signal quantification. To mitigate this, in this study careful laser power adjustments were made to minimize photobleaching and thereby preserving the integrity of fluorescent reporters. Even with adjusted laser power, allowing the host plant to grow and develop while being imaged is essential for live-cell imaging. To this end, a chambered cover-glass was used in combination with growth medium and agar. This allowed the plant to grow under sterile conditions without the need for external interference. Tissue depth-related variations in fluorescent signal are mitigated in this study due to controlled comparisons within the same optical layer. This eliminates the influence of tissue depth on signal intensity, ensuring reliable data of the same area. Depth-related variations as a result of sample drift are corrected for by imaging a stack of optical slices around the initial depth of interest. This ensures that corrections for z-drift become possible in the later analysis. The use of the DR5revV2::n3GFP-nls and TCSn::ntdTomato-nls double has allowed us to image auxin and cytokinin signalling concurrently. This has not been used previously to study hormone signalling in plant nematode interactions. Hence, we were able to determine on a cellular level that auxin signalling correlates with a high level of cytokinin signal, something that would otherwise have stayed unnoticed. So, in conclusion, the auxin-cytokinin double reporter system combined with live-cell imaging is a valuable tool as shown in this study to obtain novel insights in the spatio-temporal dynamics of hormone signalling domains in nematode parasitism. In addition, our analyses show that even an intractable biological system as feeding cell induction - characterized by non-uniformity and unpredictable expansion rates and directions - is amenable for quantitative live-cell imaging. This shows that our approach offers also opportunities to study other complex plant-microbe interactions with capricious developmental patterns at the cellular level.

Material and Methods

Plant material

Seeds containing a DR5revV2::n3GFP-nls and TCSn::ntdTomato-nls transcriptional fusion were available in a Col-0 background where the auxin-responsive promoter DR5 (Ulmasov et al. 1997) drives the expression of n3GFP and the cytokinin responsive promoter TCSn (Zürcher et al. 2013) the expression of ERFP. Both fluorophores are expressed with a nuclear localisation signal (NLS) to concentrate the fluorescent signal in the nucleus for enhanced sensitivity and accurate quantification irrespective of cell size or vacuolization status (Fisher et al. 2018). The seeds were stratified at 4°C for 4 days and subsequently vapour sterilized (50 ml H2O, 40 ml NaOCl 5%, 4.4 ml HCl 25%) for a duration of 3.5 hours. Seeds were plated on solid mKNOP medium (KNOP minerals, 10% sucrose, 0.8% Daichin agar, pH 6.4) (Sijmons et al. 1991). The plates were incubated horizontally at 21°C under 16 h light/8 h dark cycle for 3 days to ensure the growth of the root into the agar medium. Subsequently, they were placed vertically to promote growth of the roots along the bottom.

Nematode hatching and sterilization

Heterodera schachtii cysts were harvested from soil and cleaned using 1% NaN_3 for 20 minutes. The cysts were subsequently incubated on a 25 µm sieve at room temperature in the dark for 4 days in a 3mM ZnCl2 solution to promote hatching of infective juveniles. To prevent contamination, the antibiotics 0.15% gentamycin (w/v) and 0.05% nystatin (w/v) were added to the solution. The freshly hatched second stage juveniles (J2) were subsequently sterilised as described previously. They were collected through purification using a sucrose (35%) gradient. The infective juveniles were surface sterilised (0.002% Triton X-100 v/v, 0.004% NaN3 w/v, 0.004% HgCl2 w/v) for 10 minutes and afterwards rinsed 3 times in sterile tap water. Next, they were suspended in 0.7% Gelrite (Duchefa biochemie) solution to a final concentration of 10 juveniles per µl for use as inoculum in nematode infection assays.

In vitro nematode infection assays

For functional validation of the auxin-cytokinin double reporter DR5revV2::n3GFP-nls/TCSn::ntdTomato-nls (Figure 1), both infected and uninfected root systems were imaged using the standard method to monitor the spatio-temporal distribution of fluorescent reporter genes in cyst nematode induced feeding sites (Shanks et al. 2016). One week old seedlings were inoculated with 50 juveniles and imaged at 0-, 1-, 2-, 3-, 4-, and 7-days post-inoculation. For each time point ≥ 25 feeding sites were selected from a set of 10 plants. Samples were stained with 100 µm propidium iodide for 10 minutes before being imaged under an inverted LEICA SP8 confocal microscope. For detection of auxin signalling, the n3GFP fluorophore was excited with a 488 nm argon laser and emission captured at 505-530nm. Cytokinin signalling was detected upon excitation of ntdTomato with a 543 nm argon laser and emission captured at 560-580 nm. Propidium iodide was excited at 488nm and emission was captured at > 650nm to visualise the cell boundaries.

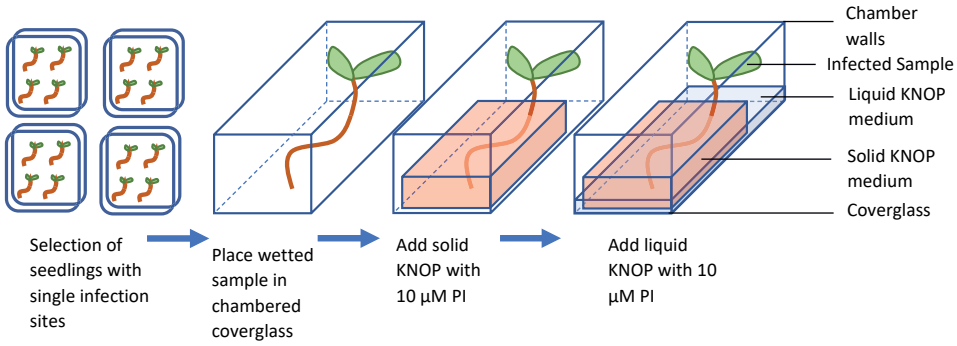


Figure 7 Sample preparation for live-cell confocal imaging. A plant is selected with a single nematode infection that just entered the sedentary stage. It is then stained with propidium iodide and put in a chambered coverglass and fixed in place by a block of solid KNOP agar supplemented with 10 µM propidium iodide. The chamber is then filled with liquid KNOP medium with 10 µM propidium iodide to facilitate staining.

In vivo live-cell imaging using confocal laser scanning microscopy (CLSM)

Plant roots with a single nematode infection were used for the in vivo live-cell imaging. Around 200 DR5v4:EGFP-nls/TCSn:ERFP-nls seeds were germinated in square plates containing mKNOP and six days afterwards inoculated with approximately 20 juveniles per plant, as described above. After 19 hours of incubation at 21°C under 16 h light/8 h dark cycle, plants were screened using a binocular for nematode juveniles that successfully invaded the roots and had transitioned from their migratory phase. During cyst nematode migration the nematode uses its stylet to penetrate the plant's cell walls and move through them. Near the vascular bundle the nematode stops migrating and enters its sedentary phase where it selects a initial syncytial cell. It probes this cell using its stylet before penetrating it and starting a cycle of feeding from the cytoplasm and injecting effector compounds (Wyss and Grundler 1992). To monitor nematodes from the start of their sedentary period, samples were collected with nematodes that were visibly probing root cells and selecting an initial syncytial cell. Up to five of these samples were taken and their roots stained with propidium iodide for 10 minutes. These seedlings were placed into sterile chambered coverglasses (Nunc™ Lab-Tek™ Chambered Coverglass) in a flowcabinet. The chambers enable the seedlings to grow in an enclosed sterile environment and the thin coverslip bottom facilitates imaging through an inverted CLSM. Subsequently, a slice of sterile solid KNOP medium supplemented with 10 µm propidium iodide was carefully placed on top of the root, leaving the shoot free, to bring it close to the imaging surface and minimize sample drift. Afterwards, the chambers were filled with 2 ml of liquid KNOP medium supplemented with 10 µm propidium iodide to maintain constant propidium iodide concentrations. Lastly, the chambers were sealed with parafilm to secure a sterile environment during imaging that still allows for gas exchange.

The prepared samples were imaged on a ZEISS LSM510/CONFOCOR2 confocal microscope equipped with a 10x air objective (NA 0.4) and an Argon ion laser. The Argon laser was used for excitation of GFP at 488 nm, and a He/Ne laser was used to excite ntdTomato at 543 nm. GFP fluorescence emission was detected with a band-pass filter 505-550 nm, while ntdTomato fluorescence was detected with a 560-600 band-pass filter. The optical slices were acquired in confocal mode (1 Airy unit) with an average of 8 scans. At 19 hours post infection

the samples were monitored for 160 hours and Z-stack images were taken every half-hour. The reporter used for auxin and cytokinin signalling was excited and measured in the same manner as described above during the nematode infection assay. All timeseries are available at the supplementary data.

Testing Sample Viability

Before live-cell imaging was performed it was confirmed that the infected seedling in the sample chamber was viable. First it was investigated what the effect of different cell-stains was on the growth of the seedlings. Arabidopsis seedlings were allowed to germinate and grow for 7 days on mKNOP medium in square plates as described above. The medium was supplemented with various concentrations of CalcoFluor White or propidium iodide to determine the effect of these cell stains on the growth of the plant. For CalcoFluor White concentrations were used of 0%, 0.1%, 1% and 10% (w/v) and for propidium iodide 0 µm, 10µm, 50µm en 100µm. Samples were incubated vertically at 21°C under 16 h light/8 h dark cycle for 7 days. In addition, infected samples in chambered coverglass were prepared as described above and stored in a climate chamber (Nunc™ Lab-Tek™ Chambered Coverglass) to assess the viability of the nematode in this setup. After three weeks the samples were visually inspected for the presence of J3 and J4 females.

Data Analysis

Picture analyses were carried out using Fiji (v2.0.0-rc-69) (Schindelin et al. 2012). CLSM allows for imaging optical slices of the sample and a stack of these optical slices is known as a z-stack. Z-stacks were taken every 30 minutes during live-cell imaging, showing the fluorescence in nuclei of infected and control root tissue as the result of promoter activity. Z-stacks consisted out of ~7 slices of 67 µm and included tissue above and below the nematode.

To monitor temporal changes in auxin and cytokinin signalling throughout the infection area, for each timepoint, the optical slice with the head of the nematode was analysed for fluorescence intensity derived from n3GFP or ntdTomato expression by calculating the mean fluorescence intensity (MI) per optical slice in the main root. This intensity was only measured for pixels selected through the use of a mask to select for nuclei. This mask was created for pixels above the noise threshold using the ‘default’ ImageJ thresholding value. Artefacts in the mask were smoothed out with the ‘smooth’ and ‘despeckle’ function and contrast was enhanced with the normalised ‘enhance contrast’ function. Jagged edges were corrected with the ‘dialate’ and ‘erode’ functions. The mean intensity (MI) of each timepoint was then normalised by division with the mean intensity of the first measurement in the time series (t=0) (RMI). In addition to the RMI the fluorescent area (FA) was defined as the sum of all fluorescent pixels as detected in fluorescent nuclei at a given timepoint for the observed optical slice. This relative mean intensity (RMI) and fluorescent area (FA) were plotted in a graph to show the overall changes in fluorescence of nematode infected root areas overtime as the result of reporter gene activity (Figure 3E and F and Figure 5E and F). This was done for three independent biological replicates.

To determine the temporal changes/dynamics in fluorescence intensities more specifically in the nematode feeding sites a single optical slice was selected from the z-stack for every

timepoint. The optical slice was chosen so that the image always contained the head of the nematode. Because small aberrations in the sample position might shift specific root segments to different optical slices, using the head of the nematode as a reference allows for the measurement to be performed on the same depth in the root even if this is not always the same depth in the z-stack. Subsequently, the feeding site was outlined manually (Region of Interest (ROI) selection) for various timepoint in a series by merging the brightfield and fluorescence images per optical slice and using that as a reference. In this way, the induction and development of the feeding cell in the selected nematode infected root area was confirmed. Between these manually outlined feeding sites at various time points, the development of the syncytium was interpolated thereby simulating syncytium growth (ROI interpolation). The mean fluorescence intensity (MI) of the reporter was then measured inside these feeding cell-enriched root tissues using the previously described mask. These mean intensities were plotted relative to the first measurement (RMI) in a graph to show the overall changes in fluorescence of feeding cell enriched root areas overtime as the result of reporter gene activity relative to the first measurement (Figure 4E and Figure 5H). This was done for three independent replicates.

For the comparison of the intensity of auxin or cytokinin signalling in the syncytial area with the signalling intensities in secondary root tips a similar approach as above was used. Using the above-described mask the intensities were collected within the outlined syncytium at one timepoint. This timepoint with the highest signalling intensity in the sample was selected based on the previous analysis (Figure 3E and F and Figure 5E and F). Similarly, the area of the secondary root tip with the highest signalling intensity was selected for root tips that were in focus and closest in time to the timepoint in which the syncytium was measured (Supplementary Figure S9). In this area the (pixel) intensities were collected and displayed in a box graph (Figure 4F and Figure 5G)

To determine how auxin and cytokinin signalling changes outside the syncytial area, a segment analysis was performed. For these spatial segment measurements, again a single slice time series was used that contains the head of the nematode. In every slice the length of the root is then marked in a rectangular area (ROI selection) and with that area the syncytial area was marked as above and filtered through the mask. For this selection the mean intensity (MI) was measured for every column of 90 µm within the rectangle. The mean signal intensity (MI) for that column is than averaged across 10 measurements over time (5 hours) of that column. This gives the average signal intensity for that particular region at that particular depth over that time period (Figure 4G and Figure 5I).

In order to determine which nuclei displayed auxin signalling, cytokinin signalling or both a binary masking method was used instead of a masking method for nuclei. Using the same single slice time series as mentioned above all auxin and cytokinin signal above the noise threshold (using the 'default' ImageJ thresholding value) was maximised while all other signal was removed to create the binary mask. The maximised auxin signal was then overlapped with the maximised cytokinin signal and pixels were coloured based on whether the maximised auxin signal, maximised cytokinin signal or both were present. This was then mapped back onto the corresponding brightfield images to display where overlap occurs (Figure 6A-D). Subsequently, the number of pixels for each signalling type were counted and displayed in a graph (Figure 6E).

To display the changes in the fluorescent area over time of auxin (Figure 5F) and cytokinin (Figure 3F) their figures were combined to display the delay in auxin signalling increase (Figure 6F). The correlation between auxin and cytokinin signalling intensity data as seen in Figure 6G is created by plotting the mean intensity of cytokinin signalling in the main root (Figure 3E) against the mean intensity of auxin signalling in the main root (Figure 5E) for all measured time points of the 3rd replicate. In Figure 6H the auxin and cytokinin mean intensity was determined in nuclei in the syncytial area and root tips. The image was thresholded to select for the top 10% of pixel intensity for both auxin and cytokinin in their respective areas. A total of 30 individual nuclei were manually selected for each group.

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Supplementary Data

Movies available at: https://tinyurl.com/msshwy8j

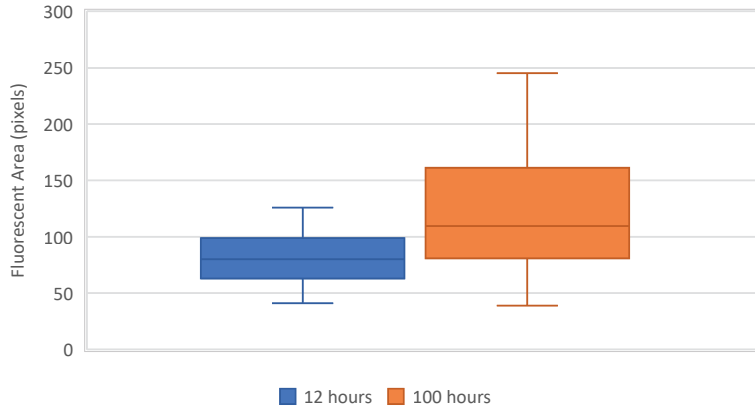


Figure S1 Sizes of the fluorescent nuclei for the 3rd replicate at 12 and 100 hours. Measurement is based on the signal from the TCSn::ntdTomato-nls reporter for cytokinin of thirty nuclei per timepoint.

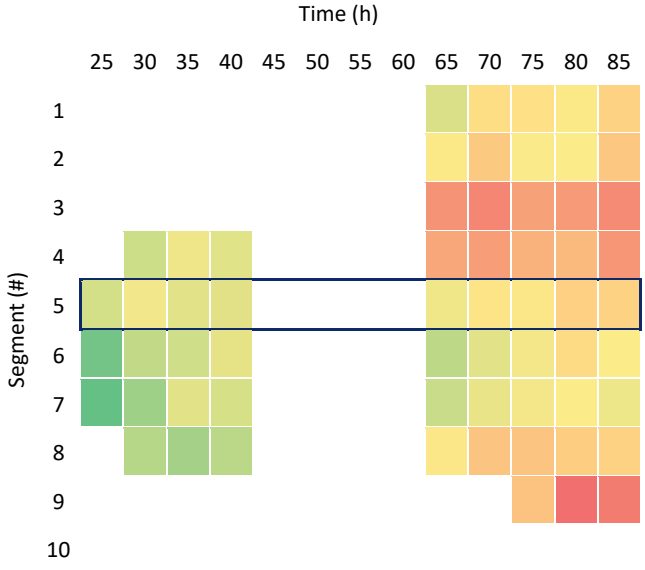


Figure S2 Heatmap of the average fluorescent intensity of the cytokinin reporter within the syncytium per root segment over time for the 1st replicate. Severe sample drift that could not be corrected makes the 45-60 range unreliable and is therefore not shown. Red indicates a higher intensity and segments are 90 µM long. The segment with the head of the nematode is highlighted in blue.

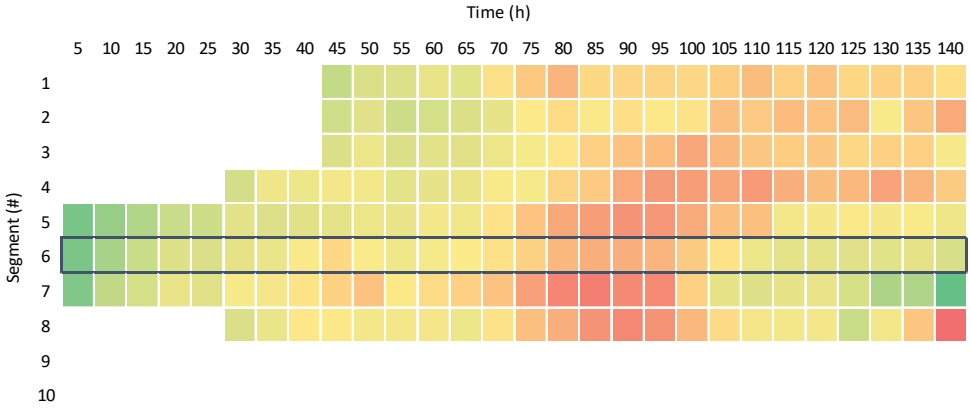


Figure S3 Heatmap of the average fluorescent intensity of the cytokinin reporter within the syncytium per root segment over time for the 2nd replicate. Red indicates a higher intensity and segments are 90 µM long. The segment with the head of the nematode is highlighted in blue.

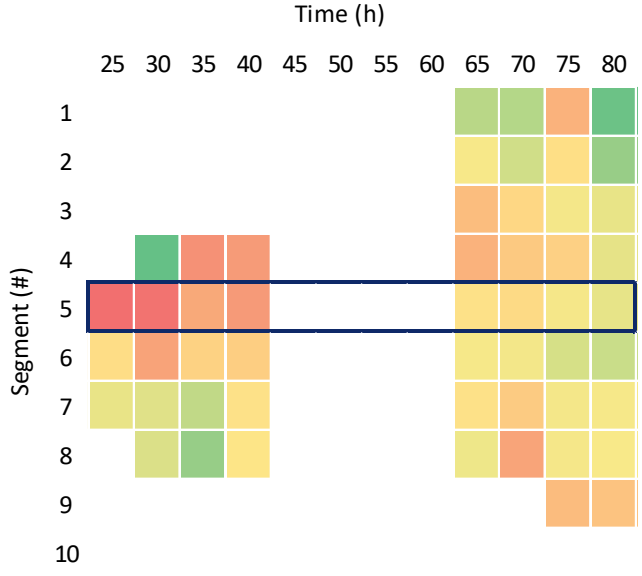


Figure S4 Heatmap of the average fluorescent intensity of the auxin reporter within the syncytium per root segment over time for the 1st replicate. Severe sample drift that could not be corrected makes the 45-60 range unreliable and is therefore not shown. Red indicates a higher intensity and segments are 90 µM long. The segment with the head of the nematode is highlighted in blue.

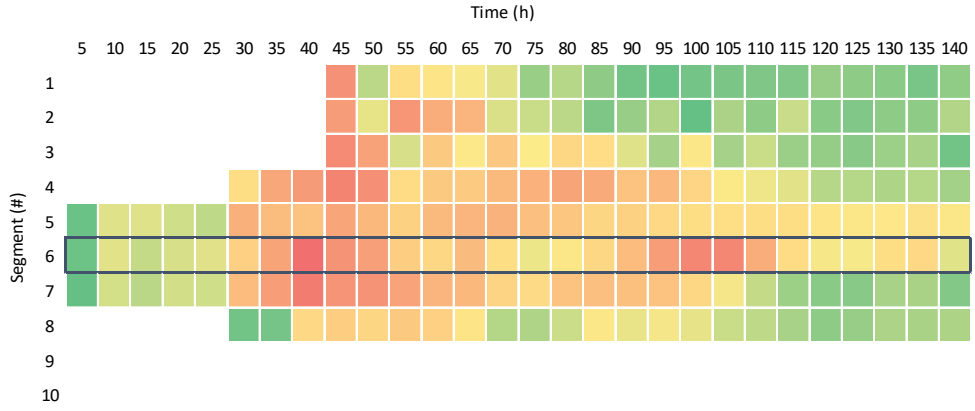


Figure S5 Heatmap of the average fluorescent intensity of the auxin reporter within the syncytium per root segment over time for the 2nd replicate. Red indicates a higher intensity and segments are 90 µM long. The segment with the head of the nematode is highlighted in blue.

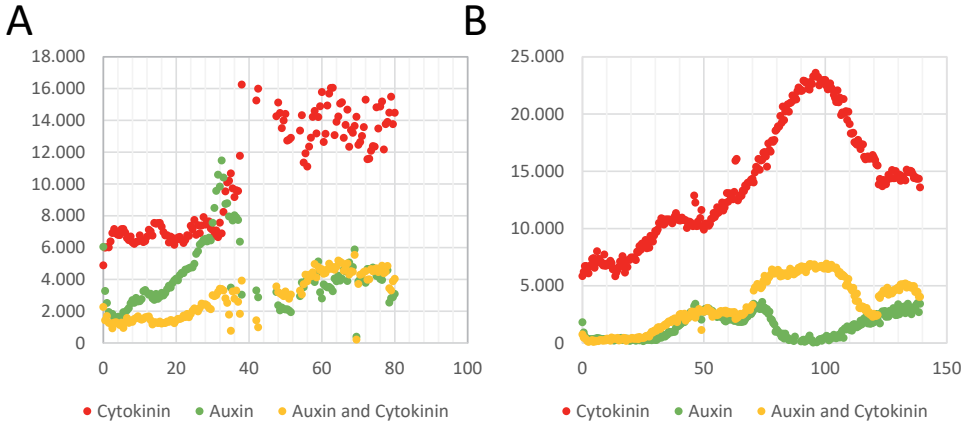


Figure S6 Fluorescent area (FA) as an indication of the number of nuclei that show auxin signalling, cytokinin signalling or both as measured in the main root over time for the 1st replicate (**A**) and the 2nd replicate (**B**). The rise in green FA signal being attributed to the developing secondary root primordia

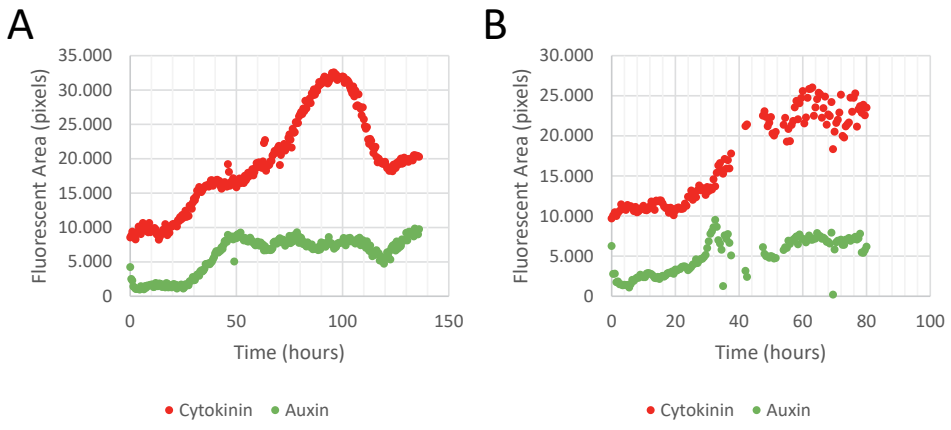


Figure S7 Fluorescent area (FA) of auxin and cytokinin in the main root showing that auxin signalling comes up later than cytokinin in the first replicate (**A**) and the second (**B**).

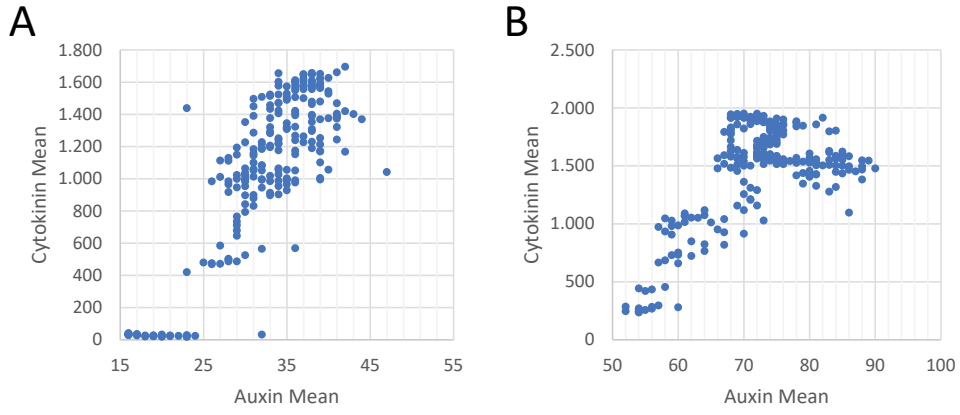


Figure S8 The mean intensity of the cytokinin signal plotted against the mean intensity of the auxin signal in the entire main root for all measured time points of the 1st replicate (**A**) and the 2nd replicate (**B**). This shows the auxin and cytokinin ratios measured at intervals of 30 min during a period of 140h.

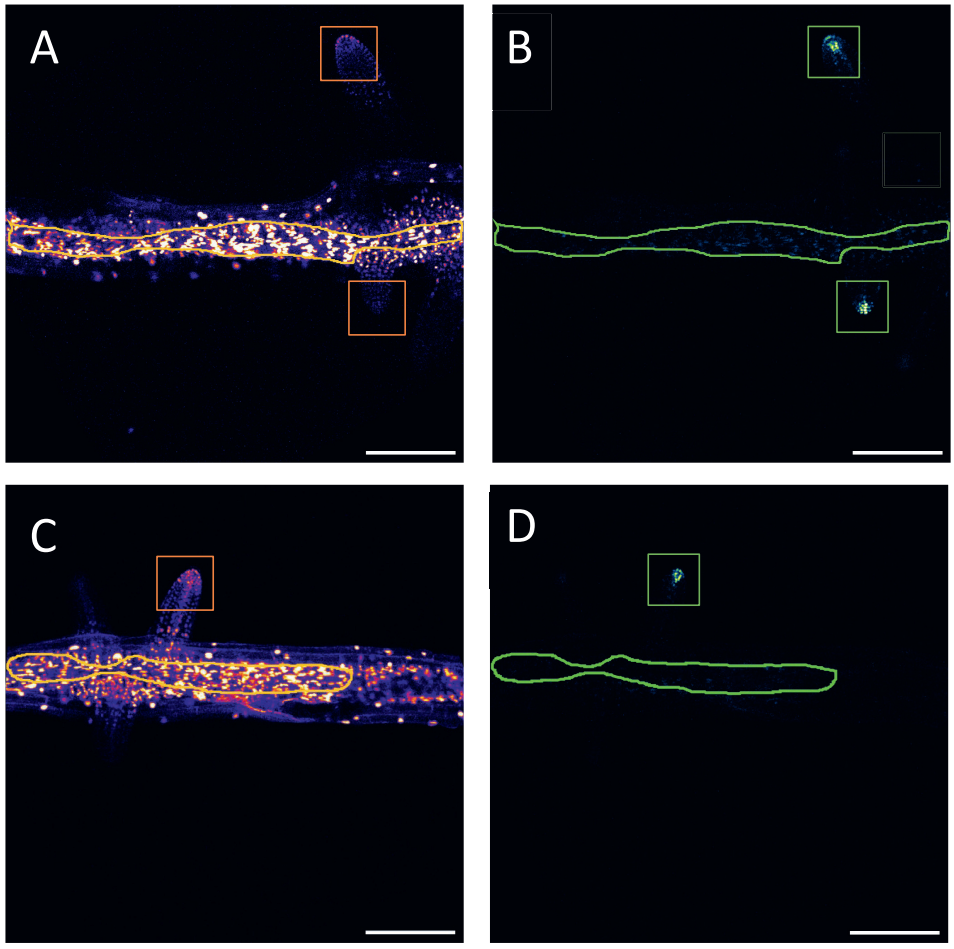
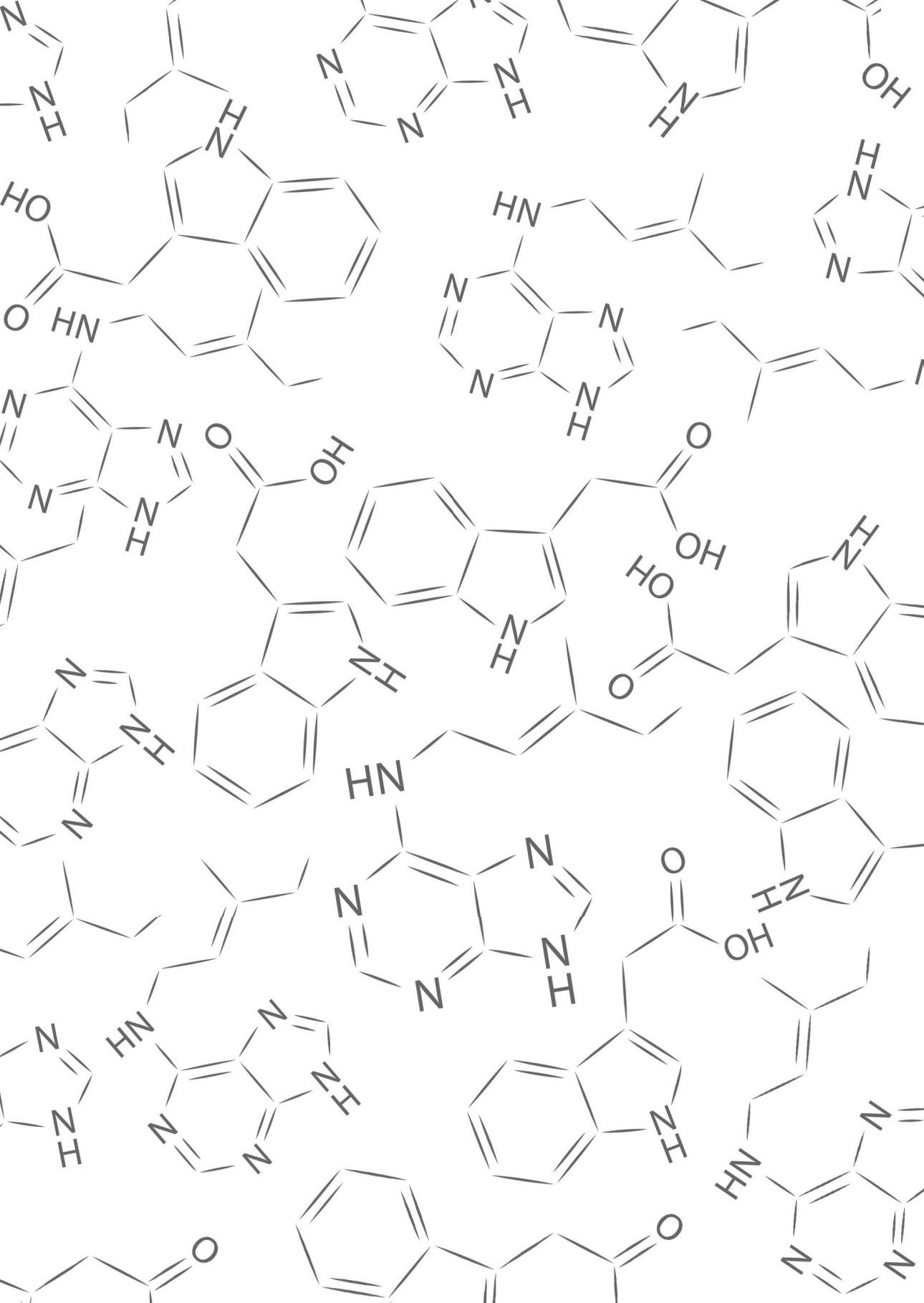


Figure S9 Examples of still frames of two movies wherein the syncytial areas and areas of secondary root tips are indicated in which the fluorescence was measured to compare the auxin and cytokinin signalling intensities between feeding sites and secondary root tips (see Figure 5G, Figure 6H). **S9A** and **S9B** show nuclei with cytokinin (**A**) and auxin (**B**) signalling of the 3rd replicate (t=113h). **S9C** and **S9D** show nuclei with cytokinin (**C**) and auxin (**D**) signalling of the 2nd replicate (t=83h). Cytokinin signalling is displayed as a blue<orange<white (fire) gradient, while for auxin signalling a is displayed as a blue<green<white (green fire blue) gradient. The scale bar represents 200µM.



Chapter 4

Biosynthesis of auxin in the potato cyst nematode *Globodera pallida*

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Abstract

The phytohormone auxin plays a crucial role in the development of so-called syncytia induced by cyst nematodes upon feeding on plant roots. However, it is unknown whether nematode-derived auxin contributes to plant parasitism by cyst nematodes. To do so, plant-parasitic nematodes should be able to produce and release auxin into the environment. Here, we investigated whether cyst nematodes are able to synthesize auxin in response to plant cues. Mass-spectrometry analyses shows that the cyst nematode Globodera pallida is able to synthesize and release the auxin indole-3-acetic acid (IAA) and that its biosynthesis is stimulated by root diffusate. Using a collection of known auxin biosynthesis genes from well-studied pathways in bacteria and plants we revealed that in the genome of G. pallida candidate genes are present for five major biosynthesis pathways which could be responsible for auxin production. RNA-seq data supported the genomic analysis and revealed the upregulation of various candidate biosynthesis genes during cyst nematode parasitism, suggesting a possible role in plant parasitism. However, genomic analysis within the phylum Nematoda showed that potential auxin biosynthesis genes are wide-spread and also occur in bacterivorous, fungivorous, entomopathogenic and animal-parasitic nematodes. Altogether, our data show that G. pallida is able to synthesize auxin and that potential auxin biosynthesis pathways are not unique for plant-parasitic nematodes. Although auxin biosynthesis may play a broader role in the life history of nematodes, a more specialized role for nematode-produced auxin in plant parasitism cannot be excluded and needs further investigation.

Introduction

Cyst nematodes are obligate biotrophic endoparasites that penetrate the roots of their host and subsequently induce the formation of complex feeding structures. Cyst nematodes move intracellularly through the root cortex and thereafter inject a suite of secretory compounds into an initial feeding cell near or within the vascular tissue. The secreted compounds modify host cells leading to a partial breakdown of cell walls and subsequent merging of neighbouring cells (Sobczak and Golinowski 2011). This process results in a structure known as a syncytium that acts as a nutrient source for the nematode. The auxin indole-3-acetic acid (IAA) is considered to play a pivotal role in syncytium formation of cyst nematodes. For example, the auxin mutant *DIAGEOTROPICA* and the double mutant of AUXIN1 *(*AUX1*)* and LIKE-AUX3 (LAX3*)* are significantly less susceptible to cyst nematode infection (Lee et al. 2011; Goverse et al. 2000). Additionally, the auxin responsive DR5 reporter has shown that syncytium formation is accompanied by a localized and transient auxin accumulation, as early as 18 hours post infection (Goverse et al. 2000; KARCZMAREK et al. 2004). IAA accumulation seems to be present in the entirety of the feeding site and during this process both reporter gene and transcriptomic studies show the expression of differentially expressed auxin related genes such as auxin response factors (ARFs), GRETCHEN HAGEN 3 (GH3) and auxin transport related genes like PIN-FORMED (PIN) and LIKE-AUX1 (LAX) (Cabrera et al. 2014; Hutangura et al. 1999; Hewezi et al. 2014; Kyndt et al. 2016). In addition, upregulation of auxin biosynthesis genes such as tryptophan decarboxylase (TDC), AMIDASE (AMI) and YUCCA (YUC) is observed in host plant cells (Chapter 2, This thesis). From this, it can be inferred that auxin accumulation in syncytia likely arises, at least partially, through changes in local auxin biosynthesis and auxin transport.

Auxin can be found in a multitude of different species ranging from plants to bacteria, fungi and even animals. Auxin biosynthesis is best studied in plants and bacteria. When considering auxin biosynthesis in plants, IAA is mainly produced from tryptophan (Trp), a product of the shikimate metabolic pathway (Maeda and Dudareva 2012). The majority of *de novo* IAA is synthesized from amino acids in the aerial part of the plant as well as the meristematic zone of the root (Ljung et al. 2005). Four routes are known for the biosynthesis of IAA in plants. For Trp-dependent IAA synthesis these pathways are: (1) the indole-3-acetamide (IAM) pathway, (2) the indole-3-pyruvic acid (IPyA) pathway, (3) the tryptamine (TAM) pathway and (4) the indole-3-acetaldoxime (IAOx) pathway (Mano and Nemoto 2012). The presence of a non-Trp dependent biosynthesis pathway has been postulated but little is known hereof (Wang et al. 2015). The IAM biosynthesis pathway was thought to be a bacteria-specific pathway until IAM was found naturally occuring in all plant species and plant genes were being identified for the conversion of IAM to IAA (Gao et al. 2020; Mano and Nemoto 2012). The IPyA pathway is the main biosynthesis pathway in plants and the majority of all IAA is produced in this manner (Mashiguchi et al. 2011). Aside from the main biosynthesis pathway IAA can alternatively be produced through the TAM pathway and requires a three step conversion from Trp to IAA involving TDC, YUCCA and AAO genes (Mano and Nemoto 2012). The IAA intermediary compound IAOx has only been found in Brassicaceae species and as such it has been concluded that the IAOx biosynthesis pathway is exclusive to this family (Sugawara et al. 2009). It is assumed that over 80% of the bacteria isolated from the rhizosphere are capable to synthesize IAA and at least five different pathways have been described for this (Patten and Glick 1996; Khalid et al. 2004; Spaepen, Vanderleyden, and Remans 2007). In bacteria the

conversion of Trp to IAA can occur through the same intermediary compounds that are found in plants (Spaepen and Vanderleyden 2011). The pathways themselves show similarity to those described in plants, although the order of intermediates can differ. An example of this is the conversion of IPyA to indole-3-acetaldehyde (IAD) by indole-3-pyruvate decarboxylase (ipdC) in bacteria (Spaepen and Vanderleyden 2011). IPyA and IAD are found in the IPyA and the TAM pathways, respectively.

In plants, auxin is a versatile molecule with a myriad of functions in both growth and development (Weijers and Wagner 2016; Vert and Chory 2011). In addition, auxin related processes have also been shown to play an important role during plant-parasite interactions and symbiotic relationships (Boivin, Fonouni-Farde, and Frugier 2016; Kohlen et al. 2018). For example, auxin plays a role in haustorium formation during infection by the hemiparasitic plant *Phtheirospermum japonicum*. Auxin is also found in animals and has been shown to be present in various gall inducing insects (Yamaguchi et al. 2012; TANAKA et al. 2013). IAA is needed for proper gall formation and is paired with an accumulation of auxin in insect galls. IAA in these insects is produced *de novo* from tryptophan through the IAOx and IAD intermediates (H. Suzuki et al. 2014b). Auxin is produced in a similar manner in silkworms (*Bombyx mori*), which are non-galling insects, thereby suggesting that it is not parasite exclusive (H. Suzuki et al. 2014a). The synthesis of auxin by plant-parasitic nematodes to manipulate its host has been discussed as well. Already decades ago, using bioassays and chemical techniques, indications were found for the occurrence of auxin in plant-parasitic nematodes (Yu and Viglierchio 1964; Viglierchio and Yu 1968). However, these findings did not lead to more detailed studies in recent years using modern techniques to investigate whether plant-parasitic nematodes can produce auxins *de novo* to facilitate plant parasitism.

In this chapter, we investigated the ability of plant-parasitic nematodes to synthesize auxin on a genomic, transcriptomic and metabolomic level. Metabolomic analyses using liquid chromatography-tandem mass-spectrometry demonstrated the presence of IAA in pre-parasitic juveniles of the potato cyst nematode *Globodera pallida*. Furthermore, it was shown that the amount of IAA measured in our analyses increased upon stimulation by plant root diffusate. Subsequently, potential homologues of known auxin biosynthesis genes were identified in a genomic screen of the *G. pallida* genome. Several candidate genes were discovered forming five potential pathways for the conversion of Trp to IAA based on both plant and microbial pathways described in other studies. The transcriptomic profiles of these candidate genes further supported their expression during infection of plant roots, indicating that they could play a role as potential auxin biosynthesis genes in plant parasitism. Genomic analysis of bacterivorous, fungivorous, entomopathogenic and animal parasitic nematodes, however, revealed a wide-spread occurrence of potential auxin biosynthesis pathways within the phylum Nematoda. Our data in conjunction with data in the literature indicates that auxin might have multiple functions in plant-parasitic nematodes. The potential role of auxin biosynthesis in plant parasitism as well as in endogenous (physiological) functions in plant-parasitic nematodes are discussed.

Results

Auxin is present in *Globodera pallida* and increases in response to root exudates

To assess and quantify the potential production of IAA by *G. pallida*, mass-spectrometry was used to identify this compound in infective second stage juveniles (pre-parasitic J2 stage). Nematodes were hatched in water and subsequently sucrose purified, surface sterilised and thoroughly washed in sterile tap water. Next, a part of the sample was plated on B5 medium to assess the presence of any remaining bacterial or fungal contamination. The absence of bacterial and fungal growth in all samples confirmed the effectiveness of the sterilisation of second stage juveniles. The viability of the nematodes was visually assessed to ensure that the amount of dead or ruptured nematodes was minimised (<1%). Samples were prepared containing: 4.000, 8.000, 40.000, 80.000 and 230.000 J2 individuals. This revealed that IAA is present in *G. pallida* in detectable levels. In addition, the quantity of IAA linearly correlates with the number of nematodes in the sample (Figure 3A). Combined, this demonstrates that surface sterilised, water-hatched second stage juveniles of *G. pallida* contain IAA, and suggests that plant stimuli are likely not required to trigger auxin biosynthesis.

In order to determine whether the amount of auxin is influenced by the perception of a host plant, *G. pallida* cysts were hatched in the presence of tomato root diffusate (TRD). A comparative analysis of water-hatched and TRD hatched juveniles demonstrated a 43% increase in the amount of IAA present in TRD hatched nematodes compared to the water hatched control (Figure 3B). To exclude a plant derived IAA contribution by TRD, we analysed its IAA content. No IAA was detected in any of the used TRD samples. For comparison, the cytokinin isopentenyl riboside (iPR) was included in our measurements as its presence in the cyst nematode *Heterodera schachtii* has been reported in a previous report (Siddique et al. 2015). Cytokinin was indeed detected in *G. pallida* and similar to IAA, cytokinin increased by 81% upon hatching with TRD (Figure 3D). Interestingly, iPR is present at lower concentrations than IAA by a factor thousand. The hormone salicylic acid (SA) was additionally included and was present in *G. pallida*. However, its concentration remained unchanged upon exposure to TRD (Figure 3C), suggesting not a uniform increase of metabolism, but a more selective effect of TRD on a specific set of metabolites. Altogether, the data indicate the induction of auxin and cytokinin biosynthesis in pre-parasitic J2 stages of *G. pallida* as a response to plant stimuli.

Subsequently, it was assessed whether auxin is not only produced but also secreted by infective juveniles and for that purpose *G. pallida* secretions were collected. TRD hatched juveniles were surface sterilised and thoroughly washed in several washing steps as described for water-hatched juveniles before an equal number was suspended in either water or TRD for 24 hours. Thereafter, the IAA concentration was measured in the nematodes as well as the liquid in which the nematodes were suspended. The auxin present in the nematodes was at comparable levels as measured before around 0,2 pmol/10.000 J2. Our preliminary data showed that auxin is present in the secretions of both water and TRD hatched nematodes (Figure 3E). Secreted auxin levels appear to be a factor thousand lower than endogenous auxin concentrations inside the nematodes (0,4 fmol/10.000 J2). However, a tenfold increase in concentration is observed in the secretion of the TRD treated sample compared to the water control (Figure 3E). Overall, it can be concluded that *G. pallida* produces IAA of which the concentration increases within nematodes in response to host plant stimuli, and

preliminary data also indicate that IAA is secreted and that IAA secretion is stimulated by root diffusate.

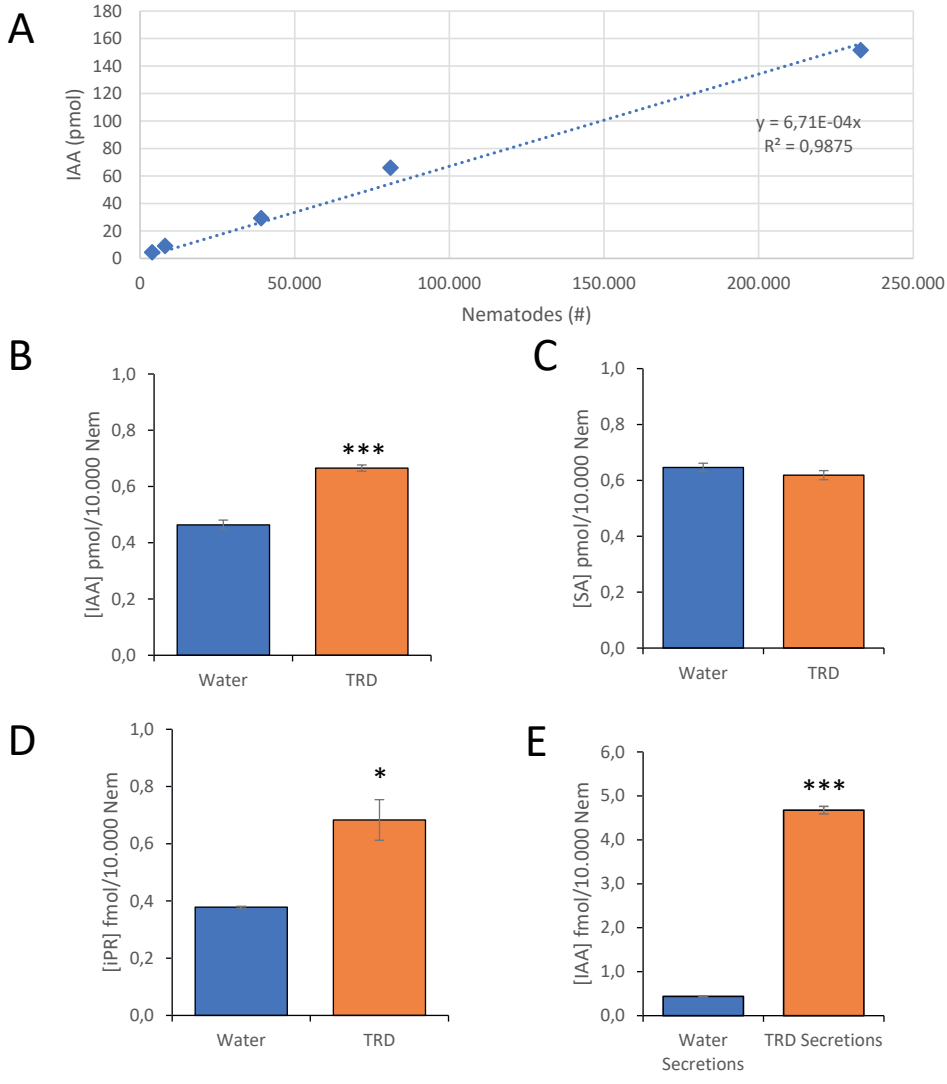


Figure 1 Auxin, cytokinin and salicylic acid quantities produced by pre-parasitic second stage juveniles of *G. pallida* as measured through MRM-UPLC-MS/MS. (**A**) Correlation of the concentration of IAA measured in an increasing number of infective nematodes obtained from a single batch of water-hatched pre-parasitic juveniles (J2). Effect of exposure to Tomato Root Diffusate (TRD) on the auxin (**B**), salicylic acid (**C**) and cytokinin (**D**) concentration shown as the average of three technical replicates of equal amounts of infective nematodes as compared to the water control. Auxin measurement is a representative dataset out of three independent biological replicates (Supplementary Figure S1), while salicylic acid and cytokinin represent one biological sample per treatment. (**E**) Concentration of IAA measured in water-induced secretions and TRD induced nematode secretions (one biological sample per treatment). Significance was determined using a student T-test. *p<0,05; ***p<0,001.

Identification of candidate auxin biosynthesis genes in *G. pallida*

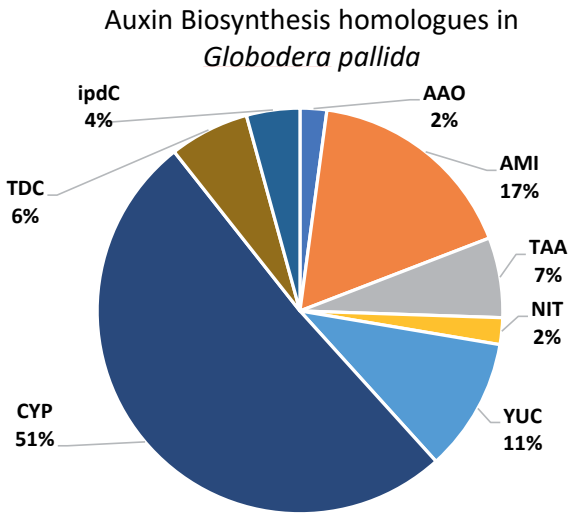
The presence of auxin in pre-parasitic second stage juveniles indicates the existence of an auxin biosynthesis pathway in *G. pallida*. However, no information is available yet about how auxin biosynthesis occurs in plant-parasitic nematodes. Auxin biosynthesis is best studied in plants and bacteria and involves multiple pathways. To identify potential auxin biosynthesis genes in *G. pallida*, a comparative genomics approach was used including 10 plant species and 10 bacteria species (Supplementary Table S1) to create Hidden Markov Model (HMM) conserved protein models for each gene in the biosynthesis pathways of plants and bacteria (63 protein sequences distributed over 11 different auxin biosynthesis genes). These HMM models were then used to scan the *G. pallida* genome to identify auxin biosynthesis candidate genes. Additionally, the tRNA isopentenyl transferase (IPT) cytokinin biosynthesis genes from *Caenorhabditis elegans* and *Arabidopsis thaliana* have been included as a previous study has shown that the cyst nematode *Heterodera schachtii* produces cytokinin using this gene (Siddique et al. 2015). The genes from the collection were grouped together for each enzyme in the plant and bacterial auxin biosynthesis pathway. In total, for the conversion of tryptophan to IAA, a total of 191 genes were identified in the *G. pallida* genome with 97 of these being annotated as cytochrome P450s. Interestingly, several potential homologues were found for the IPT gene.

Following the initial screening of the 191 genes, a comprehensive analysis utilizing the Pfam database was undertaken to ascertain the presence of catalytic domains associated with these genes. This analysis revealed that 47 of the 191 genes contain functional protein domains that correspond to their plant or bacteria counterpart. These 47 genes cover a total of 10 enzymatic steps in auxin biosynthesis (Figure 2C), each characterized by protein domains corresponding to their putative function (Figure 2A). It is noted that genes identified through the Hidden Markov Model (HMM) scan, and simultaneously found to contain expected functional protein domains, are categorized here as homologous. The majority of these genes are homologues of the Cytochrome P450 (CYP) gene family, which is not surprising considering its role with metabolites in general and the size and prevalence of this gene family in all (multi)cellular organisms (Nelson 1999) (Figure 2B). Interestingly, putative homologous sequences for enzymes in all four major auxin biosynthesis routes from plants could be found in the genome of *G. pallida* (Figure 1C). Homologous sequences for the enzymes AMIDASE (AMI), YUCCA (YUC), Indole-3-acetaldehyde oxidase (AAO) and NITRILASE (NIT), all involved in the last step of their respective auxin biosynthesis route in plants, could be identified. From these, the largest number of homologues could be found for the AMI gene (8). While various YUC (5) and CYP (24) genes were identified it was not possible to differentiate between the different YUC or CYPs in the biosynthesis pathway (Figure 2C). For example, the YUC responsible for the conversion of TAM to IAD or the YUC catalysing the reaction of IPyA to IAA both lead to the identification of the same potential homologues in *G. pallida*. In addition, these protein families have members that are not involved in auxin biosynthesis, which hampers a further differentiation within these families. Overall, 47 candidate auxin biosynthesis genes were identified in *G. pallida* on the basis of sequence similarity and functional domain prediction, providing evidence for the presence of potential auxin biosynthesis pathways in *G. pallida* (Supplementary Table S2).

A

Name	Homologues	HMM E-value	Pfam E-value	Domain
AAO	1	9,0E-172	1,1E-20	Aldehyde oxidase and xanthine dehydrogenase, a/b hammerhead domain
AMI	8	2,4E-25 - 3,5E-34	2,9E-56 - 4,5E-78	Amidase
TAA	3	2,0E-5 - 4,0E-36	3,2E-17 - 6,2E-59	Aminotransferase class I and II
NIT	1	1,3E-14	8,6E-63	Carbon-nitrogen hydrolase
YUC	5	1,8E-13 - 4,5E-34	1,8E-63 - 4,5E-157	Flavin-binding monooxygenase-like
CYP	24	1,6E-13 -2,7E-56	3,4 E-12 – 4,1E-101	Cytochrome P450
TDC	3	4,8E-133 - 6,4E-152	4,6E-74 - 2,3E-148	Pyridoxal-dependent decarboxylase conserved domain
ipdC	2	3,0E-22 - 4,2E-27	1,0E-21 - 2,0E-25	Thiamine pyrophosphate enzyme, C-terminal TPP binding domain

B



C

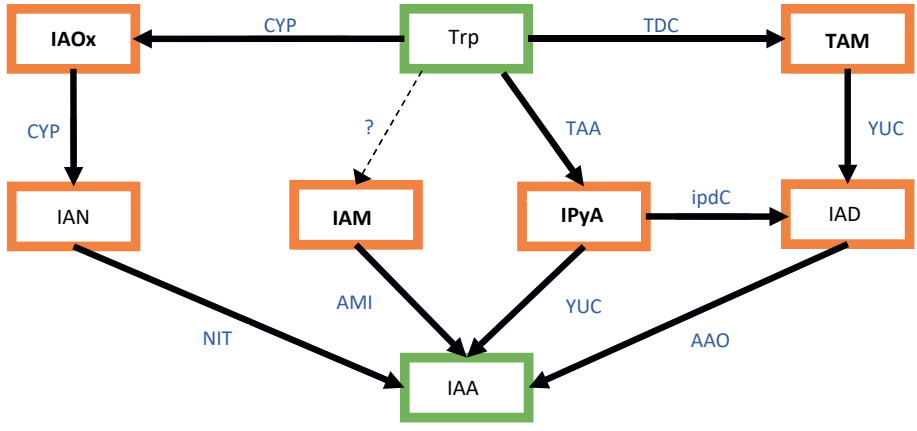


Figure 2 Identification of potential auxin biosynthesis genes and possible pathways in *G. pallida*. (**A**) Overview of the different auxin biosynthesis gene homologues found in the *G. pallida* genome (D383, van Steenbrugge et al. 2023) after a Hidden Markov Model search followed by a functional domain analysis. Potential homologues are named after their original search query and possess identical functional domains as predicted through Pfam with the indicated E-value for both the HMM scan and Pfam analysis (highest and lowest found). (**B**) Distribution of the number of auxin biosynthesis homologues detected in *G. pallida* as % of the 47 found genes. (**C**) Potential auxin biosynthesis pathways in *G. pallida* based on mapping of the identified homologues on the five main pathways in plants and bacteria. Precursor and end products are displayed with a green border and intermediate compounds with an orange border. Blue shows the homologues identified in *G. pallida* that potentially facilitate the displayed conversion. Dotted line and question mark denote a hypothetical expected conversion step in nematode auxin biosynthesis.

Abbreviations: TAM – Tryptamine, IAM – Indole-3-acetamide, IPyA – Indole-3-pyruvic acid, IAOx – Indole-3-acetaldoxime, Trp – Tryptophan, IAD – Indole-3-acetaldehyde, IAN – Indole-3-acetonitrile, IAA – Indole-3-acetic acid

Our bioinformatic pipeline was not only able to detect candidate auxin biosynthesis genes belonging to the last step of the four auxin biosynthesis routes (i.e. IAOx, TAM, IAM and IPyA (Figure 1C)), but it could also identify homologues that could facilitate the conversion of tryptophan to the intermediate compounds thereby forming a complete pathway. It is noted that for the IAM pathway a candidate gene for the last step (AMI) was detected, but that the homologue for the iaaM gene was absent in *G. pallida*. Several candidate iaaM genes were discovered in the HMM scan but did not have a matching predicted functional domain in our Pfam analysis. The iaaM gene facilitates the conversion of tryptophan to IAM in bacteria. IAM occurs also in plants while no iaaM gene has been detected and it is assumed that in plants the conversion of tryptophan to IAM is catalyzed by another gene than in bacteria (Kasahara 2016). Similar to plants, it is here postulated that the absence of the iaaM gene in *G. pallida* does not necessarily obstruct the conversion of tryptophan via IAM into IAA. Potential auxin biosynthesis genes for conversion between intermediate compounds from bacterial pathways were also found. Two putative homologues for indole-3-pyruvate decarboxylase (ipdC) have been identified which, in bacteria, produce the auxin intermediate indole-3-acetaldehyde (IAD) from the auxin intermediate (IPyA) (Spaepen and Vanderleyden 2011). This enzyme could account for an additional biosynthesis route next to the four pathways known in plants by following the IPya pathway and half way branching off into the TAM pathway. In conclusion, our genomic analyses indicate that in theory five auxin biosynthesis pathways could be active in *G. pallida* and may contribute to the conversion of tryptophan into auxin as described (Figure 2C).

Expression and upregulation of potential auxin biosynthesis genes in different life stages

To investigate whether the auxin biosynthesis candidate genes detected in the *G. pallida* genome are also expressed, transcript abundance of the 47 candidate auxin biosynthesis genes was analysed. Therefore, their transcription dynamics were retrieved from a publicly available expression atlas of various *G. pallida* life stages (i.e. egg, J2, 7dpi, 14dpi, 21dpi, 28dpi and 35 dpi (Cotton et al. 2014)). For all 47 candidate genes transcripts were found thereby confirming the expression of the selected putative auxin biosynthesis genes (Supplementary Table S3). Hierarchical clustering shows that the expression patterns of auxin biosynthesis candidate genes in the parasitic stages are more similar to each other than to eggs and pre-parasitic juveniles (Figure 3). In addition, the expression patterns of the genes in males form

a clearly separated group, in line with the distinctive migratory lifestyle of males as compared to immobile sedentary females. Within these groupings, some heterogeneity is observed: one male sample deviates from the other two male samples and at three time points the parasitic stages do not cluster together, indicating the experimental and/or biological variation. Similarly, heterogeneity is observed in expression within a group of similar genes. For example, YUC, TAA, and CYP genes do not all cluster together based on the expression profile in different stages. Altogether, it is concluded that the candidate auxin biosynthesis genes are expressed during the life cycle of *G. pallida* and expression levels vary between life stages.

Next, the correlation between expression patterns of candidate genes within the same pathway was studied across different life stages. Therefore, candidate genes were grouped together within the same pathway based on similarities in their expression pattern using a Pearson correlation (Supplementary Table S4). The expression profile of genes within each pathway with the highest Pearson correlation were selected as the most promising candidates for that pathway (Figure 4). The potential IAOx pathway consists of two CYP450P genes and one NIT gene where the NIT1 gene and CYP8 cluster close together in terms of expression (Figure 4A). Although CYP9 differs significantly from the other two in terms of expression level, the changes in expression follow a similar trend over life stages as NIT1 and CYP8. The potential TAM pathway consists of three genes of which AAO1 and YUC2 share a similar expression pattern, while the expression profile of TDC3 deviates (Figure 4B). The three genes in the TAM pathway seem to be lowly expressed during the egg and J2 phases, whereas specifically TDC3 is induced during infection with a sharp drop at 28 dpi which recovers slightly at 35 dpi. The potential IAM pathway lacks a possible gene for the conversion of Trp to IAM and as such only the expression of the potential AMI genes is considered, which facilitates the conversion of IAM to IAA. The different potential AMI genes display a variety in expression levels, with AMI1 showing relatively high TPM values compared to the other seven AMI genes (Figure 4C). The most similar expression patterns between potential auxin biosynthesis genes are found within the IPyA pathway (Figure 4D). The expression levels of both YUC3 and TAA3 are low in eggs and pre-parasitic second stage juveniles after which a steep rise is observed during the first 14 days followed by a decline (Figure 4D). Additionally, the potential IPyA pathway contains the branching point for what we refer to here as the IAD pathway and consists of TAA1, ipdC1 and AAO1 (Figure 4E). The selected genes in this pathway display a repetitive rise and decline in expression across the various life stages. The correlation between the expression of ipdC1 and AAO1 is one of the lowest (0,39) together with AAO1 and TDC3 (0,37) (Figure 4F). These correlations further show that genes in the IAOx and IPyA pathway correlate better in terms of expression when compared to the TAM and IAD pathway. Overall, this analysis shows that there is a level of similarity in expression patterns within potential auxin biosynthesis pathways, especially for the putative IAOx and IPyA pathways.

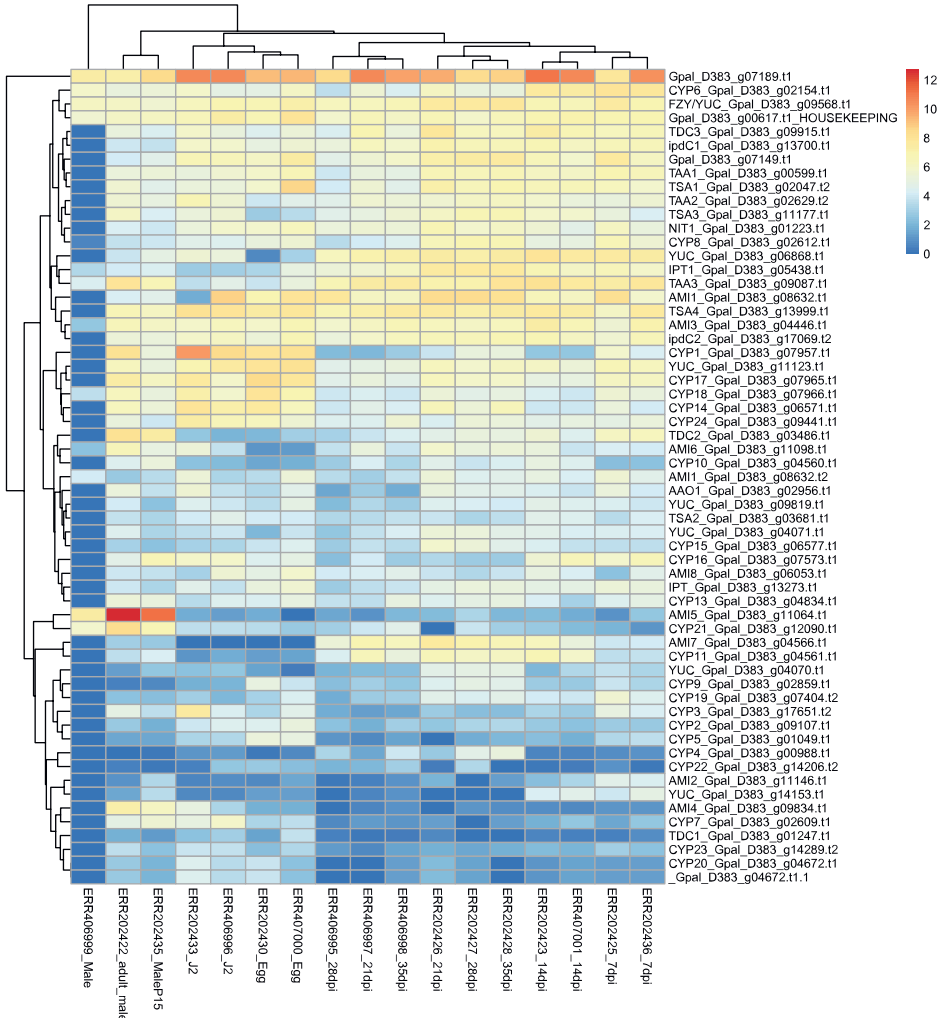
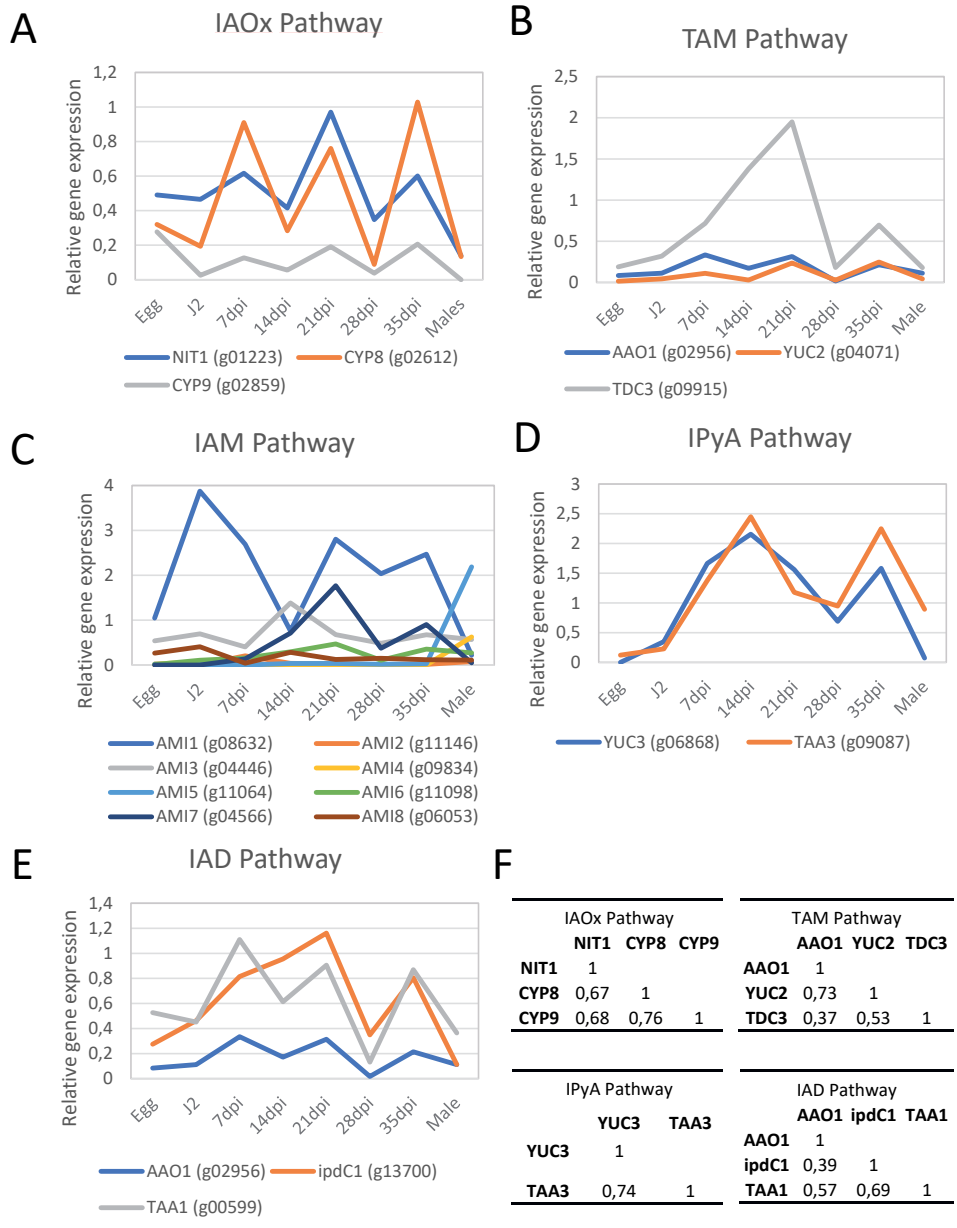


Figure 3 Identification and hierarchical clustering of transcripts from the 47 potential auxin biosynthesis genes present in the *G. pallida* genome. Gene expression levels are shown as $\log_2$ transcripts per million (TPM) values in a heatmap. Transcript data were retrieved from the gene expression atlas of Cotton et al. 2014. Samples obtained from different life stages and time points (days post infection (dpi)) as well as the gene transcripts are ordered according to a hierarchical clustering The household gene cyclic AMP-dependent transcription factor ATF-4 (g00617) is included as a visual reference (marked HOUSEKEEPING).



F

IAOx Pathway

	NIT1	CYP8	CYP9
NIT1	1		
CYP8	0,67	1	
CYP9	0,68	0,76	1

TAM Pathway

	AAO1	YUC2	TDC3
AAO1	1		
YUC2	0,73	1	
TDC3	0,37	0,53	1

IPyA Pathway

	YUC3	TAA3
YUC3	1	
TAA3	0,74	1

IAD Pathway

	AAO1	ipdC1	TAA1
AAO1	1		
ipdC1	0,39	1	
TAA1	0,57	0,69	1

Figure 4 Expression pattern of potential auxin biosynthesis genes in various life stages of *G. pallida* relative to the household gene ATF-4 (g00617). Candidate genes with the highest Pearson correlation are shown for an auxin biosynthesis pathway based on similarity in transcript abundance. (**A**) the IAOx pathway, (**B**) the TAM pathway, (**D**) the IPyA pathway and (**E**) the IAD pathway. (**C**) For the IAM pathway only AMI genes were found and their expression pattern is displayed in various life stages of *G. pallida*. (**F**) Highest positive Pearson correlations between potential auxin biosynthesis genes per pathway.

Auxin biosynthesis genes are wide-spread within the phylum Nematoda

To explore how widespread the potential auxin biosynthesis genes and possible pathways are in the phylum Nematoda, HMM scans were conducted with the same models that were used to identify candidate auxin biosynthesis genes in *G. pallida* (Figure 2A). The HMM scans were carried out on a comprehensive panel of plant-parasitic, animal parasitic and free-living nematode proteomes ranging across all clades in the phylum for which genomes were available. By mapping these candidate biosynthesis genes on the known auxin biosynthesis pathways in plants and bacteria as a model, it was determined whether a species could have the required genes to form a hypothetical auxin biosynthesis pathway. Considering the best hits from the HMM scans, it is noteworthy that the E-values of the HMM hits for all nematode species closely approximate those of the candidate biosynthesis genes in *G. pallida* that were identified through our Pfam analysis (Supplementary Table S5, Figure 2A). This suggests that *G. pallida* is not an exception within the phylum Nematoda with regard to the occurrence of potential auxin biosynthesis genes. The HMM scan reveals that all nematode species tested contain candidate genes for potential auxin biosynthesis pathways (Table 1). In total, 17 of the 30 nematode species have potential candidate biosynthesis genes for all five biosynthesis pathways. An exception among the plant-parasitic nematodes being the *Meloidogyne* species which seem to miss potential genes for the TAM and IAD pathways. This is due to the absence of potential candidates for the AAO gene that catalyses the last step in the auxin biosynthesis; the conversion of IAD to IAA. It is noted, that this gene seems to be absent in several animal parasitic nematodes as well. *Soboliphyme baturini* seems to be the least-equipped nematode species to synthesize auxin, and possesses only two potential auxin biosynthesis pathways; the IAM and IAOx pathway. In conclusion, our data indicate that potential auxin biosynthesis pathways are wide-spread within the phylum Nematoda and are present in plant-parasitic, bacterivorous, fungivorous, entomopathogenic and animal parasitic nematodes.

No indications for evolutionary trajectories specific for plant-parasitic nematodes

To study whether candidate auxin biosynthesis genes in *G. pallida* might have been recruited via horizontal gene transfer from e.g., bacteria or plants a BLAST search was performed. This BLAST search was carried out for the nucleotide sequences of the 47 potential auxin biosynthesis enzymes against the NCBI database of plants, bacteria, nematodes, fungi, and non-redundant protein sequences. The highest similarities for the 47 candidate genes from *G. pallida* were found with genes in other nematode species. BLAST searches against the plants, bacteria, fungi, and non-redundant protein sequences databases did result in hits but with a significantly lower similarity (data not shown), indicating that horizontal gene transfer from other kingdoms is an unlikely scenario. In addition, no evidence was found for evolutionary adaptations leading to the secretion of auxin biosynthesis enzymes as is seen for chorismate mutase (Vanholme et al. 2009). Chorismate mutase is a metabolic enzyme that converts chorismate into prephenate and is encountered in various plant-parasitic nematode species in which it is expressed in the subventral glands and preceded by a secretory signal peptide (Gheysen and Mitchum 2011; Jones et al. 2003). Analysing the candidate genes from *G. pallida* that might facilitate the last step in auxin biosynthesis (AMI, NIT, YUC, AAO) did not result in the detection of any predicted signal peptide (data not shown). In conclusion, no indications were found for evolutionary processes specific for *G. pallida*.

Table 1 Overview of five potential auxin biosynthesis pathways in plant-parasitic (red highlight), free-living (blue highlight) and animal parasitic (yellow highlight) nematodes based on the detection of candidate auxin biosynthesis genes in genome sequences using an HMM scan. Green slots indicate the putative presence of a complete auxin biosynthesis pathway based on the identification of potential auxin biosynthesis genes for each gene within that pathway. Orange slots indicate that a potential pathway might be incomplete due to the absence of at least one candidate gene within that pathway (which gene is mentioned in text).

		Auxin Biosynthesis pathways				
	Nematode Species	**IAM**	**IPyA**	**IAOx**	**TAM**	**IAD**
Plant Parasitic	*Bursaphelenchus xylophilus*					
	Ditylenchus destructor					
	Ditylenchus dipsaci					
	Globodera pallida					
	Globodera rostochiensis					
	Heterodera glycines					
	Meloidogyne arenaria				AAO	AAO
	Meloidogyne enterolobii				AAO	AAO
	Meloidogyne floridensis				AAO	AAO
	Meloidogyne graminicola				AAO	AAO
	Meloidogyne hapla				AAO	AAO
	Meloidogyne incognita				AAO	AAO
	Meloidogyne javanica				AAO	AAO
Free-living	*Acrobeloides nanus*					
	Caenorhabditis elegans					
	Halicephalobus mephisto					
	Pristionchus pacificus					
Animal Parasitic	*Ancylostoma caninum*					
	Ascaris lumbricoides					
	Anisakis simplex					
	Brugia malayi				AAO	AAO
	Haemonchus contortus					
	Loa loa				AAO	AAO
	Onchocerca flexuosa				AAO	AAO
	Romanomermis culicivorax				AAO	AAO
	Soboliphyme baturini		TAA, YUC		YUC	YUC
	Steinernema carpocapsae					
	Strongyloides stercoralis					
	Trichinella spiralis				AAO	AAO
	Trichuris trichiura					

Discussion

Here, we investigated the ability of plant-parasitic nematodes to synthesize auxin on a genomic, transcriptomic and metabolomic level. We show that IAA can be detected in *G. pallida* using mass-spectrometry and that its concentration increases upon contact with plant stimuli. Preliminary data suggests that auxin is secreted and that exposure of nematodes to root exudates may result in a tenfold increase of auxin in secretions when compared to water hatched juveniles. Potential auxin biosynthesis genes were identified using a collection of known homologues in bacteria and plants, leading to the identification of candidate genes for all steps in five potential auxin biosynthesis pathways (four plant and one bacterial pathway). The expression of these candidate genes was verified in an RNA-seq dataset and varied for most genes between developmental stages. Our bioinformatic analysis revealed that potential auxin biosynthesis genes are widespread within the phylum Nematoda and occur in free-living, plant- and animal-parasitic nematodes.

The presence of IAA in its biologically active form in *G. pallida* is supported by previous data on plant-parasitic nematodes. Several decades ago the presence of auxin has been reported in egg masses and 2nd stage juveniles of the root-knot nematode *M. incognita* and larvae of *M. hapla* (Viglierchio and Yu 1968; Yu and Viglierchio 1964). This was shown with elementary verification methods such as chromatography, the Avena first internode test to determine if it is a growth substance, as well as Ehrlich and Salkowski chemical tests to determine whether it is an indole compound (Yu and Viglierchio 1964; Viglierchio and Yu 1968; Chapter 2, this thesis). A more recent, preliminary report used mass-spectrometry to detect IAA and conjugated forms of IAA in secretions of *M. incognita* and *H. schachtii* as well as biosynthesis intermediates in their lysates (J. De Meutter et al. 2005). In this research we have demonstrated the presence of IAA in *G. pallida* as well and have identified potential biosynthesis genes. Our bioinformatic approach has shown that auxin biosynthesis candidate genes are widespread throughout the phylum Nematoda. Potential auxin biosynthesis genes are present in plant-parasitic nematodes but also in animal parasitic and non-parasitic free-living nematodes (Table 1). With this data it is tempting to speculate that these nematodes too might produce auxin. However, candidate genes for auxin synthesis steps could not always be found in each nematode species. This may be due to the absence of a particular biosynthesis route of which the AAO gene is the most absent auxin biosynthesis gene amongst the investigated species and is absent in all studied *Meloidogyne* species (Table 1). AAO acts as an aldehyde oxygenase for the conversion of IAD to IAA in the last step of the TAM pathway. However, recently an aldehyde dehydrogenase (AO) was found to catalyse the same reaction of IAD to IAA in *Pseudomonas syringae* (McClerklin et al. 2018). In addition, AO mediated auxin biosynthesis contributed to virulence of *P. syringae*. AOs are conserved throughout evolution and are mainly known for their function in detoxifying endogenous aldehydes (Shortall et al. 2021). Moreover, AOs are studied in *C. elegan*s to evaluate the effect of alcohol and alcoholism (Alaimo et al. 2012). The IAA synthesis properties of AO give rise to the possibility that not AAO is used in *Meloidogyne* species but instead AO catalyses the last step of the TAM pathway. A short BLAST search further supports this through the presence of several putative *AO* genes in *M. graminicola* and *M. enterolobii*.

Upregulation of the expression of potential auxin biosynthesis genes during infection was found in all five putative auxin biosynthesis pathways in *G. pallida*. However, changes in the

expression levels of potential auxin biosynthesis pathways could not directly be associated with the rise and decline of auxin signalling during feeding cell development. Previous reports have shown that auxin signalling peaks during the initial phases of the infection (KARCZMAREK et al. 2004). Auxin signalling reaches its maximum level between 1 and 5 dpi and hereafter stays at a steady level for several days (Goverse et al. 2000). At 10 dpi and later time points no signal can be detected (KARCZMAREK et al. 2004). Also considering the upregulation of individual candidate auxin biosynthesis genes no correlations are observed with reported levels of auxin signalling during the initial phases of syncytia development. Apart from pitfalls in such comparisons, there are more aspects to consider. The production rate of a metabolic compound in a pathway, such as IAA, is in part determined by its rate limiting step (Zhao 2012). When the expression of a non-rate limiting enzyme is increased this does not necessarily affect the quantity of the produced compound. As such, if the auxin biosynthesis gene in *G. pallida* is not the rate limiting step, then its upregulation is a poor indicator of the production rate of IAA. Therefore, the expression of the biosynthesis genes should always be taken in consideration together with the other enzymes in the same pathway. The production of auxin is further complicated by substrate availability and the presence of multiple possible biosynthesis routes. While the substrates and intermediate compounds of all these auxin biosynthesis pathways can be converted to IAA, it is not necessarily their predetermined fate as the conversion to compounds other than IAA is possible. For example, tryptamine is an intermediary compound of the TAM auxin biosynthesis pathway but can alternatively be converted into serotonin by tryptamine 5-hydroxylase in rice (Kang et al. 2007). Additionally, IAOx and IAN of the IAN pathway are intermediates in the production of camalexin in Arabidopsis which acts as a phytoalexin to deter bacterial and fungal pathogens (Mucha et al. 2019). As such, it is clear that these metabolites should not be viewed as only intermediary compounds of auxin but as intermediary compounds for a diverse number of processes. The multifaceted nature of these compounds highlights the complexity of IAA metabolism. This further complicates investigations into the role of both the auxin biosynthesis genes as well as potential intermediary compounds in the biosynthesis of auxin in *G. pallida*. Taken together it can be concluded that the interpretation of expression patterns of biosynthesis genes should be taken into the context of the entire pathway (e.g. rate limiting steps) and that auxin intermediates may have more functions than their conversion into auxin.

Auxin has long been thought to play a crucial role during nematode infection. During nematode infection auxin is suspected to be involved in processes such as hypertrophy, cell wall ingrowths, and cell cycle activation (de Almeida Engler et al. 1999; Gheysen and Mitchum 2011). The accumulation of auxin is thought to mainly be the result of manipulation of auxin transport and the induction of plant derived local biosynthesis (Kyndt et al. 2016; R. Suzuki et al. 2022). Our data suggest, however, that plant-parasitic nematodes might have an additional third option to increase auxin levels in plant roots through the secretion of nematode-derived auxin. The quantity of IAA measured in second stage juveniles of *G. pallida* (~10^{-16} mol/J2) greatly exceeds that of a single plant cell as Arabidopsis root apex protoplast cells contain ~10^{-19} mol IAA (Petersson et al. 2009). This indicates that the nematode contains sufficient IAA to be biologically relevant. Preliminary data on the secretion of IAA suggest that the excreted quantity per juvenile is roughly three orders of magnitude lower than what is present in the body of the nematode (Figure 1E). However, these values stay within the range of ~10^{-19} mol/J2 and thereby in the range of biological relevance. Taken together, a

biologically relevant amount of IAA has been found in *G. pallida*, that may contribute to the development of syncytia via a yet unknown secretion mechanism.

Similar to auxin, the concentration of cytokinin (IPR) in second stage juveniles increases upon exposure to plant exudates (Figure 1D), but is three orders of magnitudes lower in *G. pallida* than the measured auxin quantities. Cytokinin has also been detected in *H. schachtii* and *M. incognita* and reported to have a secretion rate of ~10^{-18} mol/J2/hr (Jan De Meutter et al. 2003). This secretion rate is relatively high considering the low amount of cytokinin in second stage juveniles detected in this study (10^{-18} mol, Figure 1D), but may in part be explained by the measurement of more types of cytokinins (Jan De Meutter et al. 2003). These secreted amounts of cytokinins were assumed to be physiologically relevant, which has been confirmed in a study in which silencing of *ISOPENTENYLTRANSFERASE*, a cytokinin biosynthesis gene, reduced the expansion of syncytia and reproduction of *H. schachtii* (Siddique et al. 2015). Interestingly, a HMM scan showed that homologues of *ISOPENTENYLTRANSFERASE* are not unique for plant-parasitic nematodes, but are also found in bacterivorous, fungivorous, entomopathogenic and animal parasitic nematodes (Supplementary Figure S8) The biosynthesis of the other measured phytohormone salicylic acid was not stimulated by plant exudates. The function of salicylic acid in *G. pallida* is not clear, but considering its occurrence and role in bacteria, fungi, and malaria parasites (Mishra and Baek 2021; Matsubara et al. 2015), this metabolite has most likely a function not directly related to plant parasitism but this needs further investigation.

Our data indicate that the ability of *G. pallida* to synthesize auxin is not a unique adaptation of plant-parasitic nematodes within the phylum Nematoda. The candidate auxin biosynthesis genes in *G. pallida* seem to have evolved via vertical evolution, because recruitment of auxin biosynthesis genes via horizontal gene transfer from e.g., bacteria or plants is not a likely scenario. First, the wide-spread occurrence of potential auxin biosynthesis genes within the phylum Nematoda does not support a scenario similar to cellulases, well known examples of putative horizontal gene transfer from bacteria to plant-parasitic nematodes (Danchin et al. 2010). Unlike the auxin biosynthesis genes, cellulases are unique for plant-parasitic nematodes and are not encountered in free-living and animal parasitic nematodes (Haegeman, Jones, and Danchin 2011). However, it is noted that recently an exception has been reported and that cellulases, probably recruited via horizontal gene transfer from slime molds, have also been identified in free-living *Pristionchus* nematodes to expand its dietary range (Han et al. 2022). Second, the potential auxin biosynthesis genes in *G. pallida* are more similar to potential auxin biosynthesis genes identified in bacterivorous, fungivorous and animal-parasitic nematodes than to their equivalents in plants, bacteria or fungi. Overall, the pattern that emerges supports the idea that auxin within the phylum Nematoda has an endogenous function in the growth and development of nematodes. Examples for the role of auxin in endogenous processes can be found in various non-plant-parasitic nematode species. Mass-spectrometry has shown the presence of auxin inside the free-living nematode *Caenorhabditis elegans* (Zhou, Zhang, and Butcher 2019). With isotope-labelled auxins it was shown that IAA acts as an intermediary compound for the production of ascarosides. Ascarosides are a family of pheromones that regulate a variety of sex-specific and social behaviours including hermaphrodite repulsion, olfactory plasticity and dauer entry and exit (Ludewig and Schroeder 2013). Aside from being used as an intermediate compound, auxin on its own seems to have a beneficial effect on the lifespan of *C. elegans* (Loose and Ghazi

2021). This ties in with data showing an increased proliferation in mammalian cell lines when treated with auxin (Cernaro et al. 2015). Although the underlying mechanisms remain unknown it is clear that auxin can serve several purposes inside a nematode unrelated to parasitism. This is also seen in other species as IAA was detected in terrestrial arthropods, including spiders, mites, crustaceans and millipedes (Tokuda et al. 2022). The concentration of IAA in arthropods seems to be higher than in plants with, on average, a tenfold difference and in some cases up to fifty times as much IAA per gram fresh weight. The precise role of auxin in arthropods is still unknown but auxin was measured in both males and females as well as in various developmental stages implying that IAA might play a role throughout the lifecycle. Altogether, a picture emerges that the roles of auxin are many and that being a plant hormone is merely one of them.

To study the relevance of auxin secretion by *G*. *pallida* in the induction of syncytia, various experiments can be considered. Analogous to classical effector studies, the spatial distribution of auxin through the nematode's body can be studied. For example, antibodies can be used to determine whether relatively high auxin concentrations are observed in secretory glands. Similarly, the spatial-temporal dynamics of the expression of potential auxin biosynthesis genes can be studied at near-cellular resolution using RNA tomography (Ebbing et al. 2018). The potential upregulation of auxin biosynthesis genes in secretory glands may point at an external role of auxin. To further identify the role of biosynthesis genes in *G. pallida,* candidate genes could be knocked down (by the lack of strategies to obtain stable knock-outs in PPN) to observe their effect on the total auxin pool in the nematode and success of infection. However, due to the putative role of auxin in both parasitic and non-parasitic functions it may be difficult to disentangle the effects of knock downs on endogenous processes and syncytium development. An alternative approach is to focus on knock downs that may prevent secretion of auxin. A preliminary BLAST search indicated the presence of potential auxin exporters in the genome of *G. pallida*. While any candidates for PIN exporters are notably absent, various potential ABCB auxin exporters could be found. Various members of the ATP-binding cassette subfamily B (ABCB)-type transporters of the multidrug resistance/phosphoglycoprotein (ABCB/MDR/PGP) protein family have been identified as auxin exporters in plants (Cho and Cho 2013). These ABCB proteins localise to the plasma membrane of the cell in a non-polar manner and maintain auxin homeostasis providing a stable and uniform auxin distribution among plant cells (Cho and Cho 2013). As such, knock downs and studying the localisation of the predicted ABCB transporters may be helpful in clarifying the relevance of auxin in syncytia development. Thus, it can be concluded that studying the role of auxin in parasitism for *G. pallida* is more complex than analyzing effector proteins due to the multiple endogenous roles auxins may have.

In summary, our data demonstrate that *G. pallida* is able to synthesize and secrete IAA, and that plant stimuli seem to enhance these processes. The auxin concentrations appear to be biologically relevant, since the secreted quantities are in the same order of magnitude as biologically relevant concentrations in plant cells (Petersson et al. 2009). However, the widespread occurrence of the auxin biosynthesis candidates within the phylum Nematoda also suggests an endogenous role for auxin outside of parasitism possibly related to vitality and ascaroside synthesis (Ludewig and Schroeder 2013; Loose and Ghazi 2021). Therefore, knocking down auxin biosynthesis and studying the potential auxin secretion mechanisms in

e.g., secretory gland cells will be crucial in elucidating its relevance for the development of syncytia induced by *G. pallida*.

Material and Methods

Auxin biosynthesis gene identification in *G. pallida*

The amino acid sequences of known auxin biosynthesis genes were collected from 10 plant and 10 bacteria species (Supplementary Table S1). Genes from different species that facilitate the same step in auxin biosynthesis were grouped together. Each such group was then aligned using MAFFT. If only one protein sequence was found that facilitates a step in auxin biosynthesis it was supplemented to form a group with the top 5 hits from a BLASTp search of that target in the National Centre of Biotechnology (NBCI) database. Potential auxin biosynthesis genes were identified by performing a Hidden Markov Model (HMM) scan on the *G. pallida* genome (van Steenbrugge et al. 2023) with each gene group of auxin biosynthesis genes in order to find patterns of homologous domains. Cygwin was used as Linux extension to perform the HMM scan using HMMER (v3.3.1). On these results a domain search was performed to select for potential *G. pallida* homologues that contain the same predicted protein domains as known auxin biosynthesis genes. CLC Main was used to perform this domain search using the Pfam database. This resulted in a list of potential homologues in *G. pallida* that have a similar structure and predicted protein domains as known auxin biosynthesis genes (Supplementary Table S2).

Homologue expression in *G. pallida*

To determine the expression of the auxin biosynthesis homologues the reads from the life-stage specific transcriptomes of *G*. *pallida* from Cotton et al. 2014 were mapped to the candidate genes. Expression is normalized to transcripts per million (TPM).

Nematode sample preparation

Cysts of *Globodera pallida* (population Rookmaker) originated from greenhouse culture and were long term stored at -80 °C. A users batch was transferred from -80°C to 4°C until use. Cysts were exposed to tap water or tomato root diffusate (TRD) at 20°C in the dark, to obtain water or TRD hatched second stage preparasitic juveniles. The TRD had been stored for over two years at 4°C since it was collected from the roots of tomato plants in a hydroponic growth chamber with ½ Hoagland solution as nutrients.

Juveniles were purified with 35% sucrose solution with a layer of water on top in conical glass centrifuge tubes. After centrifugation at 2000 rpm in a Hereaus Multifuge 3 S-R for 3 minutes the juveniles collected at the interface of sucrose and water from which they were aspirated with a glass Pasteur pipet and transferred to a new centrifuge tube. Juveniles' pellets were resuspended and washed 3 times in tap water, followed by a wash in sterile tap water, by centrifugation at 3000 rpm. Juveniles were transferred to a sterile 1,5 mL Eppendorf tube containing 4 µL of 10% Tween-20, to prevent juveniles from sticking to the tube wall, and washed with sterile tap water three more times. To surface sterilize the juveniles, they were

incubated in the following solutions consecutively: 0,5% (w/v) streptomycin-penicillin solution for 20 minutes, 0,1% (w/v) ampicillin-gentamycin solution for 20 minutes, sterile tap water for 5 minutes, 0,1% (v/v) chlorhexidin solution for 3 minutes. Finally, juveniles were washed at least 3x in sterile tap water before their surface sterility was checked. Besides this, all juvenile samples at this stage were checked for their vitality visually under a binocular.

Sterility check

A small aliquot of the juvenile suspension after surface sterilization was transferred to a B5 medium plate (containing 3,29 g/L Gamborg B5 including Vitamins and salts, 20 g/L Sucrose, pH 6,2; 1,5 % Bacto-agar, Difco) and was incubated in the dark at 20°C for 1 week. After one week the plate was checked for bacterial and fungal growth.

Collecting nematode secretions

TRD hatched J2's of *Globodera pallida* (population Rookmaker) were collected and surface sterilized as described above. Sterility check, vitality check and counting were performed as described above. Equal number of J2's were incubated in 0,5 mL sterile tap water and TRD in sterile 1,5 mL Eppendorf tubes separately on a rotator (Agar scientific, AR 384) at 20°C in the dark for 24 hours. After 24 hours, a small amount of the suspension was visually inspected for nematode vitality. The J2 were spun down and the supernatant, containing the secretions, was transferred to a new sterile 1,5 mL and stored at -80°C until auxin measurement by UPLC-MS/MS.

Extraction of phytohormones

Juveniles were lysed in a buffer according to Holterman et al. 2006 with some modifications. Lysis took place at 20°C and 1200 rpm in a Thermomixer (Eppendorf, Hamburg, Germany) during approximately 7 hours. Lysed juvenile samples were spun down at max. speed for 3 minutes and the supernatant transferred to a new sterile 1,5 mL Eppendorf tube. Lysed juvenile samples were aliquoted into three technical replicates for UPLC-MS/MS and stored at -80°C until use. For the extraction of phytohormones from J2's of *G. pallida*, ~30.000 individuals were used per sample.
Per sample, 1 mL of 100% methanol (MeOH) containing stable isotope-labeled internal standards (IS, Supplementary Table S6) at an end concentration of 100 nM per standard per sample. Samples were extracted and measured as previously described (Gühl et al. 2021; Schiessl et al. 2019). All solvents were evaporated in a speed vacuum system (SPD121P, ThermoSavant, Hastings, UK) at RT and the residue stored at -20˚C until further analysis.

Detection and quantification of phytohormones by Liquid Chromatography-Tandem Mass-Spectrometry

Sample residues were dissolved in 100 µL of acetonitrile/water (acid hormones) or MeOH/water (CKs) (0,1% formic acid) (20:80, v/v), and filtered through a 0,45 mm Minisart SRP4 filter (Sartorius, Goettingen, Germany). Analyses of plant growth regulators was performed by comparing retention times and mass transitions with those of unlabeled standards (Supplementary Table S6) using a Waters XevoTQs mass spectrometer equipped

with an electrospray ionization source coupled to an Acquity UPLC system (Waters, Milford, USA) as previously described (Gühl et al. 2021; Schiessl et al. 2019). Chromatographic separations were conducted on an Acquity UPLC BEH C18 column (100 mm; 2,1 mm; 1,7 mm; Waters, USA) by applying an acetonitrile/water (0,1% formic acid) or methanol/water (0,1% formic acid) gradient. The column was operated at 50˚C with a flow rate of 0,5 mL•min^{-1}. The column was equilibrated for 30 min using either solvent composition at the start of a run. The acetonitrile/water (0,1% formic acid) gradient started from 20% (v/v) acetonitrile, increasing to 70% (v/v) acetonitrile in 17 min. To wash the column, the water/acetonitrile gradient was increased to 100% (v/v) acetonitrile in a 1,0 min gradient, which was maintained for 1,0 min before going back to 20% acetonitrile using a 1,0 min gradient, prior to the next run. The methanol/water (0,1% formic acid) gradient started from 5% (v/v) methanol, increasing to 70% (v/v) methanol in 17 min. To wash the column, the water/methanol gradient was increased to 100% (v/v) methanol in a 1,0 min gradient, which was maintained for 1,0 min before going back to 5% methanol using a 1,0 min gradient, prior to the next run. The sample injection volume was 3/5 µL (acids/cytokinin, respectively). The mass-spectrometer was operated in positive and negative electrospray ionization mode when required. Cone and desolvation gas flows were set to 150 and 1000/800 l•h^{-1}, respectively. The capillary voltage was set at 3,5/3,0 kV, the source temperature at 150˚C, and the desolvation temperature at 550˚C. The cone voltage was optimized for each standard compound using the IntelliStart MS Console (Waters, Milford, USA). Argon was used for fragmentation by collision-induced dissociation. Multiple reaction monitoring (MRM) was used for quantification (Guhl et al., 2021). Parent–daughter transitions for the different (stable isotope labeled) compounds were set using the IntelliStart MS Console. MRM transitions, cone voltage and collision energy selected for compound identification and quantification are shown in Supplementary Table S6. To determine sample concentrations, a 10-point calibration curve was constructed for each compound ranging from 1 µM to 190 pM and each dilution also contained a known amount of an appropriate deuterium-labelled internal standard.

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Supplementary Data

Supplementary Tables available at: https://tinyurl.com/m9srztnj

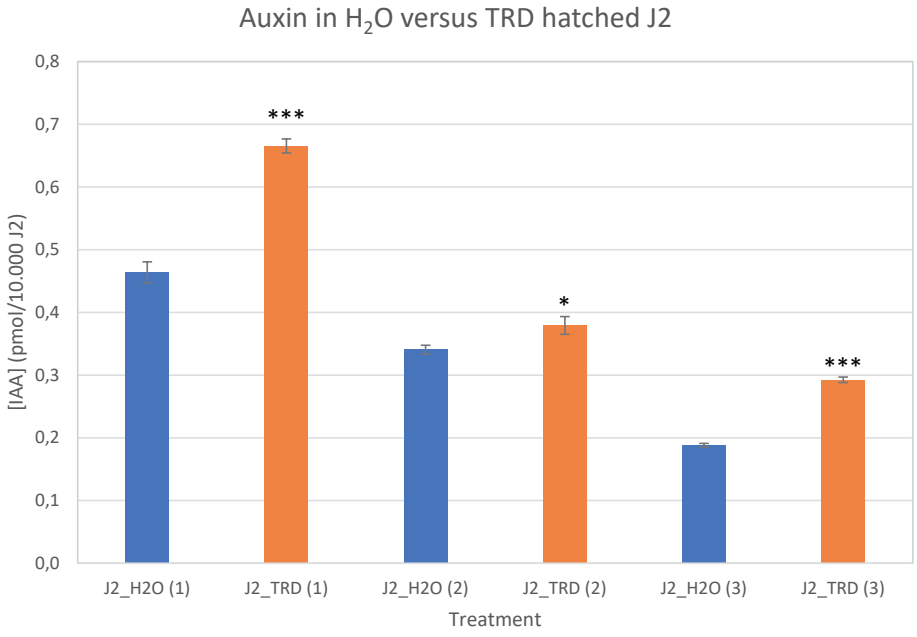
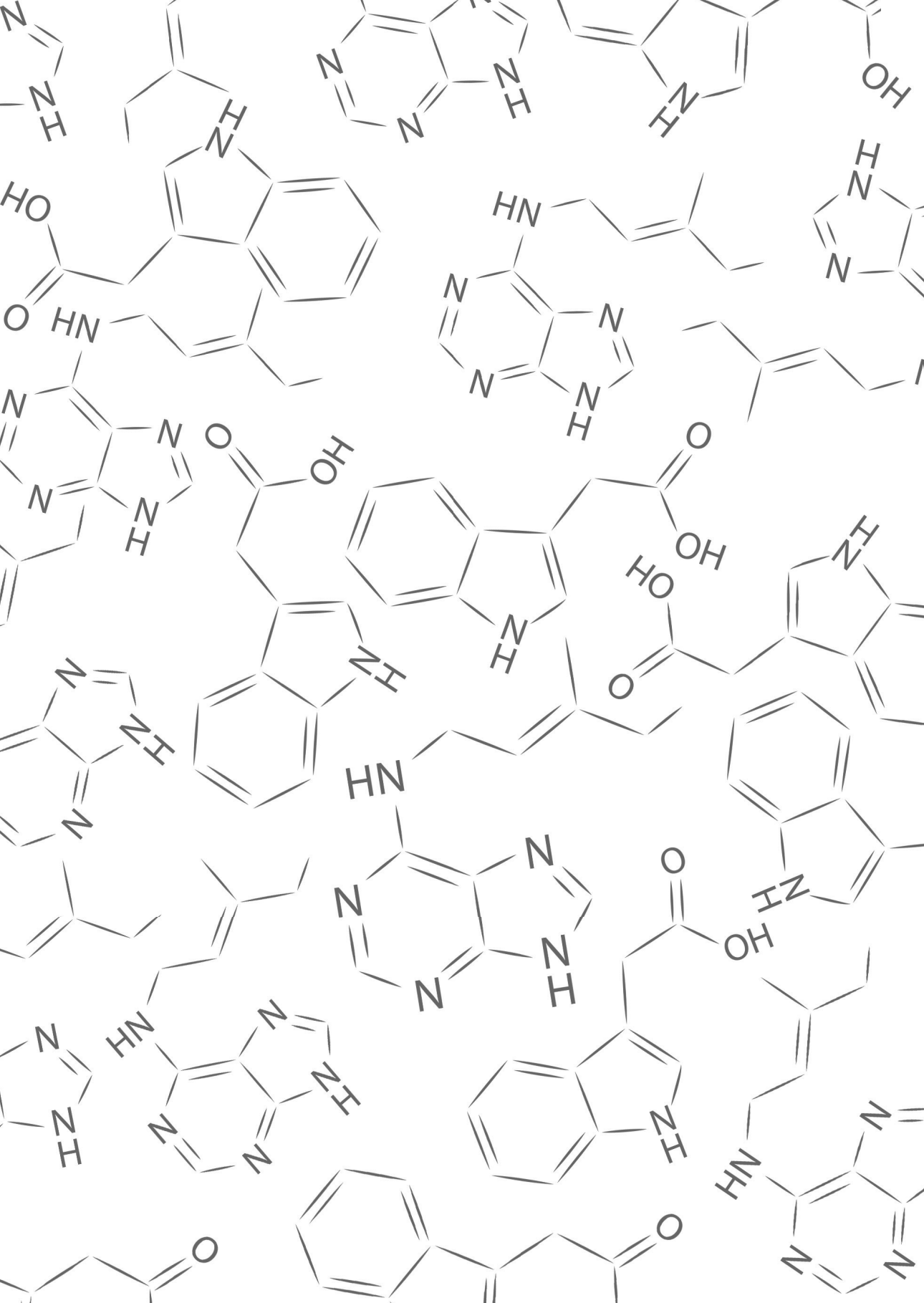


Figure S1 Concentration of the auxin IAA from three biological replicates in second stage juveniles hatched in either water or Tomato Root Diffusate (TRD). Significance was determined using a student T-test. *p<0,05; ***p<0,001.



Chapter 5

PLETHORA transcription factors contribute to gall formation induced by root-knot nematodes

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Abstract

Root-knot nematodes are sedentary endoparasites that induce giant cells in the roots of their host as a result of mitosis without cytokinesis leading to multinuclear and hypertrophied cells. However, little is known about the underlying pathways resulting in the development of these highly specialized feeding cells. Since the developmental programs of lateral root formation and giant cell development seem to overlap, we investigated the role of PLETHORA (PLT)3, PLT5 and PLT7 in the infection process of *Meloidogyne incognita* on *Arabidopsis thaliana* roots. The transcription factors PLT3, PLT5 and PLT7 are essential in the development and spacing of root primordia as well as lateral root emergence. Hence, we hypothesised that PLT3, PLT5 and PLT7 play a role in the development of root-knot nematode feeding sites. The *plt3plt5plt7* triple mutant showed a consistent decrease in the number of galls and less adult females with egg masses. Furthermore, infection of the *plt3plt5plt7* triple mutant led to the formation of an aberrant gall phenotype at 42 days post inoculation. Reporter studies showed that in the triple mutant PIN3-EGFP expression disappears after 3 days post inoculation and that auxin signalling recorded by DR5-EGFP is enhanced. Preliminary data of single PLT mutants reveal that PLT5 mutants bear more galls and more females with egg masses than wild type, while single mutants of PLT3 and PLT7 display more galls as well, but less females with egg masses. Overall, our findings demonstrate that PLT transcription factors are able to affect different aspects of the infection process, i.e., gall induction, gall morphology and the development of egg-producing females. In addition, considering the prominent role of auxin in gall formation, our data indicate that regulation of the auxin response by PLTs may contribute to the development of normal feeding sites induced by *M. incognita*.

Introduction

Plant-parasitic nematodes (PPN) are a major agro-economical pest that cost the agroindustry worldwide an estimated amount of 157 billion dollars annually (Singh, Singh, and Singh 2015). One of the most severe species is the sedentary root-knot nematode *Meloidogyne incognita*. This nematode is ranked as one of the most rapidly spreading biological threats in agricultural fields, which can be attributed to its wide host range of more than 1000 plant species from 200 different genera (Perry, Moens, and Starr 2009). Second stage juveniles (J2) of *Meloidogyne incognita* penetrate their host through the root tip directly above the elongation zone (von Mende 1997). They subsequently move intercellularly between cortical cells towards the root meristem. There, they enter the vascular cylinder from below and migrate upwards to establish a feeding site by injecting salivary compounds in surrounding cells (Rosso et al. 2011). These feeding sites consist of several cells that develop around the head of the nematode and go through a round of mitosis without cytokinesis leading to binucleate cells (Jones and Payne 1978). Subsequently they undergo several rounds of nuclear division and DNA amplification that result in large multinuclear and hypertrophied cells designated as giant cells. Typically, five to seven giant cells are created by the nematode and can increase up to 100 times in size. The tissue surrounding the giant cells is subjected to hyperplasia and form together with the giant cells the typical gall or knot structure (Favery et al. 2016). Feeding on these giant cells allows the nematode to develop into their adult female stage and produce offspring as a cluster of eggs held within a gelatinous matrix.

The phytohormone auxin, indole-3-acetic acid (IAA), is known to play an important role in the development of feeding sites induced by root-knot nematodes. A localized and transient accumulation of auxin is observed as early as 18 hours post infection during feeding site formation through the expression of the auxin responsive reporter DR5 (KARCZMAREK et al. 2004). IAA accumulation seems to occur in the entire gall and seems to reach its peak 3-5 days post infection (Absmanner, Stadler, and Hammes 2013). The auxin accumulation has been thought to be responsible for various processes during infection such as hypertrophy, cell wall ingrowths, and cell cycle activation (de Almeida Engler et al. 1999; Gheysen and Mitchum 2019). This is demonstrated by infection of several auxin mutants, including *dgt*, *pin1*, and *arf7/arf19,* which are all significantly less susceptible to root-knot nematode infection (Olmo et al. 2017; Goverse et al. 2000; Kyndt et al. 2016) . The accumulation of auxin seems to be induced through various processes. Most notably through the manipulation of polar auxin transport via import and export proteins. For example, a strong expression of the auxin influx reporters *AUX1* and *LAX3* is observed in giant cells during infection (Mazarei et al. 2003; Kyndt et al. 2016). Similarly, the expression and localisation of the PIN auxin efflux proteins are affected. Among others, PIN3 expression is activated at the basipetal side of galls probably to direct the auxin flow from neighbouring cells towards the developing giant cells and PIN7 expression is repressed in giant cells to likely prevent auxin export (Kyndt et al. 2016).

Perception of auxin in plant cells leads to the transcription of various auxin responsive genes through the activation of the TIR1-AFB signalling pathway. Transcription of auxin response genes is facilitated by the AUXIN RESPONSE FACTOR (ARF) transcription factor family (Li et al. 2016). An example of auxin response genes is the LATERAL ORGAN BOUNDERIES-DOMAIN (LBD) gene family. LBDs are a family of transcription factors that play a crucial role in the growth and development of plants (Okushima et al. 2007). Of note is that the transcription

factor *LBD16* is induced by either ARF7 or ARF19 and leads to divisions in the xylem pole pericycle for lateral root formation. It has been shown that LBD16 is important for gall formation induced by root-knot nematodes by infection of the *35S::LBD16-SRDX* overexpression line, in which no feeding sites could be established, and the *lbd16* knockout mutant which shows a lower infection rate as well (Cabrera et al. 2014; Olmo et al. 2017). LBD genes play a role in the development of lateral roots and have been shown to induce the expression of PLETHORA (PLT) transcription factors (Feng et al. 2012). As such, this leads to a signalling network being active during lateral root development involving IAA, ARFs, LBDs and PLTs.

PLT genes are a family of transcription factors that develop tissues in roots and shoots as well as control outgrowth and patterning of organ primordia (Prasad et al. 2011; Hofhuis et al. 2013). Specifically, PLT3, PLT5 and PLT7 play an important role during lateral root formation in the development and spacing of primordia as well as lateral root emergence (Du and Scheres 2017). This is demonstrated by the *plt3plt5plt7* triple mutant in which lateral root emergence is severely restricted and that develops a main root that is lined with clustered lateral root primordia in developmental arrest (Hofhuis et al. 2013). PLT3, PLT5 and PLT7 operate downstream of ARF7 and ARF19 and interestingly, might regulate the localisation and expression of PIN proteins, specifically PIN1 and PIN3 (Prasad et al. 2011; Du and Scheres 2017). In addition, mutations in genes compromise lateral root development, as seen in knock-out lines of PLT3, PLT5 and PLT7 (Abril-Urias et al. 2023; Olmo et al. 2020; Cabrera et al. 2014). Interestingly, such lateral root mutants reduce in many cases also the susceptibility to root-knot nematodes, indicating that the developmental programmes underlying secondary root formation and gall formation have overlapping components. These observations together with the link between LBDs, Polar Auxin Transport (PAT) and PLTs raises the question whether or not PLTs are involved in the regulation of gall formation upon nematode parasitism of plant roots.

Here, we investigate the role of PLT3, PLT5 and PLT7 in the infection process of the root-knot nematode *M. incognita*. Infected Arabidopsis single *plt3*, *plt5*, *plt7* and triple *plt3plt5plt7* mutant lines were used to study their effect on gall formation and nematode reproduction. Data show that both the single and triple mutants have a noticeable effect on the number of galls and adult females with egg masses. With the exception of the single *plt5* mutant, all mutations led to the formation of aberrant gall morphologies. To explore the hypothesis that the phenotypes obtained are associated with changes in auxin homeostasis, the *plt3plt5plt7* triple mutant line harboring the *PIN3* reporter gene was used for *M. incognita* infection. A repression of *PIN3* expression was observed in this mutant background, which was accompanied with an increase in auxin signalling within the feeding sites. Overall, our data indicate that PLT genes are required for proper infection, affect the expression of *PIN3* and modulate the auxin response. Our data show that in the context of gall formation, PLT3, PLT5 and PLT7 operate in a different manner than described for lateral root formation. In contrast to their functioning in canonical lateral root emergence, the three PLTs function in a non-redundant manner with regard to gall formation, development of females with egg masses and aberrant gall morphologies. Lastly, we discuss the developmental pathways putatively recruited by root-knot nematodes and the involvement of PLT3, PLT5 and PLT7 in these pathways. The emerging pattern seems that root-knot nematodes recruit components of

developmental programs underlying lateral and adventitious root formation as well as pathways involved in tissue regeneration and post-embryonic organogenesis.

Results

Mutants of PLT3, PLT5 and PLT7 affect the number of galls and females with egg masses
To determine whether the *PLT3*, *PLT5*, *PLT7* genes play a role in feeding site formation by the root-knot nematode *M. incognita* an *in vitro* infection assay was performed on Arabidopsis mutants. Because PLT3, PLT5 and PLT7 are known to act partially redundant during lateral root emergence, the *Arabidopsis thaliana* triple mutant line *plt3plt5plt7* was first tested (Hofhuis et al. 2013). The *plt3plt5plt7* line was infected with second stage juveniles of *M. incognita* and the formation of galls was monitored and recorded over the course of three weeks. A pronounced decrease of up to 50% was seen in the *plt3plt5plt7* mutant as compared to the wild type control *A. thaliana* Col-0 (Figure 1A). Little to no galls could be observed during the first week of infection. After the first week, infection sites started to develop into galls but the numbers remained reduced compared to the wild type control (Figure 1A). Infection of the triple mutant *plt3plt5plt7* showed a consistent pattern across four biological repeats (Supplementary Figure S1-4). Subsequently, the contribution of each gene to the observed phenotype in the *plt3plt5plt7* mutant was assessed by testing the single *plt3*, *plt5* and *plt7* mutants. In three of the four independent biological replicates, an increase in the number of galls (up to 50%) was observed for the *plt5* single mutant compared to wild type at all measured time points (Figure 1A, Supplementary Figure S1-4). However, in one replicate of the *plt5* single mutant a significant lower number of galls was found compared to wild type. Infection data on the *plt3* and *plt7* single mutants suggest a similar trend as seen in most replicates for *plt5*, both showing an increased number of galls (Supplementary Figure S2). Preliminary data on the *plt3plt7* double mutant revealed a neutral phenotype, as in this mutant line the number of galls is comparable to those of the wild type (Supplementary Figure S3). So, our data suggest that a complicated interaction between the PLETHORA transcription factors occurs, wherein mutant analyses reveal three different phenotypes with regard to the number of galls induced by *M. incognita*. The combined absence of PLT3, PLT5 and PLT7 in the triple mutant leads to a decrease in gall formation, while preliminary data on single mutants reveal an increase in gall numbers. The *plt3plt7* double mutant displays a third, neutral phenotype showing no deviation from wild type. Altogether, our data suggest that PLT3, PLT5 and PLT7 act with regard to gall formation in a non-redundant and non-additive manner.

To assess the effect of PLTs on the development of nematodes, the amount of egg producing females was measured at six weeks after infection. These bioassays demonstrate that the triple mutant *plt3plt5plt7* shows not only a decrease in number of galls as described above but also a reduction in the development of females with egg masses (Figure 1B). A similar correlation between the number of galls and egg masses was observed for the *plt5* single mutant. The *plt5* mutant shows an increase in gall numbers as well as in the number of females with egg masses (Figure 1B, Supplementary Figure S2). This correlation is also seen for the replicate where a decrease in galls was observed compared to the wild type as the number of females with egg masses dropped accordingly (Supplementary Figure S4). Interestingly, preliminary data of the *plt3* and *plt7* single mutants indicate that an increase in

gall numbers is not associated with a rise in females with egg masses. The number of egg masses in the *plt3* and *plt7* mutants was significantly lower than in the wild type and *plt5* mutant (Supplementary Figure S2), indicating that in these mutant lines the functioning of the giant cells as food source is attenuated. Thus, in the triple mutant *plt3plt5plt7* and single *plt5* mutant, gall formation is positively correlated with the development of egg masses, whereas preliminary data of the single plt3 and plt7 mutants suggest that gall formation and proper nematode development are uncoupled. These data suggest that PLT5 seems to have a different mode of action than PLT3 and PLT7 in the context of nematode parasitism.

The number of nematodes that penetrated the *plt3plt5plt7* mutant were measured to exclude the possibility that the observed decrease in infection is due to a decrease in number of nematodes attracted to the roots or due to reduced root penetration. Remarkably, there was a significant decrease in the number of penetrated nematodes of roughly 20% observed between wildtype Col-0 and the *plt3plt5plt7* mutant (Figure 1C). However, this difference does not seem to match the magnitude in which the decrease in gall formation occurs in the *plt3plt5plt7* mutant. This indicates that the decrease in gall formation cannot be fully explained by differences in attraction or penetration of the root by the nematode. Similarly, the higher number of galls formed in the *plt5* mutant cannot be attributed to an increase in juveniles that penetrate the root as no significant difference was found in the number of nematodes in the root (Figure 1C). Altogether, our data suggest that the PLTs are not redundant during infection and affect the infection process in a non-additive manner. The single mutant data indicate that PLT5 acts differently than PLT3 and PLT7. The single mutant data show for all three transcription factors an increase in gall numbers compared to wild type, however, for the *plt5* mutant a positive correlation between gall numbers and egg-producing females is observed, whereas this correlation is negative for *plt3* and *plt7*.

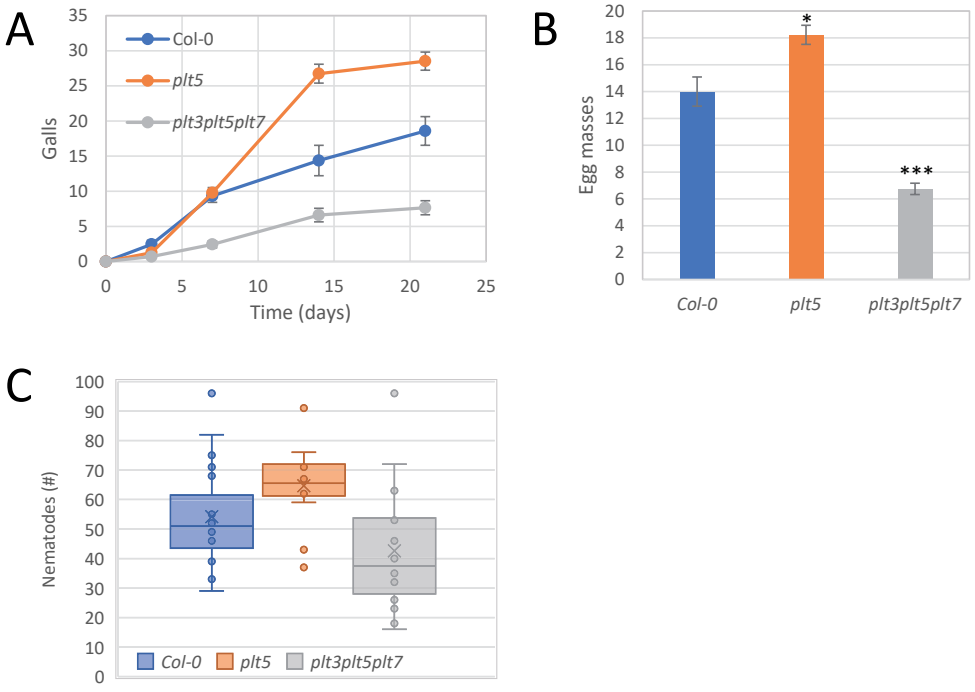

Figure 1 Infection assay using CRISPR lines of *plt5* and *plt3plt5plt7* showing the difference in (**A**) number of galls over the course of three weeks and (**B**) the number of females with egg masses developed at six weeks after infection. (**C**) Nematode attraction and penetration assay showing the number of nematodes in the root system of Col-0, *plt5* and *plt3plt5plt7* Arabidopsis plants after one week post infection. Error bars represent standard error of at least n=24 samples. Displayed is a subset of a measurement of Supplementary Figure S2. A total of four independent biological replicates were carried out (Supplementary Figures S1-4). Significance was determined using a one-way ANOVA. *p<0,05, ***p<0,001

PLETHORA mutants show aberrant gall morphologies

To determine whether gall morphology is affected by the PLT mutants, the gall phenotype was studied over the course of the infection process. The gall morphology was recorded at 3, 7, 14, 21 and 42 DPI for the *plt3*, *plt5* and *plt7* single mutants together with the *plt3plt7* double mutant and the *plt3plt5plt7* triple mutant. The time series revealed that the galls from all mutants do not display any abnormal phenotypes for the first three weeks after infection, but deviating phenotypes were observed at 42 DPI (Figure 2). With the exception of the *plt5* mutant, all tested mutants showed an aberrant gall morphology. The deviations in gall structures are characterised by a severe increase in size due to vigorous callus-like growth and are accompanied by dark coloration of the galls. The *plt5* mutant formed galls comparable to wild type Col-0, while the single *plt3* and *plt7* mutants, the double *plt3plt7* mutant and the triple *plt3plt5plt7* mutant show similar distinct gall morphologies. The gall phenotypes of the single mutants *plt3* and *plt7* further emphasizes the non-redundant nature of the PLTs in the

context of gall formation. It further shows that the aberrant phenotypes are not associated with the observed differences in correlations between gall numbers and egg masses. Despite the distinct gall morphologies, the *plt3plt7* and *plt3plt5plt7* mutants show similar to the *plt5* mutant a positive correlation between the number of galls and egg masses. In addition, the plt3 and plt7 mutants show a reduction in egg masses while the number of galls increase and have similar aberrant phenotypes as the double and triple mutants. Overall, a distinct gall phenotype of increased size and irregular shape is observed at 42 DPI for single mutants of PLT3 and PLT7, but not PLT5 in line with a different mode of action as observed for gall numbers and egg masses.

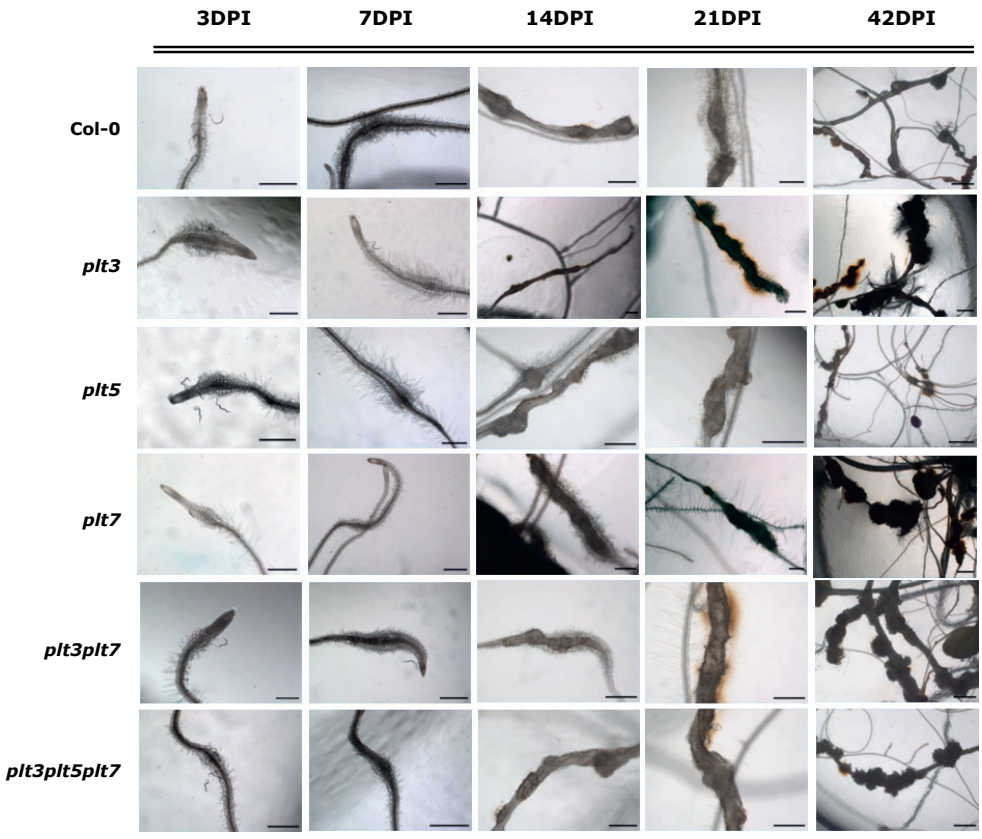

Figure 2 Morphology of PLT mutants during six weeks of infection. The Col-0 and *plt5* show regular sized gall formation whereas the *plt3* and *plt7* single mutants, the *plt3plt7* double mutant and the *plt3plt5plt7* triple mutant develop significantly larger callus-like galls. Representative images were taken from 75+ observations over three independent biological replicates. Scale bar for 3-21 DPI is 0,5 mm and for 42 DPI 2 mm.

DR5v2::EGFP reporter activity is enhanced in *plt3plt5plt7* mutant galls

PLETHORA genes are known to regulate the expression and localisation of PIN proteins during lateral root formation (Du and Scheres 2017). PLT3, PLT5 and PLT7 specifically have been shown to affect the functioning of PIN1 and PIN3 thereby regulating polar auxin transport in roots. In order to determine whether PLT genes affect the auxin response during infection, the expression of a DR5v2::EGFP reporter was studied first in the *plt3plt5plt7* mutant at 1, 3, 7 and 14 DPI. An increase in GFP expression in both Col-0 and *plt3plt5plt7* galls was observed as a result of nematode infection from 3 DPI and onwards (Figure 3). However, this increase seems further enhanced in the galls of the *plt3plt5plt7* mutant. To quantify this difference, the average GFP signal (mean intensity) was compared between galls from the Col-0 background and the *plt3plt5plt7* background under identical imaging conditions. This analysis shows that there is a significant higher level of GFP inside galls formed in the *plt3plt5plt7* mutant (Figure 4). This suggests a higher accumulation of auxin in galls of *M. incognita* when these *PLTs* are mutated, which was also reported for lateral root primordia (Du and Scheres 2017). Thus, these data suggest that PLTs regulate and restrict the auxin response in galls.

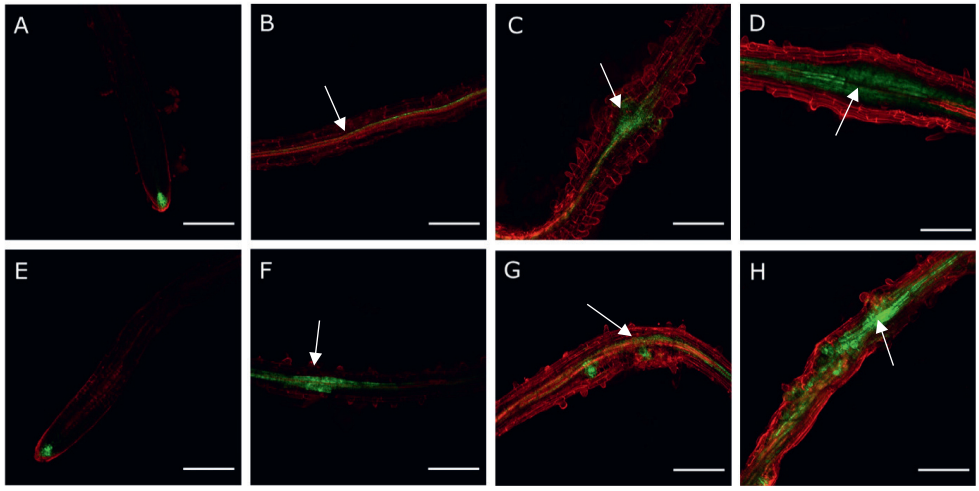

Figure 3 Confocal imaging showing the expression of DR5v2-EGFP in galls of wildtype Col-0 and *plt3plt5plt7* mutants. CLSM images depicting the localization of DR5v2-EGFP (green) in the root tip (**A**) of wildtype Col-0 and galls at 3 DPI (**B**), 7 DPI (**C**) and 14 DPI (**D**). CLSM images depicting the localization of DR5v2-EGFP in the root tip (**E**) of the *plt3plt5plt7* mutant and galls at 3 DPI (**F**), 7 DPI (**G**) and 14 DPI (**H**). Cells walls are stained with propidium iodide (20 µM) (red), arrows indicate the location of the feeding site and the scale bar represents 200 µm. Representative images were taken from 90+ observations over three independent biological replicates.

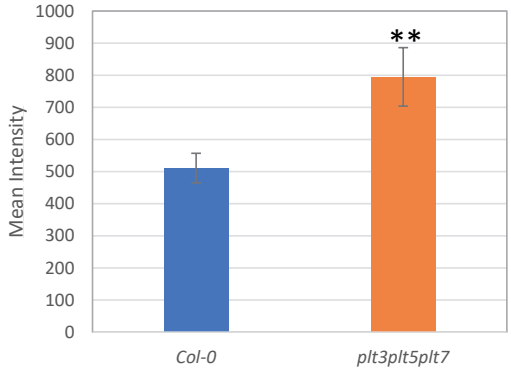

Figure 4 Analysis of the mean intensity of the GFP signal from images of DR5v2-EGFP expression in Col-0 and *plt3plt5plt7* galls at 14 DPI. Sample size was at least n=19 for each genotype. Significance was determined using a two tailed homoscedastic T-test. **p<0,01

PIN3 expression is strongly reduced in galls after 3 DPI in *plt3plt5plt7* triple mutant

To determine whether PIN1 and PIN3 expression is affected by PLETHORA proteins in nematode feeding sites causing the observed increase in auxin signalling, the expression of PIN3::PIN3-EGFP and PIN1::PIN1-EGFP was investigated next in a Col-0 and *plt3plt5plt7* mutant background over the course of a week during nematode infection. PIN3 was naturally expressed in the vasculature and the columella of the roots of Arabidopsis and upon infection its expression increases in the feeding sites (Figure 5A-C). This is in agreement with previously published results that show the role of PIN3 in the infection process (Kyndt et al. 2016). Expression of PIN3 in uninfected root tips of the *plt3plt5plt7* mutant is comparable to that of the Col-0 background (Figure 5D). GFP fluorescence is observed inside the gall, but also outside at the basipetal side. A very strong to complete reduction of the PIN3 signal was observed upon nematode infection of the *plt3plt5plt7* mutant compared to wild type Col-0 at 7 DPI, but not 3 DPI (Figure 5F). This shows that *PIN3* loses expression in the combined absence of PLT3, PLT5 and PLT7 during gall formation. PIN1 is naturally localised in the vasculature of the plant and its expression could indeed be visualised in the root tip (Figure 6A). In earlier reports the importance of PIN1 during nematode infection was mentioned (Kyndt et al. 2016; Goverse et al. 2000; Grunewald et al. 2009). However, in this study the expression of PIN1 seemed to be absent in most galls for both Col-0 and *plt3plt5plt7* mutants at 3 and 7 DPI (Figure 6 B-C, E-F). Only in a minority of the samples *PIN1* expression (2/30 at 3 DPI) was observed to be present, specifically in root terminal galls (Supplemental Figure S4). Overall, our data suggests that PLTs affect *PIN3* expression during nematode infection.

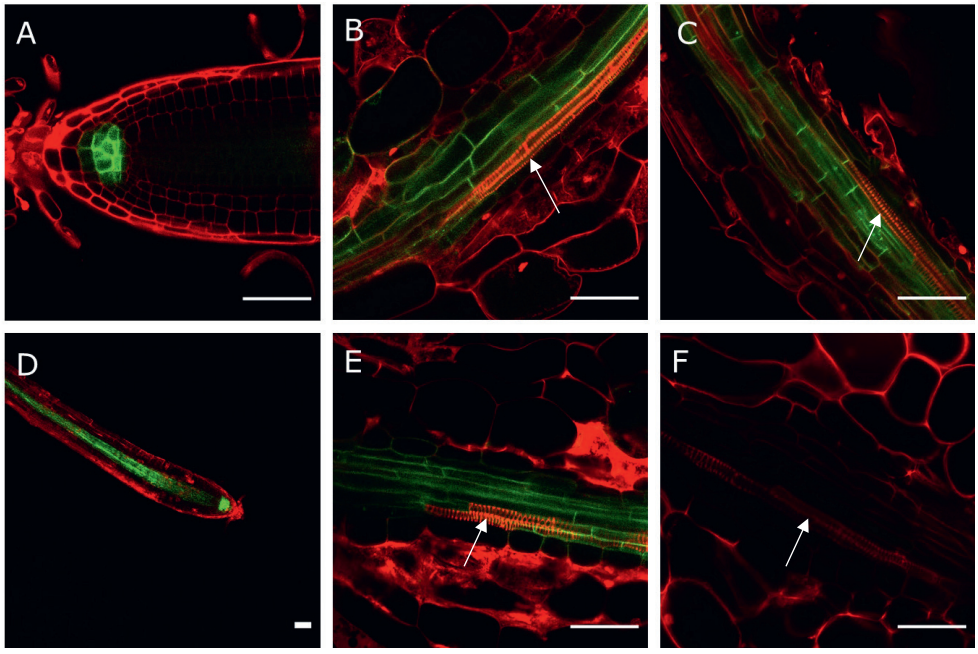

Figure 5 Confocal imaging showing the expression of PIN3::PIN3-EGFP in galls of wild type Col-0 and *plt3plt5plt7*. CLSM images depicting the localization of PIN3-EGFP (green) expressed under its native promotor in the root tip (**A**) of wild type Col-0 and galls at 3 DPI (**B**) and 7 DPI (**C**). CLSM images depicting the localization of PIN3-EGFP expressed under its native promotor in the root tip (**D**) of the *plt3plt5plt7* triple mutant and galls at 3 DPI (**E**) and 7 DPI (**F**). Cells walls are stained with propidium iodide (red), arrows indicate the location of the xylem and the scale bar represents 50 µm. Representative images were taken from 90+ observations over three independent biological replicates.

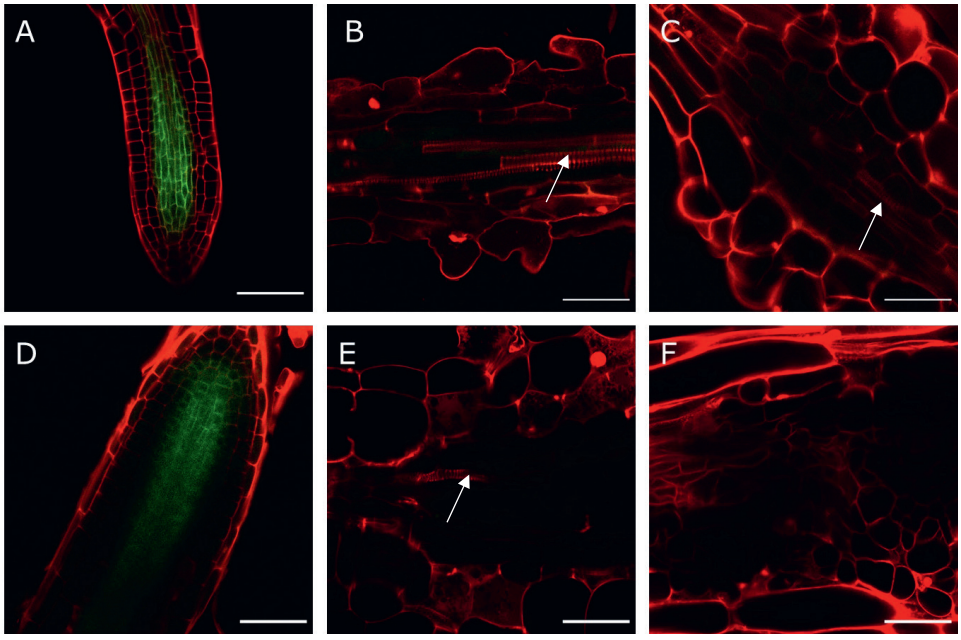

Figure 6 Confocal imaging showing the expression of PIN1::PIN1-EGFP in galls of Col-0 and *plt3plt5plt7*. CLSM images depicting the localization of PIN1-EGFP (green) expressed under its native promotor in the root tip (**D**) of wild type Col-0 and galls at 3 DPI (**B**) and 7 DPI (**C**). CLSM images depicting the localization of PIN1-EGFP expressed under its native promotor in the root tip (**D**) of *plt3plt5plt7* and galls at 3 DPI (**E**) and 7 DPI (**F**). Cells walls are stained with propidium iodide (red), arrows indicate the location of the xylem and the scale bar represents 50 µm. Representative images were taken from 90+ observations over three independent biological replicates.

Discussion

Our data show that the PLETHORA transcription factors PLT3, PLT5 and PLT7 contribute to different aspects of feeding site development of the root-knot nematode M. incognita in Arabidopsis roots. Infection of the plt3plt5plt7 triple mutant shows a consistent reduction in gall numbers and the development of females with egg masses. Preliminary data show that single mutations have a noticeable effect on the infection success, indicating that the PLTs in the context of gall formation operate in a non-redundant manner. In addition, our data suggests that PLT3 and PLT7 have a different role in the infection than PLT5 as for the single mutants of the PLT3 and PLT7 a decrease in the number of egg masses was seen but not in PLT5. This observation is corroborated by the formation of aberrant gall phenotypes in single mutants of PLT3 and PLT7, but not of PLT5. Furthermore, in the plt3plt5plt7 triple mutant PIN3 expression is strongly reduced in galls after 3 DPI, which is accompanied with enhanced auxin signalling. Altogether our data indicate that PLTs influence different aspects of the infection process, wherein modulation of the auxin response via regulating PIN3 expression may play a role.

PLT3, PLT5 and PLT7 genes do seem to, at least partially, perform different functions during root-knot nematode infection. Infection of the single mutants of PLT3, PLT5 or PLT7 result in a higher number of formed galls; however, for plt3 and plt7 mutants a lower rather than a higher number of egg mass developing females is observed. The number of egg masses is an indication of the quality of the feeding sites and therefore it suggests that feeding sites are easier established in the plt3 and plt7 mutants but with possible intrinsic defects resulting in a decreased development of females with egg masses. However, the data from the plt3 and plt7 mutants represent a single biological replicate and should be interpreted with care, also considering the inconsistent data from the four independent biological replicates of the single mutant plt5. This variability of the plt5 data might be attributed to the dynamic nature of transcription factors (Swift and Coruzzi 2017), wherein the knock-out of a transcription factor may lead to instable protein complexes leading to enhanced phenotypic variability. Nevertheless, it appears that PLT3, PLT5 and PLT7 are operating in a non-redundant and non-additive manner with regard to the number of galls and females with egg masses as well as gall phenotype. This is in contrast to their role in lateral root emergence where they act in a redundant manner (Du and Scheres 2017; Hofhuis et al. 2013). However, it is noted that with regard to the positioning of lateral root primordia and lateral roots along the primary root (rhizotaxis), the three PLTs operate also in a non-redundant and non-additive manner (Du and Scheres 2017).

PLT3/PLT5/PLT7 regulate the expression of PIN1 and PIN3 in canonical lateral root development. This was clearly demonstrated for the *plt3plt5plt7* mutant during lateral root primordia development where the polar localization of PIN1 to the plasma membrane was severely impaired and the overall level of expression for both *PIN1* and *PIN3* was significantly reduced (Du and Scheres 2017). When considering the role of PINs during nematode infection it seems that PIN3 is active in neighbouring cells at the basipetal side of the gall, but not inside the feeding site during early infection (Kyndt et al. 2016). However, this changes later on as PIN3 becomes active in giant cells and surrounding cells at later stages. PIN1 has been reported to be highly expressed in young galls to at least 7 DPI, but since gall size and nematode development was not influenced by the *pin1* mutation it was concluded that PIN1 is not required for proper gall formation (Kyndt et al. 2016). As such, it appears that PIN3 and not PIN1 is more important in creating and controlling auxin maxima during gall formation (Kyndt et al. 2016). As shown here, the absence of PLT3, PLT5 and PLT7 strongly reduces the expression of PIN3 after 3 DPI, and therefore might explain the increase in auxin signalling in the *plt3plt5plt7* triple mutant observed at 14 DPI. It is noted, that in our experiments PIN1 expression was mainly absent in galls at both 3 and 7 DPI and observed in only a minority of the samples. PIN1 expression was specifically observed in root terminal galls suggesting that the location of the feeding site is important for PIN1 expression. Also, in root-knot nematode galls of tomato plants PIN1 expression has been shown to be absent (Meidani et al. 2021). Overall, our data indicate that PLETHORA genes are required to correctly distribute auxin within galls, most likely via regulating the expression of PIN3. With regard to the increased auxin signalling in the *plt3plt5plt7* mutant, it would be interesting to see if this is directly caused by the dysregulation of PIN3. To test this, DR5-EGFP can be studied in a *plt3plt5plt7* background complemented with a PIN3 construct driven by a PLT promotor. However, complementation experiments might be a concern because it was shown that pPLT7::PIN1 was unable to complement the phenotype of the *plt3plt5plt7* mutant during LRP formation (Du and Scheres 2017). As an alternative, studying the expression of DR5-EGFP in a *pin3*

mutant background might prove equally useful to further pinpoint the role of PLTs in regulating auxin responses during root-knot nematode infection.

The relationship between lateral root formation and feeding site development has long been suggested and is, in part, based on overlap in expression patterns of e.g., auxin responsive genes, auxin transporters and cell cycle regulators (Abril-Urias et al. 2023; Olmo et al. 2020; Goverse et al. 2000; Cabrera et al. 2014; Mathesius 2003). Lateral root (LR) formation can be divided into various stages with the first stage being LR positioning (Malamy and Benfey 1997). This stage includes mechanisms to position, specify and activate lateral root founder cells (LRFC) from which the LR develops. Auxin signalling is an important aspect of LRFC positioning and specification and during LRFC specification, an accumulation of auxin induces the expression of ARF7 and ARF19. In turn ARF7 and ARF19 regulate the expression of PLT3, PLT5 and PLT7 through the use of the LATERAL ORGAN BOUNDERIES-DOMAIN (LBD) (Feng et al. 2012). PLT3, PLT5 and PLT7 are required for development and spacing of primordia as well as lateral root emergence (Hofhuis et al. 2013) and function downstream of ARF7 and ARF19. The double mutant *arf7arf19* fails to accomplish the first asymmetric cell divisions necessary for the development of lateral root primordia and, similar to the triple *plt3pl5pl7* mutant, lacks almost completely the formation of lateral roots (Wilmoth et al. 2005). However, *arf7/arf19* double mutants do not show any impairment in gall formation by root-knot nematodes (Olmo et al. 2020). This implies that in the context of gall formation the PLT3/PLT5/PLT7 transcription factors are not activated via this canonical lateral rooting pathway and are induced via a pathway independent of ARF7 and ARF19.

Besides the canonical lateral root pathway, plants have evolved a variety of alternative developmental programmes leading to the outgrowth of roots, also from non-root tissues (Sheng et al. 2017; Liu et al. 2018; Ge et al. 2019). The *plt3plt5plt7* triple mutant has been reported to form hardly any lateral roots due to developmental arrest of lateral root primordia when grown under standard conditions (Hofhuis et al. 2013). Yet, during infection of *M. incognita* the number of secondary roots increases significantly in the *plt3plt5plt7* triple mutant (data not shown). Also, when plants are grown in soil or upon wounding the *plt3plt5plt7* triple mutant forms secondary roots (Sheng et al. 2017). These secondary roots are formed though a different initiation pathway compared to lateral roots and might be attributed to 'adventitious lateral rooting' mediated by WOX11 (Sheng et al. 2017; Ge et al. 2019). Adventitious lateral root formation initiation is characterised by the induction of LBD16 through WOX11 while in lateral root formation this is through ARF7 and ARF19 (Sheng et al. 2017). It has been shown that LBD16 is important for gall formation during root-knot nematode infection by the *35S::LBD16-SRDX* overexpression line in which no feeding sites could be established and by the *lbd16* knockout mutant which shows a lower infection rate (Cabrera et al. 2014; Olmo et al. 2017). As such, LBD16 provides a molecular link between secondary root formation and gall formation. In a similar way connects LBD16 the development of galls in leaves and the outgrowth of adventitious roots from leaf explants., being equivalent in morphology to giant cells in root-galls (Olmo et al. 2017). Here also LBD16 - essential for adventitious rooting - appeared to be required for feeding site formation as root-knot nematodes could not develop in 35S::LBD16-SRDX leaves (Olmo et al. 2017). Altogether, it is clear that LBD16 is a key factor in the development of lateral roots, adventitious lateral roots and adventitious roots from leaf explants as well as in the formation of galls from root and leaf explants.

An intriguing question is how LBD16 and PLT3, PLT5 and PLT7 are connected in the context of gall formation. It is noted that several LBD family members have been identified as direct promoter binding proteins for PLT3, PLT5 and PLT7, but not LBD16 (Du and Scheres 2017). Furthermore, compared to canonical lateral root formation (Hofhuis et al. 2013; Banda et al. 2019; Du and Scheres 2017), relatively little is known about the cellular processes mediated by PLT3, PLT5 and PLT7 genes during adventitious rooting processes (Liu et al. 2018). While the various stages in LR formation have been studied in detail, the function of PLT3, PLT5 and PLT7 during AR primordium formation and AR outgrowth have hardly been addressed (Shanmukhan et al. 2021; Liu et al. 2018). Besides developmental programmes involved in rooting processes, it has been suggested that modules from pathways implicated in root apical meristem (RAM) maintenance are also involved in gall formation (Olmo et al. 2020). Key transcription factors (SCR, SCZ and SHR) implicated in RAM significantly contribute to successful root-knot nematode infections as established by testing mutant lines (Olmo et al. 2020). Interestingly, galls do also share molecular components, including LBD16, with developmental pathways involved in root regeneration, callus induction on auxin-rich media and upon wounding. Triple mutants demonstrated that PLT3, PLT5 and PLT7 are required for adventitious rooting (Liu et al. 2018; Shanmukhan et al. 2021), wound-induced callus formation (Ikeuchi et al. 2017) and de novo shoot regeneration from callus on media (Kareem et al. 2015). Taken together, the PLT3, PLT5 and PLT7 transcription factors are essential in several plant developmental pathways, which may explain the profound effect of PLT3, PLT5 and PLT7 on gall formation and root-knot nematode development as observed in this study. To further characterise the aberrant gall formation phenotype obtained in the PLT mutants, sectioning followed by microscopic inspection might provide insights into the nature of the enlarged galls at the cellular level. Determining whether the giant cells have enlarged in addition to the observed increased in size of the surrounding tissue by extra cell divisions or hypertrophy might provide further insight into the mechanisms underlying this phenomenon.

In sum, our data demonstrate that PLT3, PLT5 and PLT7 are involved in regular gall formation and root-knot nematode development. While it is clear that PLT3, PLT5 and PLT7 play a role during nematode infection it remains unclear when they come into play. A time series expression analysis and reporter studies are needed to elucidate the temporal and spatial expression patterns of the *PLT* genes. The PLETHORA genes encode transcription factors that induce a variety of downstream targets. Elucidating which of these targets is affected during nematode infection might lead to a greater understanding of the role of the PLTs during infection. Comparative transcriptomics using RNA-seq between wild type galls and galls from a plt3plt5plt7 mutant will give a target list of affected genes and might reveal relevant signalling pathways. At present the PLT3, PLT5 and PLT7 transcription factors cannot be linked to a specific plant developmental pathway recruited by root-knot nematodes. The emerging pattern based on data obtained in this study and previous research is that root-knot nematodes are able to recruit multiple, partially overlapping pathways, wherein PLT3, PLT5 and PLT7 may play a role. It will be interesting to study in which pathways PLT3/PLT5/PLT7 and LBD16 are connected in the context of gall formation. Finally, the fact that gall formation is not fully compromised in the triple mutant *plt3plt5plt7* indicates that root-knot nematodes are also able to recruit PLT3/PLT5/PLT7 independent pathways.

Material and methods

Plant materials and growth conditions

Seeds from the wild type *Arabidopsis thaliana* ecotype Colombia 0 (Col-0) (Alonso et al. 2003) and PLT mutant lines were kindly provided by Prof. Dr. ir. Ben Scheres, Plant Developmental Biology Group, Wageningen University Research, the Netherlands (Du and Scheres 2017). They were cold-stratified at 4°C for 4 days to promote and synchronize germination rates and subsequently vapour sterilised in 0,7 M NaOCL and 1% HCL in tap water for 4 hours. Single sterilised seeds were placed in a six well plate (Falcon) containing 4 ml per well of Murashige and Skoog medium with vitamins 4,7 g/L (Duchefa biochemie), 2% sucrose and 0,8% Daichin agar (Duchefa biochemie) set to pH 5,7. The seeds were allowed to germinate in a plant growth chamber at 21°C under a 16h light/8h dark cycle for 7 days. Individual one-week-old seedlings were subsequently transferred to a separate well in a new six well plate containing MS medium and were incubated for 7 more days at 21°C under a 16h light/8h dark cycle and then used in nematode infection assays.

Nematode collection, hatching and surface sterilisation

Eggs from the root-knot nematode *Meloidogyne incognita* strain 'Morelos' (kindly provided by INRAE, Sophia Antipolis, France) were collected from a greenhouse culture on tomato. Roots were treated with 0,05% v/v NaOCl for three minutes and subsequently rinsed with tap water and the collection of eggs on a 25 µM sieve. To surface sterilize, eggs were treated with a solution consisting of 1% NaN3 (w/v) for 20 minutes under constant stirring. To hatch, eggs were rinsed thoroughly with sterile tap water and subsequently placed on a 100 µm sieve in a glass Petri dish with an antibiotic solution (0,15% gentamycin w/v, 0,05% nystatin w/v) in the dark at room temperature for 4 days. Next, juveniles were collected and purified using a 35% sucrose solution (Warmerdam et al. 2018). For surface sterilisation, J2 were treated with a $HgCL_2$ solution (0,002% Triton X-100 w/v, 0,004% NaN3 w/v, 0,004% HgCl2 w/v) after which the nematodes were thoroughly rinsed three times with sterile tap water. Surface sterilised juveniles were prepared for inoculation by making a nematode suspension in 0,7% Gelrite (v/v) to obtain a homogenous density of the nematode inoculum.

Nematode infection assay

Fourteen-day-old seedlings of *Arabidopsis thaliana* wild type and mutant genotypes grown in six-well plates were inoculated with a Gelrite suspension of approximately 180 juveniles of infective surface sterilized *M. incognita* juveniles. Nematodes were applied near the main root tip of each plant using a repetitive pipet (Eppendorf Multipette® Plus). Inoculated plants were incubated in a growth chamber at 21°C under a 16h light/8h dark cycle (Warmerdam et al. 2018). The infection rate was assessed by counting the number of galls, developing females and egg masses at 1, 3, 5, 7, 14, 21 and 42 days post inoculation. Samples were observed using a binocular microscope (Wild Heerbrugg) and pictures were made using an AxioCam MRc5. Statistical analysis was performed on the data using SPSS Statistics 23 (IBM®) with an ANOVA test to determine significance.

In vivo confocal microscopy of nematode infected roots

The reporter lines PIN1::PIN1-EGFP, PIN3::PIN3-EGFP, Dr5v2::EGFP in a Col-0 and *plt3plt5plt7* background (kindly provided by Prof. Dr. ir. Ben Scheres, Plant Developmental Biology, WUR) were used to monitor the spatial-temporal dynamics of their promoter activity in roots upon nematode infection by the detection of EGFP fluorescence (either free or as fusion protein). Infected and mock-inoculated root systems were separated from the shoot and incubated in propidium iodide (Sigma-Aldrich, 20 μM) for several minutes to stain the cell walls for contrast followed by direct observation under an inverted confocal microscope (LSM510, Zeiss) after preparation on an object slide. Root samples were excited using a 488 nm argon laser and emission was captured using a 505-545 nm bandwidth filter and imaged with pinhole 1. Z-stack and image analysis was done using the LSM image browser software (Zeiss).

Quantification of EGFP levels

To quantify changes in auxin signalling between DR5v2::EGFP in Col-0 and the *plt3plt5plt7* mutant the mean intensity (MI) of the fluorescent signal was measured and analysed using Fiji (v2.0.0-rc-69) (Schindelin et al. 2012). Images with identical microscopy settings such as magnification and pinhole were compared for the visualisation of DR5v2::EGFP expression. Images were taken at 14DPI at a single depth with the setting described above and were taken at the depth with the highest visible signal. For the analysis, first the default signal threshold was imposed on each picture to remove noise from the measurement. Subsequently, the intensity of the EGFP signal was averaged to obtain the mean intensity (MI) for that image. Lastly, the MI of all Col-0 and *plt3plt5plt7* infection sites were averaged to obtain Figure 4. Sample size was at least n=19 both Col-0 and *plt3plt5plt7*. Significance of differences observed was determined using a two-tailed homoscedastic T-test ($p<0,05$).

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Supplementary Data

Figures S1-S4 represent four independent biological replicates

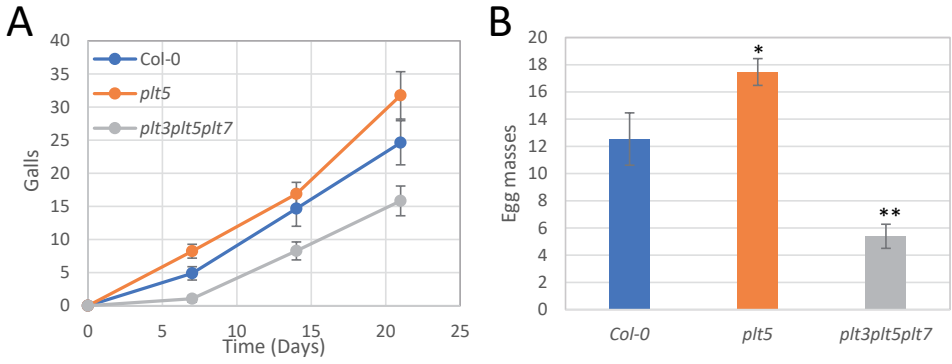


Figure S1 Root-knot nematode (*M. incognita*) infection assays using CRISPR lines of *plt5* and *plt3plt5plt7* showing differences in (**A**) number of galls observed on roots of *in vitro* grown plants at dpi = 7, 14, 21 and (**B**) the number of females with egg masses developed at six weeks post inoculation (dpi = 42). Col-0 was used as wild type control. Error Bars represent standard error. Significance was determined using a one-way ANOVA (n=24; *p<0,05 **p<0,01) in a single experiment.

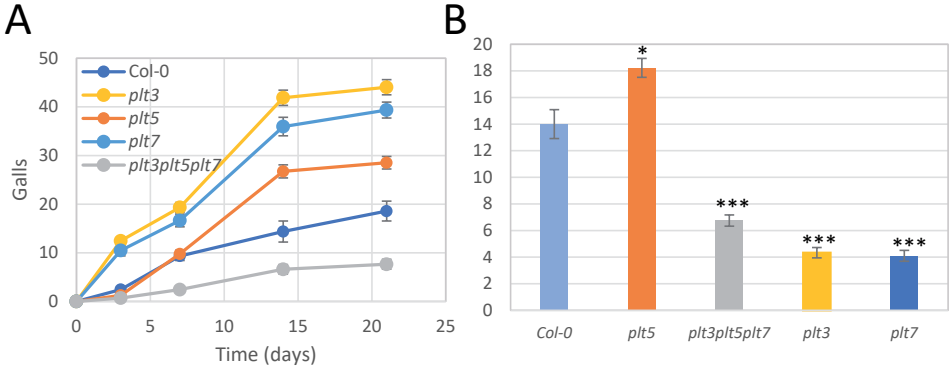


Figure S2 Root-knot nematode (*M. incognita*) infection assay of *plt5*, *plt3* and plt7 single and *plt3plt5plt7* triple mutants using CRISPR lines showing the difference in (**A**) number of galls observed on roots of *in vitro* grown plants at dpi = 3, 7, 14, 21 and (**B**) the number of females with egg masses developed at six weeks post inoculation (dpi = 42). Col-0 was used as wild type control. Error Bars represent standard error. Significance was determined using a one-way ANOVA (n=24; *p<0,05 ***p<0,001) in a single experiment.

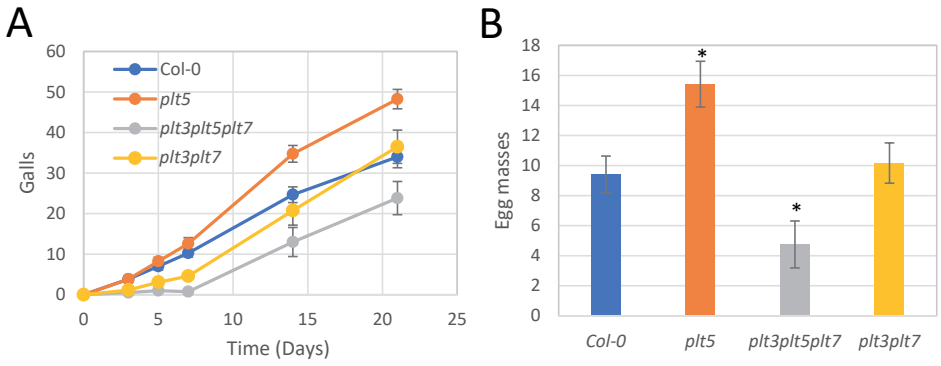


Figure S3 Root-knot nematode (*M. incognita*) infection assay of *plt5*, *plt3plt7* and *plt3plt5plt7* using T-DNA insertion lines showing the difference in (**A**) number of galls observed on roots of *in vitro* grown plants at dpi = 3, 5, 7, 14, 21 and (**B**) the number of females with egg masses developed at six weeks post inoculation (dpi = 42). Col-0 was used as wild type control. Error Bars represent standard error. Significance was determined using a one-way ANOVA (n=24; *p<0,05) in a single experiment.

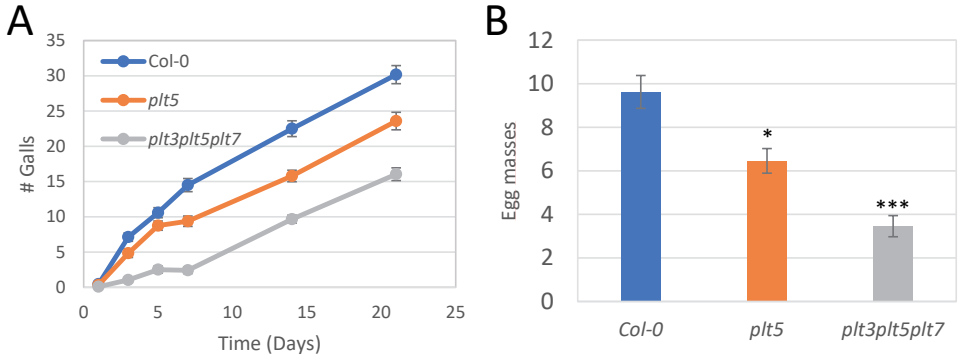


Figure S4 Root-knot nematode (*M. incognita*) infection assay of *plt5* and *plt3plt5plt7* triple mutants using CRISPR lines showing the difference in (**A**) number of galls observed on roots of *in vitro* grown plants at dpi = 1, 3, 5, 7, 14, 21 and (**B**) the number of females with egg masses developed at six weeks post inoculation (dpi = 42). Col-0 was used as wild type control. Error Bars represent standard error. Significance was determined using a one-way ANOVA (n=24; *p<0,05 ***p<0,001) in a single experiment.

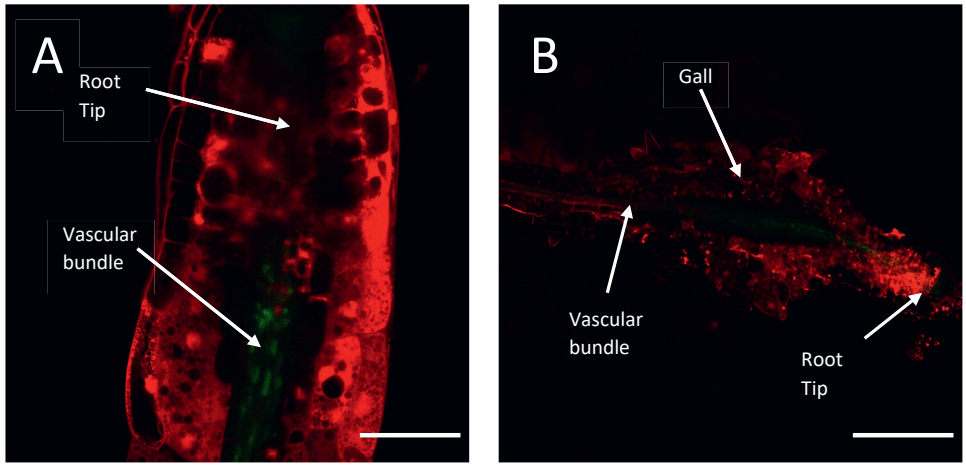
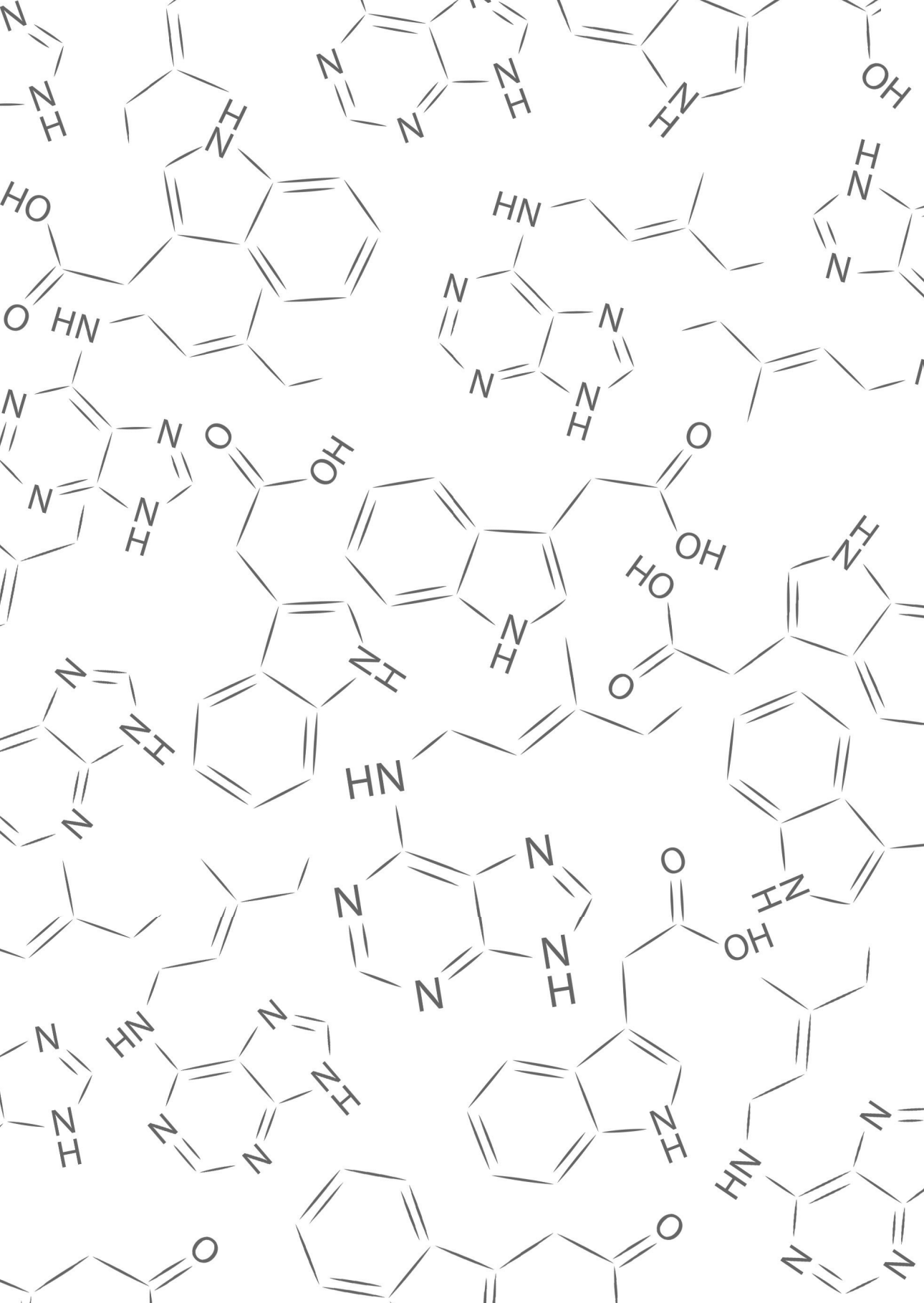


Figure S5 Confocal imaging showing the occasional expression of PIN1::PIN1-EGFP in wild type Col-0 galls infected with *M. incognita*. CLSM images depicting the localization of PIN1::PIN1-EGFP (green) in root terminal galls of the wild type Col-0 at 1 dpi (**A**) and 3 DPI (**B**). No expression was observed at 7dpi (data not shown). Cells walls are stained with propidium iodide (20 µM) (red) and the scale bar represents 50 µm and 200 µm, respectively.



Chapter 6

General Discussion

Introduction

Plant-parasitic nematodes are a significant threat to plants and cause extensive damage to agricultural crops. Understanding the infection mechanisms of these nematodes is crucial for developing effective strategies to mitigate their impact. Various plant-parasitic nematodes have evolved sophisticated strategies to invade plant roots and establish feeding sites, enabling them to extract nutrients, develop and reproduce. Plant-parasitic nematodes worldwide cost the agroindustry an estimated amount of 157 billion dollars annually (Singh, Singh, and Singh 2015; Nicol et al. 2011). Among the various types of nematodes, cyst and root-knot nematodes are well-known for their ability to cause significant damage to their host (J. T. Jones et al. 2013). Cyst nematodes move intracellularly through the plant's cortex and select an initial syncytial cell near or within the vascular bundle to initiate the formation of a feeding site (Bohlmann and Sobczak 2014). They inject a suite of secretory compounds known as effectors into the initial syncytial cell to alter the hosts metabolism which ultimately leads to the formation of a syncytium. Partial breakdown of the neighbouring cell walls and the subsequent fusion of their cell membranes leads to the formation of the syncytium (Sobczak and Golinowski 2011). In contrast, second stage juveniles of root-knot nematodes move intercellularly through the vascular bundle where they select up to 10 individual cells and transform these into giant cells. They inject salivary compounds that induce cell division without cytokinesis leading to the development of this type of feeding site (Gheysen and Mitchum 2011). Despite the fact that cyst and root-knot nematodes develop distinct feeding structures they do share common characteristics. For example, both form large multinucleate cell conglomerates with proliferated endoplasmic reticulum as a result of reprogramming of normal root cells (Eves-van den Akker, Lilley, Ault, et al. 2014). To form such a unique plant organ, cyst nematodes and root-knot nematodes manipulate developmental and physiological processes in normal plant roots at the onset of parasitism. This is accompanied by dramatic changes in gene expression and hormone balance. Key players are the phytohormones auxin and cytokinin. Here, I will discuss the various implications of auxin and cytokinin in nematode feeding site induction and development in the context of our current understanding of their contributions to nematode infections. Furthermore, recommendations are given based on the findings obtained in this thesis to address further technical, methodological and biological challenges involved in research on plant-parasitic nematode feeding structures.

Accumulation of auxin and cytokinin in feeding sites of root-knot and cyst nematodes

Auxin and cytokinin are both known to accumulate during infection of cyst and root-knot nematodes (KARCZMAREK et al. 2004). In Chapter 3 a temporal expression map could be made of auxin and cytokinin signalling during cyst nematode infection with the use of an auxin-cytokinin double reporter combined with live-cell imaging. This expression pattern has shown that cytokinin signalling increases rapidly and precedes auxin signalling. Auxin signalling starts with a lag phase and is induced after the first contours of the syncytial area have become visible. However, after the induction of both, auxin and cytokinin signalling were simultaneously observed in nuclei of the syncytial area during infection. It was also revealed that cytokinin signalling is relatively high in nematode infection sites compared to signalling maxima in root tips, while in contrast auxin signalling is relatively weak. Interestingly, preliminary data from the auxin-cytokinin double reporter suggests that the auxin-cytokinin ratio in the feeding sites of the root-knot nematode *Meloidogyne incognita* differs greatly from that of the cyst nematode *Heterodera schachtii* with auxin signalling being more strongly induced (Figure 1). Our data match a report based on transcriptome data showing that cytokinin-induced genes are enriched in syncytia compared to auxin inducible genes, indicating that cytokinin signalling is dominant over auxin signalling during cyst nematode infection, while the opposite is seen during root-knot nematode infection (Cabrera, Fenoll, and Escobar 2015). The difference in accumulation of auxin and cytokinin between cyst and root-knot nematodes might be explained by variation in the mechanisms underlying the accumulation of these hormones. Plant-parasitic nematodes seem to have developed sophisticated mechanisms to manipulate the levels and distribution of auxin to establish successful infections and create suitable feeding sites as described (Chapter 2).

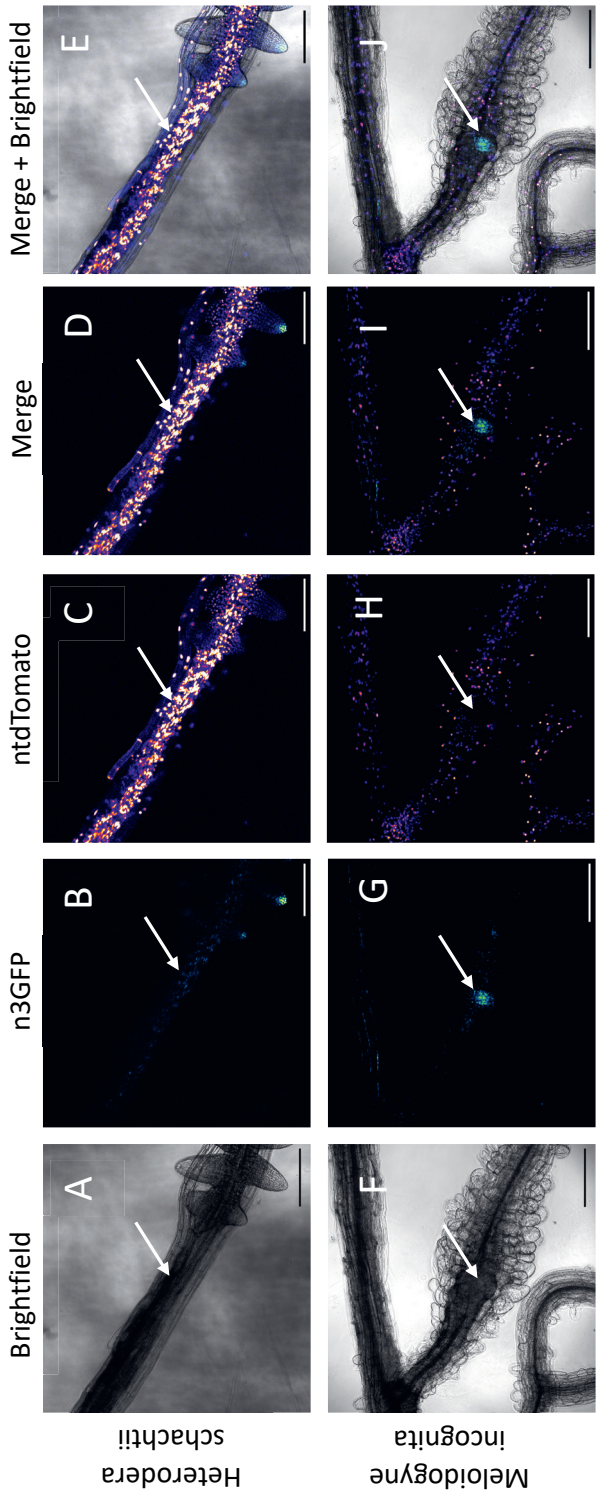


Figure 1 Visualisation of marked differences in cytokinin and auxin signalling through the use of the double reporter DR5revV2::n3GFP-TCSn::ntdTomato in root segments of Arabidopsis infected with the cyst nematode *Heterodera schachtii* (**A-E**) and the root-knot nematode *Meloidogyne incognita* (**F-J**). (**A**) Bright field image of the *H. schachtii* infected root segment, including secondary roots. (**B-D**) Three sequential optical slices of the same *H. schachtii* infected root segment obtained by CLSM showing auxin (blue<green<white (green fire blue) gradient) and cytokinin signalling domains (blue<orange<white (fire) gradient). (**E**) Overlay of the auxin and cytokinin signalling domains and brightfield image. Images taken from Chapter 3 supplementary data MovieS3_Rep3 at 97 hours. Scale bar represents 200 µm. Arrows indicate the head of the nematode. (**F**) Bright field image of the root-knot infected root segment. (**G-I**) Three sequential optical slices of the same *M. incognita* infected root segment obtained by CLSM showing auxin (blue<green<white (green fire blue) gradient) and cytokinin signalling domains (blue<orange<white (fire) gradient). (**E**) Overlay of the auxin and cytokinin signalling domains and bright field image. Images taken at 7DPI. Scale bar represents 200 µm. Arrows indicate the location of giant cells.

Mechanisms underlying auxin accumulation in feeding sites of cyst and root-knot nematodes

In theory, three distinct mechanisms might be employed by plant-parasitic nematodes to manipulate auxin levels: manipulation of polar auxin transport, manipulation of local auxin biosynthesis, and secretion of auxin. In this thesis insight was gained regarding the relevance of these strategies.

Modification of auxin transport within feeding sites

The process of auxin transport is thought to influence the induction and development of nematode feeding sites (Grunewald et al. 2009; Kyndt et al. 2016). PIN proteins facilitate auxin export and the AUX1/LIKE AUX1 (AUX/LAX) family acts as import carriers. Cyst and root-knot nematodes manipulate auxin transport by altering the expression and localization of PIN proteins (Grunewald et al. 2009; Kyndt et al. 2016). For example, during cyst nematode infection, PIN1 and PIN7 are absent from the feeding site, indicating a decrease in downward auxin flow (Grunewald et al. 2009). On the other hand, PIN3 and PIN4 are highly expressed and localized towards the lateral sites of the syncytium. These changes suggest that cyst nematodes block the downward flow of auxin and redistribute it laterally to facilitate auxin accumulation in the feeding site. The change in PIN localisation is reported to occur between 2 and 5 dpi (Grunewald et al. 2009) and is in agreement with the time frame that was observed for the rise in auxin signalling with live-cell confocal imaging of feeding cell development of *H. schachtii* (Chapter 3). Similar to PINs, the AUX/LAX importers seem to be relevant for root-knot nematode infection as mutations in AUX1 and LAX3 bear significantly fewer and smaller galls (Kyndt et al. 2016). In the case that hindering of polar auxin transport towards the root tip would be the sole inductor of the observed auxin accumulation, it might be reasonable to postulate that the rise in auxin signalling in developing feeding sites induced by cyst nematodes would first be observed near the rootward side of the syncytium and would subsequently be observed more at the shootward side. However, no such gradient is observed (Chapter 3). Live-cell confocal imaging shows an increase in auxin signalling that occurs simultaneously across the entire syncytial area (Chapter 3). An alternative explanation, albeit not mutually exclusive, is that the manipulation of PINs by the cyst nematode contributes to auxin accumulation by limiting the efflux of auxin that is produced by local biosynthesis or injected by the nematode.

Induction of auxin biosynthesis within feeding sites

Cyst and root-knot nematodes may also induce auxin biosynthesis within feeding sites (Chapter 2). This has been scarcely studied but might offer insight in the auxin dynamics observed in Chapter 3. Literature indicates that metabolomic precursors of auxin like tryptophan and indole-3-acetonitrile concentrations increase in feeding sites of cyst and root-knot nematodes, which suggests the induction of local auxin biosynthesis (SETTY and WHEELER 1968; Yu and Viglierchio 1964). Furthermore, transcriptome analyses have revealed the upregulation of various auxin biosynthesis genes during nematode infection (Chapter 2). Additionally, the Ethylene Response Factor 9 (ERF109) promotes auxin biosynthesis by binding to the promoters of YUC2 and ASA1 and has been suggested to regulate local auxin biosynthesis at the nematode infection site as DR5::GUS expression was significantly lower in

erf109 mutants (Guarneri et al. 2023). This suggests that auxin accumulation in syncytia is at least partially the result of local auxin biosynthesis. In Chapter 3, a simultaneous and ubiquitous increase in auxin is observed throughout the syncytial area. The local induction of auxin biosynthesis may also explain the simultaneous increase in auxin signalling across the area. As such, our data supports a role for the induction of local auxin biosynthesis. Local auxin biosynthesis is known to act as a key regulator in plant root development (Brumos et al. 2018). It can act separately or in conjunction with polar auxin transport and is involved in processes such as wound regeneration and ethylene responses (Brumos et al. 2018; Matosevich et al. 2020). For example, induction of multiple local auxin sources occurs during root tip regeneration (Matosevich et al. 2020). This regeneration largely relies on cell expansion and cell division. Auxin is specifically activated in wound-adjacent cells and regulates cell expansion, cell division rates and the transcription factor ERF115 that is known to be involved in regeneration (Hoermayer et al. 2020). Cyst nematodes migrate intracellularly through the root in a destructive manner while root-knot nematodes cause wounding near the root tip. With the link between wounding and nematode infection, wound induction might play a role in establishing an auxin accumulation.

Auxin injection into feeding sites by plant-parasitic nematodes

In Chapter 4, our investigations have unveiled that a plant-parasitic nematode, specifically *G. pallida*, possesses the active form of auxin in pre-parasitic juvenile homogenate and the solution in which such infective juveniles were incubated. These findings suggest that infective juveniles from *G. pallida* may secrete auxin to reprogram plant cells in the vascular bundle. This discovery introduces the possibility of nematode-derived auxin serving as a signalling molecule in the formation of feeding sites. One conceivable scenario involves the nematode injecting its auxin into the initial syncytial cell (ISC) during the early stages of the infection cycle, potentially triggering a cascade of reactions leading to the subsequent expansion of the syncytium. However, data from Figure 5 in Chapter 3 does not support the notion of an early induction of auxin signalling near the head of the nematode on that scale. Instead, auxin signalling becomes evident after the first contours of the syncytial area becomes visible with bright-field microscopy and appears to rise simultaneously across the entire syncytial area. Nonetheless, this observation does not exclude a role for nematode-secreted auxin in syncytia formation, as auxin signalling near and around the head of the nematode is relatively high in comparison to the edges, but needs further investigation.

The role of auxin within the phylum Nematoda

Recent research has expanded our understanding of auxin, traditionally viewed as a plant hormone, and revealed its influence in non-plant organisms, such as terrestrial arthropods (Tokuda et al. 2022). This broader role challenges the conventional perspective that confines auxin's function solely to the plant kingdom. In the context of nematode infection, the emphasis has historically been placed on auxin's accumulation during the formation of feeding sites. Expanding upon the non-plant presence of auxin, analyses in Chapter 4 have identified the presence of potential auxin biosynthesis genes in nematodes other than the plant-parasitic ones. This discovery supports the idea that synthesis of auxin is not a unique trait of plant-parasitic nematodes, suggesting a broader role within the phylum Nematoda. As such, it is vital to discern between the possible roles of nematode-derived auxin. It remains

challenging to distinguish whether nematodes utilize auxin for parasitic purposes or that auxin facilitates endogenous functions for nematode development, functioning, and survival. It is plausible that nematode-derived auxin may be employed for both purposes, further complicating the differentiation between endogenous and parasitic roles.

Various endogenous processes in non-plants have been identified which auxin can affect or play a role in. For example, the non-plant-parasitic nematode, *Caenorhabditis elegans*, also contains auxin. In *C. elegans*, auxin acts as an intermediary compound for the production of ascarosides, pheromones regulating sex-specific and social behaviors (Zhou, Zhang, and Butcher 2019). Additionally, auxin appears to impact the lifespan of *C. elegans*, pointing towards potential physiological benefits (Loose and Ghazi 2021). Similarly, mammalian cell lines demonstrate increased proliferation upon auxin treatment, showing auxin's involvement in diverse biological contexts (Cernaro et al. 2015). Interestingly, auxin has been investigated for the prevention of diabetic complications in rats (Al-Malki 2022). The administration of auxins demonstrated potential therapeutic effects, including the reversal of oxidant/antioxidant imbalance, mitigation of oxygen free radicals, inhibition of advanced glycation end products formation, and prevention of inflammatory mediator release. Auxin's presence in terrestrial arthropods, including spiders, mites, crustaceans, and millipedes, adds further complexity (Tokuda et al. 2022). While the exact roles of auxin in arthropods remain uncertain, the higher concentrations of IAA compared to plants and its presence throughout various developmental stages suggest a multifaceted role. In conclusion, the conventional notion of auxin as a plant hormone has evolved. The revelation of auxin's presence and potential functions in non-plant organisms, especially nematodes and arthropods, broaden our perspective of auxin's role. However, the potential dual role of auxin in nematodes for both endogenous as well as parasitic functions hamper the elucidation of its functions. While auxin's contributions to plant growth and development are well-established, it is increasingly apparent that auxin serves various purposes, such as an intermediary biosynthesis compound or as a potent antioxidant, across a diverse array of organisms. The emerging insight into the multifaceted roles of auxin warrants further investigation to unravel its complexities and shed light on the broader biological significance of this hormone.

Reciprocal auxin signalling between nematodes and plants

In plant-pathogen relationships the role of auxin may be greater than just regulating growth related processes. It might instead function as a reciprocal signalling molecule for both host and parasite during infection. Microbial synthesis of IAA has long been known to occur and increasing evidence suggests that IAA can act as a reciprocal signalling molecule in plant-microbe interactions (Spaepen and Vanderleyden 2011). For example, the phytopathogen *Agrobacterium tumefaciens* induces the production of auxin in plants through the transference of the T-DNA region of the Ti plasmid for the development of crown galls on roots (Mashiguchi et al. 2019). In turn IAA acts as a signal molecule in *A. tumefaciens* and inhibits vir gene and chv gene expression through competition with the phenolic signal compound that interacts with the VirA/G system (Liu and Nester 2006). Similarly, expression of a gene encoding an ABC-type auxin transporter was found to be specifically up-regulated by IAA in *A. tumefaciens* (Yuan Ze-Chun et al. 2008). Some indications have been found to suggest that IAA may act as a reciprocal signalling molecule in plant-parasitic nematodes as well. On one hand, our investigations show that *G. pallida* is able to synthesize auxin and also

to secrete auxin (Chapter 3). Moreover, biochemical assays from several decades ago using the Ehrlich and Salkowski chemical tests have suggested the presence of auxin in various cyst and root-knot nematodes (Yu and Viglierchio 1964; Viglierchio and Yu 1968; Hanschke, Borriss, and Kampfe 1975; Agarwal et al. 1985; Ganguly and Dasgupta 1987) and more recently, it was reported that auxin, mainly in conjugated form, could be detected using mass-spectrometry inside *Heterodera schachtii* and *Meloidogyne incognita* (De Meutter et al. 2005). On the other hand, IAA found in plant root exudates induce chemotaxis in the root-knot nematode *Meloidogyne incognita* (R. H. C. Curtis 2008). This is supported by the fact that auxin is able to bind to the chemosensory organs, amphids and phasmids of nematodes as was demonstrated for *Meloidogyne incognita* (R. Curtis 2007). Additionally, it was shown that the surface lipophilicity of *Globodera rostochiensis* decreased when coming into contact with IAA which was suggested to be important for the nematode to adjust to the host's environment (AKHKHA et al. 2002; 2004). Overall, it can be seen that auxin can function in a reciprocal signalling relationship between plants and nematodes during infection.

Mechanisms underlying cytokinin accumulation in feeding sites

Cytokinins are plant hormones that play a crucial role in various physiological processes, including cell division, shoot and root development, and responses to environmental stresses (Vankova 2014). In the context of plant-parasitic nematodes, specifically cyst and root-knot nematodes, cytokinins have been implicated in the formation of feeding sites. The accumulation of cytokinins (Chapter 3) in feeding sites is thought to be crucial for the nematodes' successful parasitism (Lohar et al. 2004; Shanks et al. 2016). In theory, three distinct mechanisms might be employed by nematodes to manipulate cytokinin levels in plant cells: manipulation of transport, induction of local biosynthesis, and secretion by the nematode.

Modification of cytokinin transport within feeding sites

Cytokinin transport plays a pivotal role in regulating cytokinin levels across various plant tissues. Despite the lack of specific research into cytokinin transport manipulation during nematode infection, a fundamental understanding of cytokinin transport in plants provides insights into potential mechanisms that might be exploited by nematodes during parasitic interactions. The transport of cytokinins within plants predominantly relies on three classes of transporters: purine permeases (PUP) and equilibrative nucleoside transporters (ENT and the G subfamily of ATP-binding cassette (ABCG)) transporters (Durán-Medina, Díaz-Ramírez, and Marsch-Martínez 2017). The process of cytokinin import is chiefly mediated by PUP and ENT transporters. These importers allow the uptake of cytokinin from external sources or transportation between plant organs. On the other hand, cytokinin export, crucial for maintaining appropriate hormone levels, is managed by ABCG transporters. These transporters ensure the efflux of cytokinins from cells into the intercellular space or the vascular system for long-distance movement. Considering the complex interplay of cytokinin transport in healthy plants, it's reasonable to hypothesize that nematodes could exploit these existing pathways to manipulate cytokinin distribution. This could potentially lead to the accumulation of cytokinins in nematode feeding sites, promoting the formation of feeding sites. A similar feat is observed during haustorium formation by the hemiparasitic plant

Phtheirospermum japonicum which transports endogenous cytokinin to modify host morphology and fitness and inducing hyperthropy (Spallek et al. 2017). Chapter 3 shows a wide-spread and steep increase in cytokinin signalling during infection. This signalling might be enhanced through modifying transport and as such, manipulation of cytokinin transporters could be an important way in which nematodes accumulate cytokinin. To comprehensively understand the role of cytokinin transporters and their potential manipulation by nematodes, investigating whether nematodes target and alter the localisation or expression of specific cytokinin transporter genes could provide valuable insights into the molecular basis of this intricate interaction.

Induction of cytokinin biosynthesis within feeding sites

In Chapter 3 a surge in cytokinin signalling throughout the entire nematode feeding site was seen during infection which can potentially be explained by the induction of localized cytokinin biosynthesis. Cytokinin biosynthesis in plants is regulated by several gene families, with isopentenyltransferase (IPT) playing a crucial role. It is noteworthy that during nematode infection, the genes associated with cytokinin biosynthesis exhibit differential expression patterns in plants infested with cyst and root-knot nematodes (Dowd et al. 2017; Shukla et al. 2018). During infections by the cyst nematode, *Heterodera schachtii*, a distinct upregulation of adenylate IPT genes, notably IPT1, is evident in the course of developing syncytia. This is in contrast to the response observed in root-knot nematode infections, wherein a parallel induction of IPT1 expression is conspicuously absent (Dowd et al. 2017). This dissimilarity in cytokinin biosynthesis profiles implies a nuanced manipulation of cytokinin levels by different kind of nematodes, meticulously tailored to align with their parasitic strategies. Furthermore, it is pertinent to mention that a comprehensive exploration of cytokinin biosynthesis in the context of nematode feeding site formation remains incomplete. Cytokinin biosynthesis is known to encompass the involvement of CYP (cytochrome P450) genes and LOG (LONELY GUY) genes (Vankova 2014). Notably, neither the CYP genes nor the LOG genes have been directly investigated with regards to their potential roles in nematode feeding site development.

In addition to the above, one should acknowledge the broader role of cytokinin biosynthesis in plant-pathogen interactions. Indeed, cytokinin levels are subject to modulation in both the cortical and epidermal cell layers during various pathogenic interactions. For instance, robust cytokinin signalling has been documented in root cortical cells during nodule initiation and development with *LjIpt2* and *LjLog4* being identified as the major contributors to this in *lotus japonicus* (Reid et al. 2017).

Cytokinin injection into feeding sites by plant-parasitic nematodes

It has been previously reported that the cyst nematode *H. schachtii* produces and secretes cytokinins, which significantly enhances its infective abilities (Siddique et al. 2015). However, whether this is a feature that other nematodes possess as well is unclear. In Chapter 4 it is also revealed that *G. pallida* contains cytokinins at relatively high levels and suggests that *G. pallida* might secrete cytokinin as well, maybe for proper feeding site formation. Additionally, a bioinformatic analysis to search for IPT genes in other nematode species according to the method in Chapter 4, shows that IPT seems to be widespread among nematode species

further supporting the idea that the use of cytokinin during infection might not solely occur in *H. schachtii* (Chapter 4, Supplementary Figure 7). Chapter 3 of this thesis, however, indicates that the injection of cytokinins may not play a sole role during the initial phases of feeding site formation. This is primarily due to the observed uniform increase in cytokinin levels across the feeding site, rather than exhibiting a slow gradient-wise distribution originating from the nematode's head. This finding suggests that injected cytokinins might not be the sole driving force behind the various stages of feeding site development. As such, the injection of nematode-derived cytokinins may contribute to the complex interplay between the nematode and its host in concert to other methods such as local biosynthesis and transport.

The role of cytokinin in the phylum Nematoda and other kingdoms

In Chapter 4 it was shown that cytokinin is present in the cyst nematode *Globodera pallida* and, may, similar to what has been demonstrated for auxin in the cyst nematode *H. schachtii*, contribute to a successful infection (Siddique et al. 2015). Other plant attacking organisms, like phytopathogenic bacteria, fungi, and gall-inducing insects have been shown to produce cytokinins as well, which underlines a role for cytokinin in plant interactions (Andreas et al. 2020). However, the cytokinin biosynthesis gene IPT is also found in the bacterivorous species *C. elegans* (Siddique et al. 2015), indicating that cytokinin might have additional endogenous functions in nematodes. Also insects, including non-gall-inducing species, have been found to possess cytokinins (Andreas et al. 2020). Moreover, not only insects but most organisms seem to synthesize cytokinin (Murofushi et al. 1999). When speculating about the potential endogenous functions of cytokinin it should be noted that the role of cytokinin in plants has encouraged people to focus on their potential effects in mammals with the aim of treating human diseases (Fathy et al. 2022). As such, it was discovered that cytokinins possess antioxidant properties and can modulate cellular aging processes in mammalian cell lines (Othman et al. 2016). Similarly, cytokinins increase stress resistance and longevity of *C. elegans* (Kadlecová et al. 2018). Furthermore, they seem to have potential neuroprotective, immunomodulatory, and anti-proliferative effects in mammalian cell lines (Fathy et al. 2022). Thus, in nematodes cytokinins might be utilized both for parasitic interactions with plants as well as endogenous physiological functions. Distinguishing between these functions might prove challenging as discussed previously for auxin.

Spatiotemporal dynamics of auxin and cytokinin signalling during feeding site development

Overlap and crosstalk between auxin and cytokinin signalling

This thesis (Chapter 3) has identified a rapid and substantial increase in cytokinin signalling as one of the earliest events in the infection process of the beet cyst nematode *H. schachtii*. This cytokinin signal reached a plateau, remaining constant throughout as feeding site formation progressed. Intriguingly, this cytokinin response was heterogeneous, encompassing various cell types, including those outside of the syncytial area. In contrast, the increase of auxin signalling manifested after an initial lag phase and only after the contours of the syncytial area were visible under brightfield microscopy. Furthermore, the auxin signal was confined to the syncytial area. This all indicates that cytokinin signalling precedes auxin signalling in syncytia, marking a paradigm shift from previous assumptions that auxin played an initiating role in syncytium development. Additionally, in contrast to developmental processes in developing vascular tissue in the root tip (Bishopp et al. 2011; Wybouw and De Rybel 2019; De Rybel et al. 2014), in the syncytial area high auxin signalling intensities within nuclei are always linked with high cytokinin signalling intensities. With these overlapping signalling domains of auxin and cytokinin revealed by the double reporter, one can speculate about how these two key plant hormones might interact and influence plant-nematode interactions.

One intriguing aspect of the crosstalk between auxin and cytokinin is the possibility that cytokinin induces auxin accumulation, which aligns with the observations in Chapters 3 during nematode infection where cytokinin signalling precedes auxin signalling in the syncytial area. In root tips, high auxin and cytokinin signalling intensities are not observed in the same cells, and signalling maxima are characterized by either high auxin/cytokinin ratios or low auxin/cytokinin ratios (Chapter 3). However, during cyst nematode infection these domains do not appear as separate and instead coincide in the syncytial area. It is as of yet unclear how auxin and cytokinin signalling interact with each other within the syncytial area. Interaction between auxin and cytokinin signalling in previous reports have unveiled a reciprocal relationship between auxin and cytokinin biosynthesis within developing root such as the root apex as well as shoot tissues, demonstrating that exogenous cytokinin application or induced cytokinin biosynthesis prompts a rapid increase in auxin production (Ohashi-Ito et al. 2019; B. Jones et al. 2010). Furthermore, reducing endogenous cytokinin levels leads to a corresponding reduction in auxin biosynthesis. In the context of plant-nematode interactions, a similar mechanism may be at play, where cytokinin signalling induces the expression of auxin biosynthesis genes in syncytial cells, leading to localized auxin biosynthesis within the feeding site. However, the reverse might also be the case during nematode infection. The auxin induced LONESOME HIGHWAY (LHW) and TARGET OF MONOPTEROS5 (TMO5)/TMO5-LIKE1 (T5L1) dimer complex, can promote the expression of cytokinin biosynthesis genes, such as LONELY GUY3 (LOG3) and LOG4, thereby facilitating localized cytokinin production (De Rybel et al. 2014). Thus, previous reports have also shown that in certain contexts auxin can stimulate cytokinin biosynthesis. This implies that auxin and cytokinin can play a pivotal role in regulating each other's biosynthesis, serving as an inducer under specific conditions. The precise molecular mechanisms by which these hormones modulate biosynthesis during nematode infection warrant further exploration, but the data thus far hint at a potential direct

and reciprocal relationship between these two hormonal pathways. However, this relation has not yet been explored in the context of plant-nematode interactions.

Another intriguing facet of the auxin-cytokinin interplay is the potential influence of cytokinin on the polar auxin transport machinery, particularly the PIN-FORMED (PIN) auxin transporters. Modification of auxin transport has been shown to occur during infection of cyst nematodes as a result of changes in polarity of PIN proteins (Grunewald et al. 2009). As such, cytokinin might play a role in affecting PIN localization during infection. Research has unveiled that cytokinin-responsive transcription factors, known as CYTOKININ RESPONSE FACTORS (CRFs), can directly regulate the expression of PIN genes (Šimášková et al. 2015). This transcriptional control, mediated through specific PIN CYTOKININ RESPONSE ELEMENT (PCRE) domains, enables cytokinin to fine-tune auxin transport capacity. By affecting PIN protein abundance and localization, cytokinin may redirect the auxin flow within syncytial cells, influencing the direction of auxin movement. This mechanism could explain the observed auxin accumulation within the developing feeding site. Cytokinin enhances the depletion of the PIN1 auxin transporter at precise polar domains, leading to a rearrangement of PIN polarities within plant cells. This selective sensitivity to cytokinin appears to be correlated with the degree of phosphorylation of PIN proteins. As such, the increase in cytokinin signalling during infection might lead to the changes in PIN polarity of the initial feeding cell as described in Grunewald et al. Importantly, it has been reported that, based on transcriptome analyses, cytokinin signalling is dominant over auxin signalling during cyst nematode infection, while this is opposite during root-knot nematode infection (Cabrera, Fenoll, and Escobar 2015). This supports our data where auxin signalling in the syncytial area is lower than cytokinin signalling (Chapter 3). Additionally, this aligns with our preliminary data showing distinct higher auxin signalling levels in galls (Figure 1). This intriguing contrast in auxin and cytokinin concentrations and/or ratios between syncytia and giant cells raises the question whether these differences may explain the development of dissimilar feeding sites by cyst and root-knot nematodes.

In summary, the signalling maxima of auxin and cytokinin seem to overlap during feeding site formation of cyst nematodes with the level of auxin signalling being lower in syncytia compared to root tips and the level of cytokinin signalling being higher. Within the syncytial area the highest level of auxin signalling is always paired with a high level of cytokinin signalling and vice versa. This is in contrast to the auxin and cytokinin signalling maxima in the stele of the root tip (Bishopp et al. 2011; Wybouw and De Rybel 2019; De Rybel et al. 2014). During vascular tissue formation sharp boundaries are generated between auxin and cytokinin signalling maxima by a genetic network in which, among others, auxin represses the cytokinin response (De Rybel et al. 2014). Apparently, this genetic network is not operating within syncytia. Similar patterns are observed in the root cap, quiescent centre and neigbouring stem cells (Fisher et al. 2018; Wu et al. 2021; Petersson et al. 2009), although the precise positions of the signalling maxima may differ depending on the reporter construct used. Also direct measurements and predictive modelling of auxin and cytokinin concentrations may deviate from the expression patterns of reporter genes (Moore et al. 2024). Nevertheless, reporter genes are widely accepted as a proxy for hormone concentrations to study the tightly regulated crosstalk between auxin and cytokinin, but should be interpreted within the appropriate context and in a comparative way. Our data indicate that the auxin-cyokinin signalling ratios in root tips revealed by the double reporter

deviate markedly from those in the syncytial area. The overlap between auxin and cytokinin signalling maxima inside the syncytial area might be the result of the induction of local auxin biosynthesis and the dysregulation of PINs by cytokinin resulting in aberrant cytokinin-auxin signalling ratios.

Advantages of quantitative live-cell imaging using an auxin-cytokinin double reporter

In the realm of plant-nematode interactions, understanding the intricate hormone signalling dynamics within host plants during nematode infection has been a longstanding challenge. Traditionally, this investigation relies on hormone reporters such as DR5::GFP or DR5::GUS for auxin (Absmanner, Stadler, and Hammes 2013; KARCZMAREK et al. 2004). Previous studies often focused on a single hormone reporter thereby leaving potential relationships between hormones unexplored. Moreover, the conventional methodology in plant nematology involves infecting numerous plants and capturing images at specific timepoints of randomly selected samples. This results in asynchronous infection stages being imaged due to variations in nematode infection speeds. Additionally, a single daily imaging session will fail to capture signalling dynamics occurring throughout the day. Furthermore, data analysis is often qualitative, relying on visual assessment of signal intensity changes between different timepoints. In contrast, the novel approach in this thesis employed a meticulous selection of infected plants to ensure uniformity in nematode infection stages at the start of the measurements. This was done by selecting plants with a single infection spot containing a single nematode. Further selection was done based on uniform behavior as they were selected to be at the end of their migratory phase and specifically had started probing cells as an indication for the formation of an infection site.

This thesis employs an auxin-cytokinin double reporter system with a nuclear localisation signal. This reporter enables the visual examination of both auxin and cytokinin signalling simultaneously in each pixel, thereby allowing the comparison in nuclei in and around the syncytial area. In addition to combining the use of an auxin-cytokinin double reporter with time-lapse microscopy, this study has introduced a semi-quantitative approach to assess hormone signalling. This departure from evaluations that rely on visual distinction for a classification of higher or lower, offers a more nuanced perspective on the signalling dynamics within the host plant during nematode infection. Through semi-quantitative measurements, this research has provided valuable insights into the relative strength of cytokinin and auxin signalling within nematode-induced feeding sites. Notably, it has been discerned that cytokinin signalling within feeding sites of *H. schachtii* exceeds the cytokinin signalling observed in root tips. This finding suggests a targeted and enhanced cytokinin response within the feeding site. In contrast, auxin signalling within these feeding sites appears comparatively weaker, highlighting the distinct signalling dynamics employed by cyst nematodes compared to normal root development. Moreover, the use of a nuclear localisation tag has allowed for a more precise demarcation of the auxin and cytokinin signalling domains.

Another novel facet of this research is the application of time-lapse microscopy with the use of fluorescent reporters within the context of nematode infection. Previous applications of time-lapse microscopy have helped in establishing fundamental knowledge about nematode infections, having used differential interference contrast microscopy to show close up movies of nematodes invading and inducing feeding cells in their host (U Wyss and Zunke 1986; Urs

Wyss 2000). Our approach includes on top of time-lapse microscopy, the use of fluorescent reporters and marks a departure from traditional static analyses in the field, due to the real-time monitoring of auxin and cytokinin signalling dynamics throughout the course of infection. Through time-lapse microscopy, this study has discerned critical temporal relationships between cytokinin and auxin signalling. Specifically, it has been observed that cytokinin signalling precedes the onset of auxin signalling during infection. This temporal information has revealed that the initiation of feeding sites also precedes auxin signalling. Furthermore, in this method one observes a single nematode and thus individual differences become clear in terms of the speed and intensity of hormonal responses over the course of infection. For example, it is noted that there is significant variation in the intensity of auxin and cytokinin signalling between samples, suggesting that the strength of signalling differs between individual nematode infections.

In conclusion, the innovative use of an auxin-cytokinin double reporter, time-lapse microscopy, and semi-quantitative measurements have significantly advanced our comprehension of auxin and cytokinin dynamics during plant-nematode interactions. This novel approach has shown that cytokinin signalling precedes auxin signalling during feeding site development, how their expression profiles are correlated in both space and time, and how cytokinin and auxin signalling intensities differ between syncytia and root tips. However, the laborious nature of this method does not advocate for its usage in large scale routine analyses. Regardless, this multifaceted study offers a new approach for investigating hormonal signalling in the context of plant-nematode interactions, offering a promising method for future research in this field.

Pitfalls and mitigations in using the auxin-cytokinin double reporter

The application of the auxin-cytokinin double reporter in the investigation of plant-nematode interactions brings forth a powerful tool for dissecting hormonal signalling dynamics within the host plant. However, it is imperative to recognize and address certain inherent limitations associated with this methodology to ensure the accuracy and reliability of the obtained results.

One notable challenge in employing the auxin-cytokinin double reporter system lies in the potential impact of laser-induced photobleaching during the measurement process, particularly when employing confocal microscopy. Photobleaching, characterized by the irreversible loss of fluorescence in the reporter proteins due to laser exposure, can compromise the precision of signal quantification. In this research, meticulous attention was devoted to mitigating this issue through the careful adjustment of laser power settings to levels that minimize photobleaching. Additionally, microscopy settings were calibrated to adequately visualize auxin and cytokinin signals from root tips prior to the start of the experiment. This strategic adjustment serves to sustain the integrity of the fluorescent reporters, thus preserving the accuracy of the data. Another noteworthy consideration is that the fluorescent reporter proteins employed in this system possess a finite half-life, approximately on the order of a day in the case of GFP (Corish and Tyler-Smith 1999). Consequently, the signal detected represents a cumulative measure of current and past gene expression activity. In instances where the double reporter signal appears static over a 24-hour period, it is essential to realize that this phenomenon does not imply an absence of

hormonal activity. Rather, it suggests a dynamic equilibrium between fluorescent reporter synthesis and degradation. As such, a stable unchanging signal of over a day, as is the case for the cytokinin signalling observed during cyst nematode infection (Chapter 3), still represents active signalling at that level.

A fundamental aspect of the auxin-cytokinin double reporter system is the inherent delay in signal production. Unlike direct hormone detection methods, this system relies on the activation of reporter genes downstream of hormone effects. Thus, the observed signal is contingent on the time required for protein synthesis following hormone exposure. Although this delay is relatively brief, in the order of minutes, it represents an intrinsic limitation that warrants consideration when interpreting data. However, as the time interval in this study is half an hour the effect is negligible. The sensitivity of the auxin-cytokinin double reporter system is dependent on various factors, including the promoter regions of the reporter genes and microscopy settings. In this research, the selected microscopy settings were thoughtfully tailored to visualize auxin and cytokinin signals at levels comparable to those in root tips. With live-cell imaging using confocal microscopy, the effect of depth on a fluorescent signal is a critical consideration. Optical sectioning, involves capturing light from a specific depth to create images of specific layers within a specimen. However, when imaging thick tissues, some of the fluorescent signal is blocked by the tissue layers above the desired optical slice, resulting in differences in signal intensity between different optical layers. In this particular study, this depth-related variation in signal is not applicable since all comparisons are made within the same optical layer. This controlled approach eliminates the influence of tissue depth on the fluorescent signal, ensuring consistent and reliable data for the investigation of hormonal dynamics.

With the use of the auxin-cytokinin double reporter the signalling events are visualized during infection. Although this is an indication of the auxin and cytokinin concentration in the cell, it is difficult to make quantitative statements about their concentrations based on the output of the reporter. This difficulty mainly arises from the fact that not all the hormone leads to signalling and that the two hormones do not lead to equal amounts of signal. Here, this issue is avoided by making statements about the relative amount of signalling (as indicated by the relative mean intensity) between two different timepoints. As such, this relative mean intensity (RMI) allows us to quantify the relative changes in reporter signals between different timepoints and areas. In conclusion, the application of the auxin-cytokinin double reporter in studying plant-nematode interactions has several limitations and pitfalls. However, by being aware of these and in using the right microscopy settings and analysis methods these can be mitigated.

Developmental pathways recruited by nematodes

Feeding site formation and lateral root development in plants are thought to be closely linked processes driven by shared molecular mechanisms (Abril-Urias et al. 2023; Lohar et al. 2004; Cabrera et al. 2014). Both involve the action of auxin, cytokinin, and specific transcription factors. For example, the LATERAL ORGAN BOUNDARIES-DOMAIN (LBD) 16 gene, pivotal in lateral root formation, is also critical for gall development induced by root-knot nematodes thereby indicating its central role in both processes (Cabrera et al. 2014). In contrast,

cytokinins exhibit divergent effects, inhibiting lateral root formation but promoting RKN-induced gall formation (Lohar et al. 2004). Shared processes might suggest that the developmental programs of lateral root development might play a role in nematode feeding site development (Cabrera et al. 2014).

Due to this potential overlap, we investigated the role of PLETHORA (PLT)3, PLT5 and PLT7 in the infection process of *Meloidogyne incognita* in Chapter 5. PLT3, PLT5 and PLT7 play an important role during lateral root formation where they are involved in the development and spacing of root primordia as well as lateral root emergence (Hofhuis et al. 2013). This is exemplified by the *plt3plt5plt7* triple mutant where lateral root emergence is severely restricted. It furthermore develops a main root that is lined with clustered lateral root primordia in developmental arrest (Hofhuis et al. 2013). It should be noted that PLT3, PLT5 and PLT7 operate downstream of ARF7 and ARF19 and might regulate the localisation and expression of PIN proteins, specifically PIN1 and PIN3 (Du and Scheres 2017). In our research, the *plt3plt5plt7* triple mutant showed a consistent decrease in the number of galls in addition to less adult females with egg masses. Additionally, the infection led to the formation of an aberrant gall phenotype at 42 days post inoculation. With reporter studies, we showed that in the triple mutant PIN3-EGFP expression disappears after 3 days post inoculation and that auxin signalling is enhanced as shown by DR5-EGFP. Preliminary data revealed that single mutants of PLT3, PLT5 and PLT7 bear more galls, with *plt5* having more females with egg masses than wild type but *plt3* and *plt7* having less. These data indicate, that the PLT genes (PLT3, PLT5, and PLT7) do not operate redundantly as they do in the emergence of lateral roots.

The non-redundant mode of action of PLT genes during root-knot nematode infection indicates that the developmental pathways of lateral root formation and giant cells are distinct. This is further supported by the ARF7/ARF19 double mutants that displays no impairment in gall formation during nematode infection as PLT3, PLT5 and PLT7 work downstream of ARF7/ARF19 (Olmo et al. 2020). This suggests that PLT activation in the context of gall formation deviates from the canonical lateral rooting pathway (Abril-Urias et al. 2023; Olmo et al. 2020; Feng et al. 2012). Aside from the canonical lateral rooting pathway an additional pathway for the development of adventitious roots exists (Bellini, Pacurar, and Perrone 2014). On the molecular level, the induction of lateral roots and adventitious roots distinguishes itself through specific pathways, with the involvement of distinct genes. In adventitious root formation, there's a distinctive involvement of WUSCHEL-Related Homeobox (WOX) genes that specify founder cells crucial for root initiation (Bellini, Pacurar, and Perrone 2014). These mechanisms intertwine with various plant hormones, such as auxins, cytokinins, ethylene, abscisic acid, gibberellins, jasmonate, brassinosteroids, and strigolactones, exerting context-dependent effects on root development (Bellini, Pacurar, and Perrone 2014). Unlike lateral roots, adventitious lateral roots follow a different initiation pathway, with WOX 11 playing a key role in 'adventitious lateral rooting'. This process is marked by the induction of LBD16 through WOX11, whereas lateral root formation relies on ARF7 and ARF19. Intriguingly, LBD16 links secondary root formation to nematode infection, as evidenced by its importance in gall formation. This connection extends to leaf explants, where LBD16 is essential for adventitious root formation and gall development, emphasizing its pivotal role across various root types (Sheng et al. 2017; Ge et al. 2019; Cabrera et al. 2014; Olmo et al. 2017).

In contrast to the extensive understanding of PLT3, PLT5, and PLT7 in canonical lateral root formation, their roles in adventitious rooting processes remain relatively unexplored, highlighting a gap in our knowledge. Moreover, it has been proposed that modules from root apical meristem (RAM) maintenance pathways also contribute to gall formation, as demonstrated by the significance of key transcription factors (SCR, SCZ, and SHR) in successful nematode infections (Olmo et al. 2020). Remarkably, galls share molecular components, including LBD16, with developmental pathways related to root regeneration, callus induction, and wound-induced callus formation. Triple mutants have further underscored the importance of PLT3, PLT5, and PLT7 in adventitious rooting, wound-induced callus formation, and de novo shoot regeneration, demonstrating their multifaceted roles in diverse plant developmental pathways (Du and Scheres 2017; Hofhuis et al. 2013). In conclusion, our experimental findings suggest that PLT3, PLT5, and PLT7 play a role in root-knot nematode infection but not through the lateral root pathway as this does not seem to be active during infection. However, the precise pathway PLT3, PLT5 and PLT7 are active in during infection remains unclear.

Future Research

Developmental stages of nematode feeding site

Plant-nematode interactions represent intricate processes involving a myriad of molecular, cellular, and physiological events. Current research on these interactions is characterized by analysis of various metrics that cover the entirety of the infection period. This includes examining the number of female nematodes, alongside assessing the size of the feeding sites as well as their morphology. However, in doing so only a generalized overview of the infection process can be acquired. Thus, in the pursuit of elucidating plant-nematode interactions, it may be beneficial to take a stepwise approach by dividing the development of nematode feeding sites into distinct stages, much like the developmental stages observed in lateral root formation and nodule formation (Malamy and Benfey 1997; Xiao et al. 2014). Nodule development in the plant-rhizobium interaction, can be divided into three overlapping stages: pre-infection, nodule initiation, and differentiation (Eckardt 2006). In the pre-infection stage, specific flavonoids released by the host roots serve as chemoattractants for the rhizobial symbiont and activate the expression of nod genes, leading to the formation of nodules. Similarly, during lateral root formation, several steps, including pericycle priming, founder cell specification, and lateral root initiation, contribute to the final outcome of lateral root emergence (Torres-Martínez et al. 2019). Dividing these processes into various stages has distinct advantages. Firstly, it provides a structured framework for a complex biological process which allows for easier identification of regulatory factors at each stage. Secondly, the division in stages allows for a clear overview of sequential developmental events giving a greater temporal resolution. Lastly, it allows for easier comparison between different species and conditions leading to greater understanding of their development.

In the context of plant-nematode interactions, it is essential to recognize that various processes are specific to particular stages of feeding site development. Investigating these processes across the entire developmental timeline can be challenging. Instead, by focusing

on specific stages, valuable insights may be gained into the mechanisms at play. The delineation of specific stages in the development of feeding sites induced by cyst and root-knot nematodes has remained elusive. However, in the endeavor to unravel the intricacies of these interactions, a framework consisting of five distinct stages can be proposed. This framework is rooted in the striking similarities observed between the feeding sites of cyst and root-knot nematodes and those induced by other plant-parasitic nematodes. By adopting this approach, one can aim to establish a common ground for understanding the intricate processes underlying feeding site formation in nematodes.

One hypothesis is that the first three stages (Figure 2) in the formation of feeding sites induced by cyst nematodes and root-knot nematodes might share common mechanisms as can be inferred from the various types of feeding cells seen within the phylum Nematoda (Smant, Helder, and Goverse 2018). For instance, the first stage could involve the induction of non-hypertrophied uninucleate nurse cells, as seen in *Mesocriconema xenoplax* (Hussey, Mims, and Westcott 1992). These cells exhibit specialized cellular alterations that may resemble the initial feeding cells selected by root-knot and cyst nematodes. The second stage might involve the formation of uninucleate giant cells without secondary cell wall ingrowths, as observed in *Meloidodera mexicana* and *Longidorus elongatus* (Hernandez et al. 2017). It is noted that the feeding site of *Longidorus elongatus* starts with the induction of a uninucleate giant cell followed by cell division giving rise to two to four enlarged daughter cells. This second phase can be considered as stage III in the development of syncytia and giant cells (Figure 2). While these uninucleate cells differ in their origin and number, the shared characteristic is the absence of secondary cell wall ingrowths. This similarity suggests a potential commonality in the mechanisms underlying giant cell formation in root-knot nematodes and cyst nematodes. These observations lead to the hypothesis that the uninucleate hypertrophied cells without secondary cell wall ingrowths may represent a steppingstone for both giant cells and syncytia. The development of syncytia, like giant cells, involves swelling of the initial feeding cells.

For root-knot nematodes the two subsequent stages are distinct from cyst nematodes. Stage IV involves the development of polynucleate giant cells without secondary cell wall ingrowths, as observed in *Xiphinema index* (Urs Wyss 1978). These multinucleate hypertrophied cells emerge from acytokinetic mitosis, a hallmark of giant cells induced by root-knot nematodes. This step represents a pivotal point where the developmental programs of giant cells and syncytia begin to diverge. In stage V, polynucleate giant cells with extensive secondary cell wall ingrowths are encountered, which are characteristic of root-knot nematodes such as *Meloidogyne* spp. These giant cells result from repeated rounds of acytokinetic mitosis and feature hypertrophy, enlarged nuclei, dense protoplasm, and extensive secondary cell wall ingrowths, ultimately distinguishing them from earlier stages.

For cyst nematodes, stage IV entails the formation of syncytia with partial cell wall dissolution. *Nacobbus aberrans*, an informative species, induces syncytia that resemble both giant cells and syncytia (Eves-van den Akker, Lilley, Danchin, et al. 2014). These syncytia result from incomplete cell fusions and may comprise more than 150 individual cells. This stage highlights the unique process of syncytium formation by cyst nematodes and is assumed to mark the divergence of giant cell and syncytium formation. Stage V involves the formation of full-grown syncytia with extensive secondary cell wall ingrowths, found in species like *Globodera*

rostochiensis and *Heterodera glycines*. These syncytia expand within the root, featuring hypertrophy, thickening of cell walls, and numerous cell fusions, primarily within the stele. These distinct steps in nematode feeding site development not only provide a roadmap for understanding the complex processes involved but also highlight the remarkable diversity in these parasitic interactions. By dissecting and comparing these stages across different nematode species, one can aim to uncover the underlying mechanisms that drive the formation of these unique feeding structures.

In summary, adopting a stepwise approach to investigate the developmental stages of nematode feeding sites, akin to nodule formation and lateral root development, holds promise for unraveling the intricacies of these interactions. By focusing on specific stages and drawing parallels to well-studied processes, we may bridge gaps in our understanding and ultimately contribute to the development of novel strategies for nematode control in agriculture.

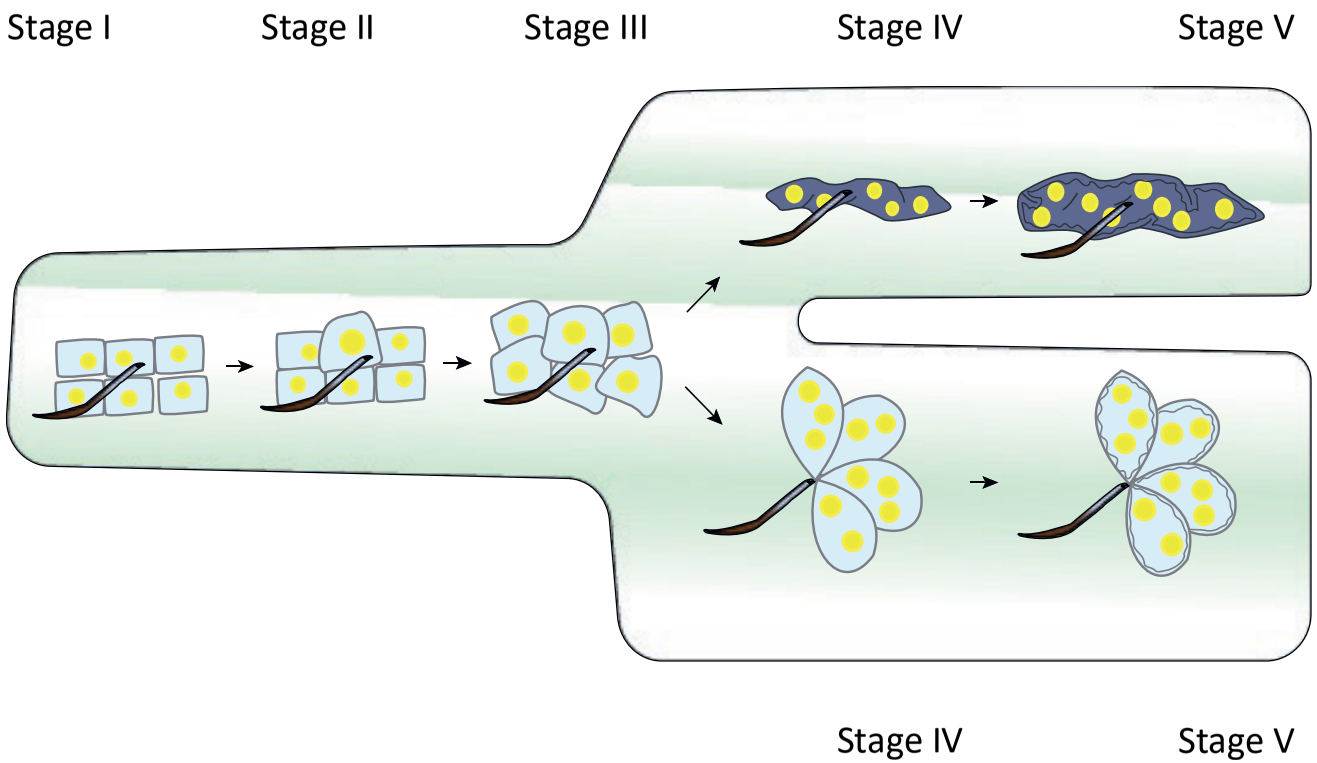


Figure 2 Postulated stages for the evolution of syncytia induced by cyst nematodes (top) and polynucleate giant cells induced by root-knot nematodes (bottom). Cells are represented in blue with yellow nuclei. Stages I-III show feeding on a single non-hypertrophied cell (stage I), induction of a single hypertrophied cell (stage II) and induction of several hypertrophied uninucleate nurse cells with enlarged nuclei. In the top row Stage IV and V show the divergence from a possible common developmental pathway into syncytia through partial cell wall dissolution with extensive cell wall ingrowths in stage V. The bottom row shows stage IV and V for the development of polynucleate giant cells with extensive cell wall ingrowth in stage V. (Further explanation see main text.)

Bridging the gap between hormone signalling and gene expression

Chapter 3 delved into the intricate signalling dynamics of auxin and cytokinin during cyst nematode infection. While we have obtained detailed spatial and temporal insights into hormone signalling patterns, there exists a critical need to link these patterns to specific gene expression events triggered by these signalling processes within the nematode feeding site. As it currently stands there is a gap between hormone signalling profiles and induced gene expression. To address this challenge, we advocate for the adoption of a state-of-the-art methodology called tomo-seq or similar technologies (Kruse et al. 2016). Tomo-seq is a method that combines traditional RNA sequencing with a microscopy-based spatial resolution. The technique involves cryosectioning of an embryo or tissue of interest, followed by RNA-seq analysis on individual sections. This approach allows one to obtain genome-wide expression data while preserving spatial information. Tomo-seq has already been successfully applied in diverse biological contexts, including the study of zebrafish embryogenesis and heart regeneration (Kruse et al. 2016). With tomo-seq, it is possible to create spatial gene expression profiles at a specific time point during cyst nematode infection. By examining a series of tomo-seq samples over the course of nematode feeding site development, one can construct a rough timeline of gene expression dynamics. This timeline will provide valuable insights into the genetic events that occur as the feeding site matures. To align the specific timepoints of the tomo-seq with the auxin signalling pattern the expression profile of the GH3 gene can be used. This alignment is feasible because the promoter region used in the creation of the auxin reporter, which facilitated the profiling of auxin signalling (Chapter 3), is based on the GH3 gene. This synergy between gene expression data and hormone signalling profiles will enable us to observe how and where specific genes are activated as the feeding site grows. In practical terms, combining live-cell imaging to monitor auxin or cytokinin signalling with selected tomo-seq time points will allow one to correlate hormone signalling dynamics with gene expression patterns. This integrated approach promises to unravel the intricate molecular processes underlying plant-nematode interactions with unprecedented detail and bridge the gap between reporter signalling dynamics and gene expression.

In conclusion, the integration of tomo-seq into our research arsenal holds the potential to bridge the gap between observed hormone signalling dynamics and gene expression events during cyst nematode infection. This innovative technique will pave the way for a deeper understanding of the molecular mechanisms governing these interactions, ultimately facilitating the development of targeted strategies for nematode control in agriculture.

A Bottom-Up Approach: Focusing on Specific Downstream Events

The hormones auxin and cytokinin exhibit a multifaceted role in plant-nematode interactions, influencing both host plant defenses and nematode parasitism strategies. While this is an intriguing avenue of research, it presents a significant challenge, as auxin and cytokinin also serve critical functions in normal endogenous plant processes essential for plant growth and development. The multifunctional roles of auxin and cytokinin in plants extend to regulating growth, development, and stress responses, indicating their indispensability for proper plant functioning and survival. Consequently, studying their specific impact on plant-nematode interactions becomes a challenging task. The traditional scientific approach involves disabling or manipulating specific components of a process to discern their individual contributions.

However, when it comes to auxin and cytokinin, the situation is complicated by their broad and pleiotropic effects on various plant functions. The pleiotropic effects of auxin and cytokinin present a significant issue when attempting to investigate their roles in plant-nematode interactions. Disrupting hormonal processes could result in a wide range of effects, potentially affecting not only the interaction under investigation but also the overall fitness and health of the plant. For example, the double *aux1/lax3* and quadruple mutant *aux1/lax1/lax2/lax3* display a marked decrease in infection but are also known to disrupt plant development (Swarup and Bhosale 2019; Mazarei et al. 2003). This makes it difficult to attribute specific changes in the interaction solely to the absence of these hormones, as the observed outcomes might be the result of pleiotropic effects rather than the direct influence of auxin or cytokinin on the interaction.

In light of these challenges, a potential solution to uncover the roles of auxin and cytokinin in plant-nematode interactions may lie in adopting a bottom-up approach. Instead of directly manipulating hormonal processes, one could start by investigating specific physiological and cellular events during infection. By starting with such events one can discover the direct factors that are responsible for the event. Subsequently, it then becomes possible to elucidate the regulators of these direct factors, followed by looking into the next regulatory layer. All these regulators of all these events will eventually converge into master regulators, most likely including hormones, and all lead further back to the nematode. For instance, the modification of cell walls is a critical process that occurs in both cyst and root-knot nematodes, but it is followed by distinct outcomes. By focusing on known factors directly associated with these processes, one can pinpoint the key components and molecular mechanisms responsible for cell wall modification. This approach provides a more targeted and manageable starting point for unraveling the intricacies of plant-nematode interactions. As deciphering these specific processes continues and their regulators become clear, the roles of auxin and cytokinin are likely to fall into place within a more defined context, thus avoiding the confounding influence of pleiotropic effects. In conclusion, the multifaceted roles of auxin and cytokinin in plant-nematode interactions pose a significant challenge when attempting to decipher their specific impact. The pleiotropic effects of these hormones on various plant processes hinder the traditional top-down approach of disabling hormonal processes to study their roles in interactions. A more promising solution may be a bottom-up approach, beginning with specific events in the interaction and identifying the genes and mechanisms responsible. This approach can lead to a better understanding of the complex interplay between hormones and plant-nematode interactions.

Conclusions and perspectives

The study of plant-nematode interactions presents a complex and multifaceted challenge, but several key insights and strategies have emerged from this study. Feeding sites of cyst and root-knot nematodes both accumulate higher levels of auxin and cytokinin than their surrounding tissue. However, the magnitude and ratio of this accumulation varies and depends on the specific type of feeding cell. Additionally, the double reporter used in this study reveals that auxin and cytokinin signalling maxima overlap during cyst nematode infection, revealing lower levels of auxin signalling in syncytia, but with comparatively more cytokinin signalling. Furthermore, the presence of potential auxin biosynthesis genes in

bacterivorous, fungivorous, entomopathogenic and animal parasitic nematodes suggests that auxin might have potential endogenous functions within parasitic and non-parasitic nematodes. Finally, our experimental findings concerning the roles of PLT3, PLT5, and PLT7 indicate that these transcription factors are able to affect different aspects of the infection process and that the regulation of the auxin response by PLTs may contribute to the development of feeding sites of *M. incognita*. Although progress has been made in unraveling the complexities of the role of hormones in plant-nematode interactions, it is imperative to acknowledge that there remain many unknown variables. As such, suggestions can be made for future research endeavors focused on elucidating the intricacies of hormonal dynamics, molecular signalling, and feeding site development. For example, by adopting structured developmental stages, integrating advanced methodologies like live-cell imaging, and focusing on specific downstream events, one might make significant strides in unraveling the complexities of plant-nematode interactions, including the source of auxin, and developing effective strategies for nematode management in agriculture.

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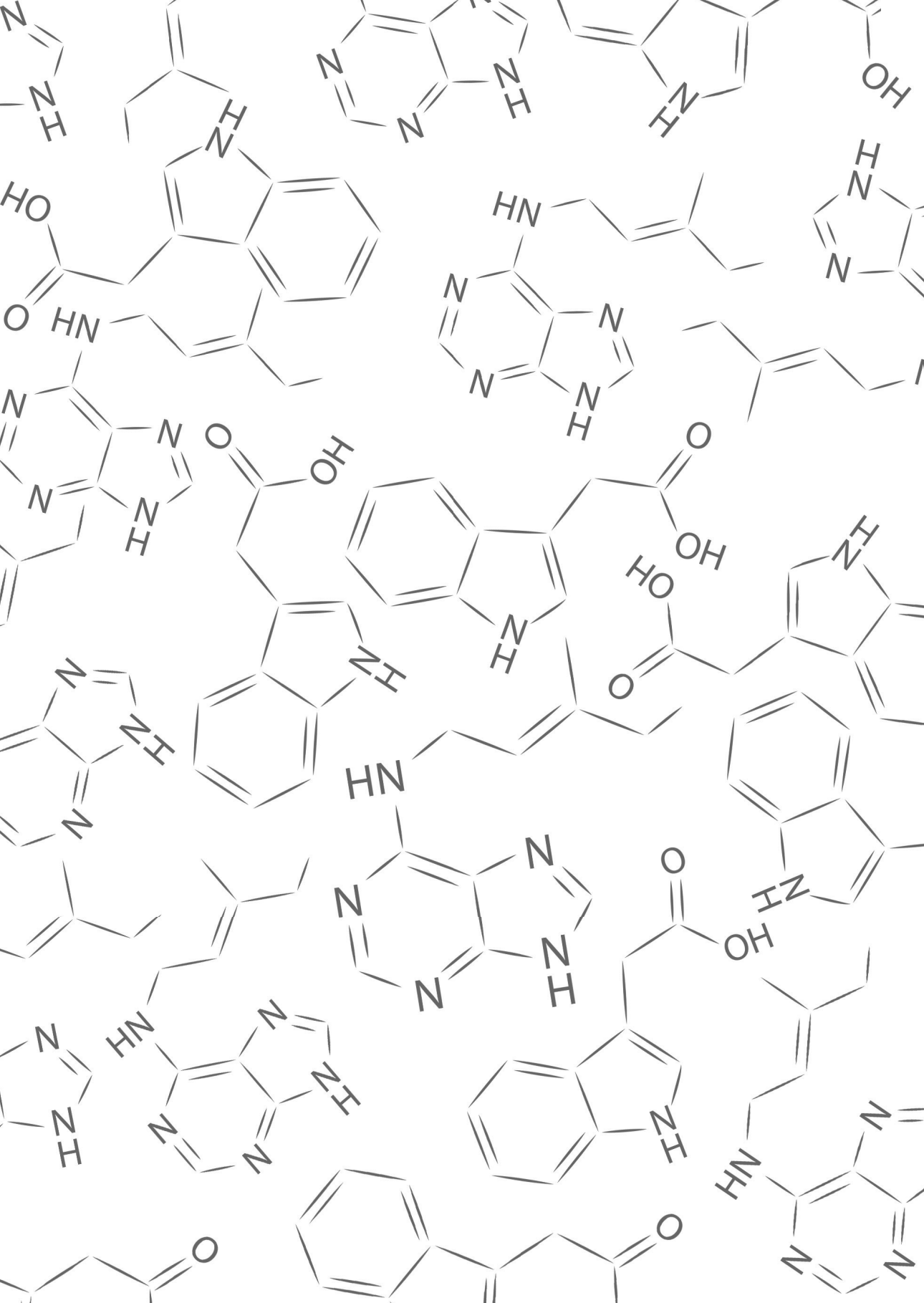

Appendix

Summary
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Summary

Cyst and root-knot nematodes are sedentary endoparasites that cause annually losses of over 150 billion dollars to crops. Therefore, they are considered as one of the most damaging plant pathogens in agricultural fields. Cyst and root-knot nematodes invade their host species via the roots to establish a feeding site that allows for growth and reproduction of the parasite called syncytia and giant cells, respectively. Syncytia are an agglomeration of cells that have fused together through enlarged plasmodesmata and partial cell wall dissolution, which function as a highly metabolically active transfer cell for nutrient uptake by the nematode. They are characterised by an expanded and dense cytoplasm, enlarged nuclei, various additional organelles, and secondary vacuoles. In contrast, root-knot nematodes induce a number of giant cells characterised as enlarged cells that have increased in size of up to 100 times due to repeated mitosis without cytokinesis and several rounds of endoreduplication. These giant cells are hypertrophied and contain multiple endopolyploidy nuclei with a high cytoplasmic density and numerous subcellular organelles. These elaborate changes in plant roots associated with cyst- and root knot nematode infection are thought to be mediated by the phytohormones auxin and cytokinin. However, the molecular mechanisms underlying their contribution at the onset of cyst- and root knot nematode parasitism is still fragmented.

In Chapter 1, background information is provided about cyst- and root knot nematodes including their mode of action and how they manipulate their host for their own benefit. Moreover, knowledge gaps are identified in particular the role of auxin and cytokinin in the establishment of a successful feeding relationship in plant roots. Finally, the scope of my thesis is outlined including the research questions and hypotheses addressed.

Next, Chapter 2 provides an in-depth literature analysis of various aspects of auxin homeostasis, including its synthesis, breakdown, transport, and signalling in plants during infection. Plant-parasitic nematodes exploit endogenous molecular and physiological pathways in the roots of their host to establish the aforementioned unique feeding structures. The plant auxin indole-3-acetic acid (IAA) is a fundamental component in the formation of these complex feeding sites. It becomes evident that plant-parasitic nematodes have evolved multiple strategies to manipulate auxin levels, thereby establishing a successful parasitic relationship with their hosts. These strategies include 1) manipulation of auxin transport to induce an accumulation, 2) increasing local auxin biosynthesis as well as 3) affecting auxin catabolism. Additionally, a potential role for other auxins, like phenylacetic acid (PAA) and indole-3-butyric acid (IBA) is proposed in the development of nematode feeding sites.

Cytokinin is another crucial plant hormone involved in feeding site development for both cyst and root-knot nematodes. Similar to auxin, cytokinin was reported to be present in the feeding sites of these nematodes and required for successful infection. The interaction between auxin and cytokinin during nematode infection is de facto unknown, but thought to be complex and multifaceted. This warranted further investigation of these hormones in the context of nematode feeding site development (Chapter 3). To do so, a method for simultaneous live-cell confocal imaging was developed to analyze the spatiotemporal signalling dynamics of the auxin and cytokinin domains during early feeding site development. With the use of a fluorescent double reporter our observations revealed that cytokinin

signalling occurs rapidly and precedes auxin signalling, with cytokinin predominantly present in nearly all cell types, including those outside the syncytial area. In contrast, auxin signalling is induced after the first contours of the syncytial area have become visible. Within the syncytial area the signalling domains of auxin and cytokinin overlap with high auxin signalling intensities in nuclei being associated with high cytokinin responses. These findings illustrate the changes in auxin and cytokinin signalling induced by the nematode and show that the live-cell imaging is a valuable tool to obtain novel insights in the spatio-temporal dynamics of hormone signalling domains during nematode parasitism.

With regards to the multiple strategies that nematodes have evolved to manipulate auxin levels, we investigated in Chapter 4 whether cyst nematodes possess the ability to synthesize auxin. Through mass-spectrometry analyses, it is revealed that the cyst nematode *Globodera pallida* indeed contains IAA. Additionally, a genomic analysis revealed that *G. pallida* contains several potential auxin biosynthesis genes that could form a hypothetical biosynthetic pathway. Furthermore, the predicted biosynthesis genes were expressed during infection suggesting a role in plant parasitism. A genomic analysis indicated, surprisingly, that potential auxin biosynthesis pathways are not unique to plant-parasitic nematodes but are also present in various nematode species, including species that are bacterivorous, fungivorous, entomopathogenic, and animal parasitic. These findings indicate a broader role for auxin in the life history and mode of action of nematodes such as metabolite synthesis and longevity.

The accumulation of auxin during cyst and root-knot nematode infection leads to the induction of auxin response genes. Chapter 5 shifts focus to the auxin induced transcription factors PLETHORA (PLT)3, PLT5, and PLT7 in root-knot nematodes. These transcription factors, known for their roles in root development, were hypothesized to contribute to the development of root-knot nematode feeding sites. The study revealed that the *plt3plt5plt7* triple mutant displays a decrease in the number of galls and fewer adult females with egg masses, suggesting the importance of PLT3, PLT5, and PLT7 combined in the formation of proper feeding sites. Moreover, PLT mutants seem to affect PIN3 localisation and suggests a role for the PLT transcription factors in establishing or maintaining auxin maxima during nematode infection.

In Chapter 6, the main findings of this thesis are discussed in the context of our current understanding of molecular plant-nematode interactions in particular the role of auxin and cytokinin in nematode feeding site development. Furthermore, recommendations are proposed for future research, including innovative steps to address technical, methodological and biological challenges intrinsic to studies on molecular plant-nematode interactions. It is suggested that adopting a stepwise approach to investigate the developmental stages of nematode feeding sites would provide a structured framework to unravel the induction and formation of complex feeding structures like syncytia and giant cells. Moreover, by combining live-cell imaging with regional RNA-seq it might be possible to bridge the gap between the observed hormone signalling dynamics and gene expression events during the onset of cyst nematode infection.

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About the Author

Matthijs Oosterbeek was born on December 19th, 1992 in Veenendaal, the Netherlands. With a specific interest in chemistry, he graduated from the gymnasium at the Marnix College in Ede.

Matthijs received his BSc in Molecular Life Sciences at the University of Wageningen with a minor in Infectious Diseases and Host Defence Mechanisms. To further explore his interest in biology at the molecular level he also completed his MSc in Molecular Life Sciences at Wageningen University with a specialisation in Physical Biology. His MSc thesis pertained to the role of the plant hormone jasmonic acid in plant-rhizobium interaction. Additionally, he investigated the role of transposable elements in rice on an epigenetic level during his internship abroad at the Sainsbury Laboratory of Cambridge University.

After receiving his master's degree, he participated in the EPS talent programme to write a PhD proposal concerning the presence of viral transposable elements in plant genomes. After the talent programme he was offered a PhD position at the laboratory of Nematology working on plant-nematode interactions. Since then, he has been working on unravelling the role of the hormones auxin and cytokinin in feeding site formation.

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