COMPARATIVE ANALYSIS OF THE ROLE OF CYTOKININ IN FEEDING SITE FORMATION INDUCED BY CYST AND ROOT-KNOT NEMATODES

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By
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COMPARATIVE ANALYSIS OF THE ROLE OF CYTOKININ IN FEEDING SITE FORMATION INDUCED BY CYST AND ROOT-KNOT NEMATODES

Presented by Carola M. De La Torre Cuba

A candidate for the degree of

Doctor of Philosophy

And hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

This dissertation is dedicated to my parents Amalia Cuba and Carlos De La Torre for their unconditional love, support and encouragement all these years.

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ABSTRACT

Cyst and root-knot nematodes are obligate plant parasites of global economic importance with a remarkable ability to reprogram root cells into unique feeding sites. Previous studies have suggested a role for cytokinin in feeding site formation induced by these two types of nematodes, but the mechanistic details have not yet been described. Using Arabidopsis as a host plant species, this study shows a comparative analysis of cytokinin genes in response to the beet cyst nematode (BCN), *Heterodera schachtii,* and the root-knot nematode (RKN), *Meloidogyne incognita*. Distinct differences in the regulation of cytokinin biosynthesis, catabolism and signaling genes in response to BCN and RKN infection were found, suggesting differential manipulation of the cytokinin pathway by these two nematode species. Furthermore, key cytokinin genes contributing to BCN and RKN disease susceptibility were identified. This study also evaluated ARABIDOPSIS HISTIDINE KINASE (AHK), *ahk2/3*, *ahk2/4* and *ahk3/4* receptor mutant lines in response to BCN and RKN infection and found a decrease in susceptibility compared to the wild type control, Col-0. An analysis of *ahk* double mutants using *CYCB1:GUS*/*ahk* introgressed lines revealed contrasting differences in the cytokinin receptors controlling cell cycle activation in feeding sites caused by RKN and BCN. Results from these studies could be used in the future to engineer novel forms of nematode resistance in crops of economic importance.

CHAPTER 1

INTRODUCTION

Economic Importance, Symptoms, and Management of Cyst and Root-knot Nematodes

Cyst and root-knot nematodes are the two most economically important plant-parasitic nematodes worldwide (Jones *et al.*, 2013). The cyst nematode (CN) genus, *Heterodera*, contains 82 nematode species each infecting a relatively narrow range of plant species (Perry & Moens, 2013). Some of the plants affected by *Heterodera* in the US include crops of major economic importance such as soybean, sugar beets, and wheat (Jones *et al.*, 2013). Symptoms of infected plants range from no obvious aboveground symptoms to chlorotic, stunted plants, and associated reductions in yield. Diagnosis of a CN problem is through soil sampling and/or visual inspection of plant root systems for the presence of "cysts" on the surface of the roots. Cysts are the adult females that ultimately protrude from roots as they mature. Each cyst contains hundreds of eggs that remain protected in the soil until conditions are favorable for infective juveniles to hatch and continue the nematode's life cycle. For this reason, CN populations are difficult to eradicate once established in the field, requiring multiple disease management strategies to mitigate crop losses. One of the most well established management strategies relies on the use of resistant varieties. For example, there is extensive use of soybean varieties resistant to soybean cyst nematode in the US. Additional methods of management include non-host crop rotation, biologicals, and nematicides (Davis & Tylka, 2000).

The root-knot nematode (RKN) genus, *Meloidogyne,* comprises 98 species, each infecting a wide range of plant species and causing over 157 billion dollars in annual crop losses globally (Jones *et al.*, 2013; Sasser 1997). Over three thousand plant species are susceptible to this pest including vegetables, ornamentals, cereals, crops and fruit trees (Abad *et al.*, 2003). RKN also have a wide geographic distribution with some species preferring cool climates (*M. hapla*, *M. naasi*, *M. chtiwoodi* and *M. fallax*) and others warmer climates (*M. incognita*, *M. javanica* and *M. arenaria*). Four species, *M. incognita*, *M. javanica*, *M. hapla* and *M. arenaria* are the most economically important worldwide (Taylor & Sasser, 1978). Of these species, *M. incognita* is the most damaging and polyphagous, being able to infect a diverse array of plants including tubers (potato, okra, yam), fruits (grapevine, papaya, mango, watermelon, coconut, citrus), vegetables (tomato, cabbage, pepper) and other crops (soybean, tobacco) (Taylor & Sasser, 1978; Onkendi *et al.*, 2014). Typical symptoms caused by RKN are deformed, galled root systems, which interferes with the normal absorption of water and nutrients leading to wilting, chlorosis, and stunting in the aboveground parts of the plant (Jones *et al.*, 2013). Additional symptoms include delayed maturity, toppling and decreased yield (Onkendi *et al.*, 2014). Symptoms vary across plant species, but their severity depends on the use of optimal management strategies, soil type, and initial population density (Jones *et al.*, 2013). Management methods include raising soil temperatures with a transparent polyethylene cover (soil solarization), use of biocontrol agents (such as *Pasteuria penetrans*, *Bacillus subtilis*, *Pseudomonas fluorescens*) and the use of resistant cultivars (Taylor & Sasser, 1978; Siddiqui & Mahmood, 1999).

Biology and Life Cycles of Cyst and Root-knot Nematodes

CN and RKN are microscopic roundworms with remarkable adaptations for plant parasitism. One such adaptation is a hollow syringe-like mouth structure or 'stylet' that the nematodes use to penetrate into root tissue, migrate within roots, secrete molecules into host cells, and sequester nutrients from the plant (Davis & Mitchum, 2005; Mitchum *et al.*, 2012). CN possess a robust stylet that allows them to migrate intracellularly causing massive damage to root tissues, while RKN have a finer stylet and carefully migrate intercellularly causing minimal damage (Perry & Moens, 2003). Another adaptation is the development of specialized esophageal gland cells, one dorsal and two subventral, which the nematode uses to produce small molecules and proteins that are secreted through the stylet into root tissues to establish a parasitic association with a host plant (Figure 1.1).

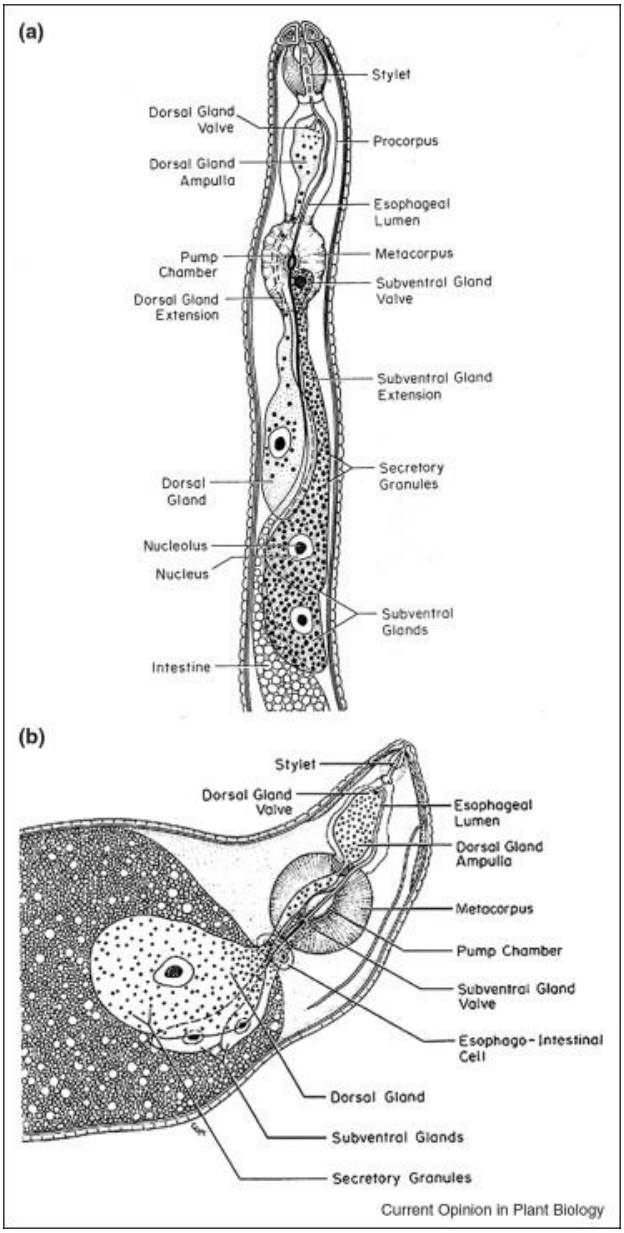

Figure 1.1. llustrations of the anterior portions of the migratory and sedentary stages of endoparasitic nematodes that contain esophageal gland secretory cells associated with the nematode stylet, a hollow oral feeding spear. (a) A migratory, infective second-stage juvenile illustrated with the two subventral esophageal gland cells packed with secretory granules. (b) A swollen female from within infected roots with reduced subventral gland cells and an enlarged active dorsal esophageal gland cell now packed with secretory granules. Reprinted with permission of Dick Hussey and reprinted from Mitchum *et al*., 2013.

The CN and the RKN life cycles (Figure 1.2) have been well studied. Infective second-stage juveniles (J2) hatch from eggs in the soil using their stylet to break through the eggshell and migrate towards host roots. Juveniles penetrate into the root by a combination of mechanical thrusts of the stylet and the secretion of cell wall degrading enzymes, and migrate either inter-cellularly (RKN) or intra-cellularly (CN), towards the vascular cylinder. Near the vascular cylinder, juveniles select a single cell (CN) or group of cells (RKN) and secrete an assortment of parasitic molecules causing remarkable transcriptional and metabolic changes to host cells allowing them to establish a permanent feeding site. Plant cyclin dependent kinases, mitotic cyclins, and cell wall modifying proteins have been shown to be upregulated during this stage (Goellner *et al.,* 2001; Mitchum *et al.*, 2012; Gheysen & Mitchum, 2011; Jammes *et al.,* 2005; De Almeida-Engler *et al.*, 1999; Barcala *et al.*, 2010; Ramsay *et al.*, 2004). CN induces partial cell wall dissolution leading to the fusion of hundreds of cells to form a multinucleated 'syncytium'. RKN induces selected cells to enlarge hundreds of times the size of a normal cell and triggers repeated nuclear divisions to form multinucleate 'giant-cells'. In addition, surrounding cells enlarge and proliferate giving rise to the characteristic 'root knot' or gall (Gheysen & Fenoll, 2002; Perry & Moens, 2013). Both types of feeding cells have a dense cytoplasm, vast amounts of organelles, and cell wall ingrowths that proliferate next to the xylem poles to facilitate nutrient and water uptake from the plant (Jones & Payne, 1978; Gheysen & Fenoll, 2002).

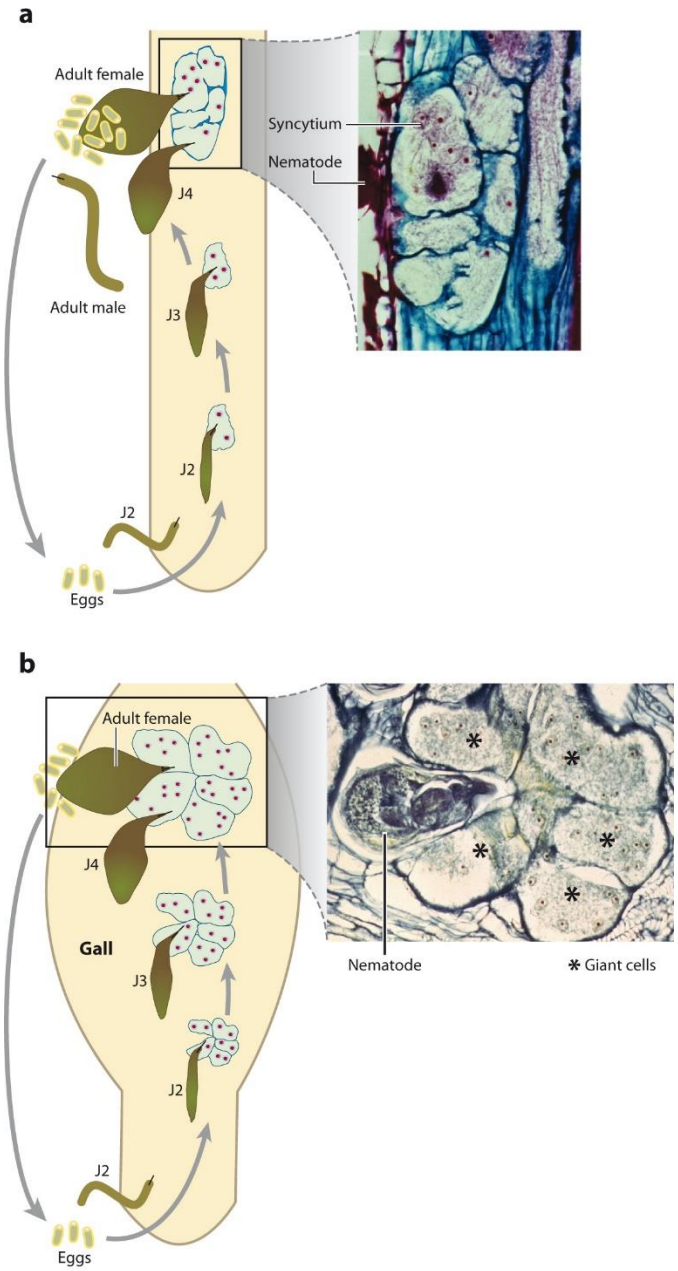

Figure 1.2. Diagrams depicting life cycles of cyst and root-knot nematodes showing photographs of longitudinal sections of nematode feeding sites. Pre-parasitic juveniles use a hollow mouth spear (stylet) to secrete small molecules into host cells near the root vasculature to developmentally reprogram them into essential feeding cells called a) syncytium in the case of cyst nematodes or b) giant cells in the case of root-knot nematodes. Surrounding tissue proliferation accompanies giant cell formation forming a gall. Abbreviations: J2, second-stage juvenile; J3, third-stage juvenile; J4, fourth-stage juvenile. Reprinted from Mitchum et al., 2012.

Syncytia and giant-cells are the only source of nutrients and water for the nematode and therefore pivotal for nematode survival. If formed properly, giant-cells and syncytia will sustain nematode growth until adulthood. CN reproduce by sexual reproduction or amphimixis. Adult males leave the roots and can follow pheromones emitted by females for mating. Females, on the other hand, remain attached to the roots and continue feeding. A single fertilized CN female can lay hundreds of eggs, most of which remain protected within the female body until favorable hatching conditions occur. The dead female body or 'cyst' is highly resistant to dry conditions and can protect the eggs in the soil for many years. In contrast to CN, most RKN species are parthenogenic, a form of asexual reproduction. This allows for rapid build-up of a RKN population within the field in a single growing season. While most CN eggs are encapsulated within the female, RKN eggs are laid outside the female body onto the surface of the gall within a gelatinous matrix to protect the eggs from desiccation. CN and RKN on average complete their life cycle in approximately 30 – 40 days (depending on the host-nematode species combination) (Perry & Moens, 2013).

Cyst and Root-Knot Nematodes Secrete Small Molecules Altering Host Cellular Processes

Successful feeding site establishment and maintenance depends on manipulation of cellular processes within the infected cell. This is accomplished through the secretion of parasitic molecules or 'effectors' by the nematode, which are mainly synthesized within the nematode esophageal glands and are transferred into the host via the stylet (Figure 1.3). Effectors are known to function both within the cytoplasm and the apoplast and are

therefore potentially secreted to either cell compartment where they trigger multiple changes in host transcription and signaling that result in the augmentation of plant development processes (Gheysen & Mitchum, 2011; Gardner *et al.*, 2015).

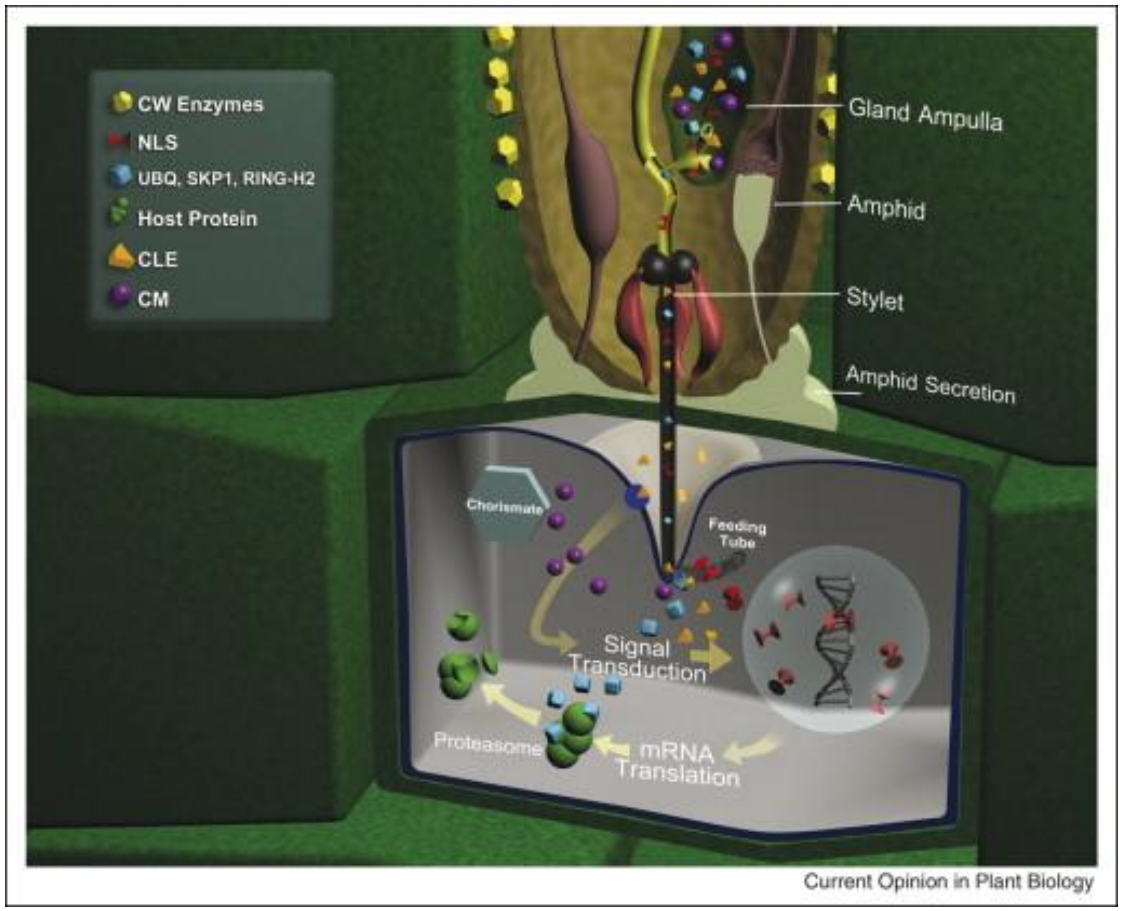

Figure 1.3. A model of potential interactions of secreted products of phytonematode parasitism genes with host plant cells. Nematode esophageal gland cell secretions are released through valves within ampulla for transport out of the stylet (feeding spear) into host tissues. Cell wall (CW)-modifying proteins (endoglucanases, pectolytic enzymes, xylanases, and expansins) may be secreted to aid the migration of infective juveniles through host plant tissues. Other nematode gland cell secretions might have multiple roles in the formation of specialized feeding cells by the nematode, including effects on host cell metabolism by secreted CM; signaling by secreted nematode peptides, such as homologs to plant CLE peptides; selective degradation of host proteins through the ubiquitin (UBQ)-proteosome pathway by ubiquitin, Skp-1, and RING-H2 secreted from the nematode; and potential effects of secreted nematode proteins that contain NLS within the host cell nucleus. Figure designed by Bill Baverstock (North Carolina State University Creative Services). Reprinted from Davis et al. (2004) with permission from Elsevier.

One of the best characterized families of cyst nematode effectors belongs to the CLE (CLAVATA3/endosperm surrounding region-related) family, a class of peptide hormones that control the balance between division and differentiation of plant meristem cell populations. Cyst nematode CLEs are composed of three main domains: an N-terminal secretion signal peptide (SP); a variable domain (VD) and a 12 amino-acid C-terminal conserved CLE domain. CLE prepropeptides produced in the dorsal gland cell are directed to the secretory pathway by the N-terminal SP. Following secretion of the propeptide to the cytoplasm of host cells, the VD is responsible for trafficking these peptides through the plant secretory pathway to the apoplast where they can acquire plant-specific post-translational modifications and spatially coincide with plant receptors to exert their function (Wang *et al.*, 2010; Chen *et al.*, 2015). Overexpression of nematode CLEs in Arabidopsis resulted in similar phenotypes observed when plant CLEs are overexpressed. Additionally, exogenous applications of synthetic nematode CLE peptides have resulted in numerous plant morphological changes reminiscent of plant CLE applications. Furthermore, nematode CLEs can partially or fully complement a *clv3* mutant, a member of the plant CLE family involved in shoot apical meristem stem cell maintenance. Nematode CLE peptides were shown to bind to plant receptors and receptor mutant plants are more resistant to cyst nematodes (Guo *et al.*, 2011; Replogle *et al.*, 2011; Replogle *et al*., 2013). Overall, these findings demonstrated that nematode CLEs can mimic plant CLEs in function. To further determine whether nematode CLEs were involved in nematode virulence, Bakethia *et al.*, 2007 used RNAi-mediated silencing to reduce HgCLE expression in soybean cyst nematode juveniles. Plants inoculated with HgCLE-silenced nematodes presented fewer cysts compared to control plants, suggesting the HgCLE

effector is required for nematode infection. Similar results were found by Patel *et al.*, 2008 using host-derived RNAi against *Heterodera schachtii* HsCLE, further supporting the role of nematode CLEs in parasitism.

RKN also secretes small peptides that may impact developmental and hormonal signaling pathways. For example, the RKN *16D10* gene encodes a predicted 13-aa secreted peptide (Huang *et al*., 2006a). *In vitro* RNAi derived post-transcriptional silencing of 16D10 by soaking pre-parasitic juveniles in dsRNA targeting 16D10 significantly decreased nematode infectivity of Arabidopsis plants. Similarly, dsRNA expression of transcripts complementary to 16D10 *in planta* significantly reduced nematode infection in Arabidopsis, grapes and potatoes (Huang *et al.*, 2006b; Yang *et al.* 2013; Dinh *et al.*, 2015), indicating an important role for 16D10 in RKN parasitism. Interestingly, 16D10 is unable to complement the *clv3* mutant phenotype suggesting a divergent function for this peptide. Additional studies showed 16D10 interacts with SCR-like transcription factors SCL6 and SCL21, with potential roles in root growth and development. Furthermore, overexpressing 16D10 in Arabidopsis plants promoted host cell proliferation. These studies suggest RKN secrete small molecules to promote cell proliferation by targeting specific host transcription factors. However, further studies are needed to determine the precise mechanism of 16D10 function in feeding site formation (Huang *et al.*, 2006a).

Recently, *INFLORESCENCE DEFICIENT IN ABCISSION* (IDA)-like genes have been found in root-knot nematode species *Meloidogyne incognita* and *Meloidogyne hapla*. Plant IDA genes encode for small peptides with a role in organ abscission and interact with LRR-RLK HAESA (HAE) and HAESA-like (HSL2) to upregulate Knotted1-like homeobox (KNOX) transcriptional factors required for cell separation. Missexpression of

IDA peptides results in enlarged cells in the abscission zone (Shi *et al.*, 2011). *M. incognita* and *M. hapla* IDA peptides are 46% similar to plant IDAs and might serve as IDA mimics (Tucker and Yang, 2013). Given that KNOX genes are expressed in giant cells (Koltai *et al.*, 2001), potentially root-knot nematodes secrete IDAs to alter IDA signaling controlling KNOX expression during feeding site formation (Tucker & Yang, 2013). However, the role of nematode IDA peptides in nematode parasitism still needs to be described.

Nematodes are also known to alter auxin signaling and transport. Grunewald *et al*. (2009) showed auxin efflux PIN proteins were altered in response to CN infection. Specifically, increased PIN1 expression allows for auxin efflux from adjacent cells into the initial syncytial cell, while PIN3 and PIN4 redistribute accumulated auxin to lateral cells. Auxin accumulation is associated with upregulation of cell wall modifying enzymes potentially resulting in cell wall dissolution required for feeding site expansion. Additionally, PIN single and double mutants are less susceptible to cyst nematode supporting the role of PIN proteins in nematode infection (Grunewald *et al.*, 2009). Similar to the interaction with CN, auxin transport plays an important role in RKN infection. Using GUS reporter genes and GFP-protein fusions, Kyndt *et al.*, 2016 examined expression patterns and localization of two auxin influx (AUX1, LAX3) and five efflux transporters (PIN1-4,7) in response to *Meloidogyne incognita* infection of Arabidopsis. AUX1, LAX3 and PIN3 shared a polarized expression pattern at the basipetal side of each gall suggesting auxin flow from the neighboring cells into the giant cells. They were also expressed in giant cells indicating additional redirection of auxin flow between each independent giant cell. Single mutants, *aux1*, *lax3* and *pin3,* presented significantly fewer nematodes, fewer galls and impaired nematode development suggesting auxin transport from the adjacent

cells into the feeding site to be required for gall formation. Similarly, PIN1 and PIN4 are expressed in giant cells but only *pin4* and not *pin1* mutants showed decreased susceptibility, suggesting PIN4 and not PIN1 is crucial for gall development. On the other hand, PIN2 and PIN7 were only expressed in cortex and surrounding cells, but not in giant cells probably to prevent excessive auxin exit and increase auxin accumulation at the feeding site.

A well-characterized cyst nematode effector known to alter host hormonal pathways is *Heterodera schachtii* dorsal gland effector protein 19C07. 19C07 physically interacts with the Arabidopsis LAX3 protein, a member of the AUX/LAX family of auxin influx transporters, which is expressed in the initial and surrounding syncytial cells (Lee *et al.*, 2011). LAX3 expression in feeding sites coincides with the upregulation of polygalacturonase, an auxin regulated cell wall loosening enzyme, to most likely facilitate the incorporation of new cells into the growing syncytium. The *lax3* mutant did not show significant reduction in nematode infection, probably due to functional redundancy, but double *aux1lax3* and quadruple *aux1lax1lax2lax3* mutants did have a negative effect, further supporting the role of auxin transport in CN feeding site formation (Lee *et al.*, 2011). Since no Hs19C07 ortholog has been found in the RKN secretome and no other RKN effectors have been reported to interact with auxin transporters, it has been suggested that auxin rearrangements in galls are independent of direct manipulation by the nematode (Kyndt *et al.*, 2016). Other than Hs19C07, *Heterodera schachtii* effector 10A07 has also been shown to target auxin signaling during parasitism. The 10A07 effector is only expressed in nematode dorsal gland cells, and is putatively secreted via the stylet into the host cytoplasm. Once inside the cell, 10A07 physically interacts with plant kinase IPK for

phosphorylation of residues Ser-144 and Ser-231 and subsequent trafficking into the host nucleus. In the nucleus, 10A07 putatively interacts with auxin negative regulator IAA16 presumably to interfere with auxin signaling during feeding site formation (Hewezi *et al.*, 2015).

In addition to secreting effectors that directly regulate phytohormone transport and signaling, prior studies suggested that these nematodes might also secrete phytohormone mimics. Auxin has been detected in secretions and lysates of CN and RKN juveniles (Setty & Wheeler, 1968; De Meutter *et al.*, 2004). In addition, cytokinins were detected at physiologically active concentrations by high performance liquid chromatography (HPLC) in CN and RKN pre-parasitic juvenile lysates and exudates (Dimalla *et al.*, 1977, De Meutter *et al.*, 2003). More recently, it was discovered that CN encodes a cytokinin biosynthesis homolog, tRNA-IPT, which is expressed at high levels in the nematode esophageal glands. Plants challenged with tRNA-IPT-silenced nematodes showed significantly fewer numbers of cysts compared to plants infected with untreated nematodes. Furthermore, CN infected roots showed high levels of CYCB1::GUS expression in syncytia, however, tRNA-IPT-silenced nematodes fail to activate expression of this reporter gene. CYCB1::GUS is a marker for cell division as it is expressed between the G2 and M cell cycle phases suggesting that CN use cytokinin mimics to activate CYCB1 in the host plant to start the establishment of a feeding site (Siddique *et al.*, 2015). Interestingly, RKN may also secrete cytokinins (Dimalla & Van Staden, 1977; De Meutter *et al.*, 2003); however the feeding sites that they induce in susceptible hosts are quite different to the feeding sites induced by CN as above described. Furthermore, RKN cytokinin biosynthesis genes remained to be identified and characterized.

Arabidopsis Cytokinin Pathways and Functions

Plant cytokinins (CKs) are N^6 adenine derivatives commonly associated with the regulation of meristematic cell division in the shoot apical meristem (SAM), the root apical meristem (RAM) and the vascular cambium. Whether or not meristematic cells proliferate or differentiate into daughter cells is tightly controlled by CK signaling pathways (Zurcher *et al.*, 2016). In Arabidopsis, plant CKs are synthesized by two distinct gene families encoding for seven AMP/ADP/ATP isopentenyltransferases (IPT) and two tRNA-Isopentenyltransferase (tRNA-IPT) genes, which utilize cytoplasmic dimethylallyl diphosphate (DMAPP) and AMP/ADP/ATP or tRNA as precursors, respectively (Kakimoto *et al.*, 2001;Takei *et al.*, 2001) (Figure 1.4). In plastids, IPT uses hydroxymethyl butenyl diphosphate (HMBDP) instead of DMAPP as the main precursor. Previous studies

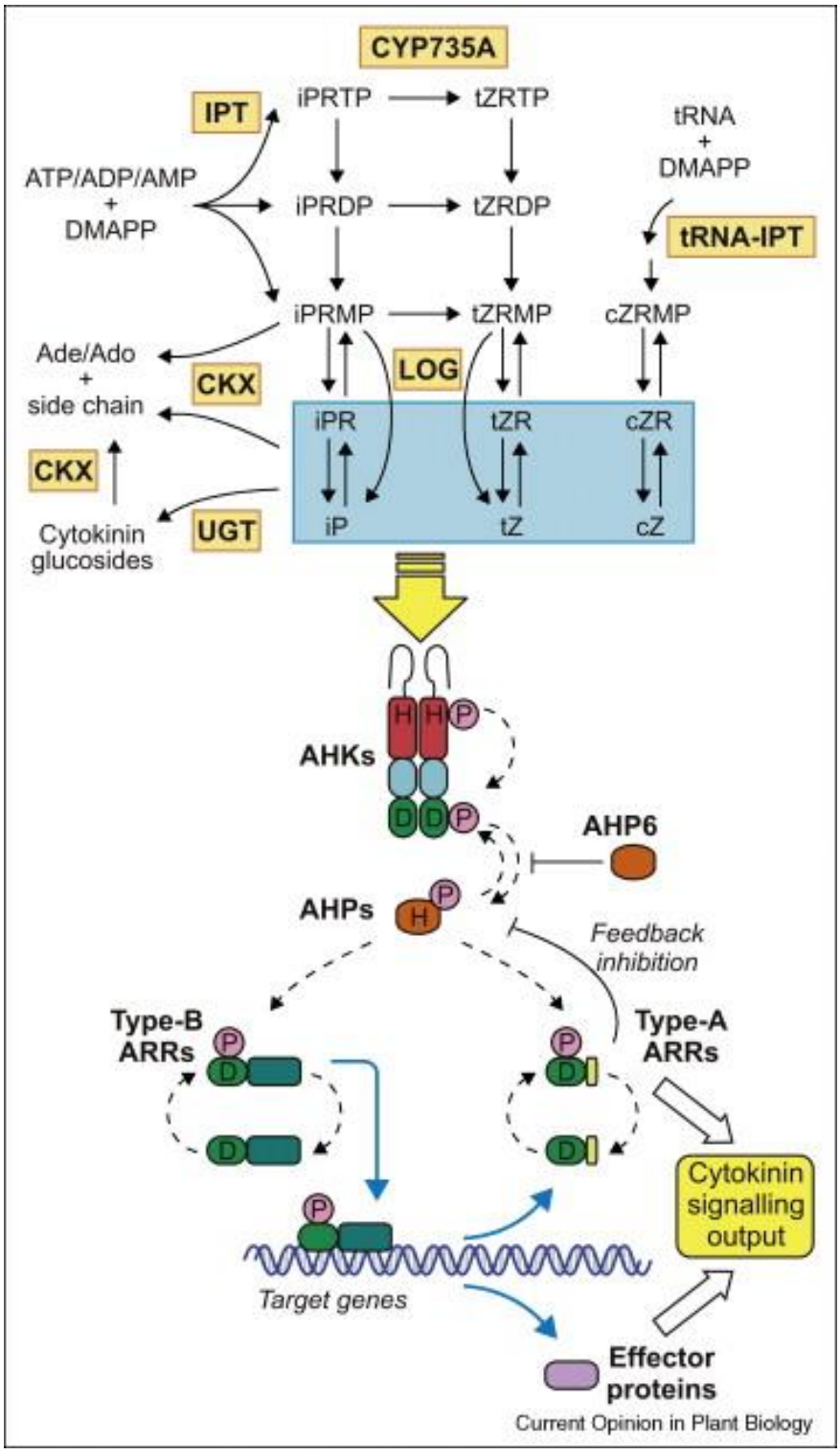
CYP735A
IPT
iPRTP
tZRTP
tRNA
+
DMAPP
ATP/ADP/AMP
+
DMAPP
iPRDP
tZRDP
tRNA-IPT
iPRMP
tZRMP
cZRMP
Ade/Ado
+
side chain
CKX
LOG
iPR
tZR
cZR
CKX
Cytokinin
glucosides
UGT
iP
tZ
cZ
AHKs
AHP6
AHPs
Feedback
inhibition
Type-B
ARRs
Type-A
ARRs
Cytokinin
signalling
output
Target genes
Effector
proteins
Current Opinion in Plant Biology

Figure 1.4. Schematic model of cytokinin metabolism and core steps of the cytokinin signaling pathway. Biosynthesis of iP-cytokinins and tZ-cytokinins is initiated by adenosine phosphate-isopentenyltransferases (IPTs) to form iP-nucleotides which can be converted to the corresponding tZ-nucleotides by cytochrome P450 monooxygenases (CYP735As). iPRTP, iPRDP and the corresponding tZ-nucleotides are dephosphorylated by phosphatases, and iPRMP and tZRMP can be directly converted to active free bases by cytokinin nucleoside 5′-monophosphate phosphoribohydrolases (LOGs). *cis*-Zeatin (cZ) cytokinins, which in some plant species are the major cytokinin metabolites, are synthesized in *Arabidopsis* exclusively by tRNA-IPTs which utilize tRNAs as prenyl acceptors. Biologically active cytokinins, highlighted in blue, are inactivated by cytokinin oxidases/dehydrogenases (CKXs) and by conjugation to sugar moieties through glycosyltransferases (UGTs). A histidine (H)/aspartate (d)-phosphorelay (indicated by dotted arrowed lines) through a two-component signaling cascade is initiated by the binding of biologically active cytokinin to a CHASE domain of histidine kinase receptors (AHKs) and autophosphorylation of a His-residue in the protein kinase domain (red). The phosphoryl group is transferred via the Asp-residue of the receptor receiver domain (green) to a conserved His of the histidine phosphotransfer proteins (AHPs). Non-activated CRE1/AHK4 possesses phosphatase activity that dephosphorylates AHPs. AHP6, lacking the conserved His-residue, inhibits the phosphoryl transfer, presumably by interacting with activated receptors and/or response regulators. AHP proteins relay the signal to B-type or A-type response regulators (ARRs). B-type ARRs, which contain a C-terminal DNA-binding domain (turquoise), are transcription factors regulating expression of their target genes including A-type *ARR*s. One function of the A-type ARRs is to repress signaling in a negative feedback loop. Together with other effector proteins, they determine the signaling output of the pathway (Werner & Schmulling, 2009). Reprinted with permission from ScienceDirect.

have shown that *IPT* genes are involved in biosynthesis of isopentenyladenines (iP) which are subsequently interconverted into *trans*-zeatins (tZ) by cytoplasmic P450 monooxygenase CYP735A (Takei *et al.*, 2004). In contrast, tRNA-IPTs are known to produce *cis*-zeatins (cZ). CK homeostasis is maintained by the action of cytokinin oxidases/dehydrogenases (CKX) which catalyze the irreversible degradation of CK molecules into an adenine and isoprenoid chain (Schmulling *et al.*, 2003). CK perception in higher plants is based on a two component system (TCS): a signaling mechanism evolutionarily conserved between eukaryotes and prokaryotes (Hwang *et al.*, 2001, Hwang *et al.*, 2002, Heyl & Schmulling, 2003). In Arabidopsis, this system consists of three Histidine Kinase membrane bound receptors (AHK2, AHK3, AHK4/CRE1), five Histidine Phosphotransfer Proteins (AHP1-5), and 10 Type A-and 11 Type B- Response Regulators (ARR) (Ueguchi *et al.*, 2001a; Ueguchi *et al.*, 2001b; Inoue *et al.*, 2001, Suzuki *et al.*, 2001; Hutchison *et al.*, 2006; Mason *et al.*, 2004; To *et al.*, 2004). CK recognition triggers autophosphorylation of the receptors. A phosphoryl group is subsequently moved into the nucleus via phosphorelay. AHPs serve as 'molecular shuttles' between the cytoplasm and the nucleus, where they physically interact with Type B–ARRs to induce the expression of CK responsive genes (Heyl & Schmulling, 2003). Finally, CK signaling is modulated by an additional class of ARRs, called Type A–ARRs, which act as negative regulators of the CK pathway by an unknown mechanism (Figure 1.4) (To *et al.*, 2004).

C**ytokinin Functions in Plant Growth and Development**

The outcome of cytokinin function in plant development, whether it is cell proliferation or cell differentiation, is controlled in a context dependent manner. In the shoot, CK is essential for maintaining stem cell activity and cell cycle progression of the shoot apical meristem (SAM). CK regulates the expression of homeodomain transcription factor WUSCHEL (WUS) in the multipotent rib meristem (RB) which in turn promotes cytokinin signaling by downregulation of several closely related Type A-ARRs in the organizing center (OC) of the SAM (Gordon *et al.*, 2009). Functional relevance of this regulation was supported by premature meristem termination of an Arabidopsis line constitutively expressing negative regulators of the cytokinin pathway (Leibfried *et al.*, 2005). CK stem cell proliferation is mediated in part through cell cycle regulatory proteins (CYCD3) that direct the entry into S-phase and DNA duplication of mitotic cells. CYCD3s promote cell division and inhibit cellular differentiation to support the proliferation of stem cells (Scofield *et al.*, 2013). Contradictory to its positive role in the shoot, CK negatively regulates root growth by inducing differentiation of cells exiting the RAM region at the transition zone. This was in part shown by the fact that cytokinin-deficient plants have larger RAMs and longer roots than normal plants (Werner *et al.*, 2009). Cell differentiation at the transition zone is controlled by transcription factors, ARR1 and ARR2, in an AHK3-dependent manner (Dello Ioio *et al.*, 2007).

Besides plant development, transcriptomic studies have previously shown CK can also affect multiple aspects of plant cell biology. Meta-analysis of 13 microarray experiments of exogenously treated Arabidopsis seedlings using 1- 20 µm of cytokinin (zeatin or benzyladenine) by either immersion or spray, revealed 226 differentially

expressed genes across all treatments. A list of these genes, also called the "golden list", has vast enrichment in categories corresponding to RNA processing, transport, hormone and secondary metabolism (Bhargava *et al.*, 2013). Additional transcriptomic studies have revealed genes associated with upregulation of sucrose transporters and defense-related genes (Brenner *et al.*, 2012, Argueso *et al.*, 2010), suggesting cytokinins can also play important roles in sink strength and immune responses, respectively.

Rationale

The phytohormone cytokinin plays a pivotal role in the regulation of cell proliferation and many different aspects of plant growth and development. Previous studies demonstrated that CNs secrete cytokinins to induce cell cycle activation of infected root cells. Nematodes that are deficient in cytokinin synthesis are less virulent and unable to successfully form feeding sites. Like CN, RKN may also secrete cytokinins, and *Lotus japonicus* hairy roots overexpressing a cytokinin oxidase gene were less susceptible to RKN infection (Dimalla & van Staden, 1977; De Meutter *et al.*, 2003; Lohar *et al.*, 2004). In an attempt to determine if cytokinin signaling was activated at the feeding site, Absmanner *et al*. (2013) evaluated cytokinin activity in syncytia and galls using the synthetic reporter gene *TCS::GFP*. No major expression was detected in either feeding site suggesting cytokinin was not involved in this process. However, in light of recent studies showing that *TCS::GFP* expression is extremely low in certain tissues known to have cytokinin activity, whether cytokinin signaling is active in feeding sites is still unclear. Given that many aspects of feeding site formation are reminiscent of processes controlled by cytokinin in normal plant cells, we set out to investigate if cytokinin can indeed play a

role in feeding site formation. Furthermore, it has not been explored whether differential regulation of cytokinin genes can explain differences in feeding site formation between cyst and root-knot nematodes. With the premise that root cells are likely to respond to endogenous changes of cytokinin directly or indirectly stimulated by the pathogen, we hypothesized that cytokinin plays a positive role in syncytium and gall formation, but differences in host cytokinin metabolism and signaling at the feeding site exist in response to each of these nematode species.

This study set out to test this hypothesis through the following aims: **Aim 1**: To compare host cytokinin biosynthesis, catabolism and receptor gene expression in BCN vs. RKN feeding sites, **Aim 2**: To evaluate host cytokinin metabolism and signaling mutants in response to CN and RKN infection, **Aim 3:** To identify key components of plant cytokinin biosynthesis, catabolism and signaling required for the interaction with BCN and RKN.

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CHAPTER 2

STUDIES ON THE ROLE OF ARABIDOPSIS CYTOKININ BIOSYNTHESIS AND CATABOLISM GENES IN RESPONSE TO INFECTION BY THE BEET CYST NEMATODE, *HETERODERA SCHACHTII,* AND THE ROOT–KNOT NEMATODE *MELOIDOGYNE INCOGNITA**

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Contributions by authors other than Carola M. De La Torre have been highlighted in the figure legends.

ABSTRACT

Cyst nematodes (CN) and root-knot nematodes (RKN) induce changes in cell cycle and differing degrees of cell division as they form specialized feeding sites from which they derive nutrients important for their growth. Recent studies have shown cyst nematodes secrete cytokinin, a phytohormone required for plant growth and development, to re-activate cell cycle of root cells destined to become a feeding site or 'syncytium' during the infection process. Although a role for nematode cytokinin has been established, whether host cytokinin metabolism is contributing to the infection process has not been studied in detail. Furthermore, given that RKN feeding sites or 'giant-cells' also require alterations to cell cycle and extensive cell division giving rise to galls, a role for cytokinin in RKN infection has long been suspected but not addressed in detail until now. To determine if host cytokinin plays a positive role in plant-nematode interactions, and identify key host cytokinin genes required for both CN and RKN susceptibility, we conducted a spatio-temporal comparative analysis of cytokinin biosynthesis and catabolism gene expression in feeding sites using promoter-GUS reporter lines. We found differential regulation of cytokinin gene expression in syncytia compared to giant-cell and galls, suggesting a targeted manipulation of cytokinin genes specific to each nematode species. To determine if cytokinin was required for both CN and RKN infection, we evaluated Arabidopsis cytokinin catabolism overexpressing line 35S:CKX2 and found significantly fewer number of cysts and galls compared to the wild type Col-0. In addition, we evaluated Arabidopsis cytokinin biosynthesis and catabolism mutants in response to CN and/or RKN infection. Biosynthesis triple mutant *ipt357* showed no significant differences in the number of cysts that developed compared to the wild type Col-0, but smaller syncytia sizes. Catabolism

double mutant *ckx5 ckx6* showed significantly fewer number of cysts and galls compared to Col-0. Overall, these studies suggest for the first time an important role for host cytokinin biosynthesis and catabolism in nematode infection.

INTRODUCTION

Cyst nematodes (CN) and root-knot nematodes (RKN) are biotrophic pathogens able to reprogram selected host cells into unique and highly metabolically active feeding sites required for the completion of their life cycle. Two key aspects of feeding site formation are cell cycle activation of initial feeding cells and division of surrounding vascular cells (de Almeida Engler *et al.*, 1999); both functions generally controlled by the phytohormone cytokinin (Schaller *et al.*, 2014; Mähönen *et al.*, 2006). However, whether host cytokinin is required for CN and RKN infection has not yet been studied in detail.

Cytokinins are N^6 adenine derivatives with multiple but opposite functions in plant development. For example, they induce cell division and growth, but also cell differentiation and even necrosis in a context dependent manner (Zürcher and Müller, 2016). The amount of cytokinin in a cell is controlled by the rate of cytokinin biosynthesis and catabolism. In Arabidopsis, the limiting step in plant cytokinin biosynthesis is catalyzed by two distinct gene families encoding for seven AMP/ADP/ATP isopentenyltransferases (IPT) genes: *IPT1*, *IPT3*-*IPT8* and two tRNA-isopentenyltransferase (tRNA-IPT) genes, *IPT2* and *IPT9* which utilize cytoplasmic dimethylallyl diphosphate (DMAPP) and AMP/ADP/ATP or tRNA as precursors, respectively (Kakimoto, 2001; Takei *et al.*, 2001). *IPT* genes are involved in biosynthesis

of isopentenyladenines (iP) which are subsequently interconverted into *trans*-zeatins (tZ) by cytoplasmic P450 monooxygenase CYP735A (Takei *et al.*, 2004), while tRNA-IPTs are known to produce *cis*-zeatins (cZ). *IPT* genes are either tissue specific or ubiquitously expressed. *IPT1* is expressed in the procambial cells of the root and cotyledons, *IPT3* is mainly expressed in phloem, *IPT4* and *IPT8* are only expressed in the endosperm during early stages, *IPT5* is expressed in the columella of the root cap soon after germination and *IPT7* is expressed in trichomes and in the root elongation zone. On the other hand, tRNA-IPTs *IPT2* and *IPT9* are ubiquitously expressed particularly in dividing tissues including the shoot and root apical meristem and the leaf primordia (Miyawaki *et al*., 2004). Analysis of the ATP/ADP IPT family suggests there is functional redundancy among members because T-DNA insertion mutants in individual *IPT* genes do not show visible phenotypes. However, higher order mutant combinations including the triple *ipt3,5,7* and quadruple *ipt1,3,5,7* mutants have smaller rosettes, longer primary and lateral roots, and reduced iP and tZ contents compared to wild type Col-0, an effect that is more prominent in the quadruple mutant (Miyawaki *et al.*, 2006). On the other hand, tRNA-IPT mutant, *ipt2,9* has smaller rosettes and roots, reduced cZ content but normal iP and tZ levels (Köllmer *et al.*, 2014).

Cytokinin homeostasis is maintained by the action of cytokinin oxidases/dehydrogenases (CKX) which catalyze the irreversible degradation of cytokinin molecules into an adenine and isoprenoid chain (Schmulling *et al*., 2003). In Arabidopsis, the cytokinin oxidase gene family is comprised of seven genes (*CKX1-7*). Similar to cytokinin biosynthesis genes, cytokinin oxidase genes have tissue-specific expression patterns. *CKX1* and *CKX2* are mainly expressed in the shoot apex and in young floral

tissues, while *CKX4* is expressed in leaf stipules, trichomes and stomata as well as in the root cap. *CKX5* is expressed in emerging leaves, pollen, stamen and the apical meristem. *CKX6* is mainly expressed in the vascular cylinder of young shoot and root tissues as well as flowers (Werner *et al.*, 2003). *CKX7* is expressed during early development in root, hypocotyl and cotyledon vasculature as well as in the mature embryo sac (Köllmer *et al.*, 2014). Analysis of various T-DNA insertion mutants did not show a visible aboveground phenotype except for double mutant combinations containing the *ckx3-1* allele. Double mutant *ckx3 ckx5* has larger floral meristems, supernumerary ovules and an increased seed set per silique suggesting a role for cytokinin degradation in reproductive development (Bartrina *et al.*, 2011).

Although cytokinins have been extensively studied in plants, they are also found in other organisms including bacteria and fungi and more recently in the beet cyst nematode (BCN), *Heterodera schachtii*, and the root-knot nematode (RKN), *Meloidogyne incognita* (Dimalla and van Staden, 1977; Powell and Morris, 1986; De Meutter *et al.*, 2003; Pertry *et al.*, 2009; Siddique et al., 2015). Interestingly, cytokinins of microbial origin promote infection of their hosts (Sakakibara, 2005; Depuydt *et al.*, 2009; Pertry *et al.*, 2010; Ueda *et al.*, 2012; Radhika *et al.*, 2015). Some plant pathogens have the ability to use cytokinin mimics to manipulate host cytokinin metabolism and signaling pathways leading to the establishment of a sink. The crown gall inducing agent, *A. tumefaciens*, transfers a portion of DNA containing a cytokinin biosynthetic enzyme (tmr) into the host nucleus to induce synthesis of cytokinin in shoot tissues. Following genomic integration, the tmr protein is translocated to the plastid. Once in the organelle, tmr synthesizes tZs using hydroxyl-methylbutenyl diphosphate (HMBDP) without the need for P450 moonoxygenase

mediated hydroxylation (Ueda *et al.*, 2012). This results in the inability of the host to self-regulate cytokinin activation causing a hyperactive state of cytokinin signaling (Sakakibara *et al.*, 2005). Like *A. tumefaciens*, the actinomycete *Rhodococcus fascians,* uses cytokinin to induce gall formation in its host, but does so through a different approach. *R. fascians* secretes a mix of methylated forms of cytokinin, highly resistant to enzymatic breakdown by plant oxidases, causing hyper-accumulation of these molecules in the host tissue. The Arabidopsis cytokinin receptor *ahk3 ahk4* double mutant is resistant to this pathogen, suggesting pathogen-derived cytokinins signal through these receptors to cause disease. Gene expression analysis of *R. fascians* infected tissue showed downregulation of host cytokinin biosynthesis genes, but upregulation of cytokinin-degrading enzymes, suggesting accumulation of pathogen-derived cytokinins at the site of infection (Depuydt *et al.*, 2008). A genome-wide analysis of differentially expressed genes in response to *R. fascians* showed downregulation of ROS producing enzymes and an upregulation of invertases, sucrose transporters and the cell cycle activator CYCD3 in response to this pathogen (Depuydt *et al.*, 2009). More recently, it was discovered that BCN have a cytokinin tRNA-IPT biosynthesis gene homolog, which is expressed at high levels in the nematode esophageal glands. Plants challenged with the tRNA-IPT-silenced nematodes showed significantly fewer number of cysts compared to plants infected with untreated nematodes. Cytokinin content in *HsIPT* silenced worms was significantly reduced for iP cytokinins, which are the predominant form of cytokinin secreted by *H. schachtii*. Furthermore, BCN infected roots showed high levels of CYCB1::GUS expression in syncytia, however, tRNA-IPT-silenced nematodes fail to activate expression of this gene. CYCB1::GUS is a marker for cell division expressed between the G2 and M cell cycle

phases suggesting that BCN use cytokinin mimics to activate CYCB1 in the host plant to start the establishment of a feeding site (Siddique et al., 2015). Altogether, these results indicated that nematode cytokinins, preferentially of iP type, synthesized by BCN *IPT* are being delivered into the host to activate cytokinin signaling required for initiation of the feeding site (Siddique *et al*., 2015).

Although BCN cytokinin plays a role in nematode infection, it was not clear whether host cytokinin is also required in this process. Given that RKN, like BCN, also induce cell division in feeding sites, and previous studies have shown *Lotus japonicus* hairy roots overexpressing a cytokinin oxidase gene have reduced levels of RKN infection (Lohar *et al*., 2004), we explored in detail the role of cytokinin in RKN susceptibility. This chapter describes the results of a spatio-temporal comparative analysis of cytokinin biosynthesis and catabolism gene expression and function in Arabidopsis in response to BCN and RKN nematode infection.

RESULTS

Reduced levels of endogenous plant cytokinin decrease susceptibility of Arabidopsis to BCN and RKN

A prior study reported a reduction in root-knot nematode induced gall formation on *Lotus japonicus* hairy roots overexpressing cytokinin oxidases *AtCKX3* and *ZmCKX1* compared to a vector control (Lohar *et al*., 2004). To determine whether cytokinin contributes to nematode susceptibility in Arabidopsis, we evaluated a cytokinin oxidase overexpressing line (*35S:CKX2*) in response to BCN and RKN infection. At 14 days post infection (dpi), fewer cysts were developing on *35S:AtCKX2* plants compared to Col-0, but

the difference was not statistically significant. However, significant reductions were found at 30 dpi (Figure 2.1). *35S:AtCKX3* and *35S:AtCKX4* lines also had significantly fewer cysts compared to Col-0 (Siddique et al., 2015).

To determine whether cytokinin contributes to RKN infection in Arabidopsis, we evaluated the *35S:AtCKX2* line using either a sand-soil mix substrate or agar medium. When grown in either soil or medium, *35S:AtCKX2* plants were significantly less susceptible than the wild type control, Col-0. Consistent with the results of infection assays in agar plate assays, *35S:AtCKX2* gall counts were reduced by 40.7% compared to gall counts in Col-0 (Figure 2.1). In addition, *35S:AtCKX2* egg counts were reduced by 58.7% (Figure 2.2), suggesting that decreasing cytokinin content in Arabidopsis, similarly to Lotus, can cause reduced nematode infection. These findings indicate that BCN and RKN require normal levels of cytokinin to successfully infect Arabidopsis and that cytokinin plays a positive role in both interactions.

Cytokinin biosynthesis (*IPT*) gene expression is differentially regulated in response to BCN and RKN

To evaluate cytokinin biosynthesis gene expression in feeding sites induced by BCN and RKN, seven *IPT:GUS* reporter lines, including adenylate *IPT* biosynthesis genes, *IPT1*, *IPT3*, *IPT5*, *IPT7*, *IPT8* and two tRNA-IPT biosynthesis genes, *IPT2* and *IPT9*, were inoculated either with BCN or RKN infective juveniles. Infected plants were harvested at different time points representing the main stages of nematode development. A histochemical β-glucuronidase (GUS) assay was conducted on infected and uninfected

plants. In the absence of nematode infection, *IPT1* was expressed in root tips and no expression was observed in root vasculature (Figure, 2.3; Miyawaki *et al.*, 2004).

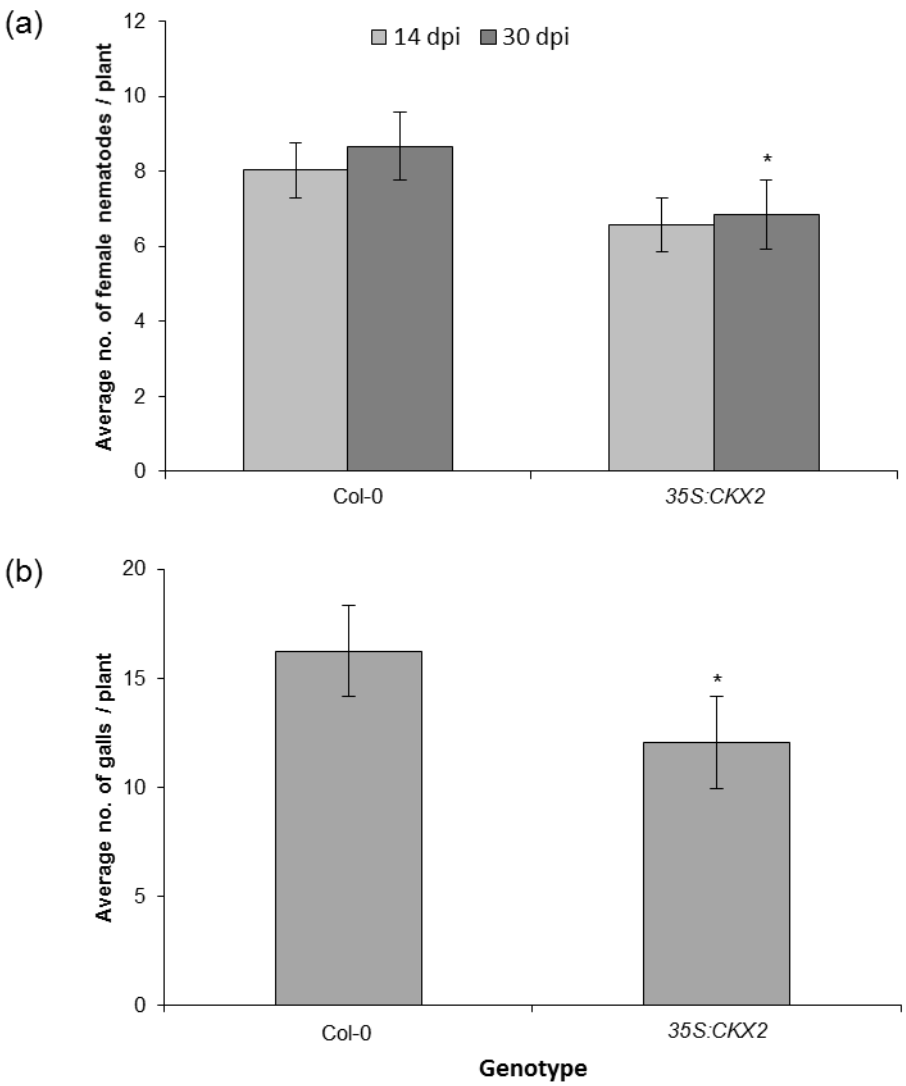

Figure 2.1. Overexpression of cytokinin oxidase 2 (*35S:CKX2*) reduces beet cyst nematode *Heterodera schachtii* and root-knot nematode *Meloidogyne incognita* infection in Arabidopsis Knop's media assays. (a) Average number of female nematodes per plant at 14 and 30 days post-inoculation (dpi), plant number (n) per line were n=33 for Col-0, n=33 for *35S:CKX2*. (b) Average number of galls per plant that developed in *35S:CKX2* and Col-0 at 40 dpi, plant number (n) per line were n= 30 for Col-0, n=36 for *35S:CKX2*. Data represent means +/- SE. Data are representative results of one out of three independent experiments. Asterisks indicate statistically significant differences compared with Col-0 using a two-tailed Student's *t*-test ($P < 0.05$).

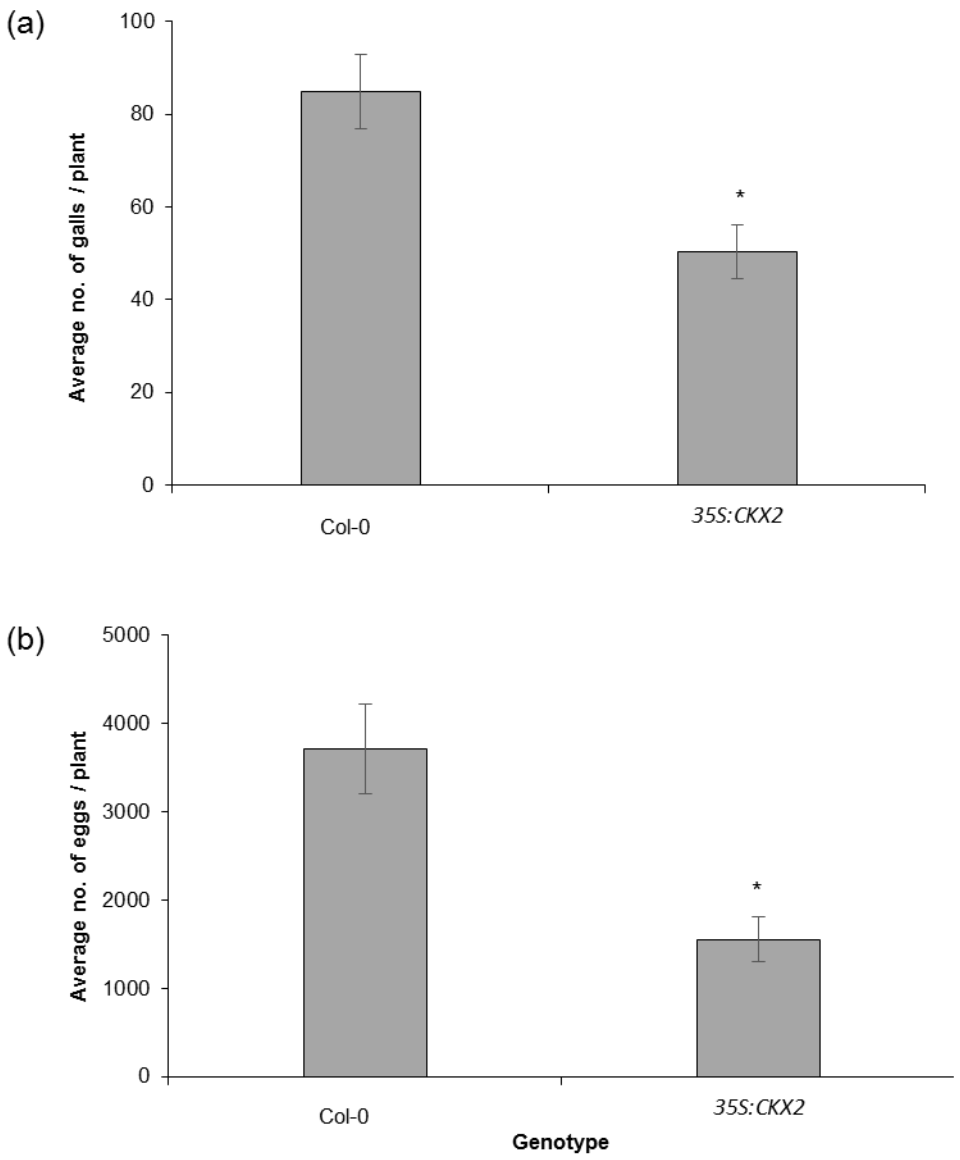

Figure 2.2. Overexpression of cytokinin oxidase 2 (*35S:CKX2*) reduces root-knot nematode *Meloidogyne incognita* infection in Arabidopsis soil assays. (a) Average number of galls per plant at seven weeks post-inoculation (wpi). Data represent mean +/- SE, n=14 for Col-0, n=12 for *35S:CKX2*. (b) Average number of eggs per plant. Total eggs per plant were harvested and counted at 7 wpi. Data represent mean +/- SE, n=14 for Col-0, n=10 for *35S:CKX2*. Data are representative results of at least two independent experiments. Asterisks indicate statistically significant differences compared with Col-0 using a two-tailed Student's *t*-test ($P < 0.05$).

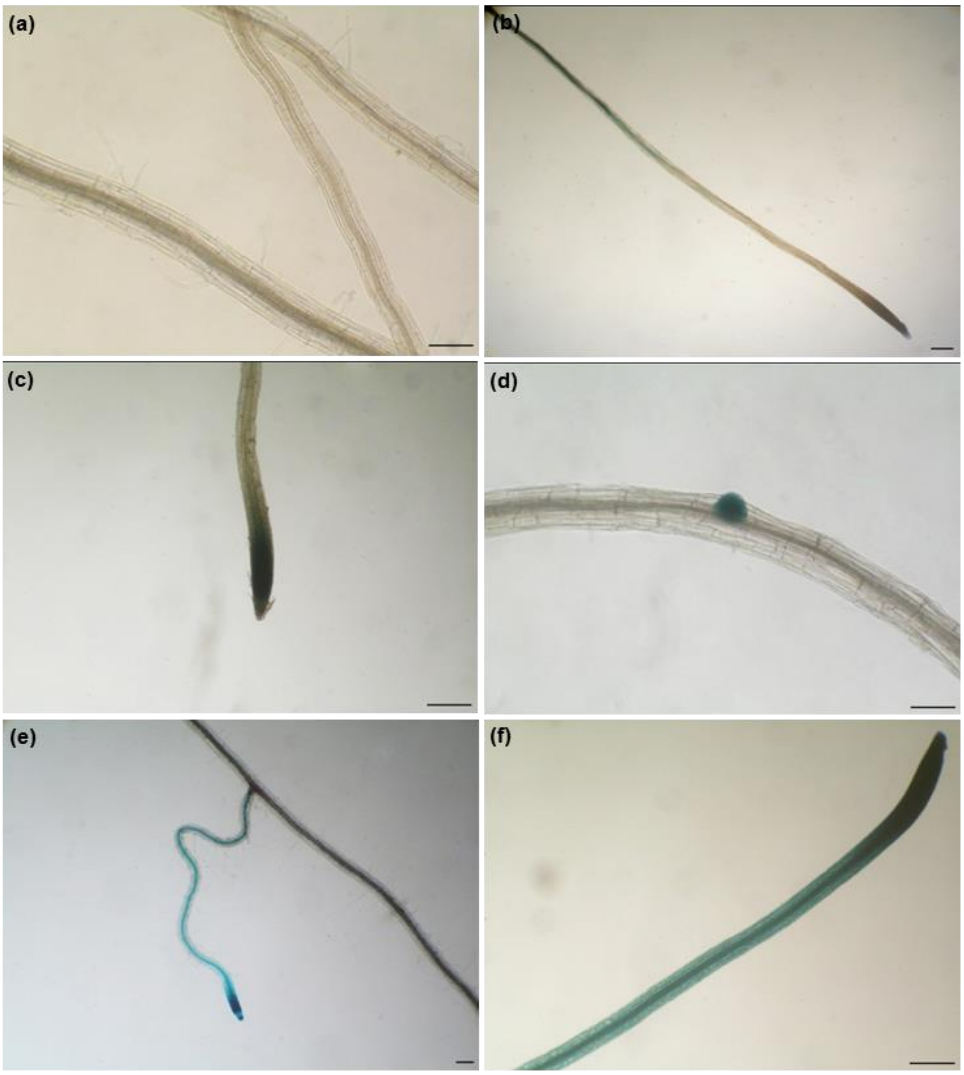

Figure 2.3. Expression of cytokinin biosynthesis genes in Arabidopsis uninoculated plants. (a) *IPT1:GUS* (b) *IPT3:GUS* (c,d) *IPT2:GUS* and (e,f) *IPT9:GUS*. Scale bar = 100 μm. Photos taken by Demosthenis Chronis.

However, as a consequence of BCN infection, GUS expression was visible in developing feeding sites (Figure 2.4a-c). In contrast to BCN infection, *IPT1:GUS* expression was not detected in galls at any time point evaluated (Figure 2.4d-f). *IPT3*, on the other hand, had a different response and was highly expressed in the root vasculature of uninfected plants (Figure, 2.3; Miyawaki *et al.*, 2004). However, when *IPT3:GUS* was challenged with either BCN or RKN, we observed downregulation in both syncytia and galls at all developmental stages (Figure 2.4 g-l). No changes in expression were found for *IPT5*, *IPT7* and *IPT8* in response to either BCN or RKN infection. Overall, these results suggest a specific regulation of certain members of the cytokinin biosynthesis gene family in response to BCN and RKN infection, with only one adenylate *IPT* gene being upregulated in response to BCN and no major upregulation of adenylate *IPT* genes in response to RKN.

BCN and RKN were also able to modulate tRNA-*IPT* gene expression. In the absence of nematode infection, *IPT2:GUS* was expressed in root tips and lateral root primordia (Figure 2.3; Miyawaki *et al*., 2004). But after nematode infection, *IPT2:GUS* was observed within developing syncytia and galls of nematode-infected roots at multiple developmental stages (Figure 2.5a-e). Cross-sections of galls revealed expression of this gene in giant-cells (Figure 2.5f). *IPT9:GUS* expression, on the contrary, was downregulated in syncytia and galls (Figure 2.5g-l), despite strong expression in the root vasculature of uninfected and infected roots. Changes in *IPT1* and *IPT3* gene expression were confirmed by qRT-PCR of BCN and RKN-infected plants compared to mock-inoculated controls (Figure 2.9).

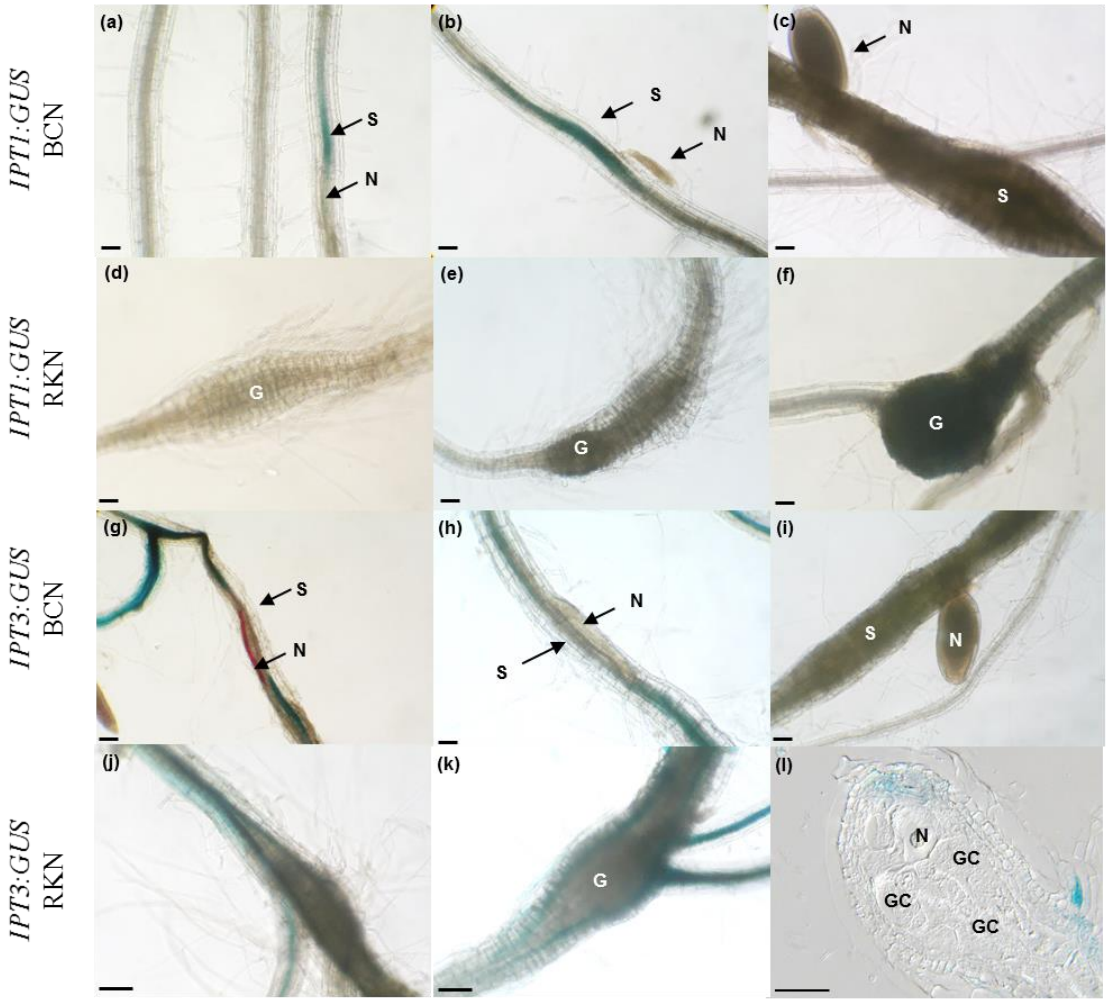

Figure 2.4. Cytokinin biosynthesis isopentenyltransferases *IPT1* and *IPT3* expression in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*. (a-c) *IPT1:GUS* and (g-i) *IPT3:GUS* expression in BCN-infected Arabidopsis roots during early and later stages of parasitism: (a,g) second-stage juveniles (J2), (b,h) third-stage juveniles (J3) and (c,i) fourth-stage juveniles (J4). (d-f) *IPT1:GUS* and (j-l) *IPT3:GUS* expression in RKN-infected Arabidopsis roots during early and later stages of parasitism: (d,j) 3, (e,k) 6 and (f,l) 11 dpi. (l) Longitudinal section of a gall showing reduced *IPT3:GUS* expression in GC. Abbreviations: N, nematode; S, syncytium; G, gall; GC, giant cell. Scale bar=100 μm, 50 μm (l). Photos taken by Demosthenis Chronis.

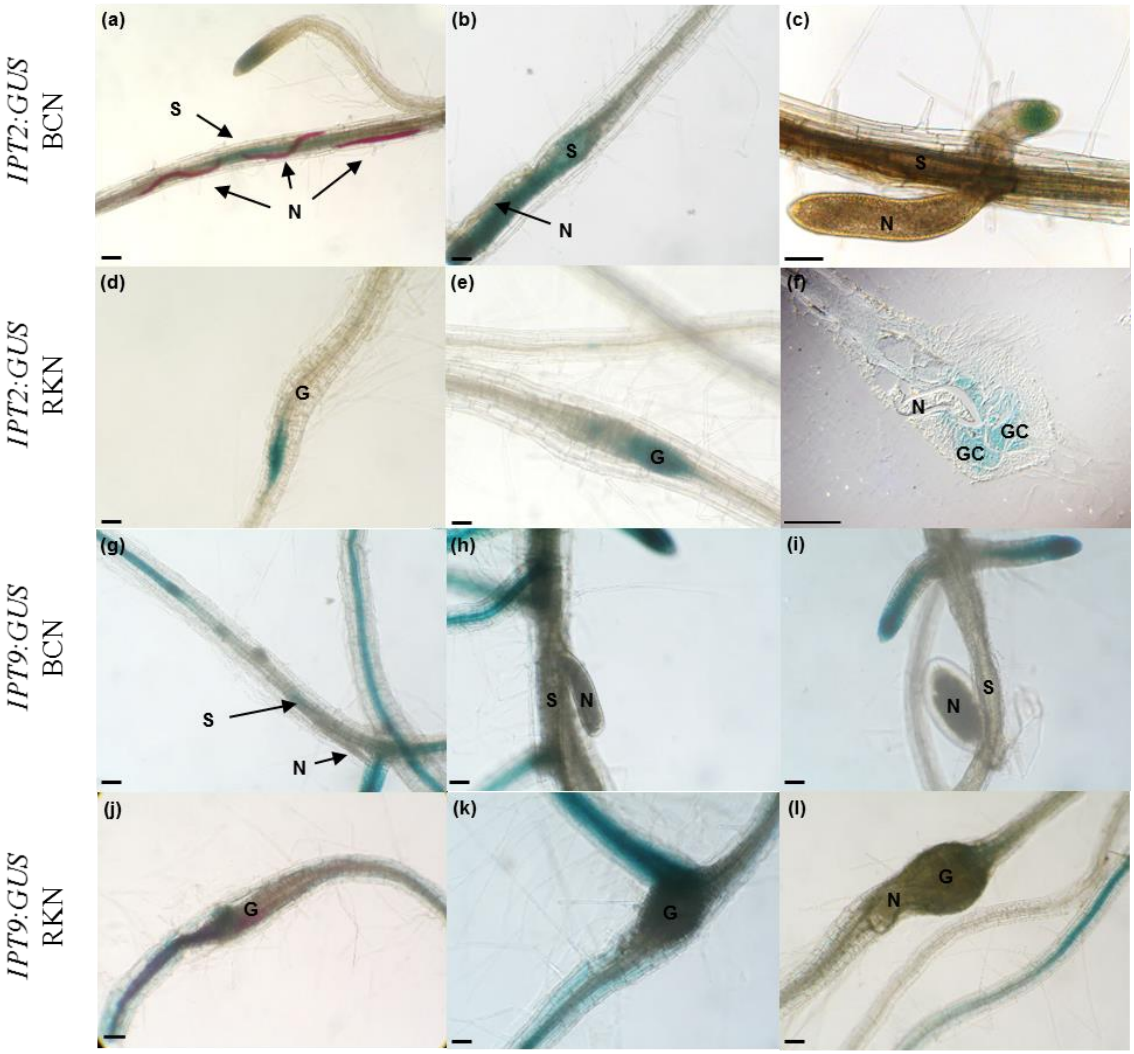

Figure 2.5. Cytokinin biosynthesis trans-isopentenyltransferases *IPT2* and *IPT9* expression in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*. (a-c) *IPT2:GUS* and (g-i) *IPT9:GUS* expression in BCN-infected Arabidopsis roots during early and later stages of parasitism: (a,g) second-stage juveniles (J2), (b,h) third-stage juveniles (J3) and (c,i) fourth-stage juveniles (J4). (d-f) *IPT2:GUS* and (j-l) *IPT9:GUS* expression in RKN-infected Arabidopsis roots during early and later stages of parasitism: (d,j) 3, (e,k) 6 and (f,l) 11 dpi. (f) Longitudinal section of a gall showing *IPT2:GUS* expression in GC. Abbreviations: N, nematode; S, syncytium; G, gall; GC, giant cell. Scale bar=100 µm, 50 µm (f). Photos except (c) were taken by Demosthenis Chronis.

Cytokinin catabolism (*CKX*) gene expression is differentially regulated in response to BCN and RKN

Cytokinin levels are tightly regulated by a family of seven cytokinin oxidases (CKX1-CKX7) involved in the degradation of cytokinin. We tested six *CKX:GUS* lines including *CKX1:GUS*, *CKX2:GUS*, *CKX4:GUS*, *CKX5:GUS*, *CKX6:GUS* and *CKX7:GUS* in infection assays with both BCN and RKN. *CKX1:GUS* and *CKX2:GUS* showed no expression in roots (Figure 2.6a-b; Werner *et al.*, 2003) and no expression in response to nematode parasitism (Figure 2.7a-h). *CKX4:GUS* showed expression in root tips of uninfected plants (Figure 2.6c; Werner *et al*., 2003), but was not expressed in syncytia or galls of nematode-infected plants (Figure 2.7i-l). *CKX5:GUS* expression in uninfected roots was limited to the root apical meristem (Figure 2.6d; Werner *et al*., 2003). However, *CKX5:GUS* was upregulated during early stages of cyst nematode infection and declined as the nematode molted into an adult stage (Figure 2.8a-c). Interestingly, *CKX5:GUS* expression was not observed in galls at any time point evaluated (Figure 2.8d-f). In contrast, *CKX6:GUS*, which was expressed in the root vasculature (Figure 2.6e;Werner *et al*., 2003), showed upregulation during early stages of syncytium development and during early and late stages of gall development (Figure 2.8g-l). *CKX7:GUS* also showed upregulation during early stages of syncytium formation and a restricted pattern of expression within galls that declined quickly (Figure 2.8m-r). A subset of genes were confirmed by qRT-PCR of BCN and RKN-infected plants compared to mock-inoculated controls (Figure 2.9).

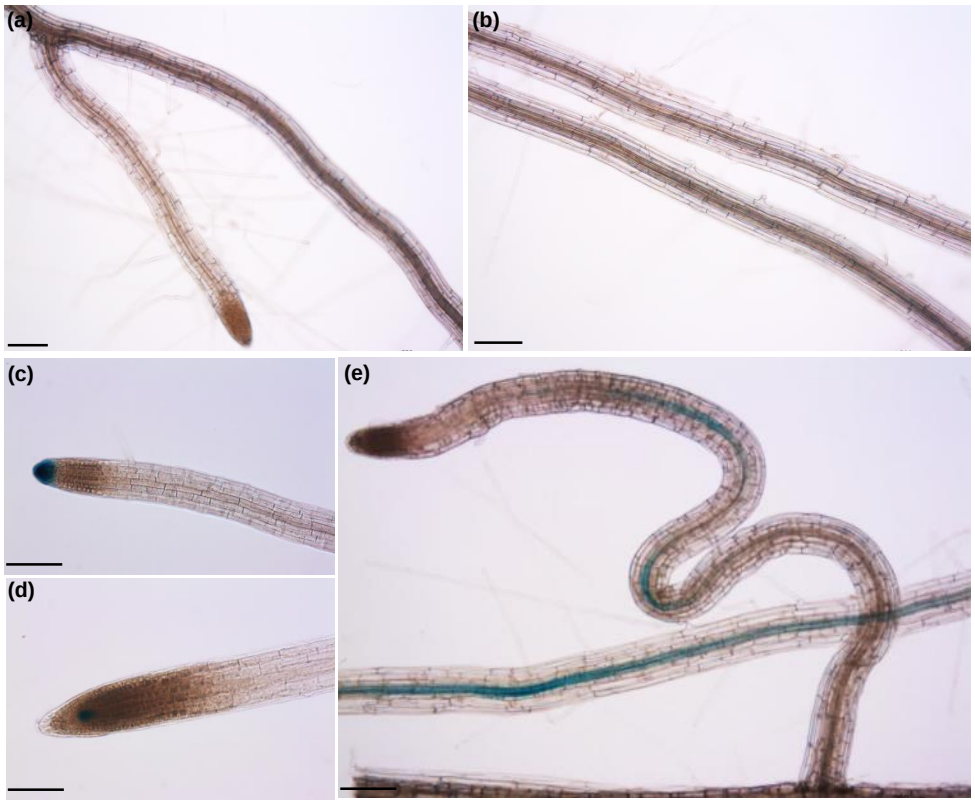

Figure 2.6. Expression of cytokinin catabolism genes in Arabidopsis uninoculated plants. (a) *CKX1:GUS* (b) *CKX2:GUS* (c) *CKX4:GUS* (d) *CKX5:GUS* and (e) *CKX6:GUS*. Scale bar = 100 μm.

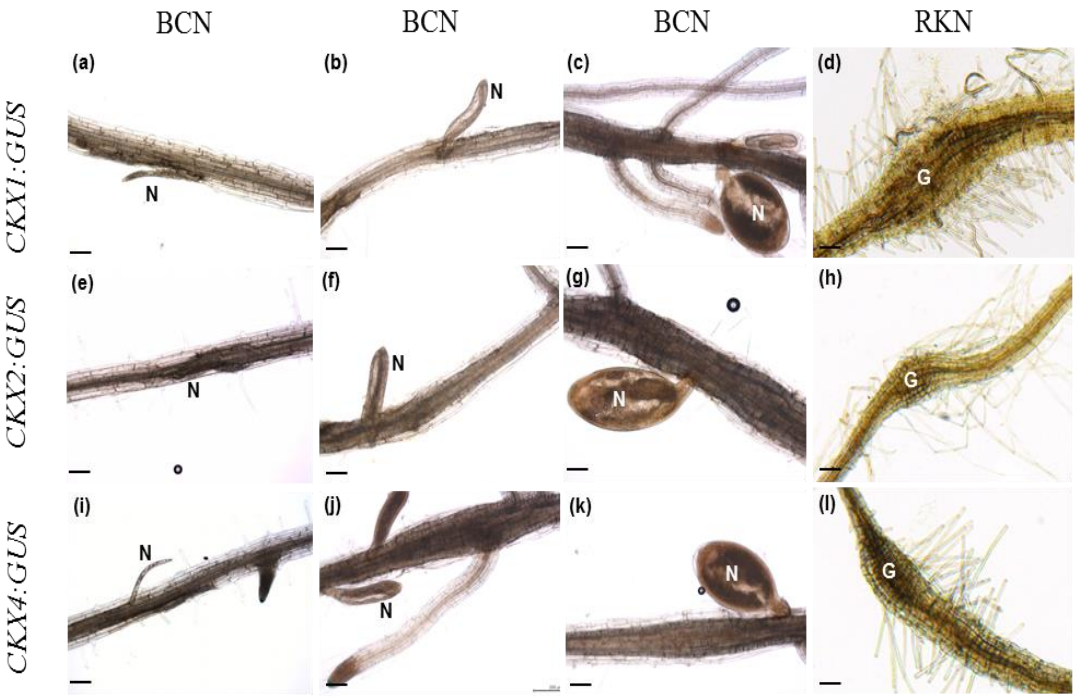

Figure 2.7. Cytokinin oxidases *CKX1:GUS, CKX2:GUS* and *CKX4:GUS* lines showing no GUS expression in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*. (a-c) *CKX1:GUS*; (e-g) *CKX2:GUS*; (i-k) *CKX4:GUS* BCN-infected Arabidopsis roots during early and late stages of parasitism: (a,e,i) second-stage juvenile (J2), (b,f,j) third-stage juvenile (J3) and (c,g,k) fourth-stage juvenile (J4). (d,h,l) *CKX1:GUS*, *CKX2:GUS*, and *CKX4:GUS* RKN-infected Arabidopsis roots. N, nematode; S, syncytium; G, gall. Scale bar=100 μm.

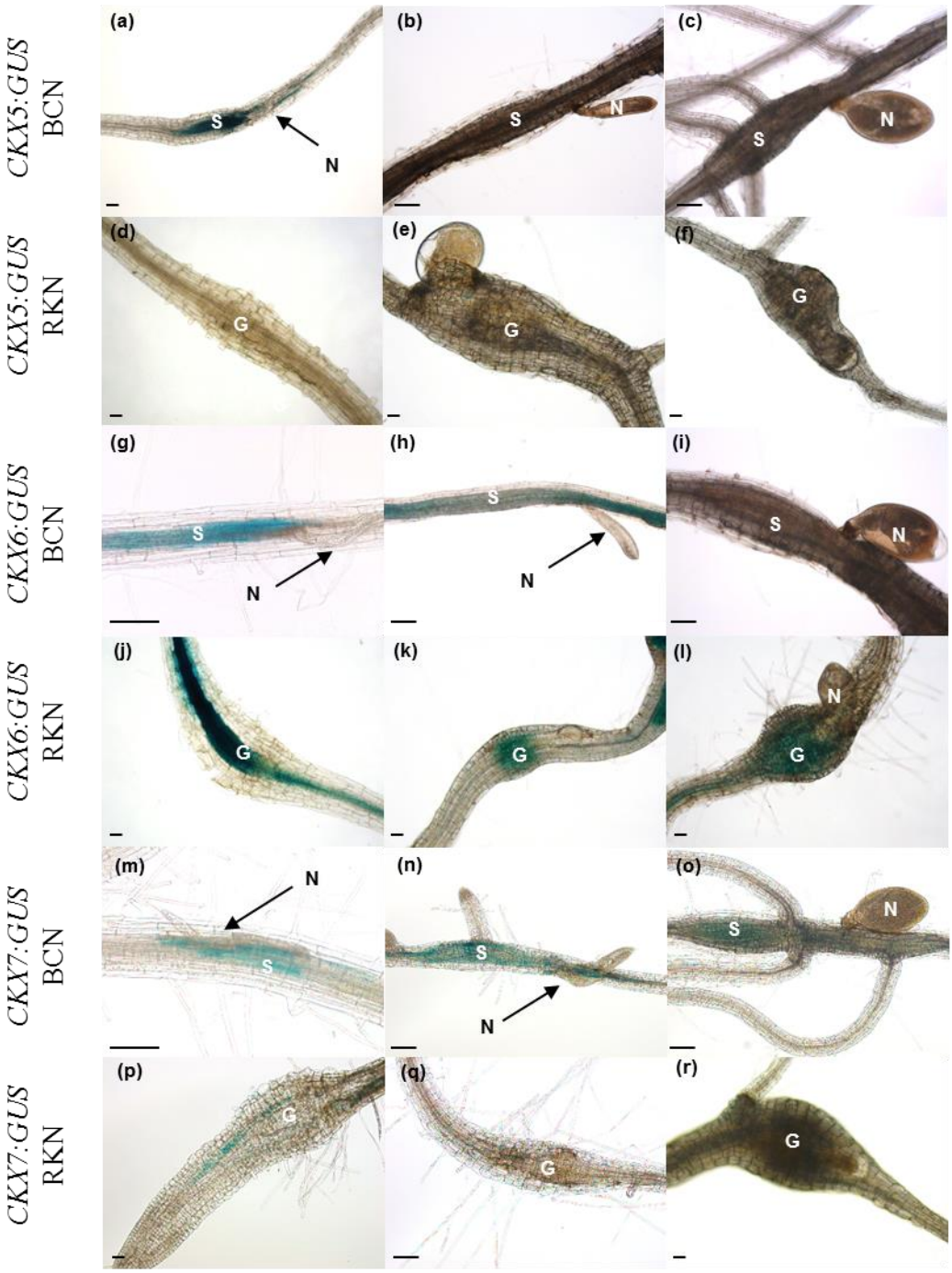

Figure 2.8. Cytokinin oxidases *CKX5*, *CKX6* and *CKX7* expression in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*. (a-c) *CKX5:GUS*, (g-i) *CKX6:GUS*, (m-o) *CKX7:GUS* expression in BCN-infected Arabidopsis roots during early and later stages of parasitism: (a,g,m) second-stage juveniles (J2), (b,h,n) third-stage juveniles (J3) and (c,i,o) fourth-stage juveniles (J4). (d-f*)* *CKX5:GUS*, (j-l) *CKX6:GUS*, (p-r) *CKX7:GUS* expression in RKN infected Arabidopsis roots during early and later stages of parasitism: (d,j,p) 3, (e,k,q) 6 and (f,l,r) 11dpi. Abbreviations: N, nematode; S, syncytium; G, gall. Scale bar=100 µm.

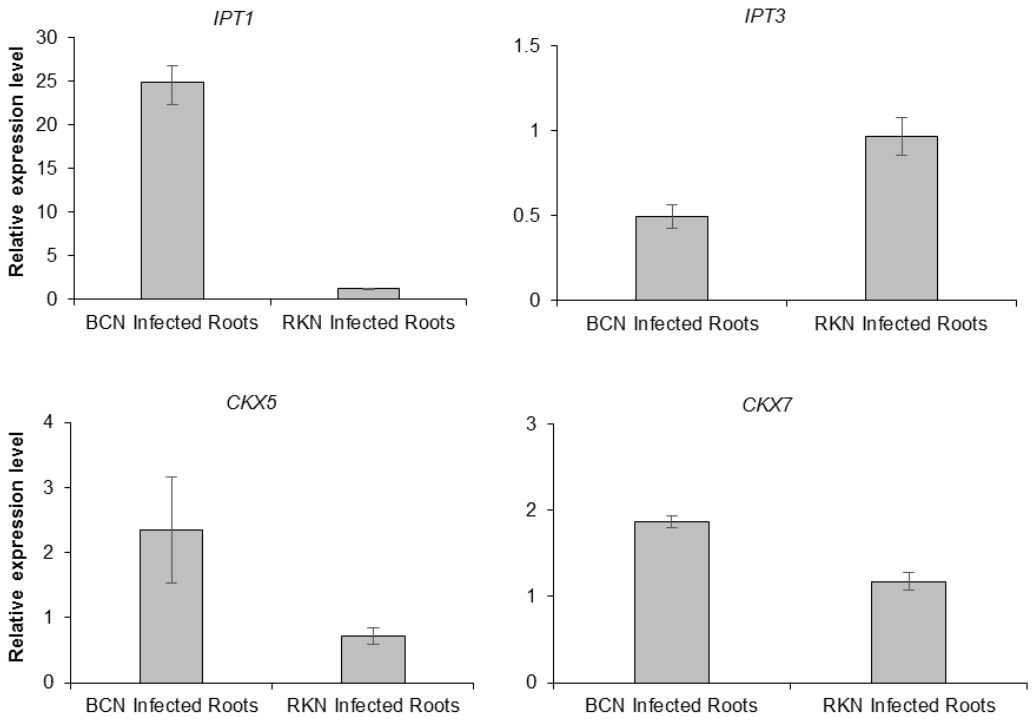

Figure 2.9. Quantification of cytokinin gene expression levels in beet cyst nematode (BCN) and root-knot nematode (RKN) infected roots. a) Transcript levels were determined by real time quantification- PCR (qRT-PCR). Average expression levels relative to mock calculated using the ΔΔCt calculation method and *AtActin* as endogenous control. *AtActin* gene (AT1G49240) was amplified using forward primer 5' AGTGGTCGTACAACCGGTATTGT and reverse primer 5' GAGGATAGCATGTGGAAGTGAGAA. Error bars represent standard error of two independent biological replicates. Four technical replicates were used per treatment.

Cytokinin biosynthesis and catabolism mutants have reduced BCN and RKN infection

The upregulation of *IPT1:GUS* in response to BCN, but not RKN, led us to evaluate *ipt1-1* in response to BCN infection; however, no phenotype was observed likely due to functional redundancy in this gene family (Figure 2.10). Therefore, we evaluated a biosynthesis triple mutant *ipt3,5,7*. Seedlings of IPT quadruple mutant *ipt1,3,5,7* are dwarf, with limited shoot and root growth, and therefore not suitable for nematode infection assays (Miyawaki *et al.*, 2006). We did not observe a significant reduction in the number of cysts developing on *ipt3,5,7* compared to Col-0, possibly due to the fact that IPT1 was still functioning in syncytia, however syncytia size was reduced (Siddique *et al.*, 2015) indicating a positive role for host cytokinin in cyst nematode feeding site formation.

The upregulation of *IPT2* in both syncytia and giant-cells led us to evaluate the Arabidopsis mutant line *ipt2-1* in response to infection by these two nematode species. The *ipt2-1* plants did not show any visible growth phenotype compared to wild type as previously described (Miyawaki *et al.*, 2006). Interestingly, when *ipt2-1* was challenged with BCN we observed significantly higher numbers of cysts than on the wild type Col-0 (Figure 2.11). *ipt2-1* root-knot infected plants also showed a trend of higher average gall number compared to Col-0; however, the lack of significance may be the result of the difficulty in detecting the small effects given the variability in gall numbers among samples (Figure 2.11). These results suggest IPT2 has a divergent role in plant-nematode interactions that is not associated with the reduced susceptibility we observed for the cytokinin biosynthesis and receptor mutants described in our previous study (Siddique *et al.*, 2015) and was not evaluated further here.

In an attempt to confirm a biological role for *CKX* gene family members upregulated by either BCN or RKN, we tested CKX mutant lines *ckx5-1*, *ckx6-2* and *ckx5-1 ckx6-2* in response to BCN and *ckx6-2* and *ckx5-1 ckx6-2* in response to RKN infection. Interestingly, we observed a significant reduction in the number of cysts and galls on the *ckx5-1 ckx6-2* mutant compared to Col-0, suggesting the importance of CKX enzymes and proper cytokinin balance for BCN and RKN infection (Figure 2.12).

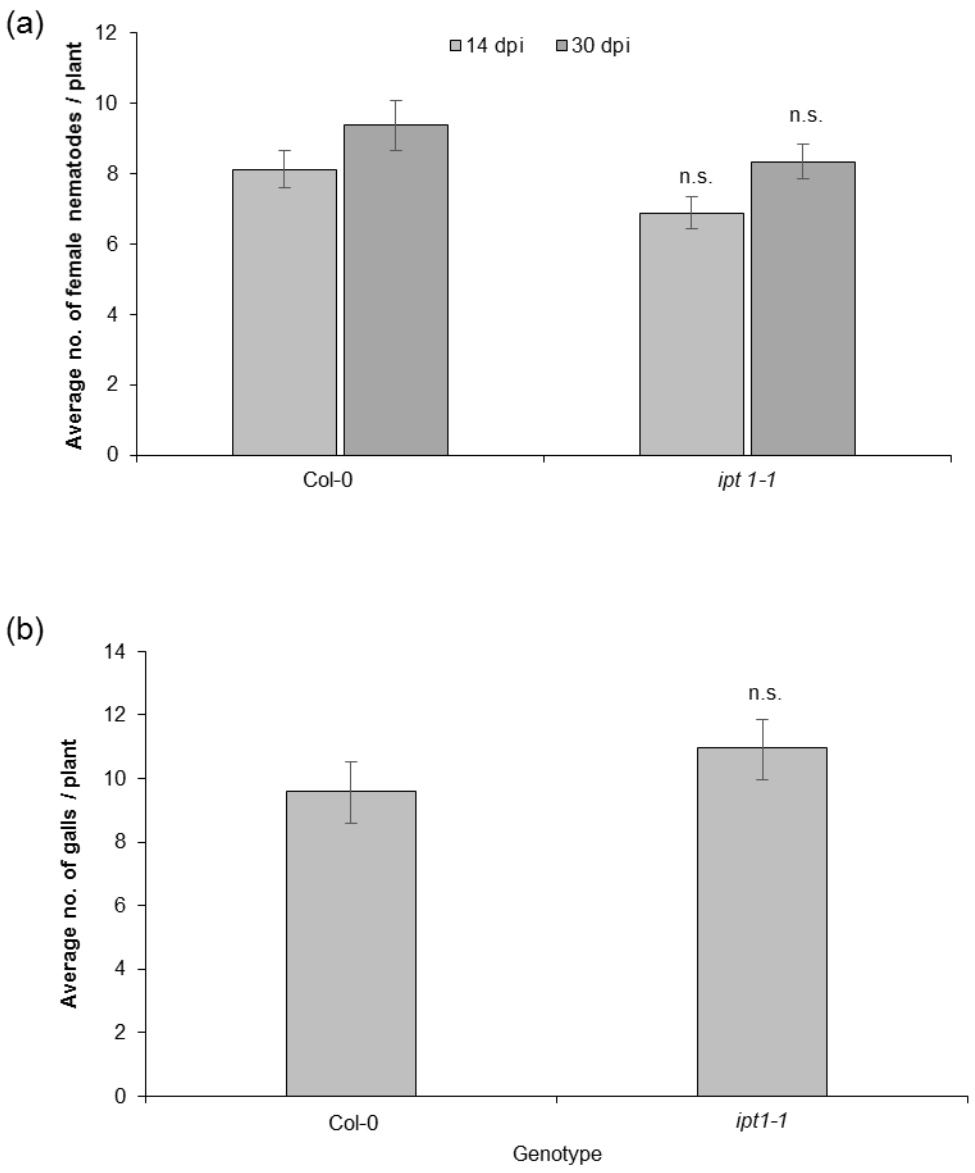

Figure 2.10. Evaluation of cytokinin biosynthesis mutant *ipt1-1* in response to beet cyst nematode *Heterodera schachtii* and the root-knot nematode *Meloidogyne incognita*. (a) Average number of *Heterodera schachtii* female nematodes per plant that developed in *ipt1-1* at 14 and 30 dpi, plant number (n) per line were n = 32 for Col-0, n = 32 for *ipt1-1*, (b) Average number of *Meloidogyne incognita* galls per plant that developed in *ipt1-1* and Col-0 at 40 dpi, plant number (n) per line were n = 31 for Col-0, n = 33 for *ipt1-1*. Error bars indicate SE. Data are representative results of one out of three independent experiments. Statistical analyses were conducted comparing *ipt1-1* with Col-0 using a two-tailed Student's *t*-test ($P < 0.05$). n.s.=non-significant differences.

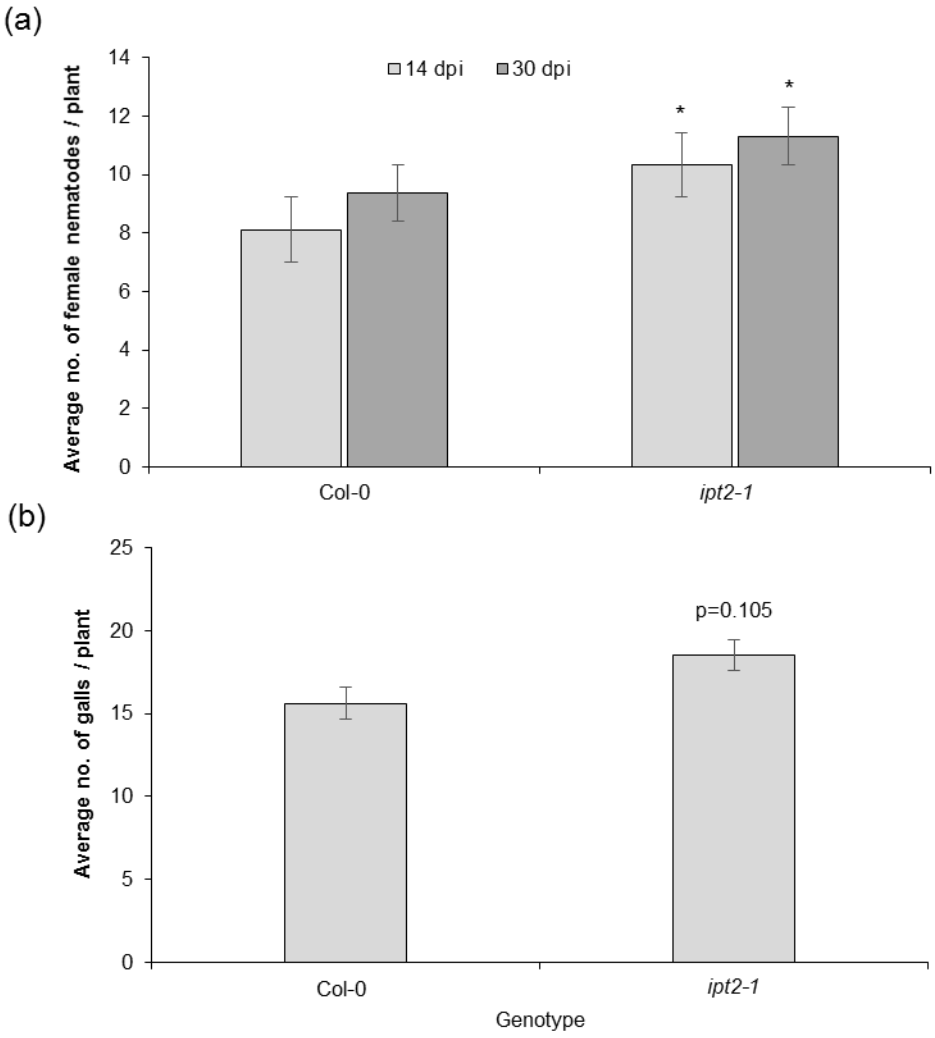

Figure 2.11. Evaluation of cytokinin biosynthesis mutant *ipt2-1* in response to beet cyst nematode *Heterodera schachtii* and the root-knot nematode *Meloidogyne incognita*. (a) Average number of *Heterodera schachtii* female nematodes per plant that developed in *ipt2-1* at 14 and 30 dpi, plant number (n) per line were n = 32 for Col-0, n = 34 for *ipt2-1*, (b) Average number of *Meloidogyne incognita* galls per plant that developed in *ipt2-1* and Col-0 at 40 dpi, plant number (n) per line were n = 32 for Col-0, n = 32 for *ipt2-1*. Error bars indicate SE. Data are representative results of one out of four independent experiments. Asterisks above columns indicate significant differences compared with Col-0 at 14 and 30 dpi by a two-tailed Student's *t*-test ($P < 0.05$).

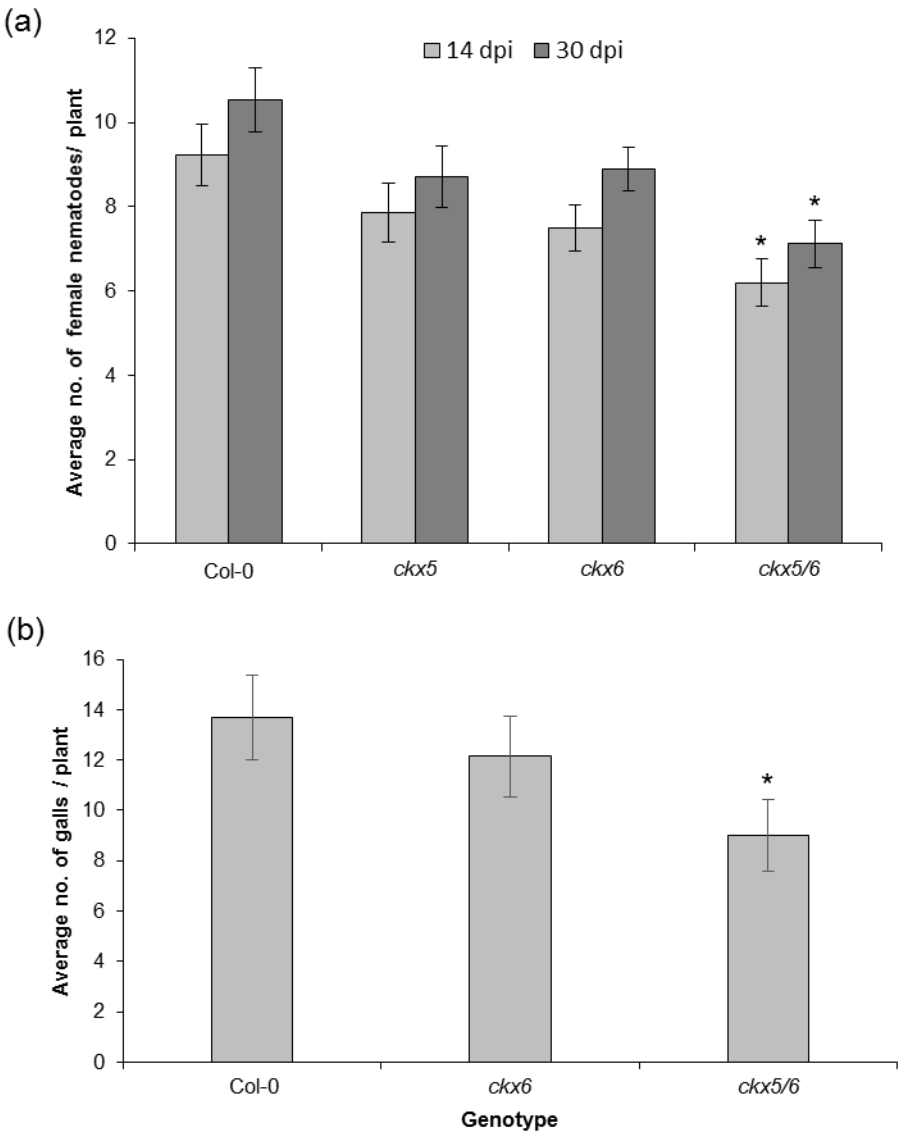

Figure 2.12. Evaluation of cytokinin oxidase mutants in response to *Heterodera schachtii* and *Meloidogyne incognita* infection. (a) Average number of *Heterodera schachtii* female nematodes per plant that developed in *ckx5* (*ckx5-1*), *ckx6 (ckx6-2)* and *ckx5/6 (ckx5-1 ckx6-2)* at 14 and 30 dpi, plant number (n) per line were n = 35 for Col-0, n = 35 for *ckx5*, n = 36 for *ckx6*, n = 35 for *ckx5/6*. (b) Average number of *Meloidogyne incognita* galls per plant that developed in *ckx6 (ckx6-2)* and *ckx5/6 (ckx5-1 ckx6-2)* at 40 dpi, plant number (n) per line were n = 29 for Col-0, n = 30 for *ckx6*, n = 28 for *ckx5/6*. Error bars indicate SE. Data are representative results of one out of three independent experiments. Asterisks above columns indicate significant differences compared with Col-0 by a two-tailed Student's *t*-test ($P < 0.05$).

DISCUSSION

CN and RKN feed as sedentary endoparasites by inducing the formation of a permanent feeding site within host roots. Although the feeding sites induced by these two types of nematodes function in a similar capacity as nutrient sinks, the molecular events leading up to their formation differ with respect to activation of the cell cycle and cell division. How these nematodes are able to alter cell cycle and induce cell division has been a long standing question in the field and the mechanistic details underlying the differences between them are still not well understood. Recent work supports a conserved role for cytokinin in CN and RKN infection (Siddique *et al*., 2015; Lohar *et al*., 2004), but whether these nematode species are manipulating common or distinct cytokinin genes has remained unknown.

In this study, through a comparative analysis using a single host plant species we demonstrate that cytokinin is a requirement of both CN and RKN interactions, but these two nematode species differentially regulate genes involved in cytokinin biosynthesis and catabolism for the establishment of syncytia and giant-cells. We first analyzed Arabidopsis GUS reporter lines of cytokinin biosynthesis genes *IPT1* and *IPT3* in response to CN and RKN infection and found commonalities, but also gene-specific differences between both nematode species. *IPT1* and *IPT3* are two of the five ATP/ADP isopentenyltransferase genes in Arabidopsis, and are involved in the production of isopenentyladenine (iP) and zeatin (z), the most abundant forms of cytokinins in higher plants (Miwayaki *et al*., 2004). In this study, we found *IPT1* to be upregulated during early stages of BCN feeding site formation. *IPT1* expression was observed in infection zones developing near the

vasculature of main roots, suggesting CN can overrule repression of this gene in mature tissue and reactivate its expression presumably to help in the growth of the feeding site. *IPT1* expression within syncytia confirms the unique nature of the feeding site, which is partially unrelated to other organs in the plant. Although the Arabidopsis *ipt1* mutant did not show reduced susceptibility to CN probably due to functional redundancy among gene family members (Miyawaki *et al.*, 2006), these results support our previous studies on the involvement of plant cytokinin biosynthesis genes in syncytium formation (Siddique *et al.*, 2015). In contrast to CN infection, RKN infection did not stimulate *IPT1* expression, unveiling what may be a key difference in cytokinin biosynthesis regulation between these two nematode species. A second member of the ATP/ADP isopentenyltransferase family, *IPT3*, displayed an expression pattern opposite to *IPT1* as it was downregulated by both nematode species at all developmental stages of feeding site growth. Although the reason why *IPT3* is downregulated in feeding sites is beyond the reach of these studies, the fact that *IPT3* is downregulated by nutrient deficiencies such as low phosphorus and low nitrogen in roots (Ramireddy *et al.*, 2014) suggests that *IPT3* gene expression might be responding to internal nutrient content changes as a result of continuous nematode feeding.

We also evaluated the family of Arabidopsis tRNA-IPT genes, *IPT2* and *IPT9*, in response to BCN and RKN. These two enzymes catalyze the biosynthesis of *cis*-zeatins, a less active form of cytokinin, with a non-exclusive role in suppressing protoxylem specification (Köllmer *et al.*, 2014). *IPT2* and *IPT9* genes have a more ubiquitous expression than other *IPT* genes. In uninoculated plants, *IPT2* is specifically expressed in root tips, whereas *IPT9* is strongly expressed in the root tips and vasculature (Miyawaki *et al.*, 2004). In this study, we observed an upregulation of *IPT2* in syncytia and giant-cells,

but a downregulation of *IPT9* in both types of feeding sites. The overall differential regulation of biosynthesis and catabolism genes by CN and RKN suggest divergent initial cues delivered by these two nematode species into the initial feeding cell(s). It is well established that CN and RKN differ in their repertoire of effector molecules and host targets (Mitchum *et al.*, 2012). Thus, each nematode species may be utilizing a distinct set of effectors to directly or indirectly target specific genes within the cytokinin pathway. Previous studies have shown CN are able to manipulate auxin transport and signaling pathways through the use of effector molecules (Lee *et al.*, 2011; Hewezi *et al.*, 2015). As more effectors are identified and characterized from both CN and RKN, additional effectors targeting hormonal pathways are likely to be identified. An alternative (but not exclusive) explanation to our findings relies on the existence of cytokinin molecules of nematode origin. Our previous studies have shown that CN have an *IPT* cytokinin biosynthesis gene homologue and are able to secrete cytokinins (Siddique *et al.*, 2015). Degradation of nematode *IPT* transcripts by dsRNA gene silencing resulted in deficient feeding sites and smaller cysts. Additionally, we showed that BCN-derived cytokinins were necessary for cell cycle activation of in initial syncytial cells (Siddique *et al.*, 2015). Considering BCN mainly secretes isopentenyladenosines and benzyladenines, while RKN mainly secretes zeatins and benzyladenines (De Meutter *et al.*, 2003), it is possible the differences we observed in cytokinin biosynthesis and catabolism gene regulation derives from differences in the type of cytokinins secreted by CN and RKN. This will be interesting to explore further. The upregulation of tRNA *IPT2* in syncytia and galls led us to evaluate the *ipt2-1* mutant to further explore the role of this gene in nematode infection. Interestingly, and contrary to our results using a triple ATP/ADP IPT mutant (Siddique et

al., 2015) we found an increase rather than a decrease in nematode susceptibility. In this study, we did not test *ipt 1-1 2-1* double mutant due to the fact that IPT1 and IPT2 belong to two distinct cytokinin biosynthesis gene families with opposite functions in nematode infection. Therefore, we did not expect to see any additive gene effect (either a decrease or further increase in susceptibility).

Although the role of IPT2 and its derived *cis* zeatin (cZ) in root pathogen infection remains to be explored, new evidence supports a role of *cZ* in the regulation of aboveground defense responses. Both exogenous pre-treatment with *cZ* and the expression of IPT2 under control of a senescence associated gene promoter (SAG-IPT2) reduced *P. syringae* symptoms (Grobskinsky et al., 2015; Schafer *et al*., 2015). Additionally, exogenous application of *cZ* to tobacco leaves increased accumulation of JA-derived metabolites (Schaffer *et al*., 2015). The *IPT2* gene may have a similar role inducing defense responses against cyst and root-knot nematode infection belowground. Further experiments using a comparative transcriptomic analysis of infected vs. uninfected *ipt2-1* and Col-0 at the site of infection could help us determine which defense pathways are being upregulated by this gene.

Proper function of cytokinin in root and shoot meristems depends heavily on a stringent regulation of cytokinin catabolism (Werner *et al.*, 2003). To identify and compare cytokinin catabolism genes involved in CN and RKN feeding site formation, we tested GUS reporter lines of cytokinin oxidase genes in response to nematode infection. Among all genes evaluated, *CKX6* was upregulated by both species and *CKX5* and *CKX7* were upregulated by BCN, but not by RKN. Interestingly, *ckx5-1 ckx6-2* but not *ckx6-2* showed a significant reduction in gall numbers compared to Col-0, suggesting CKX5 could

possibly complement CKX6 function in the *ckx6-1* mutant. Considering that CKX enzymes have distinct substrate specificities, these results may suggest a difference in the cytokinin pool accumulating in syncytia vs. galls. Our previous studies showed an increase in cytokinin content in BCN-infected roots with a higher percent of iP and tZ cytokinins. Biochemical characterization of AtCKXs expressed in transgenic tobacco showed CKX5, CKX6 and CKX7 have mild activities against these types of cytokinins compared to other CKXs, such as CKX2 and CKX4 (Galuszka *et al.*, 2007). Potentially, upregulation of *CKX5*, *CKX6* and *CKX7* and not *CKX2* and *CKX4*, coupled with the local upregulation of biosynthesis genes *IPT1* and *IPT2* and secretion of nematode-derived cytokinins, may allow for a steady increase in iP and tZ cytokinin accumulation in syncytia explaining the high levels previously observed. CKX7 activity has also been correlated with a strong activity in *cis* zeatin degradation (Köllmer *et al.*, 2015), which supports the lower levels for this type of cytokinin found in BCN-infected roots (Siddique *et al.*, 2015). In this study, we discovered *ckx5-1 ckx6-2* mutants are less susceptible to both CN and RKN parasitism supporting a role for CKX enzymes in nematode infection. Presumably, misregulation of cytokinin degradation in syncytium and galls affects proper feeding site development and the ability of the nematodes to reach adulthood. Future experiments targeting cytokinin oxidases genes at feeding sites may be used to develop plants with resistance to both CN and RKN.

MATERIALS AND METHODS

Nematode Cultures

BCN (*Heterodera schachtii*) nematode cultures were maintained on greenhouse-grown sugarbeet plants (*Beta vulgaris* cv. Monohi). RKN (*Meloidogyne incognita*)

nematode cultures were maintained on tomato (*Lycopersicon esculentum* cv. Tiny Tim) and egg plant (*Solanum melongena* cv. Black Beauty).

Plant Material

IPT:GUS reporter lines were described by Miyawaki *et al* (2004). *CKX:GUS* (*CKX1:GUS – CKX6:GUS*) reporter lines were described by Werner *et al.* (2003). *CKX7:GUS* was reported in Köllmer *et al*. (2014). The Arabidopsis cytokinin biosynthesis mutant lines used in this study: *ipt1-1, ipt2-1, ipt3,5,7,* were described in Miyawaki *et al*., 2006. Lines *ckx5-1, ckx6-2* and *ckx5-1/ckx6-2* were described in Bartrina *et al*., 2011. Seeds were surface-sterilized with chlorine gas as previously described (Wang *et al.*, 2011) and cold-stratified for 3 days at 4ºC. Seeds were plated on 0.5% Murashige Skoog (MS) medium MS Basal Salts (Caisson Laboratories, North Logan, UT, USA), 2% sucrose and 0.8% Type A Agar (Sigma, St. Louis, MO, USA) and placed in a growth chamber set at 22ºC under constant light for seven days. Seedlings were transplanted from the medium using forceps to trays of commercially available potting mix (SunGro) containing 50-60% horticulture grade vermiculite, Canadian sphagnum peat moss, horticulture grade perlite and dolomitic limestone. Trays of seedlings were covered with a plastic dome and placed in a walk-in growth chamber set at 22ºC, 70% RH and 14 h photoperiod for 6 weeks. The plastic dome was removed four days after transplanting. Plants were bottom-watered with tap water every 3 or 4 days and fertilized with a commercially available soluble fertilizer, Miracle-Gro, (N-P-K: 24-8-16) every 2 weeks.

Inoculation of Arabidopsis reporter lines

For inoculations with BCN, surface-sterilized seeds were plated in square plates on Knop's medium prepared with 0.8% Daishin agar (Brunschwig Chemie, http://www.brunschwig-ch.com/) (Sijmons, 1991). Plates were positioned vertically in a growth chamber set at 24ºC, 70% RH with a 12 h photoperiod with a light intensity of 100-150 µmol/m^2/s. Two days before inoculation, cyst nematode eggs were isolated from pot cultures and J2s hatched at 28ºC as previously described (Mitchum *et al.*, 2004). J2s were surface-sterilized in a solution containing 0.004% mercuric chloride, 0.004% sodium azide, and 0.002% Triton X-100 for 8 min, washed with sterile water five times and suspended in 0.1% agarose. Nematodes were pelleted at 2,000 rpm per 30 sec between washes. Roots of ten-day-old seedlings were inoculated with 50 surface-sterilized J2s. Plates were sealed with parafilm and placed back in the growth chamber until harvesting.

For inoculations with RKN, surface-sterilized seeds were plated one seed per well in 6-well plates (GUS reporter lines) or glass-bottom microwell dishes (MatTek Co., Ashland, MA) (*TCSn:GFP*) containing Knop's medium prepared with 0.8% Daishin agar. Plates were wrapped twice with Parafilm to avoid dehydration and placed in a growth chamber set to the conditions above. Four days before inoculation, RKN eggs were harvested from pot cultures by rinsing soil from the roots and releasing eggs from egg masses by agitating roots in 10% sodium hypochlorite for four minutes. The egg suspension and plant debris was poured over a stack of sieves: 850 µm (No.20), 250 µm (No.60), 75 µm (No. 200) and 25 µm (No. 500). Eggs collected on the No. 500 sieve were rinsed under tap water for five min and set up to hatch as previously described (Mitchum *et al.*, 2004). J2s were surface-sterilized according to above except the incubation step in

sterilization solution was for five minutes. Fourteen-day-old seedlings were inoculated with 1000 surface-sterilized J2s per plant. Plates were sealed with parafilm and placed back in the growth chamber until harvesting.

Histochemical *β*-glucuronidase (GUS) assay

Nematode-inoculated and mock-inoculated seedlings were transferred from six-well plates into plates containing GUS solution [100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), 1 mM 5-bromo-4chloro-3-indolyl glucuronide, pH 7.0, 50 mM NaCl, 0.06% Triton X-100, 2 mM potassium ferricyanide). Plates were vacuum-infiltrated twice for10 min and incubated at 37ºC overnight. Seedlings were washed three times with 70% ethanol at intervals of 30 minutes, and incubated in 70% ethanol at 4ºC overnight to remove all remaining chlorophyll prior to evaluation under an upright stereoscope. Representative samples were excised using micro scissors and forceps (DUMOXEL #4) and placed in 12-well plates containing 70% ethanol. At least 12 seedlings were analyzed per line per time point.

Fixation, embedding and sectioning of GUS-stained Arabidopsis roots

Root segments containing syncytia or galls were embedded in 1.5% low melting agarose by carefully dragging each root with forceps through a 250 µl drop of agarose placed inside a Petri dish. Agarose blocks containing root sections were cut and transferred to 12-well plates containing 4% paraformaldehyde (PFA) in a 1X phosphate-buffered saline (PBS) solution (11.9 mM phosphate buffer containing KH_2PO_4 and Na_2HPO_4 137 mM NaCl, 2.7 mM KCl, pH 7.4). Tissues were vacuum-infiltrated three times for five

minutes. Following the last infiltration, PFA was replaced with new solution and tissues were incubated for 17 h at room temperature and then at 24 h at 4ºC. After incubation, tissue was gradually dehydrated starting with 1X PBS solution for 15 min twice, and replacing the solution with 30%, 40%, 50%, 60% and 70% ethanol for 30 min each. Agarose blocks were placed between foam pads of a biopsy cassette, submerged in 70% ethanol, and sent to the MU Histology Core for paraffin embedding and sectioning at 10 μm. Mounted sections were placed on a slide warmer at 40ºC overnight for adhesion. Slides were vertically submerged in xylene for 10 min and then laid flat to dry before mounting sections in Shandon consul mount and coversliped for imaging.

Brightfield microscopy

Root pieces containing syncytia or galls were placed on a plain microscope slide (Fisher Scientific, Hampton, NH) in a drop of sterile distilled water and gently covered with a microscope cover glass (Fisher Scientific, Hampton, NH). Photos of whole roots and root sections containing feeding sites were taken with a digital Nikon COOLPIX5000 camera manually attached to a Nikon Eclipse TS100 (Melville, NY, USA) inverted microscope (*IPT:GUS*). Additional photos were taken with an Olympus Vanox AHBT3 microscope (*CKX:GUS*) or a Leica DM5500B upright microscope (*AHK4:GUS*) equipped with a Leica DFC295 color digital camera at the MU Cytology Core.

Infection assays

Knop's medium

Sterilized seeds were plated one seed per well in sterile 12-well plates (BD Biosciences, http://www.bdbiosciences.com/) containing modified Knop's medium and 0.8% Daishin agar following a randomized block design. Plates were sealed with parafilm to avoid dessication and kept in a growth chamber at 24ºC, 65% RH in a 12 h photoperiod with 100-150 µmol/m^2/s average of light intensity. Fourteen-day-old seedlings were inoculated with 1,000 surface-sterilized J2 resuspended in 25 µl of 0.01% sterile agarose and returned to the chamber. Galls were counted at 43 dpi. Thirty-six plants were evaluated per genotype per experiment and each experiment was independently repeated 3 times.

Sand-soil based substrate

Surface-sterilized seeds were imbibed in sterile water and placed at 4ºC for 48 h under constant rotation. Seeds were plated on ½ MS, 2% sucrose medium in Petri plates and kept at 22ºC in a Percival chamber set to constant light. Seven-day-old seedlings were transferred to individual pots filled with riversand: Fafard Growing mix (3:1) and placed in trays. Pots were bottom watered for about two minutes until the soil surface looked wet. Trays were completely covered and placed in a growth chamber for 5 days. Covers were then cracked for two days. On day seven after transplanting, the cover was completely removed. Fourteen-day-old seedlings were inoculated with 1,500 root-knot nematode eggs. A 200 µl pipette tip was used to punch 4 holes around each plant. Each plant was inoculated with 1 ml (1,500 eggs/ml) of egg inoculum suspended in sterile water. Tube was agitated by pipetting up and down before inoculating each plant and strongly agitated between trays.

Plants were fertilized once a week with Miracle-Gro, (N-P-K: 24-8-16). At 42 dpi, roots were carefully removed from the pots and gently rinsed in a bucket of water. Galls were counted under a stereomicroscope. Each root system was then cut into small pieces with scissors and placed in a 50 ml tube with 10% bleach and agitated for 4 min. The root slurry was poured over a nested stack of sieves (no. 20/60/200/500) while rinsing with a hand sprayer to release egg masses. Eggs were rinsed from the no. 500 sieve into a round bottom tube or small beaker and the volume was adjusted to 25 ml with water. Two ml of acid fuchsin was added and the sample was microwaved for 45 sec and then cooled to RT. One ml of solution was placed into a Huxley slide and the eggs were counted.

RNA isolation and quantitative real-time PCR

One hundred wild type Col-0 seedlings were grown on Knops medium and inoculated with beet cyst nematode, *H. schachtii* or root-knot nematode, *M. incognita*, juveniles. Root segments containing syncytium or galls were harvested at 6 dpi and immediately placed in RNA*later* stabilization solution (Qiagen, Hilden, Germany) following manufacturer's instructions. Tissue was stored at -80ºC until further processing. Each sample was homogenized in liquid nitrogen using a mortar and pestle and total RNA was isolated with NucleoSpin RNA Plant Kit (Macherel-Nagel, Düren, Germany) following user manual instructions. cDNA was synthesized using First Strand cDNA Synthesis Kit following manufacturer's guidelines (Roche Diagnostics, Mannheim, Germany). cDNA was used in subsequent relative quantitative real time PCR (qRT-PCR) reactions using primers in Table 2.1. qPCR reactions were prepared with template and a non-template control containing 15 µl of SYBR Green PCR Master Mix (Applied

Biosystems, Foster City, CA), 5 µl of 0.2 µM primers and 5 µl of 1:10 diluted cDNA in 96 well plates. qPCR was carried out with the following conditions: 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min using an Applied Biosystems 7500 Real Time PCR system. A dissociation curve analysis was conducted for each primer set. Four technical replicates per each of two biological replicates were used. *CKX5* and *CKX6* primer sequences were described by Nishiyama *et al*. (2011).

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Table 2.1. Primer sequences used in this study.

Name	Sequence (5' - 3')	Reference
IPT1-F	AGA GAT CAC AAC GAA TCA GAT TAC GT	This study
IPT1-R	ATG ACG CCG AGG AGA TGG T	This study
IPT2-F	GAA ATC AAA GCT TGC CGT TGA T	This study
IPT2-R	GCA TTG CGT CGG CGT TA	This study
IPT3-F	AAC CGC CGC GAA TTA CTG	This study
IPT3-R	TTG GAA GCT TTC CAC GGT TT	This study
CKX5-F	CCA TGG TCC TCA AAT TAG TAA CG	Nishiyama et al., 2011
CKX5-R	TCT GAG CAT CTC ATC ACC TCT C	Nishiyama et al., 2011
CKX7-F	CAC CAG AGC TAG GGT TTT GC	Nishiyama et al., 2011
CKX7-R	CAT CGA ACT CGG TGT ATA CTA CTC TT	Nishiyama et al., 2011

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CHAPTER 3

COMPARATIVE ANALYSIS OF CYTOKININ SIGNALING ACTIVATION IN FEEDING SITES BY THE BEET CYST NEMATODE *HETERODERA SCHACHTII* AND THE ROOT–KNOT NEMATODE *MELOIDOGYNE INCOGNITA* *

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Carola D. Dowd, Demosthenis Chronis, Zoran S. Radakovic, Shahid Siddique, Thomas Schmülling, Tomáš Werner, Tatsuo Kakimoto, Florian M. W. Grundler, and Melissa G. Mitchum. Divergent regulation of cytokinin biosynthesis, signaling and catabolism genes underlying differences in feeding sites induced by cyst and root-knot nematodes.

Contributions by authors other than Carola M. De La Torre have been highlighted in the figure legends.

ABSTRACT

During the infection process, cyst and root-knot nematodes form characteristic feeding sites in host roots from which they retrieve nutrients throughout their entire life cycle. Using a single host, *Arabidopsis thaliana*, we have previously shown cytokinin biosynthesis and catabolism play a role not only in the interaction with the beet cyst nematode (BCN) *Heterodera schachtii*, but also with the root-knot nematode (RKN) *Meloidogyne incognita*. Here, we demonstrate cytokinin receptors *AHK2*, *AHK3* and *AHK4/CRE1* are differentially regulated in feeding sites induced by both nematode species. Furthermore, evaluation of Arabidopsis Histidine Kinase (AHK), *ahk2/3*, *ahk2/4* and *ahk3/4* receptor mutant lines in response to cyst and root–knot nematode infection revealed these lines are significantly less susceptible to BCN and RKN, suggesting a requirement for cytokinin signaling in feeding site formation. An additional in-depth analysis of *ahk* double mutants using *CYCB1:GUS/ahk* introgressed lines, revealed contrasting differences in cytokinin receptors controlling cell cycle activation in feeding sites caused by BCN and RKN.

INTRODUCTION

Cyst nematodes (CN) and root-knot nematodes (RKN) are biotrophic pathogens that induce the formation of sophisticated feeding sites, called syncytia or giant-cells, respectively, in susceptible hosts. A syncytium results from the coalescence of multiple cells which had previously undergone partial cell wall dissolution, whereas giant-cells are formed by the massive expansion of individual cells selected for feeding (Mitchum *et al.*, 2012). Additionally, cells surrounding either the syncytium or giant-cells undergo different degrees of cell division. Whereas cells surrounding a syncytium divide to a limited extent before incorporation into the feeding site, cells neighboring giant-cells divide uncontrollably to form characteristic 'knots' or galls on roots. Since nematode success heavily depends on these processes, the mechanistic details determining these two possible outcomes have long been an outstanding question in our field of study.

Cytokinin is a phytohormone well known for its involvement in cell division of root, shoot and vasculature meristematic cells. Cytokinins are typically synthesized in apical meristems and transported through xylem or phloem to the place of action, but can be locally synthesized. However, whether a cell responds or not to external cytokinin stimuli depends on the activation of membrane bound cytokinin receptors. In fact, cytokinin biosynthesis *IPT* gene expression does not necessarily overlap with cytokinin signaling, and in many tissues cytokinin is being made, but not perceived because the cytokinin perception mechanism is turned off (Zürcher and Müller, 2016). In Arabidopsis, there are three Histidine Kinase cytokinin receptors: AHK2 (At5g35750), AHK3 (At1g27320), and AHK4/CRE1 (At2g01830), which autophosphorylate in the presence of

cytokinin and subsequently release a phosphoryl group towards the nucleus via phosphorelay. Arabidopsis Histidine Phosphoproteins (AHPs) serve as 'molecular shuttles' between the cytoplasm and nucleus, where they physically interact with type-B Arabidopsis response regulators (type-B ARRs) to induce the expression of CK responsive genes (Heyl and Schmülling, 2003).

Cytokinin receptors AHK2, AHK3 and AHK4/CRE1 belong to a closely related gene family of histidine kinases, which share 52-54% sequence identity (Ueguchi *et al.*, 2001). AHK structure-function analysis revealed each protein contains five domains including: a transmembrane segment, extracytoplasmic domain, transmitter, receiver-like, and receiver domains (Ueguchi *et al.*, 2001). Ligand binding occurs at the CHASE domain (cyclase/histidine kinases associated sensor extracellular) contained within the extracytosolic portion, while intracellular signal transmission is possible through a phosphoryl group transfer from a conserved histidine to an aspartate residue within the transmitter and receiver domains, respectively (Ueguchi *et al.*, 2001, Kieber and Schaller, 2014).

AHK2, AHK3 and AHK4/CRE1 possess common expression patterns *in planta*, but also organ and tissue specific differences. *AHK2* and *AHK3* transcripts are ubiquitously expressed with *AHK3* having stronger expression levels than *AHK2* in all tissues. On the other hand, *AHK4* is preferentially expressed in roots, with weaker expression in stems and flowers, and low or undetectable expression in leaves (Ueguchi *et al.*, 2001, Higuchi *et al.*, 2004). *AHK2* and *AHK3* are expressed in the lower part of the root apical meristem (RAM), while *AHK4* is restricted to the upper part of the RAM (Nishimura *et al.*, 2004). At the cell

level, AHK receptors are mainly found in the endoplasmic reticulum, where they are thought to interact with receptors from other signaling pathways (Wulfetange *et al.*, 2011).

AHK2, AHK3 and AHK4/CRE1 have somewhat overlapping functions in shoot and root growth. Under normal conditions, all AHK single *ahk2-2, ahk3-3, cre1-12* and double mutant: *ahk2-2 cre1-12*, *ahk3-3 cre1-12* shoots are phenotypically similar to the wild type, Col-0; only *ahk2-2ahk3-3* has significantly smaller shoots. Similarly, AHK single and double mutants have no obvious root phenotypes (Higuchi *et al.*, 2004). However, after exogenous cytokinin treatment, AHK2 and AHK3 single mutants have slight reductions of root growth, an effect that is slightly more pronounced when combined with AHK4/CRE1. Interestingly, the *ahk2-2 ahk3-3* double mutant exhibits a normal or mild cytokinin-induced inhibition of root growth compared to the other two mutant combinations (Higuchi *et al.*, 2004). A similar effect has been seen using single and double mutant allele combination *ahk2-1 ahk3-1*, suggesting roots of *ahk2 ahk3* mutants, where AHK4 is active, can still respond to cytokinin (Nishimura *et al.*, 2004). Based on this evidence and AHK expression pattern analyses, it is generally accepted that AHK2 and AHK3 have stronger roles in shoot development, while AHK4 is the predominant cytokinin receptor in root growth. Cytokinin triple mutant combination (*ahk2-2 ahk3-3 cre1-12*), is sterile, has significantly smaller shoots and roots than Col-0 and has complete insensitivity to cytokinin (Higuchi *et al.*, 2004, Nishimura *et al.*, 2004). Primary response genes Type A-ARRs, which are normally upregulated under cytokinin treatment, do not show any expression changes in triple mutant plants exposed to cytokinin (Higuchi *et al.*, 2004). Cytokinin content has been measured for some mutant allele combinations and a significant increase in the amounts tZ and its derivatives, but not for iP or its derivatives was found

for the *ahk2-5 ahk3-7*; *ahk2-5 cre1-2*, and *ahk3-7 cre1-2* double mutants. The triple mutant *ahk2-5 ahk3-7 cre1-2* has increases of both tZ and iPs, suggesting an involvement of cytokinin signaling in the control of steady state cytokinin levels in cells (Riefler *et al.*, 2006).

AHK binding affinity for cytokinin has also been well characterized. Heterologous expression of AHK4 in an *Escherichia coli* system and *in vitro Saccharomyces pombe* binding assays, have shown AHK4 is able to bind cytokinins as aminopurines (isopentenyl adenine, trans-zeatin, benzylaminopurine), and diphenylureas (thidiazyron), but not cytokinin ribosides (Yamada *et al.*, 2001). Live cell binding assays have shown AHK3 and AHK4 can bind trans-zeatin and isopentenyladenines, and both have similarly high affinities for trans-zeatin, but AHK4 has higher affinities for isopentenyladenines than AHK3 (Romanov *et al.*, 2006, Stolz *et al.*, 2011). On the other hand, AHK3 has higher affinity for dihydrozeatin than AHK4 (Romanov *et al.*, 2006). Similarly to AHK4, AHK2 has high affinities for isopentenyl adenine and trans-zeatin (Stolz *et al.*, 2011). While AHK receptors have several degrees of affinities for free bases, they are unable to bind cytokinin conjugates. Recent crystallization of the AHK4 protein revealed that the cytokinin binding site located within the CHASE domain is only 20 amino acid in length, which may explain size exclusion and no affinity of AHK receptors for cytokinin conjugates of bigger size (Hothorn *et al.*, 2011, Lomin *et al.*, 2012).

Our previous experiments demonstrated that plants overexpressing a cytokinin oxidase enzyme are less susceptible to BCN and RKN infection, suggesting cytokinin is playing a positive role in the interactions with these pathogens (Chapter 2, Siddique *et al*., 2015). We have also learned that cytokinin biosynthesis and catabolism genes are

expressed in syncytia and galls induced by BCN and RKN, respectively, and that mutants thereof are less susceptible to both species, indicating local cytokinin metabolism is required for feeding site formation. In the present chapter, we established an important role for cytokinin signaling in nematode parasitism and identified key differences in the regulation of cytokinin receptor genes that may underlie dissimilarities in feeding site formation by BCN and RKN through a comparative spatio-temporal analysis in the same host plant species. Understanding species-specific mechanisms of nematode parasitism and the essential plant factors required for completion of the nematode life cycle may lead to more effective control strategies.

RESULTS

Cytokinin receptors are differentially regulated in response to BCN and RKN

To determine if cytokinin receptor genes are regulated during the interaction with BCN and RKN, we analyzed *AHK* expression in syncytia and galls using *AHK2:GUS*, *AHK3:GUS* and *AHK4:GUS* reporter lines. GUS expression was observed in roots of all uninfected *AHK:GUS* lines as previously described (Figure 3.1a-c, Higuchi *et al.*, 2004). BCN inoculated plants showed expression of *AHK2:GUS*, *AHK3:GUS* and *AHK4:GUS* in feeding sites during early development at 3 dpi, and the expression continued in subsequent stages at 5 and 14 dpi (Figure 3.1). Similarly, roots containing parasitic RKN juveniles showed GUS expression in early galls (3 dpi) for *AHK2:GUS* and *AHK4:GUS* (Figure 3.2a-b) However, as galls continued to develop, we observed differential expression of the *AHK* promoter-reporter fusion genes compared to BCN. Even though all of these receptors

were expressed in roots of the uninfected plants, *AHK4:GUS* was strongly downregulated in developing galls (Figure 3.3g-i) while *AHK2:GUS* and *AHK3:GUS* remained expressed (Figure 3.3a-f). Cross-sections through galls showed expression of *AHK2:GUS* and *AHK3:GUS* in both giant-cells and surrounding gall tissues (Figure 3.3c,f). To further corroborate *AHK4:GUS* downregulation, we analyzed ARR15, a type-A ARR downstream component of the cytokinin signaling pathway whose expression is regulated by AHK4 (Kiba *et al.*, 2002).

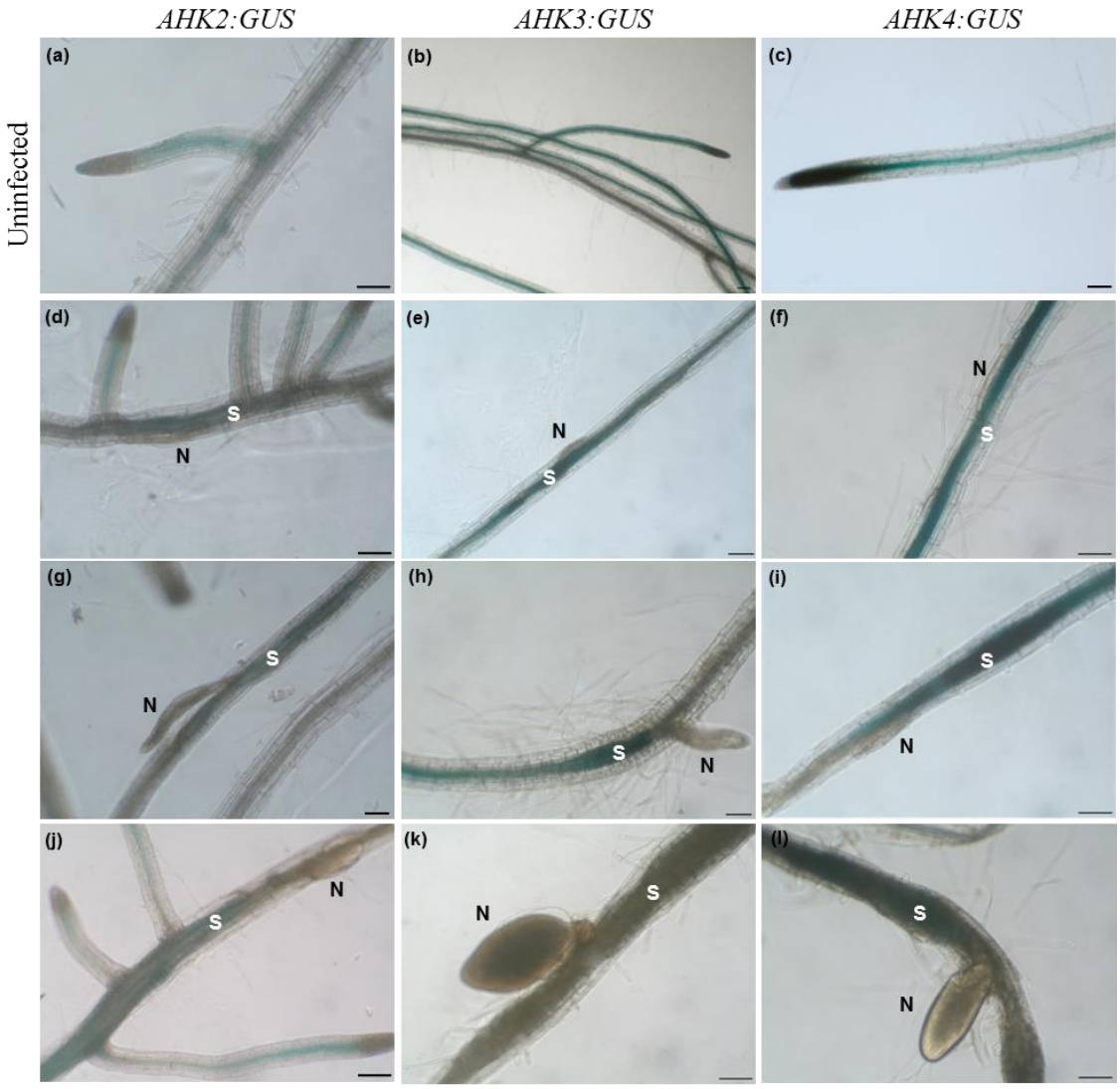

Figure 3.1. Histidine kinase receptor *AHK2*, *AHK3* and *AHK4* expression in response to the beet cyst nematode *Heterodera schachtii* during early and late stages of parasitism. Uninfected roots (a-c); 3 (d-f), 5 (g-i), and 14 dpi (j-l) Abbreviations: N, nematode; Syn, syncytium. Scale bar= 100 µm. Photos were taken by Demosthenis Chronis and published in Siddique *et al.*, 2015.

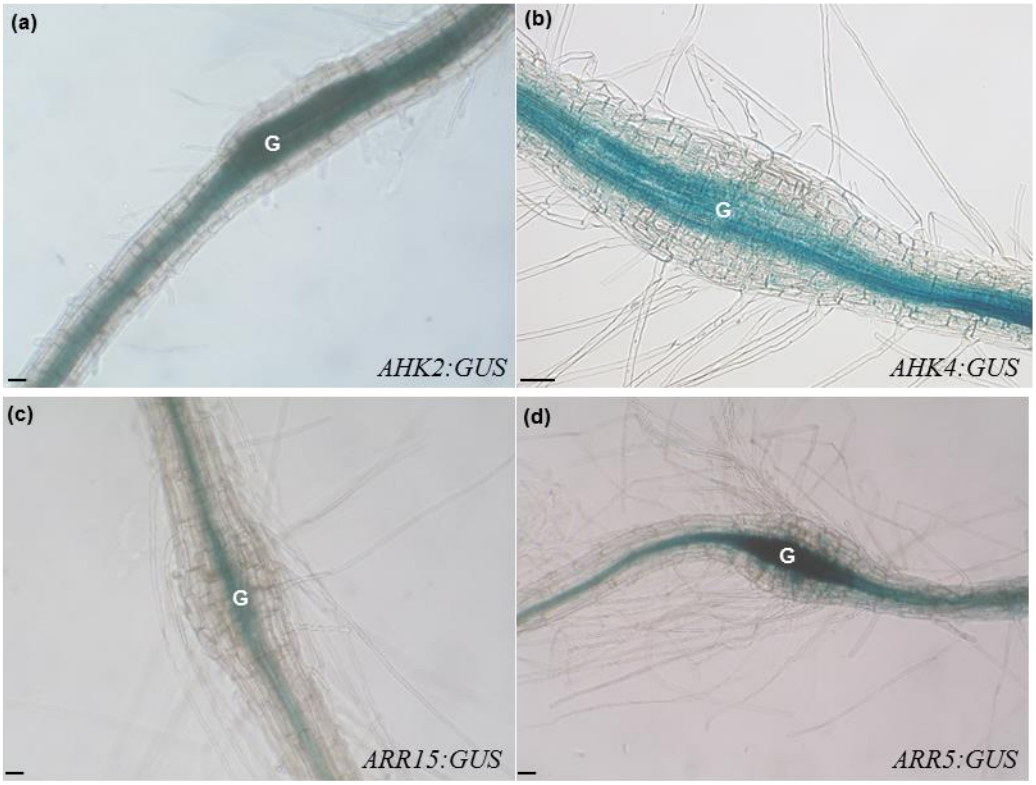

Figure 3.2. Expression of (a) *AHK2:GUS*, (b) *AHK4:GUS*, (c) *ARR15: GUS* and (d) *ARR5:GUS* lines at early stages of gall formation (3 dpi) following infection with the root-knot nematode *Meloidogyne incognita*. Abbreviations: G, gall. Scale bar = 100 µm.

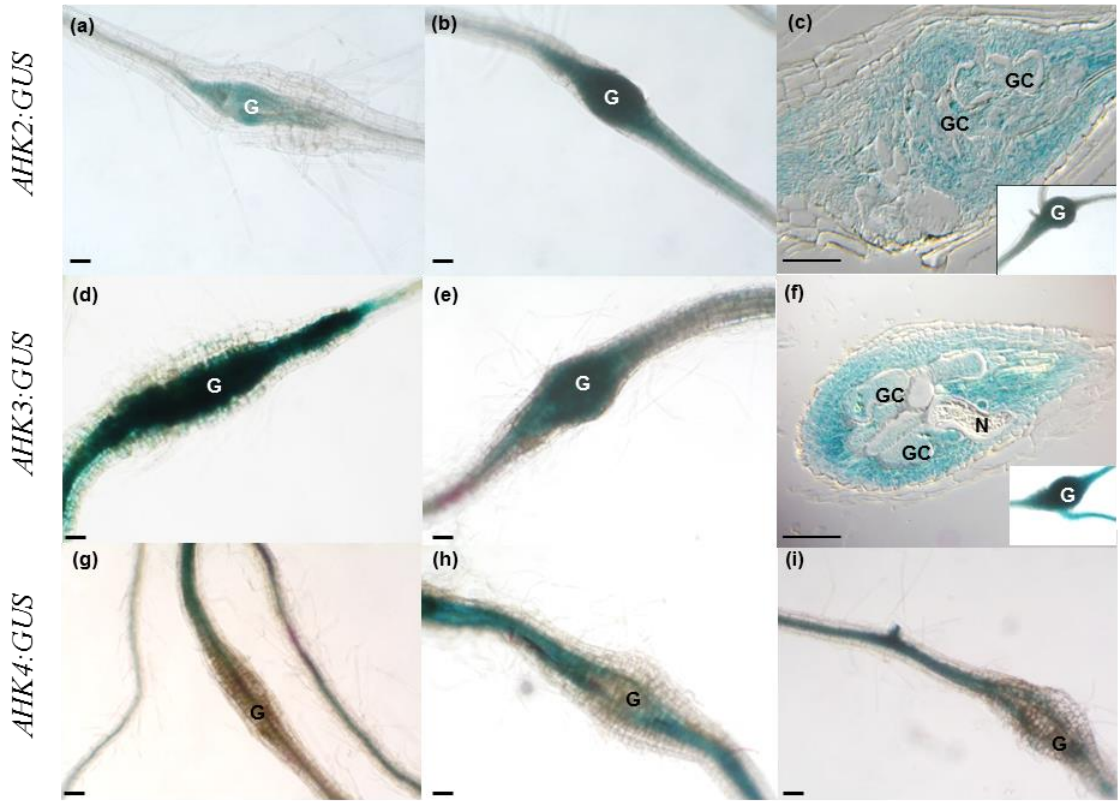

Figure 3.3. Histidine kinase receptor *AHK2*, *AHK3* and *AHK4* expression in response to the root-knot nematode *Meloidogyne incognita*. (a-c) *AHK2:GUS*; (d-f) *AHK3:GUS*; (g-i) *AHK4:GUS* during early and later stages of parasitism. (c,f) Longitudinal section of galls either (c) *AHK2:GUS* or (f) *AHK3:GUS* expression in GC. Abbreviations: N, nematode; G, gall; GC, giant cell. Scale bar=100 μm, 50 μm (c,f). Photos were taken by Demosthenis Chronis.

ARR15:GUS plants were evaluated in response to cyst and root-knot nematode infection. In uninfected roots, *ARR15:GUS* showed a low level of expression in the root vasculature. Upon *H. schachtii* infection, *ARR15:GUS* showed increased expression in developing syncytia during J2 and J3 developmental stages with a decline in expression when nematodes reached the J4 developmental stage (Figure 3.4a-c). Opposite to *H. schachtii*, no GUS expression was detected in giant-cells or galls (Figure 3.4d-f), resembling the results observed for *AHK4:GUS*. *AHK4* and *ARR15* expression was confirmed using qRT-PCR with specific primers (Table 3.1). An additional type-A ARR gene, *ARR5*, whose regulation is independent of AHK4 was also evaluated. *ARR5:GUS* was expressed in the root vasculature and root tips and showed early upregulation in response to both BCN and RKN further suggesting differential regulation of type-A *ARR* genes in galls (Figure 3.4g-l).

Cytokinin signaling is activated at BCN and RKN feeding sites

The final step in the cytokinin phosphorelay signaling cascade is controlled by transcriptional type B-response regulators (type-B ARRs). To determine if cytokinin downstream signaling plays a role in nematode feeding site formation, Absmanner *et al*. (2013) tested a *Two Component signaling Sensor* (TCS:GFP) (Müller and Sheen, 2008) in response to CN and RKN infection as a measure of cytokinin activity within these cells.

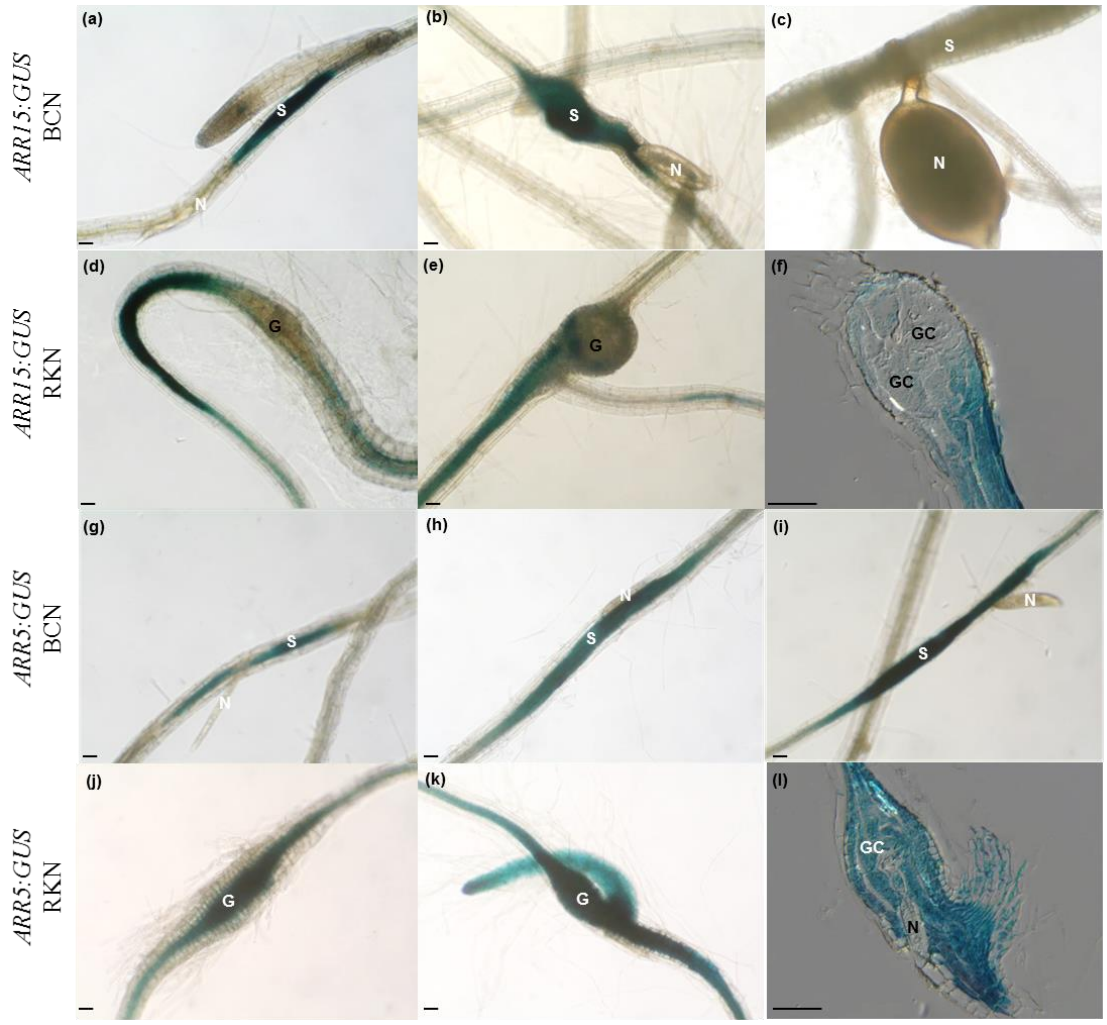

Figure 3.4. Cytokinin type A - Arabidopsis response regulators (ARR) *ARR15* and *ARR5* expression in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*. (a-c) *ARR15:GUS* and (g-i) *ARR5:GUS* expression in BCN-infected Arabidopsis roots during early and later stages of parasitism: (a,g) early and (b,h,c,i) later stages. (d-f) *ARR15:GUS* and (j-l) *ARR5:GUS* expression in RKN-infected Arabidopsis roots during (d,j) early and (e,k) later stages. (f) Longitudinal section of a gall showing reduced expression of *ARR15:GUS* expression in GC. (l) Longitudinal section of a gall showing *ARR5:GUS* expression in GC. Abbreviations: N, nematode; S, syncytium; G, gall; GC, giant cell. Scale bar=100 μm, 50 μm (f,l). Photos were taken by Demosthenis Chronis.

Table 3.1. Quantification of cytokinin gene expression in beet cyst nematode (BCN) and root-knot nematode (RKN) infected roots.

	Tissue	Replicate	ΔΔCt	Exp. Rel. To Mock	ΔΔCt+s	ΔΔCt-s	Range (min)	Range (max)	Avg. Exp. Rel. To Mock
AHK4	BCN Infected Roots	1	0.8111	0.5699	1.01298	0.6092	0.4955	0.6556	**0.93**
AHK4	BCN Infected Roots	2	-0.3708	1.2931	-0.2271	-0.5145	1.1705	1.4285	
AHK4	RKN Infected Roots	1	1.7827	0.2906	1.9739	1.5916	0.2545	0.3318	**0.48**
AHK4	RKN Infected Roots	2	0.5908	0.6639	0.7696	0.4120	0.5865	0.7516	
ARR15	BCN Infected Roots	1	-4.599	24.2346	-4.3302	-4.8678	20.1154	29.1974	**21.10**
ARR15	BCN Infected Roots	2	-4.1674	17.9687	-4.0039	-4.3309	16.0441	20.1242	
ARR15	RKN Infected Roots	1	0.7255	0.6048	0.9991	0.4519	0.5003	0.73109	**0.74**
ARR15	RKN Infected Roots	2	0.195	0.8736	0.4009	-0.0109	0.7573	1.00765	

No TCS activity was detected in feeding sites induced by these two nematodes, suggesting either type-B ARRs are not active in feeding cells or their activity was below the TCS detection threshold. Since recent studies have reported limitations regarding the use of TCS:GFP as a cytokinin marker and weak GFP expression was found in certain tissues where cytokinin is reported to have strong activity (Zürcher *et al*., 2013), it was unclear whether cytokinin downstream signaling was indeed active during gall and syncytium formation. To further elucidate cytokinin status in feeding sites and to determine if temporal differences in downstream cytokinin signaling existed between RKN and BCN, we evaluated a novel version of TCS:GFP, TCSn:GFP, which has higher sensitivity and stronger expression across multiple plant tissues (Zürcher *et al*., 2013). For this, we carried out *in vivo* imaging of gall and syncytium formation in *TCSn:GFP* infected lines starting 18 h after infection, monitoring each infection site daily until 10 dpi. Galls induced by root-knot showed early upregulation of *TCSn:GFP* (Figure 3.5), with expression increasing over time until the nematode reached maturity (Figure 3.6a-c). Similarly, we observed GFP in developing syncytia as early as 2 dpi with a decline around 10 dpi when the nematodes molted into an adult stage (Figure 3.6d-f) (this study, Siddique *et al*., 2015). Altogether, these findings suggest downstream cytokinin signaling, specifically members of the type-B ARR family, are not only activated in response to BCN, but also in response to RKN infection and no major differences in the time of activation exists between these two species.

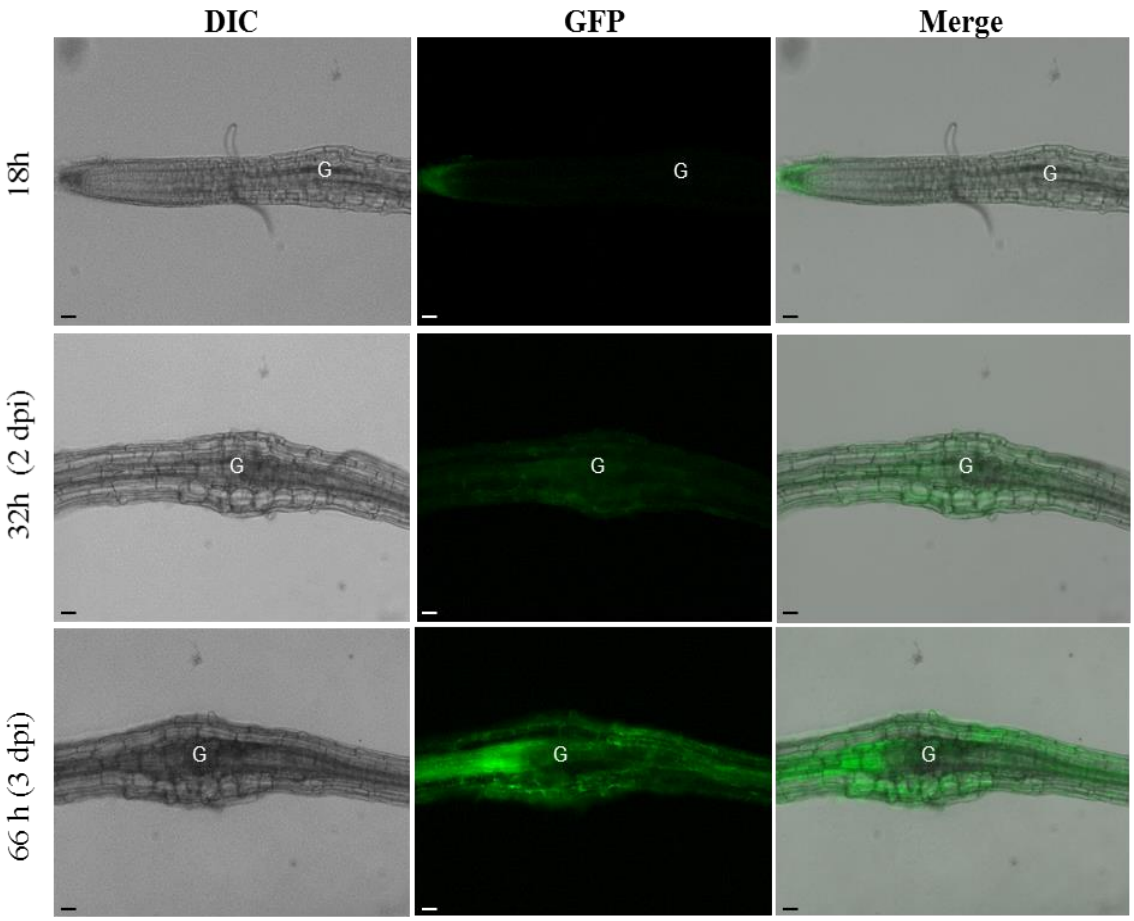

Figure 3.5. *TCSn:GFP* expression in developing galls formed by the root-knot nematode *Meloidogyne incognita*. Abbreviations: G, gall. Scale bar=100 µm.

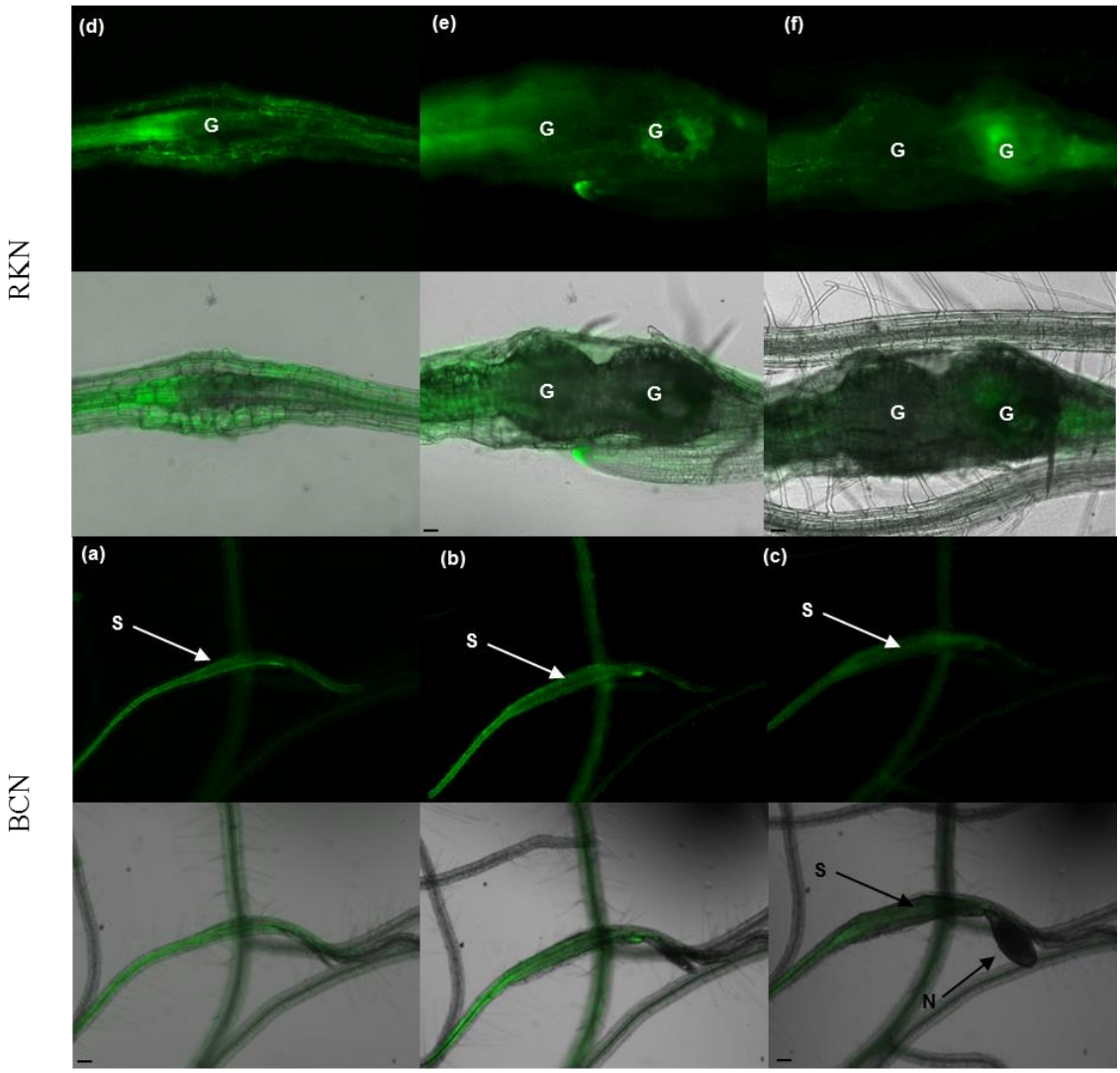

Figure 3.6. Expression of *TCSn:GFP* in response to the root-knot nematode (RKN) *Meloidogyne incognita* and the beet cyst nematode (BCN) *Heterodera schachtii*. (a-c) GFP expression in RKN-infected Arabidopsis roots showing two galls developing during early and later stages of parasitism: (d) 3, (e) 8 and (f) 13 dpi (d-f) GFP expression in BCN-infected Arabidopsis roots during early and later stages of parasitism: (a) second-stage juveniles (J2), (b) third-stage juveniles (J3) and (c) fourth-stage juveniles (J4). Abbreviations: N, nematode; S, syncytium; G, gall. Scale bar = 100 µm.

Cytokinin signaling is required for BCN and RKN parasitism

To determine the functional significance of the AHK receptors in RKN parasitism, we tested AHK receptor mutant lines. Single, *ahk2-2*, *ahk3-3*, *ahk4/cre1-12*, and double, *ahk2-2 ahk3-3* (*ahk2/3*), *ahk2-2 cre1-12* (*ahk2/4*), and *ahk3-3 cre1-12* (*ahk3/4*) mutant lines were infected with BCN or RKN following standard procedures and cyst and gall numbers were counted. Single and double *ahk* mutants, *ahk2-2 cre1-12* (*ahk2/4*), *ahk3-3 cre1-12* (*ahk3/4*) have shoots and roots of comparable sizes to Col-0; while *ahk2-2 ahk3-3* (*ahk2/3*) has smaller leaves and shorter stems than Col-0, without compromising root size (Higuchi *et al.*, 2004). Triple mutants are dwarf, with limited shoot and root growth, and therefore not suitable for nematode infection assays (Higuchi *et al.*, 2004; Riefler *et al.*, 2006).

We did not observed significant differences for any of the single receptor mutants, except for *ahk3-3* in response to BCN (Figure 3.7); however, double receptor mutants showed significantly fewer cysts and galls (Figure 3.8). At 30 dpi, the *ahk3/4* double mutant showed the fewest number of cysts of all double mutant combinations, and was significantly different from *ahk2/3* and *ahk2/4* (Figure 3.8a). Differently from cyst nematode, the *ahk2/3* double mutant showed the fewest number of galls of all double mutant combinations, and was significantly different from *ahk2/4* and *ahk3/4* (Figure 3.8b). Overall, these results are consistent with the differential expression seen for *AHK4* compared to *AHK2* and *AHK3* throughout syncytium and gall development (Figure 3.1, 3.3). To determine if cyst nematode decreased susceptibility could be explained by an abnormal feeding site growth, our collaborators measured sizes of both syncytia and female nematodes across all mutants and compared them to Col-0. Similar to our results, they

found that infected *ahk* mutant lines contained significantly smaller syncytia and females compared to the control line, Col-0 (results published in Siddique *et al*., 2015). A parallel analysis of root-knot infected mutant lines was more challenging due to the fact that giant-cells and adult nematodes are surrounded by cortical tissue trapped within the gall. Therefore, we decided to conduct a paired analysis and measured not only gall sizes, but also nematode developmental stage. We observed delayed nematode development and smaller galls for all mutant lines, as nematodes growing in mutant lines could not reach adulthood as fast as nematodes growing in Col-0 (Figure 3.9a-b).

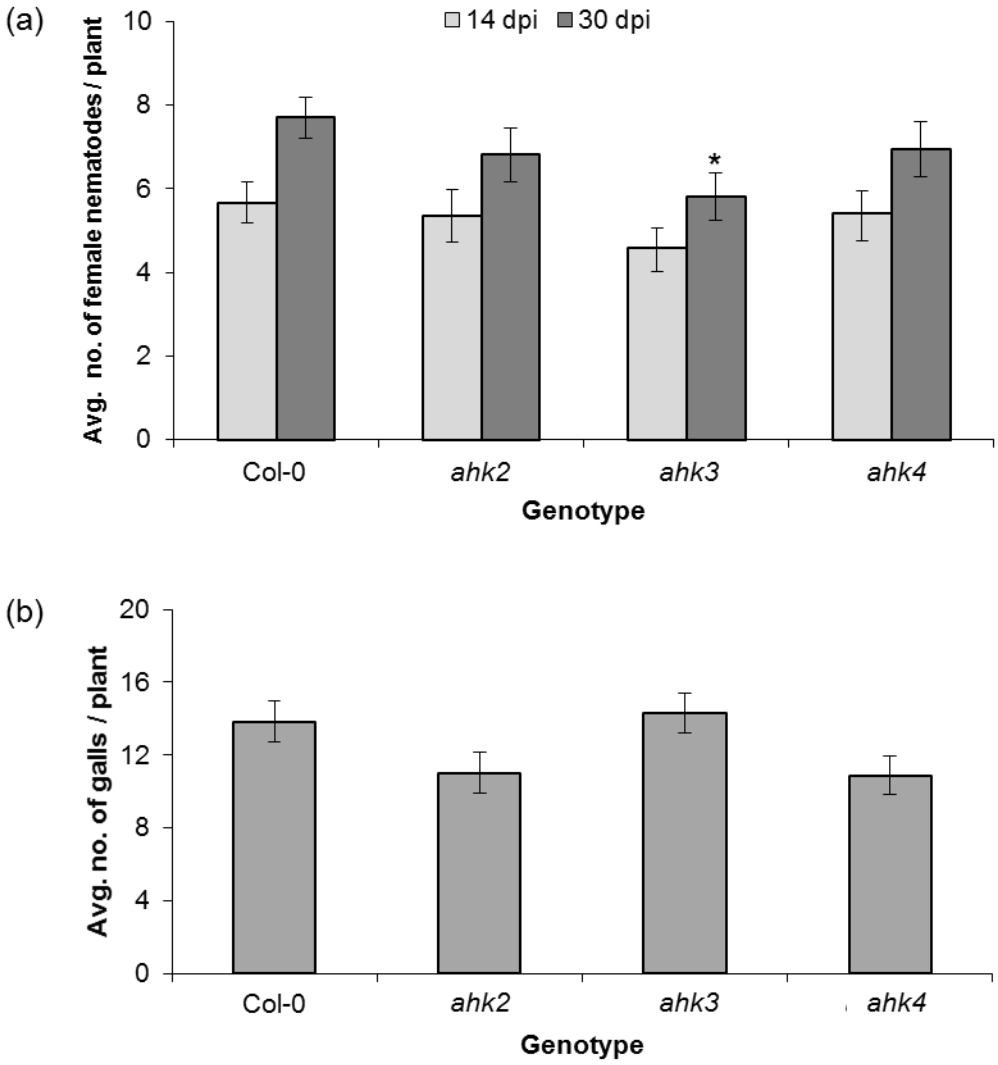

Figure 3.7. Evaluation of cytokinin single receptor mutants in response to *Heterodera schachtii* and *Meloidogyne incognita* infection, (a) Average number of *Heterodera schachtii* female nematodes at 14 and 30 dpi, plant number (n) per line were n=36 for Col-0, n=36 for *ahk2*(*ahk2-2)*, n=34 for *ahk3*(*ahk3-3)*, n=36 for *ahk4*(*cre1-12)*. (b) Average number of *Meloidogyne incognita* galls per plant at 40 dpi, plant number (n) per line were n=30 for Col-0, n= 35 for *ahk2*(*ahk2-2)*, n= 36 for *ahk3*(*ahk3-3)*, n= 29 for *ahk4*(*cre1-12)*. Error bars indicate SE. Data are representative results of one out of at least three independent experiments. Asterisk represent significant differences using a two-tailed Student's *t*-test ($P < 0.05$).

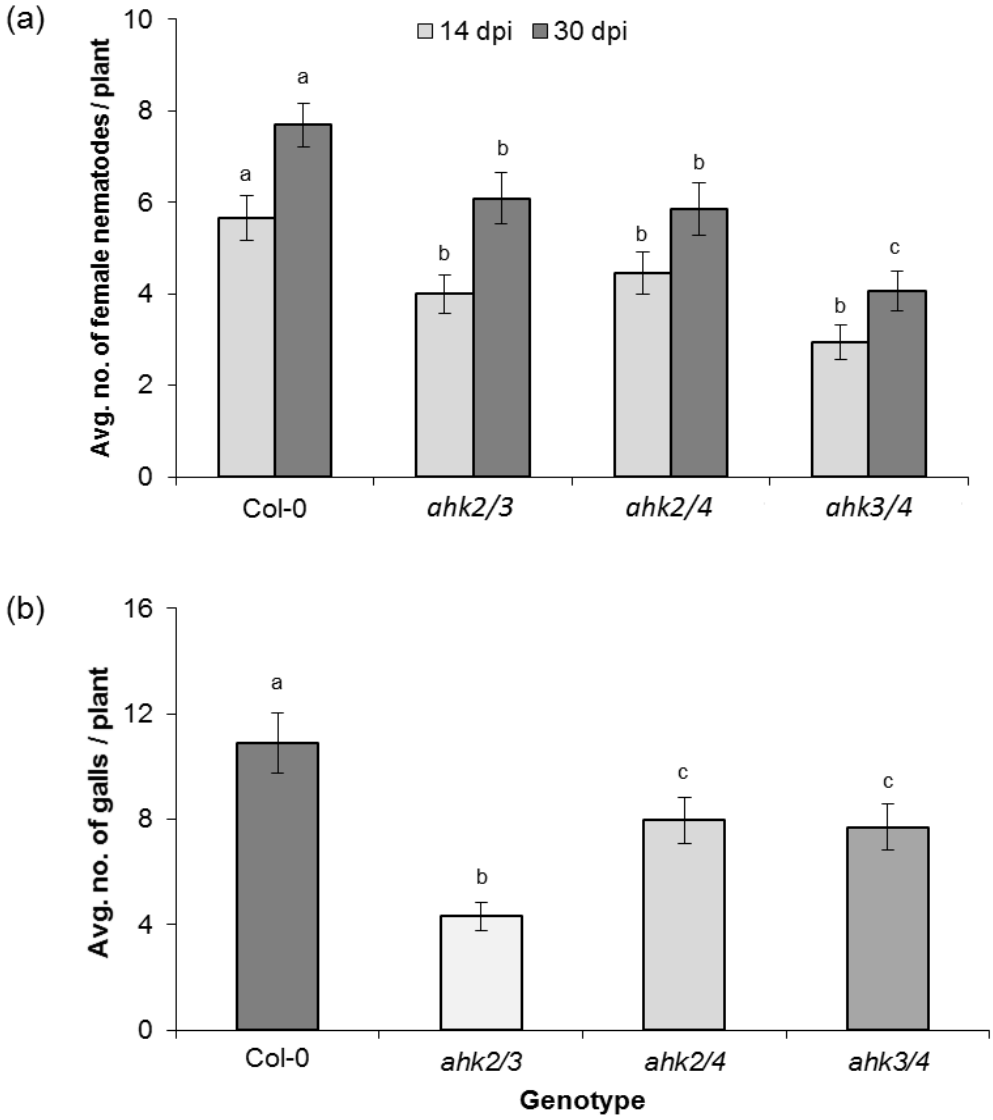

Figure 3.8. Evaluation of cytokinin double receptor mutants in response to *Heterodera schachtii* and *Meloidogyne incognita* infection. (a) Average number of cysts that developed in *ahk2-2/ahk3-3* (*ahk2/3*); *ahk2-2/cre1-2* (*ahk2/4*); *ahk3-3/cre1-12* (*ahk3/4*) at 14 and 30, plant number (n) per line were n=36 for Col-0, n=34 for *ahk2/3*, n=35 for *ahk2/4*, n=31 for *ahk3/4*. (b) Average number of galls per plant that developed in *ahk2-2/ahk3-3*; *ahk2-2/cre1-2*; *ahk3-3/cre1-12* at 40 dpi, plant number (n) per line were n= 26 for Col-0, n=29 for *ahk2/3*, n=26 for *ahk2/4*, n=23 for *ahk3/4*. Error bars indicate SE. Data are representative results of one out of at least three independent experiments. Different letters above columns indicate significant differences by a two-tailed Student's *t*-test ($P < 0.05$).

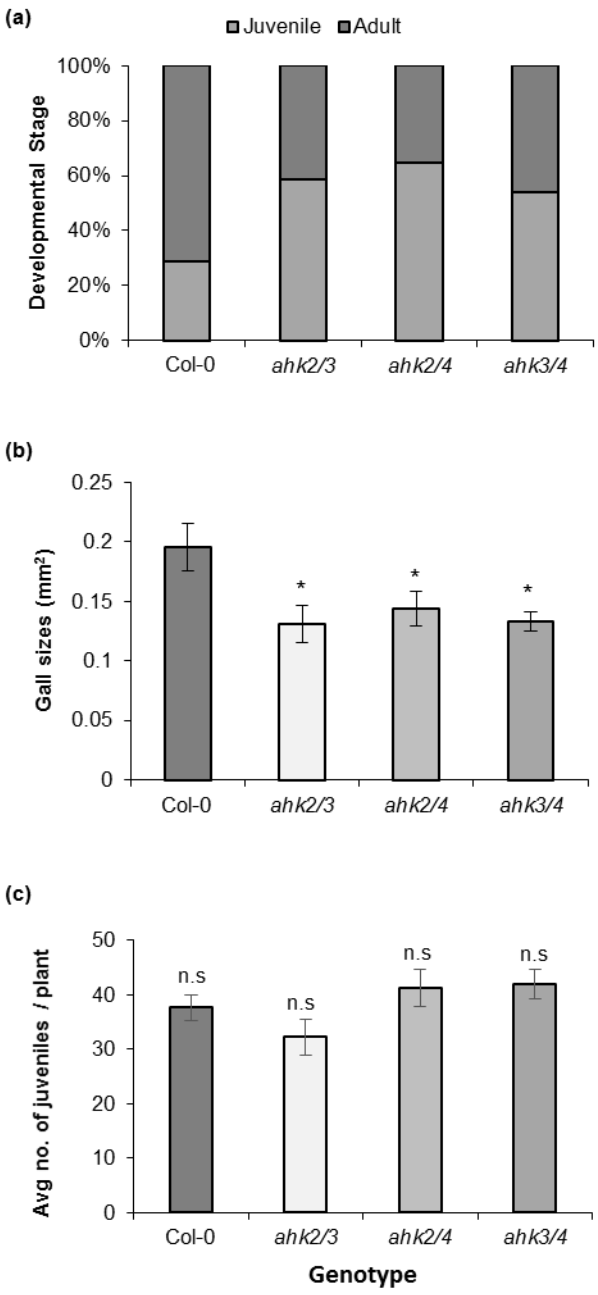

Figure 3.9. Evaluation of cytokinin double receptor mutants in response to *Meloidogyne incognita* infection. (a) Percentage of nematodes that remained juvenile or became adult in each line at 11 dpi, developing galls were marked at 4 dpi and followed during course of infection. Gall numbers per line were 7 for Col-0; 17 for *ahk2/3*; 17 for *ahk2/4*; 13 for *ahk3/4*. (b) Average gall sizes that developed per plant after 20 dpi. Gall numbers per line were 25 for Col-0; 10 for *ahk2/3*; 34 for *ahk2/4*; 28 for *ahk3/4*. (c) Average number of juveniles that penetrated per plant in each line; n= 10 for Col-0, n=9 for *ahk2/3*, n=12 for *ahk2/4*, n=10 for *ahk3/4*. Error bars indicate SE. Data are representative results of one out three independent experiments. Asterisks indicate significant differences by a two-tailed Student's *t*-test ($P < 0.05$).

Lastly, we wanted to determine if decreased root-knot susceptibility was due to reduced attraction, penetration or feeding site impairment. Therefore, we counted the number of juveniles that entered the roots at 3 dpi. No significant differences were found among genotypes (Figure 3.9). Altogether, these results suggest that cytokinin signaling is not required during nematode attraction or penetration, but rather is required for successful feeding site formation.

Cell cycle activation in root-knot nematode galls is mediated by cytokinin receptors

A concurrent study conducted by our collaborators showed cytokinin controls cell cycle reactivation in syncytia, and that this is mainly regulated through cytokinin receptors AHK3 and AHK4 (Siddique *et al.*, 2015). To determine if cell cycle reactivation is dependent on cytokinin signaling in forming galls and whether receptor specificity varies between syncytia and galls, we evaluated expression of the *cyclin B1;1:GUS* gene (*CycB1;1*) in *ahk2/3*, *ahk2/4* and *ahk3/4* double mutants in response to root-knot nematode infection. *CycB1;1* is highly expressed during the G2-to-M phase transition and therefore was used here as a mitotic marker. Evaluation of *CycB1;1:GUS* transformed Col-0 roots showed strong expression in early developing galls (4 dpi) and expression was sustained through 11 dpi (Figure 3.10a-c). *CycB1;1:GUS* expression was also often detected in receptor mutant combinations *ahk2/4* and *ahk3/4* (Figure 3.10g-l), but was frequently absent in *ahk2/3* (Figure 3.10d-f). To determine if significant differences existed between Col-0 and the receptor mutants, we quantified the number of galls in each mutant background expressing *CycB1;1:GUS*. We observed significant reductions across all lines at 5 and 7 dpi, and a higher significant decrease for *ahk2 ahk3* at 7 dpi (Figure 3.10m-n),

suggesting AHK2 and AHK3 are the major receptors for cell cycle activation in developing galls.

DISCUSSION

In this chapter, through a comparative analysis using a single host plant species we demonstrate that cytokinin signaling is a requirement of both cyst and root-knot nematode interactions, but these two nematode species differentially regulate receptor genes involved in the establishment of syncytia and giant-cells. We evaluated single and double AHK receptor mutants against *H. schachtii* and *M. incognita* infection. No significant differences in infection by either nematode species were observed for the single receptor mutants compared to wild type plants, likely due to functional redundancy; however, the *ahk3/4* double mutant showed significantly fewer cysts compared to wild type plants and the *ahk2/3* and *ahk2/4* double mutants. Conversely, the *ahk2/3* double mutant showed significantly fewer galls compared to the wild type control and the *ahk2/4* and *ahk3/4* double mutants. Overall these findings suggest that cytokinin signaling is not only required for successful Arabidopsis infection by both nematode species, but this signaling is controlled by distinct receptors and possibly distinct downstream components in each plant-nematode interaction.

The observed reductions in gall number and nematode development compared to Col-0, were not explained by a decrease in nematode penetration efficiency, suggesting cytokinin signaling is necessary for feeding site formation rather than for initial infection of the host plant. No significant differences were found across double mutants in terms of nematode development or gall size, suggesting that even though fewer galls are formed in

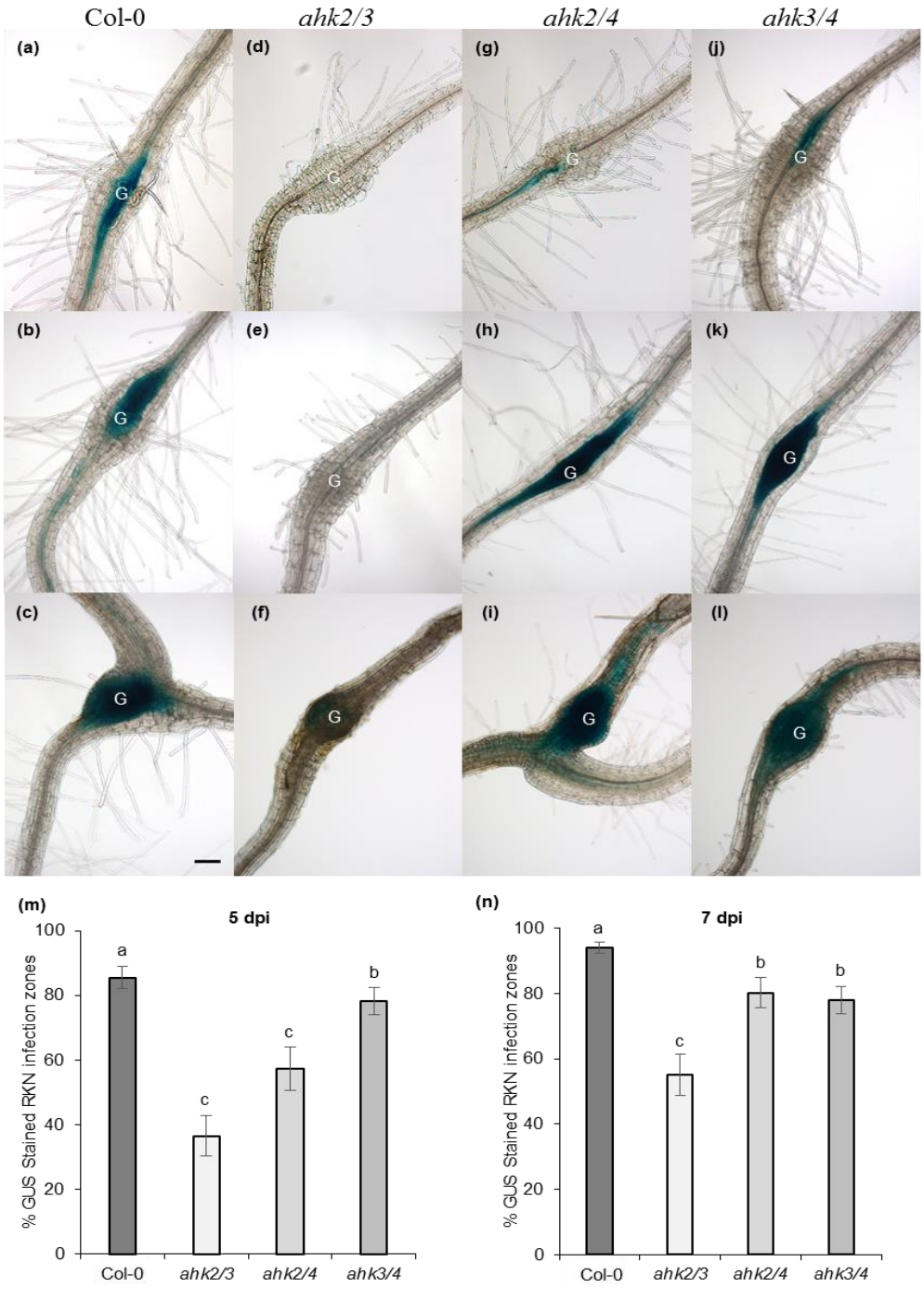

Figure 3.10. *CyclinB1;1* (*CycB1;1*) GUS expression in root-knot nematode galls developing in Arabidopsis wild type Col-0 (a-c) and cytokinin receptor mutants *ahk2/3* (d-f), *ahk2/4* (g-i), *ahk3/4* (j-l) at 4, 7 and 11 dpi (top-bottom). Abbreviations: G, gall. Scale bar=100 µm. Quantification of root-knot nematode galls expressing *CycB1,1:GUS* at 5 and 7 dpi. Error bars indicate SE. Data are representative result of one out of two independent experiments with n=28 for Col-0, n=29 for *ahk2/3*, n=28 for *ahk2/4* and n=29 for *ahk3/4*. Different letters above columns indicate significant differences by a two-tailed Student's *t*-test ($P < 0.05$). Scale bar=100 µm.

the *ahk2/3* mutant compared to *ahk2/4* and *ahk3/4*, the nematodes that are able to infect *ahk2/3* can still develop and form galls of comparable size to the nematodes growing in *ahk2/4* and *ahk3/4*. This suggests that receptors AHK2 and AHK3 play a role in early rather than late gall developmental stages. Consistent with this, *AHK2* and *AHK3* were expressed in galls over consecutive developmental stages from early stages until galls reached full maturity, whereas *AHK4/CRE1* showed downregulation in galls following the initial stages of gall formation. The expression of the type-A *ARR* gene *ARR15* and the cytokinin oxidase *CKX7* gene depends on AHK4/CRE1, both of which showed downregulation in expanding galls suggesting a reduced function of the AHK4/CRE1 receptor in response to RKN. These results support the stronger phenotypes observed for the *ahk2/ahk3* double mutant compared to *ahk2/ahk4* and *ahk3*/*ahk4*. However, we cannot exclude a role for AHK4/CRE1 during the initial stages of gall formation due to the expression of *AHK4/CRE1* in early galls and only partial reduction in gall formation on the double mutants.

To further analyze the role of AHK receptors in early gall formation, we evaluated the function of cytokinin receptors in gall cell cycle activation. We tested *CycB1;1:GUS*, introgressed into Col-0, *ahk2/3*, *ahk2/4* and *ahk3/4* lines and measured the number of infection sites expressing GUS. CycB1;1 is a cell cycle marker highly active in dividing cells expressed in galls (De Almeida *et al.*,1999). Evaluation of *CycB1;1:GUS* transformed Col-0 plants, showed *CycB1;1* expression in the majority of galls at 5 dpi and at 7 dpi. In contrast, cytokinin receptor mutants had a significantly lower number of expressing sites compared to Col-0. The *ahk2/ahk3* mutant showed the greatest reduction in expression among all mutant combinations. These results strongly suggest cytokinin signaling is

required for cell cycle progression in developing galls, and that *ahk2* and *ahk3* had a stronger effect on *CycB1;1* expression in galls than *ahk4*. Lack of cell cycle *CycB1;1* expression in galls could explain the fewer galls formed in the *ahk2/ahk3* mutant, further supporting the importance of AHK2 and AHK3 in gall formation. This is in stark contrast to our previous studies showing that AHK3 and AHK4/CRE1 act as the main receptors involved in the interaction with BCN (Siddique *et al.*, 2015).

The differences observed in cytokinin signaling between cyst and root-knot nematodes may explain the underlying cellular changes induced by these two pathogens to form quite distinct feeding sites. Whereas cyst nematodes induce limited cell division prior to incorporation of cells into the syncytium and require a normal activation of cytokinin signaling, root-knot nematodes induce an abnormal increase in cell division giving rise to the characteristic galls reminiscent of a hyperactivated state of cytokinin signaling. How root-knot achieves hyperactivation of cytokinin signaling is not yet understood, but presumably the observed differential regulation of AHK receptors in the same tissue could be involved. In roots, AHK4 is the main receptor involved in cytokinin perception as *ahk4* mutants have a clear delay in cytokinin responsiveness to exogenous applications, and exhibit longer roots compared to *ahk2* and *ahk3* mutants, which behave similarly to the wild type (Nishimura *et al.*, 2004, Higuchi *et al.*, 2004). AHK4 has also been associated with downregulation of cytokinin signaling under environmental stresses (Franco-Zorrilla *et al.*, 2002) and the direct control of negative regulators of cytokinin signaling (Kiba, 2002). In this study, we observed a predominant role for AHK2 and AHK3, but not AHK4 in gall formation. Root-knot may be downregulating AHK4, the main receptor involved in cytokinin sensitivity in roots, in order to avoid a negative feedback which would have

naturally occurred over time as result of an extensive exposure to cytokinin. This would result in partial cytokinin insensitivity and the abnormal growth observed in gall tissue, which is not observed in cyst nematode feeding sites. Future studies focusing on the identification of root-knot nematode cytokinin and the downstream components regulated by each AHK cytokinin receptor may uncover the role of differential cytokinin signaling in gall formation.

MATERIALS AND METHODS

Nematode cultures

BCN (*Heterodera schachtii*) nematode cultures were maintained on greenhouse-grown sugarbeet plants (*Beta vulgaris* cv. Monohi). RKN (*Meloidogyne incognita*) cultures were maintained on tomato (*Lycopersicon esculentum* cv. Tiny Tim) and eggplant (*Solanum melongena* cv. Black Beauty).

Plant material

AHK:GUS reporter lines were described by Miyawaki *et al.* (2004). The Arabidopsis cytokinin receptor mutant lines used in this study included *ahk2-2*, *ahk3-3*, *cre1-12* (*ahk4*), *ahk2-2 ahk3-3*, *ahk2-2 cre1-12*, *ahk3-3 cre1-12* and were described by Higuchi *et al.* (2004). *TCSn::GFP* lines were described in Zurcher *et al*., (2013). *Cycb1;1* introgressed lines were described in Siddique *et al*., (2015). Seeds were surface-sterilized with chlorine gas as previously described (Wang *et al.*, 2011) and cold-stratified for 3 days at 4ºC. Seeds were plated on 0.5% Murashige Skoog (MS) medium MS Basal Salts (Caisson Laboratories, North Logan, UT, USA), 2% sucrose and 0.8% Type A Agar

(Sigma, St. Louis, MO, USA) and placed in a growth chamber set at 22ºC under constant light for seven days. Seedlings were transplanted from the medium using forceps to trays of commercially available potting mix (SunGro) containing 50-60% horticulture grade vermiculite, Canadian sphagnum peat moss, horticulture grade perlite and dolomitic limestone. Trays of seedlings were covered with a plastic dome and placed in a walk-in growth chamber set at 22ºC, 70% RH and 14 h photoperiod for 6 weeks. The plastic dome was removed four days after transplanting. Plants were bottom-watered with tap water every 3 or 4 days and fertilized with a commercially available soluble fertilizer, Miracle-Gro, (N-P-K: 24-8-16) every 2 weeks.

Inoculation of Arabidopsis reporter lines

For inoculations with BCN, surface-sterilized seeds were plated in square plates on Knop's medium prepared with 0.8% Daishin agar (Brunschwig Chemie, http://www.brunschwig-ch.com/) (Sijmons, 1991). Plates were positioned vertically in a growth chamber set at 24ºC, 70% RH with a 12 h photoperiod with a light intensity of 100-150 µmol/m^2/s. Two days before inoculation, cyst nematode eggs were isolated from pot cultures and J2s hatched at 28ºC as previously described (Mitchum *et al.*, 2004). J2s were surface-sterilized in a solution containing 0.004% mercuric chloride, 0.004% sodium azide, and 0.002% Triton X-100 for 8 min, washed with sterile water five times and suspended in 0.1% agarose. Nematodes were pelleted at 2,000 rpm per 30 sec between washes. Roots of ten-day-old seedlings were inoculated with 50 surface-sterilized J2s. Plates were sealed with parafilm and placed back in the growth chamber until harvesting.

For inoculations with RKN, surface-sterilized seeds were plated one seed per well in 6-well plates (GUS reporter lines) or glass-bottom microwell dishes (MatTek Co., Ashland, MA) (*TCSn:GFP*) containing Knop's medium prepared with 0.8% Daishin agar. Plates were wrapped twice with Parafilm to avoid dehydration and placed in a growth chamber set to the conditions above. Four days before inoculation, RKN eggs were harvested from pot cultures by rinsing soil from the roots and releasing eggs from egg masses by agitating roots in 10% sodium hypochlorite for four minutes. The egg suspension and plant debris was poured over a stack of sieves: 850 μm (No.20), 250 μm (No.60), 75 μm (No. 200) and 25 μm (No. 500). Eggs collected on the No. 500 sieve were rinsed under tap water for five min and set up to hatch as previously described (Mitchum *et al.*, 2004). J2s were surface-sterilized according to above except the incubation step in sterilization solution was for five minutes. Fourteen-day-old (GUS reporter lines) or ten-day-old (*TCSn:GFP*) seedlings were inoculated with 1000 surface-sterilized J2s per plant. Plates were sealed with parafilm and placed back in the growth chamber until harvesting.

Histochemical *β*-glucuronidase (GUS) assay

Nematode-inoculated and mock-inoculated seedlings were transferred from six-well plates into plates containing GUS solution [100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), 1 mM 5-bromo-4chloro-3-indolyl glucuronide, pH 7.0, 50 mM NaCl, 0.06% Triton X-100, 2 mM potassium ferricyanide). Plates were vacuum-infiltrated twice for10 min and incubated at 37ºC overnight. Seedlings were washed three times with 70% ethanol at intervals of 30 minutes, and incubated in 70% ethanol at 4ºC overnight to remove all remaining chlorophyll prior to evaluation under an upright stereoscope. Representative

samples were excised using micro scissors and forceps (DUMOXEL #4) and placed in 12-well plates containing 70% ethanol. At least 12 seedlings were analyzed per line per time point.

Fixation, embedding and sectioning of GUS-stained Arabidopsis roots

Root segments containing syncytia or galls were embedded in 1.5% low melting agarose by carefully dragging each root with forceps through a 250 µl drop of agarose placed inside a Petri dish. Agarose blocks containing root sections were cut and transferred to 12-well plates containing 4% paraformaldehyde (PFA) in a 1X phosphate-buffered saline (PBS) solution (11.9 mM phosphate buffer containing KH_2PO_4 and Na_2HPO_4 137 mM NaCl, 2.7 mM KCl, pH 7.4). Tissues were vacuum-infiltrated three times for five minutes. Following the last infiltration, PFA was replaced with new solution and tissues were incubated for 17 h at room temperature and then at 24 h at 4ºC. After incubation, tissue was gradually dehydrated starting with 1X PBS solution for 15 min twice, and replacing the solution with 30%, 40%, 50%, 60% and 70% ethanol for 30 min each. Agarose blocks were placed between foam pads of a biopsy cassette, submerged in 70% ethanol, and sent to the MU Histology Core for paraffin embedding and sectioning at 10 µm. Mounted sections were placed on a slide warmer at 40ºC overnight for adhesion. Slides were vertically submerged in xylene for 10 min and then laid flat to dry before mounting sections in Shandon consul mount and coversliped for imaging.

Brightfield microscopy

Root pieces containing syncytia or galls were placed on a plain microscope slide (Fisher Scientific, Hampton, NH) in a drop of sterile distilled water and gently covered with a microscope cover glass (Fisher Scientific, Hampton, NH). Photos of whole roots and root sections containing feeding sites were taken with a digital Nikon COOLPIX5000 camera manually attached to a Nikon Eclipse TS100 (Melville, NY, USA) inverted microscope (*AHK2:GUS*, *AHK3:GUS*). Additional photos were taken with a Leica DM5500B upright microscope (*AHK4:GUS*) equipped with a Leica DFC295 color digital camera at the MU Cytology Core.

Widefield fluorescence microscopy

TCSn:GFP seedlings were grown in 35mm glass bottom microwell dishes (MatTek Co., Ashland, MA) containing 2 ml of Knop's medium for seven days and inoculated as described above. *TCSn:GFP* lines infected with cyst or root-knot nematodes were monitored daily for 14 days starting hours after inoculation. Syncytia and galls forming in *TCSn:GFP* infected lines were marked and labeled in the bottom of the plate. Photos were taken using an Olympus IX70 inverted microscope with Orca ER digital camera at the MU LSC Cytology Core.

Infection assays

Knop's medium

Sterilized seeds were plated one seed per well in sterile 12-well plates (BD Biosciences, http://www.bdbiosciences.com/) containing modified Knop's medium and

0.8% Daishin agar following a randomized block design. Plates were sealed with parafilm to avoid dessication and kept in a growth chamber at 24ºC, 65% RH in a 12 h photoperiod with 100-150 µmol/m^2/s average of light intensity. Fourteen-day-old seedlings were inoculated with 1,000 surface-sterilized J2 resuspended in 25 µl of 0.01% sterile agarose and returned to the chamber. Galls were counted at 43 dpi. Thirty-six plants were evaluated per genotype per experiment and each experiment was independently repeated 3 times.

Sand-soil based substrate

Surface-sterilized seeds were imbibed in sterile water and placed at 4ºC for 48 h under constant rotation. Seeds were plated on ½ MS, 2% sucrose medium in Petri plates and kept at 22ºC in a Percival chamber set to constant light. Seven-day-old seedlings were transferred to individual pots filled with riversand: Fafard Growing mix (3:1) and placed in trays. Pots were bottom watered for about two minutes until the soil surface looked wet. Trays were completely covered and placed in a growth chamber for 5 days. Covers were then cracked for two days. On day seven after transplanting, the cover was completely removed. Fourteen-day-old seedlings were inoculated with 1,500 root-knot nematode eggs. A 200 µl pipette tip was used to punch 4 holes around each plant. Each plant was inoculated with 1 ml (1,500 eggs/ml) of egg inoculum suspended in sterile water. Tube was agitated by pipetting up and down before inoculating each plant and strongly agitated between trays. Plants were fertilized once a week with Miracle-Gro, (N-P-K: 24-8-16). At 42 dpi, roots were carefully removed from the pots and gently rinsed in a bucket of water. Galls were counted under a stereomicroscope. Each root system was then cut into small pieces with scissors and placed in a 50 ml tube with 10% bleach and agitated for 4 min. The root slurry

was poured over a nested stack of sieves (no. 20/60/200/500) while rinsing with a hand sprayer to release egg masses. Eggs were rinsed from the no. 500 sieve into a round bottom tube or small beaker and the volume was adjusted to 25 ml with water. Two ml of acid fuchsin was added and the sample was microwaved for 45 sec and then cooled to RT. One ml of solution was placed into a Huxley slide and the eggs were counted.

Penetration assay

Arabidopsis seedlings were grown in 6-well plates and inoculated with nematodes according to the protocol described above. Three days after inoculation, plants were carefully removed and placed into 1% bleach for 4 minutes. Plants were briefly rinsed to remove excess bleach, placed into 1/25 dilution of acid fuschin solution (250 ml lactic acid, 750 ml water, 0.35 g acid fuchsin) and microwaved for 3 minutes and 30 sec. After cooling down inside the fume hood, seedlings were stored in 12-well plates containing 95% ethanol until further analysis. Each seedling was placed on a microscope slide and the roots were carefully spread out for counting. Stained nematodes were counted using a Nikon Eclipse TS100 inverted microscope. Twelve to twenty-four plants were used per genotype per experiment. Each experiment was repeated 3 times.

Nematode development assessment

Experiments were conducted following the infection assay protocol and conditions above described. Developing galls were marked in the bottom of each well plate three days after inoculation. Photos of marked galls were taken at 11 dpi using Leica M205 FA stereoscope with a color digital camera at the MU Cytology Core. Pictures of each gall

were evaluated for the presence of juvenile or an adult nematode. The number of adults and juveniles were counted and the adult/juvenile ratio was calculated per genotype. This experiment was independently replicated 3 times using eight to thirty-three galls per genotype per experiment.

RNA Isolation and Quantitative Real-Time PCR

One hundred wild type Col-0 seedlings were grown on Knops medium and inoculated with beet cyst nematode, *H. schachtii* or root-knot nematode, *M. incognita*, juveniles. Root segments containing syncytium or galls were harvested at 6 dpi and immediately placed in RNA*later* stabilization solution (Qiagen, Hilden, Germany) following manufacturer's instructions. Tissue was stored at -80ºC until further processing. Each sample was homogenized in liquid nitrogen using a mortar and pestle and total RNA was isolated with NucleoSpin RNA Plant Kit (Macherel-Nagel, Düren, Germany) following user manual instructions. cDNA was synthesized using First Strand cDNA Synthesis Kit following manufacturer's guidelines (Roche Diagnostics, Mannheim, Germany). cDNA was used in subsequent relative quantitative real time PCR (qRT-PCR) reactions using primers in Table 3.2. qPCR reactions were prepared with template and a non-template control containing 15 µl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5 µl of 0.2 µM primers and 5 µl of 1:10 diluted cDNA in 96 well plates. qPCR was carried out with the following conditions: 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min using an Applied Biosystems 7500 Real Time PCR system. A dissociation curve analysis was conducted for each primer set. Four technical replicates per each of two biological replicates were used

Table 3.2. Primer sequences used in this study.

Name	Sequence (5' - 3')	Reference
AHK4-F	ACC GTT GCT AAG TGG AGT GG	This study
AHK4-R	CTA ACC GGT GAA GGC TCT CC	This study
ARR15-F	CCG GTG AAA TTA GCT GAT GTGA	This study
ARR15-R	TTT TGG TTT TTC CTT CTT CAG CTT	This study

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CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

Cyst and root-knot nematodes are plant parasites of major economic importance that form close interactions with their hosts in order to establish a nutrient sink. Previous studies have shown cyst nematodes secrete cytokinins to activate the cell cycle of initial feeding cells (Siddique *et al*., 2015). This dissertation has extended these studies and determined that in addition to nematode cytokinin, host cytokinin biosynthesis and catabolism genes also contribute to cyst nematode infection. We have further discovered a role for cytokinin signaling in beet cyst nematode (BCN) infection.

In the future, it will be interesting to determine how cytokinin signaling intersects with other hormone pathways at the feeding site during infection. Previous studies have shown a cross-talk between cytokinin and CLE signaling during plant development. In the shoot apical meristem (SAM), homeodomain transcription factor WUSCHEL (WUS) was shown to repress Type-A ARRs, presumably upregulating cytokinin signaling (Leibfried *et al*., 2005). In turn, cytokinin regulates WUS expression using a CLV-dependent and independent pathway to regulate stem cell displacement during growth (Gordon *et al.*, 2009). In the root vascular cylinder, a member of the CLE family, CLE10, has been shown to repress the expression of the Type –A ARRSs ARR5 and ARR6, to inhibit protoxylem vessel formation (Kondo *et al.*, 2011). These findings suggest cytokinin functions downstream of CLE signaling in meristematic tissues. Past studies have shown the importance of the CLE receptors in cyst nematode feeding site formation (Guo *et al.*, 2011; Replogle *et al.*, 2011, Replogle *et al.*, 2013). Based on these studies in addition to the

findings of this dissertation, it is possible that CLE signaling spatiotemporally upregulates cytokinin signaling at the feeding site to start cell cycle activation of newly incorporated syncytial cells. Simultaneous studies of CLE and cytokinin signaling at the feeding site could help to elucidate the interplay between these two pathways.

Alternatively, CLE and cytokinin interplay might be controlled by the nematode to downregulate defense response in the host. Links between cytokinin and defense pathways in Arabidopsis have been recently found. Argueso *et al*., 2012 reported that plant cytokinins can lead to increased defense responses to a virulent strain of *Hyaloperonospora parasitica* through a salicylic acid (SA) dependent process. Choi *et al* 2010, found a downstream Type B-ARR component of the cytokinin pathway, ARR2, which can physically interact with the SA pathway regulator TGA3 to induce the expression of pathogenicity related (PR) genes. Interestingly, cytokinin-hypersensitive Type A-ARR Arabidopsis mutants, partially impaired in the ability to downregulate cytokinin signaling, have been reported to exhibit reduced cyst nematode susceptibility probably due to enhanced defense responses (Shanks *et al.*, 2016). Presumably, nematodes could be using CLE signaling to alter cytokinin signaling regulation and counteract the upregulation of defense responses.

To determine if cytokinin function in parasitism is limited to cyst nematode infection, this dissertation has further evaluated the role of cytokinin in response to root-knot gall formation. Similar to CN, we found a requirement of cytokinin degradation during RKN infection. But quite interestingly we discovered main differences in the type of cytokinin receptors mediating the interaction with cyst and root-knot. While AHK3 and AHK4 are the main receptors for cell cycle activation in the syncytium, AHK2 and AHK3

are the main receptors for activation of cell cycle in galls. A summary of these results is displayed as a comparative model between BCN and RKN in Figure 4.1. The functional divergence of cytokinin receptors is of main interest since these receptors are generally thought to be redundant in plant growth and development. Determining the downstream components controlled by different types of receptors can help elucidate the role of this specific requirement.

Previous studies have shown that pathogens can alter host cytokinin contents by secreting cytokinin into the host tissue (Pertry *et al*., 2009; Pertry *et al.*, 2010, Radhika *et al.*, 2015, Siddique *et al.*, 2015). The types and amounts of these contents vary depending on the pathogen, suggesting parasites use cytokinin in different ways to achieve niche establishment. In fact, the leafy gall actinomycete, *R. fascians*, induces accumulation of cytokinins by secreting a methylated form of *cis*-zeatin resistant to host degrading enzymes (Pertry *et al*., 2009; Radhika *et al*., 2015). In contrast, the cyst nematode *H. schachtii* secretes isopentenyladenine, which has high levels of activity in plants (Siddique *et al*., 2015). In both cases, hormonal accumulation leads to activation of cytokinin signaling and the formation of unique types of feeding sites. Isolation of RKN cytokinins *in vitro* from pre-infective juveniles revealed a mix of molecules with reported different activity levels and resistance to plant oxidases (De Meutter *et al*., 2003). Whether these molecules are in fact secreted into the host tissue to cause disease has not yet been evaluated. In the future, profiling cytokinin levels in RKN-infected tissues will elucidate the mechanism behind the role of root-knot nematode cytokinin in this host-pathogen interaction. Furthermore, the RKN cytokinin biosynthesis gene(s) should be identified and characterized. RNAi against this gene could help determine if RKN cytokinins are indeed required for infection.

Further studies on the role of cytokinin signaling in nematode infection can help us understand how these pathogens establish infection in susceptible hosts. BCN species, *Heterodera schachtii*, and RKN species, *Meloidogyne incognita*, both infect the model plant *Arabidopsis thaliana* without requiring major differences in growing conditions, serving as an excellent pathosystem for comparative analyses. In the future, the sequences of Arabidopsis cytokinin genes contributing to disease susceptibility can be used to identify orthologs in crops of economic importance. Genetic manipulation of cytokinin gene expression through the use of tissue-specific promoters could be valuable to enhance plant resistance to both nematode species without compromising major effects on plant growth and yield.

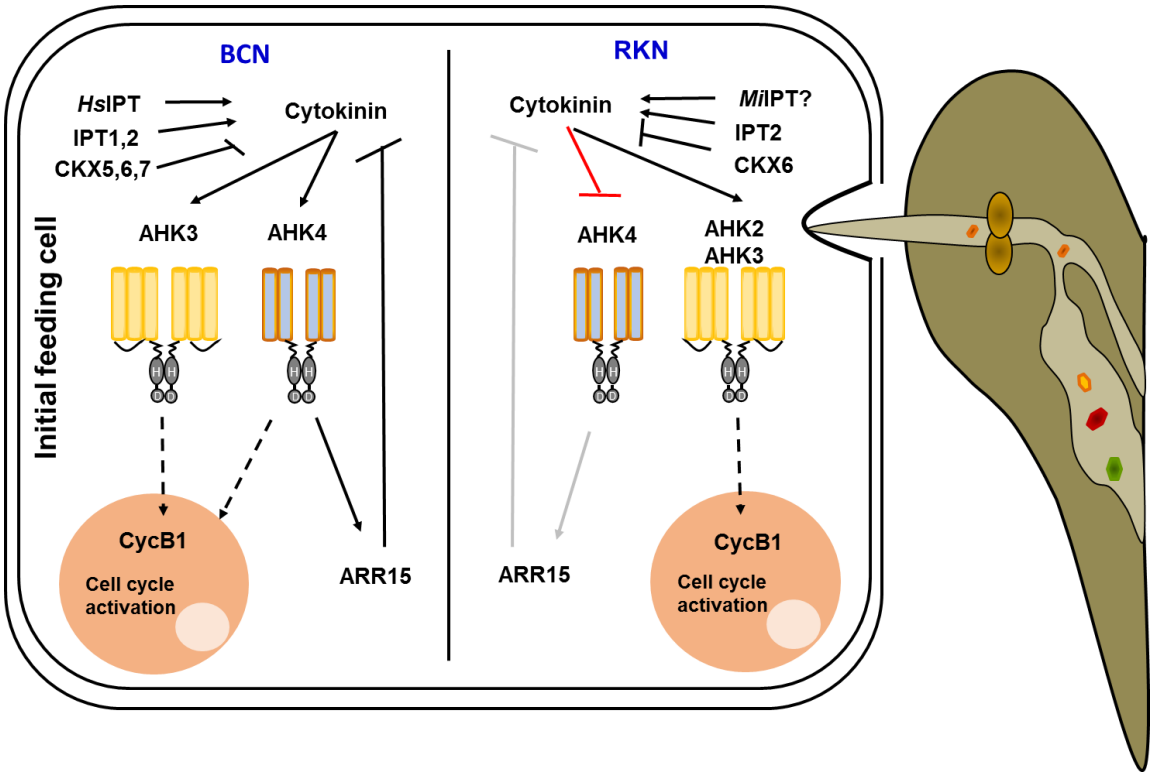

Figure 4.1. Model depicting divergent regulation of cytokinin metabolism and signaling in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*. Cytokinin is synthesized by the host or the nematode and recognized by receptors AHK3 and AHK4 in the case of syncytium formation and AHK2 and AHK3 in the case of gall formation. Upon cytokinin binding, autophosphorylation of cytokinin receptors initiates a phosphorelay resulting in nuclear upregulation of *CycB1;1*, which in turn activates cell cycle of infected cells. Cyst nematode AHK4-mediated activation of cytokinin signaling upregulates cytokinin negative regulator ARR15. Compared to cyst, downregulation of AHK4 and ARR15 in galls (right) may affect the ability of the host plant to self-regulate cytokinin activity resulting in hyperactivation of cytokinin signaling and over proliferation of cortical cells. Host cytokinin biosynthesis genes *IPT1, IPT2* and catabolism genes *CKX5*, *CKX6* and *CKX7* are upregulated in syncytia (left) while *IPT2* and *CKX6* are upregulated in galls (right). Abbreviations: *Hs*IPT: *Heterodera schachtii* isopentenyltransferase; *Mi*IPT: *Meloidogyne incognita* isopentenyltransferase; *IPT*: *Arabidopsis thaliana* isopentenyltransferase; *CKX*: *A. thaliana* cytokinin oxidase/dehydrogenase, ARR: Arabidopsis response regulator; CycB1;1: Cyclin B1;1; AHK: Arabidopsis Histidine Kinase receptor; H: Histidine residue; D: Aspartic acid residue. Dotted lines indicate multiple steps in the signaling pathway.

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VITA

Carola De La Torre Cuba was born in Lima, Peru, as the second daughter of economists Carlos De La Torre and Amalia Cuba. As a child, she visited agricultural fields around the country with her parents and became motivated by their work on agricultural and rural development. At age 16, she was accepted to La Molina National Agrarian University in Peru where she studied Biology with a minor in Biotechnology. During her sophomore year, she volunteered at the Microbial Ecology and Biotechnology 'Marino Tabusso' Laboratory where she conducted a research project on the characterization of bacterial strains with antifungal properties. It was then that she was introduced for the first time to plant pathology by her mentors, Dr. Doris Zuñiga and Dr. Tomas Melgarejo. A few years later she was selected as an international fellow to conduct a bachelor thesis at the International Potato Center (CIP) under the direction of Dr. Luis Salazar in Lima, Peru. Soon after completing her thesis, Carola was hired at the same center to work on a papaya disease management project in small rural farms in Peru where she further developed an interest in mitigating losses due to crop disease. Her motivation and enthusiasm to generate new technologies of pathogen control led her join the Dr. Lewandowski Laboratory at The Ohio State University to pursue a M.Sc. in Plant Pathology in 2007 and later join the OSU Plant Transformation Laboratory to work in crop improvement under the supervision of Dr. John Finer. At that time, she realized she wanted to deepen her knowledge of disease susceptibility in order to generate novel and durable ways of pathogen control. It was then that she decided to pursue a Ph.D. in Plant, Insect and Microbial Sciences at the University of Missouri-Columbia and joined the laboratory of Dr. Melissa Mitchum to study the molecular mechanisms of nematode pathogenesis.