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Studies on fatty acid *de novo* synthesis and metabolism in free-living and parasitic nematodes and their feeding sites in plants

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Abbreviations

ACC	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
ADP	adenosinediphosphate
ATP	Adenosine-5'-triphosphate
Ce	*Caenorhabditis elegans*
CGI	Comparative gene identifier
CoA	Coenzyme A
Col-0	Columbia 0
CPT	Carnitine palmitoyl transferase
DAG	Diacylglycerol
DGDG	Digalactosyldiacylglycerol
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPI	Days post inoculation
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ER	Endoplasmic reticulum
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
G3P	Glycerol-3-Phosphate
GC	Gas chromatography
Glc	Glucose
Hs	*Heterodera schachtii*
J1,2,3,4	Juvenille stages
kb	Kilobase pairs
L1,2,3,4	Larval stages
LB	Lysogeny Broth
LC-MS	Liquid chromatography coupled to mass spectrometry
LPA	Lysophosphatidic acid
Lyso-PC	Lysophosphatidylcholine
m/z	Mass-to-charge ratio
MGDG	Monogalactosyldiacylglycerol
mmBCFA	Mono-methyl branched chain fatty acid
mol %	Molar percentage
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MUFA	Monounsaturated
ORF	Open reading frame
PA	Phosphatidic acid
PC	Phosphatidylcholine
POD	Polarity and osmotic deficient
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
SDP	Sugar dependent
SPT	Spirotetramat
SPT-enol	Spirotetramat-enol
TAG	Triacylglycerol
VLCFA	Very long chain fatty acids
WT	Wild type

Fatty acids are abbreviated X:Y, where X represents the number of carbon atoms, and Y   represents the number of double bonds.

1 Introduction

1.1 Fatty acids and life

Fatty acids participate in membrane biogenesis, store energy, serve as signaling molecules and modulate growth and development. They are ubiquitously present in bacteria, plants, animals and their metabolism involves conserved mechanisms. Fatty acids are either synthesized *de novo* by fatty acid synthase (FAS) or taken up with the diet. Fatty acids from both routes may be further modified and assembled into more complex lipids (Figure 1-1) (Dowhan and Bogdanov, 2002; Eyster, 2007; Haucke and Di Paolo, 2007). Depending on the lifestyle of the organisms the dependency on both routes varies considerably. Whereas most animals rely on both routes, photoautotrophic organisms, like most plants, can synthesize all required fatty acids *de novo (*Li-Beisson *et al.,* 2010). On the contrary, fatty acid auxotrophy has been reported in obligate mutualistic and parasitic interactions (Xu *et al.,* 2007; Wewer *et al.,* 2014). The auxotrophy was linked to the absence of functional orthologs for FAS in the fully sequenced genomes of the respective fungi. The propensity of a gene to be lost during evolution depends on its biological importance (Krylov *et al.,* 2003). The evolutionary pressure on genes involved in metabolic processes is reduced when the host can supply the corresponding molecules. In contrast, genes important for beneficial interaction (e.g. effectors) may be preferentially retained and diversified (Jones and Dangl, 2006). Thus, lipid physiology in interactions is shaped by the symbionts´ genomes that determine their metabolic repertoire. This has important practical consequences since it allows the reconstruction of this repertoire using genomic information.
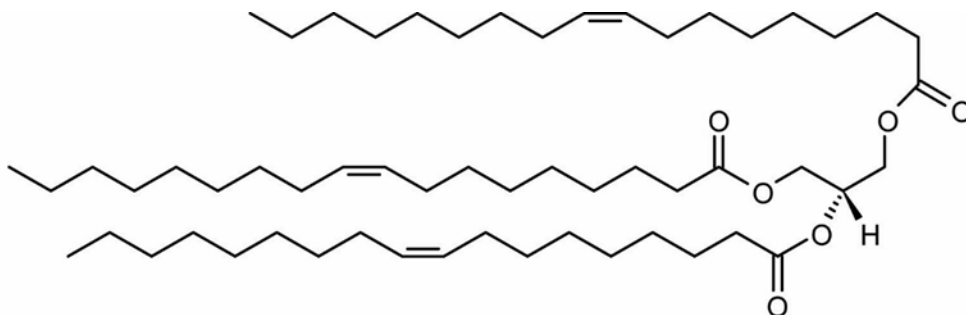
Figure 1-1   Representive structure of a glycerolipid.

Shown is the storage lipid triacylglycerol (tri18:1) that is composed of a glycerol backbone esterified to three fatty acyl groups.

1.2   Nematodes

Nematodes are ubiquitously present round worms that have diverse lifestyles including bacterial feeding as well as plant and animal parasitic species. The popular model species *Caenorhabditis elegans* is well studied and it provides a framework for key biosynthetic pathways. This framework may then be applied to other, less-well studied nematodes for which the genomic information is lacking. Although cyst nematodes (*Heterodera* and *Globodera sp.*) are important plant parasites causing yield loss in field crops, they belong to the group of less-well studied nematodes (Bird and Kaloshian, 2003). Despite the differences in lifestyle, the life cycle of both nematode species shares common features. Their life cycle is characterized by distinct stages of development divided by molts during which the old cuticle is shed and replaced. In the free living nematode *C. elegans* the different stages are referred to as larval (L1-L4, Figure 1-2) whereas they are called juvenile stages (J1-J4, Figure 1-3) in cyst nematodes.
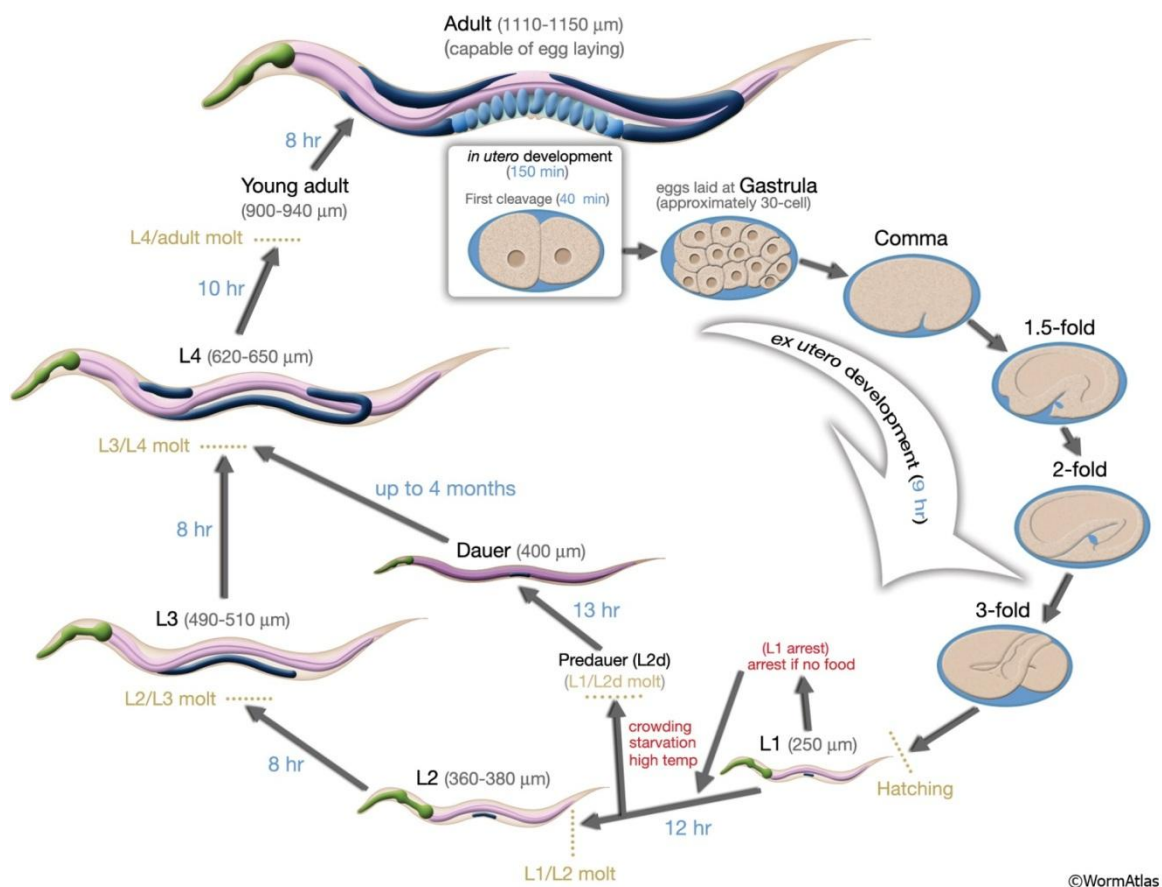
Figure 1-2   Life cycle of *C. elegans*.

After embryonic development that occurs within the egg *C. elegans* mobile L1 larvae hatch. Development of L1 is initiated by nutional cues and follows four molts before reaching adulthood. Adult hermaphrodites are againcapable of egg laying. WormAtlas, Altun, Z.F., Herndon, L.A., Crocker, C., Lints, R. and Hall, D.H. (ed.s) 2002-2015.

After hatching from the egg as L1 and J2 respectively, the nematodes will actively search for a suitable nutrient source.

Until finding this source, the nematodes will not develop and stored lipids serve as a source of energy (e.g. adenosine-triphosphate, ATP) which is required for locomotion (Story, 1984). Feeding on suitable nutrients initiates the development of *C. elegans* L1 trough nutrient derived signaling.

After completing the final molt, nematodes become adults. *C. elegans* reproduces mostly hermaphroditic whereas *H. schachtii* develops male and female nematodes. While females of *H. schachtii* stay sedentary throughout their life cycle, the males become vermiform to initiate in mating. Fertilized females develop into cysts that are filled with eggs. The cyst protects the unhatched nematodes that remain dormant until conditions become favorable.
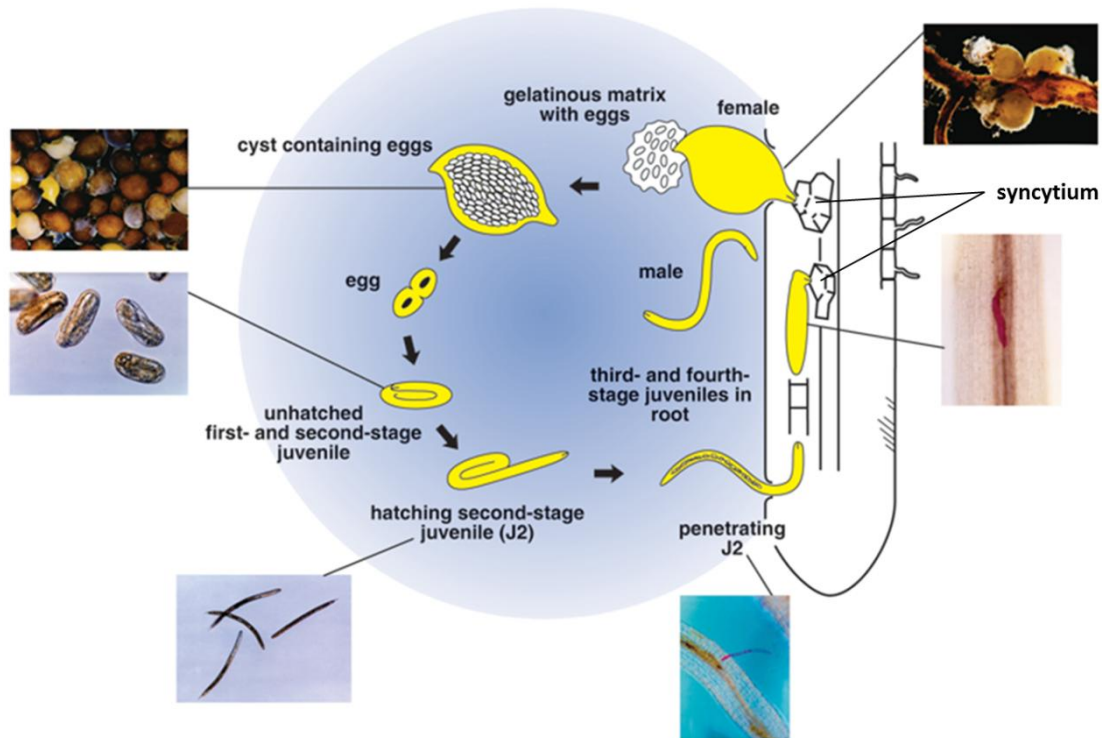
Figure 1-3   **Life cycle of cyst nematodes**

After hatching from the egg J2 will invade the root of host plants and induce a feeding site containing a syncytium. Feeding from the syncytium is essential to for the development of male and female nematodes. Adult males become vermiform and fertilize the females that develop into cyst containg eggs. Drawn by Dirk Charlson, Iowa State University

1.3   Fatty acid synthesis and metabolism in nematodes

Growth and development of nematodes is linked to the biosynthesis and metabolism of fatty acids. *C. elegans* uses *de novo* synthesized fatty acids as well as dietary fatty acids for lipid biosynthesis (Perez and Van Gilst, 2008). As depicted in Figure 1-4, fatty acid *de novo* synthesis is fueled by acetyl-CoA. The required carbon may originate from the glycolysis or B-oxidation. The rate limiting step in fatty acid *de novo* synthesis is carried out by acetyl-CoA carboxylase (ACC). ACC uses CO_2 to carboxylate acetyl-CoA yielding malonyl-CoA. Malonyl CoA is used as the chain extender in fatty acid *de novo* synthesis by fatty acid synthase (FAS) as well as for the elongation of fatty acids. Additionally, malonyl-CoA controls mitochondrial fatty acid β-oxidation by regulating carnitine palmitoyl transferase (CPT) activity (Watts, 2009). Knock down of ACC and FAS by RNAi in *C. elegans* lead to larval arrest, defects in triglyceride synthesis, molting, and reproduction (Li and Paik, 2011). Albeit contributing only minor to the total lipid pool, lipid *de novo synthesis* is essential for the development of *C. elegans* (Brock *et al.,* 2007). Nevertheless, the majority of fatty acids used for lipid biosynthesis are derived from bacterial lipids taken up with the diet likely because resorption of dietary lipids has energetic advantages as compared to its *de novo* synthesis. Additionally, β-oxidation of fatty acids can fuel ATP synthesis (Lodish *et al.,* 2000).
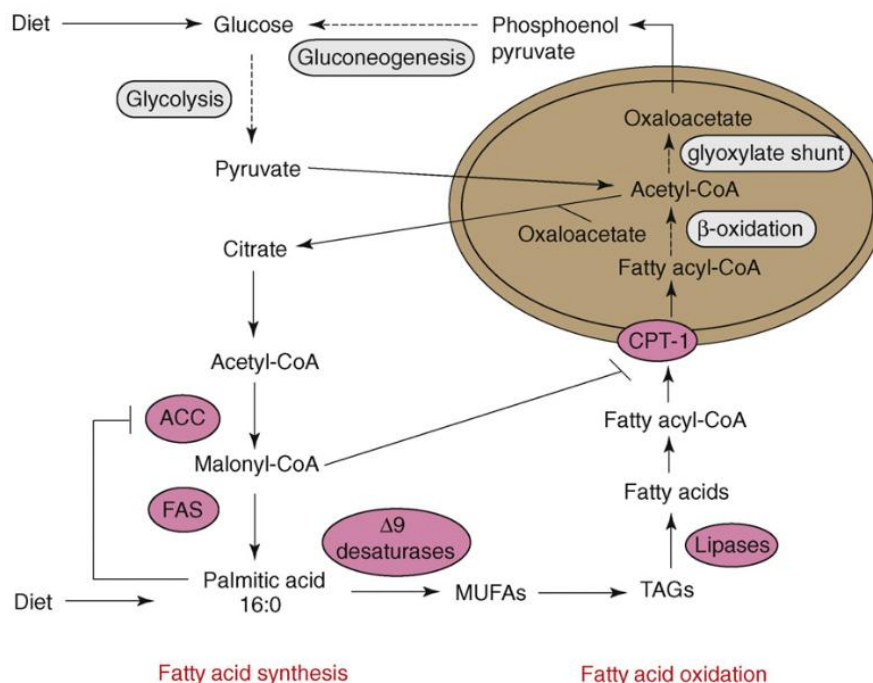
Figure 1-4   Fatty acid metabolism in *C. elegans*.

Fatty acid synthesis requires acetyl CoA from citrate that can be derived from glucose or fatty acids. Acetyl CoA is carboxylated by ACC to yield malonyl-CoA. Malonyl CoA functions as the fatty acid chain extender by FAS and regulated β-oxidation through inhibition of CPT-1. Figure taken from Watts (2009).

Fatty acids may also be desaturated giving rise to monounsaturated fatty acids (MUFA) that are precursors for polyunsaturated fatty acid (PUFA) biosynthesis. Together these fatty acids are required for the synthesis of more complex lipids including phospholipids, storage lipids and sphingolipids *(*Watts, *2009).*

Similar, migratory plant parasitic nematodes are also able to obtain fatty acids from their host and possibly synthesize specific fatty acids (Krusberg, 1967). However, it is not known if similar metabolic pathways and the required proteins exist in plant parasitic nematodes.

Cyst nematodes are sedentary plant parasites that have specialized feeding behavior. They hatch from eggs as J2 larvae and will search for a host plant. During this stage, J2 will neither feed nor develop. Stored lipids serve as a source of energy which is required for locomotion. Accordingly, lipid store content is proportional to the infectivity of the J2 larvae (Story, 1984). Also, J2´s seem to be able to generate energy from stored lipids, likely through β -oxidation. Thus, at least J2 can metabolize lipids. The lipids J2´s must have been supplied maternally so from the previous nematode generation. This nematode generation was exclusively nourished by plant derived nutrients.

H. schachtii exclusively feeds from a syncytium that forms the core of the feeding site. The syncytium develops after the nematode has selected an initial cell through progressive cell wall dissolution and protoplasmic fusion of neighboring cells. The syncytium shows a high metabolic activity and specific ultra-structural changes including central vacuole breakdown, plastid and mitochondria endoreplication as well as a proliferating endomembrane system and lipid droplet accumulation. Distinct is the temporal shift from a rough ER to a smooth ER and the formation of cell wall ingrowths during syncytium development (Golinowski *et al.,* 1996, Sobczak and Golinowski, 2011). The syncytium shares characteristics with transfer cells (Rodiuc *et al.,* 2014).

The syncytium is fueled by phloem derived solutes stimulating high metabolic activity (Böckenhoff *et al.,* 1996; Hofmann *et al.,* 2007). High metabolic activity is further characterized by a distinct transcriptomic signature, an increase of amino acids and phosphates (e.g. glycerol-6-phophate, phosphoenolpyruvate) and the accumulation of starch in syncytial plastids (Hofmann *et al.,* 2008; Szakasits *et al.,* 2009; Hofmann *et al.,* 2010;). However, starch accumulation in plastids seemed to be associated with necrotic tissue (Sobczak and Golinowski, 2011). Interestingly, although the transcriptomic signature is very different as compared to other tissues some processes seem to be similar occurring in seeds and pollen (Szakasits *et al.,* 2009). Both organs require lipid *de novo* synthesis and triacylglycerol is essential for their normal development (Zhang *et al.,* 2009).

Feeding in *H. schachtii* occurs in cycles and contains a phase without nutrient uptake (Wyss and Grundler, 1992; Böckenhoff *et al.,* 1996). During this time the syncytium is supposed to be "refilled" with phloem derived solutes leading to an expansion (Bohlmann and Sobczak, 2014). As such, the syncytium requires a flexible cell wall architecture to cope with the changes in pressure (Davies *et al.,*

2012). *H. schachtii* uses its stylet to deliver gland secretions. Part of these secretions form the feeding tube that is connected to stylets orifice. The feeding tube is used to withdraw nutrients from a zone of modified cytoplasm that is free of larger organelles (Wyss and Grundler, 1992). Both secretions and the feeding tube associate with membranous structures (especially ER) within the syncytium (Grundler *et al.,* 1999).

Together this indicates multiple roles for plant lipids in the syncytium. Plant lipid metabolism is well studied in *A. thaliana* (Li-Beisson *et al.,* 2010) that is also a suitable host for *H. schachtii* (Sijmons *et al.,* 1999)
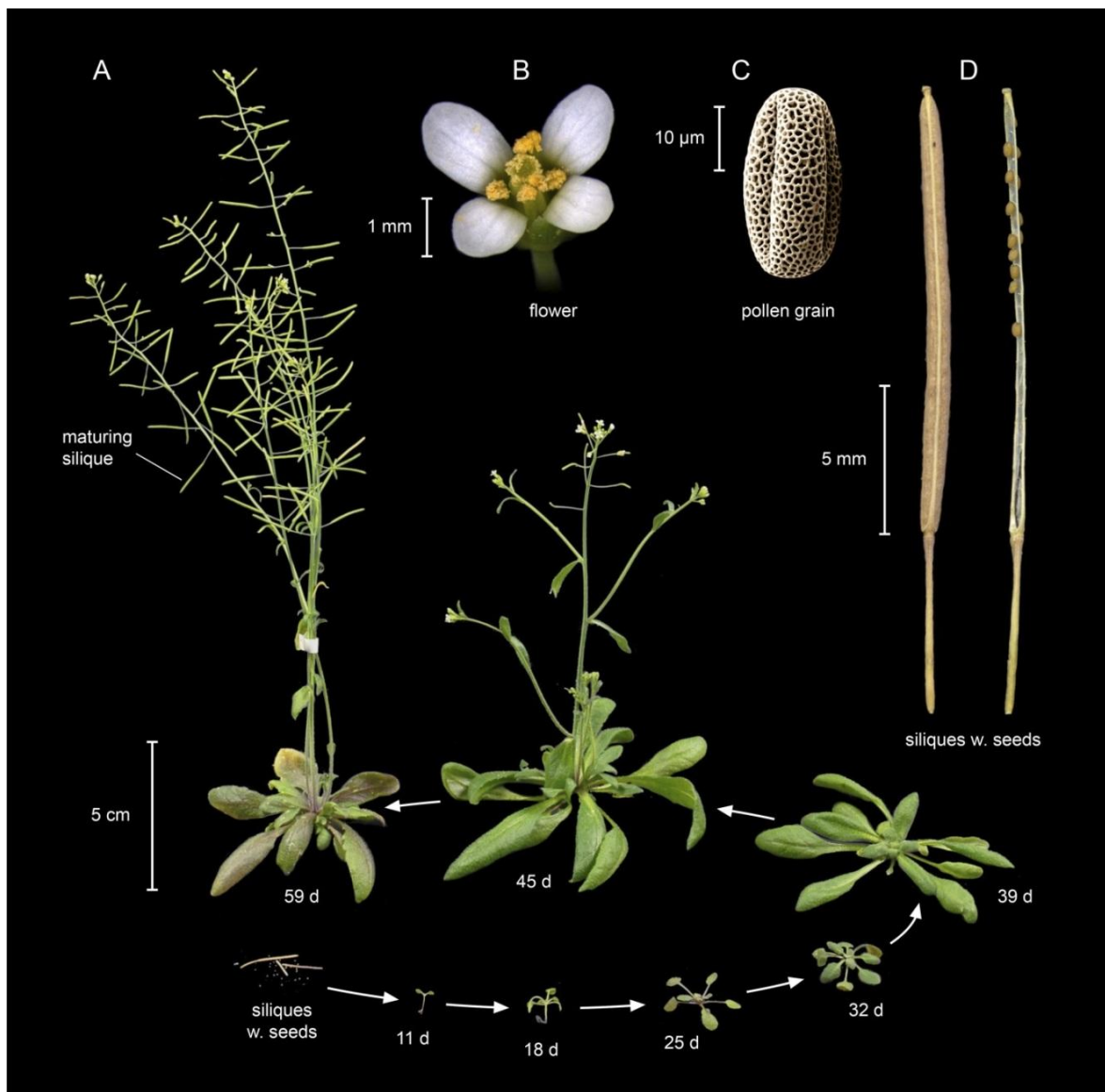

Figure 1-5 **Lifecycle of *Arabidopsis thaliana*.**

Arabidopsis thaliana is used as model species for plant biology and is a suitable host for plant parasitic nematodes. After germination and 6 weeks of vegetative growth, flowers fertilized by pollen give rise to siliques filled with seeds. Figure taken from Krämer (2015).

In *A. thaliana*, fatty acid *de novo* synthesis occurs in the plastids. However, extraplastidial pathways are also involved in lipid biosynthesis. The most abundant class of lipids are the glycerolipids that form important components of plasma membranes, endomembrane, organelles and energy stores. Extraplastidial membranes contain relative large amounts of phospholipids, but also sterol lipids and sphingolipids. These lipids are mainly synthesized in the ER. Organelles, like plastids synthesisze and contain galactolipids. Triacylglycerol (TAG) represents the main form of lipid-bound carbon reserves containing 3 fatty acids esterified to a glycerol backbone. TAG is made in the ER and accumulates in droplets that bud off from the ER membrane. TAG may be liberated from lipid droplets by lipolysis and fatty acids are broken down by β -oxidation in the peroxisome (Li-Beisson *et al.,* 2010). The fate of peroxisomes in nematode induced syncytia is not characterized. Nonetheless, modulation of plant peroxisomes in giant cells by sedentary root-knot nematodes has been described (Dinh *et al.,* 2014). However, it is not known if feeding cyst nematodes encounter lipids in host-derived nutrients. Furthermore, none of those nutrients has been defined yet.

Therefore, probing these two routes (*de novo* and dietary) for their involvement in fatty acid metabolism in plant parasitic nematodes is essential to get a better understanding of their specific biology.

1.3.1   Probing fatty acid *de novo* synthesis in nematodes

Probing protein function is generally done by deactivating it and observing the resulting phenotype. Disrupting gene activity can be accomplished using genetic tools (e.g. genomic knock-out, RNAi) or using small molecule inhibiotors. Small molecule inhibtors are small organic compounds that specifically interfere with a proteins function. The advantage of small molecule inhibitors is the spatio-temporal control of its action without genetic transformation of the target organism. The disadvantage of small molecule inhibitors lies in their target specificity. Therefore, before a small molecule inhibitor can be used to study gene function it is important to show its specific mode of action.

In an attempt to study fatty acid *de novo* synthesis in cyst nematodes we were interested to find a small molecule that inhibits ACC activity. Spirotetramat (SPT) belongs to the class of cyclic keto enols and is used as a systemic insecticide for crop protection. Within the plant, SPT is hydrolyzed to the corresponding SPT-enol. SPT-enol is thought to be the active form of SPT. SPT-enol is transported via the plant assimilate transport system, the phloem, and therefore distributed throughout the plant. In insects and mites SPT-enol suppresses fatty acid biosynthesis by inhibiting the action of ACC (Nauen *et al.,* 2008; Brück *et al.,* 2009).Target identification in insects and mites is further supported by studies on the structurally related keto-enol Spiromesifen (Karatolos *et al.,* 2012) suggesting a common mode of action.

Therefore, we were interested to determine the mode of action of spirotetramat on nematodes to develop a tool to probe the importance of fatty acid *de novo* biosynthesis.

1.3.2   Probing nutritional supply of fatty acid in cyst nematodes

To investigate a potential role of lipids in the nutrient source of cyst nematodes we studied feeding sites of *Heterodera schachtii* induced in *A. thaliana*. The sole purpose of the feeding site is to provide nutrients for the nematode. Structure and function of the feeding site are preserved in this system and in the field. The feeding site facilitates nematode maturation and the development of the next generation of infective J2´s. Thus, the system is as natural as it needs to be, to study its principle function, which is the nourishment of the nematode (Sijmons *et al.,* 1991; Golinowski *et al.,* 1996).

As outlined above, in plants, specific lipid biosynthetic reactions occur in distinct sub cellular compartments. Furthermore, the different organelles and subcellular structures contain specific lipids. This allows quantitative correlation between specific lipids and ultra-structural features. As described above, the ultrastructure of feeding sites induced by *H. schachtii* in *A. thaliana* is well established. Therefore, we aim to characterize glycerolipid metabolism of nematode induced feeding sites.

We quantitatively characterize the glycerolipidome of feeding sites and compare it to uninfected root tissue. Then we identify differential expressed transcripts that correspond to the involved syncytial glycerolipid pathways. Finally we select candidate genes and study their role in *H. schachtii-A.thaliana* interactions.

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2 Spirotetramat inhibits acetyl-CoA carboxylase activity in nematodes

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2.1 Significant statement

Spirotetramat (SPT) is a systemic insecticide targeting acetyl-CoA carboxylase (ACC). Nematode control is difficult and chemical control often affects non-target organisms adversely. In this paper we show that spirotetramat acts as a developmental inhibitor of free-living and parasitic nematodes by targeting nematode ACC. Importantly, spirotetramat action depends on nutrient availability which is known to regulate the initiation of development. This is the first report of ACC as a pharmacological target for nematodes and has broad implications for applied and basic science.

2.2 Abstract

Spirotetramat (SPT) is used as systemic pesticide targeting acetyl-CoA carboxylase (ACC) of insects and mites. In this article we show that SPT acts as a developmental inhibitor for the free-living nematode *C. elegans* and the plant parasitic nematode *H. schachtii*. SPT treatment leads to growth arrest and disruption of the life cycle. We also show that SPT does not affect non-developing stages suggesting that developmental initiation is required for SPT to act. Furthermore, SPT specifically affects lipid stores and fatty acid composition and inhibits nematode ACC activity. Last we show that *H. schachtii* also expresses an ACC and its knockdown by RNAi partly mimics the effects of SPT, supporting the conclusion that SPT acts on the common nematode target ACC.

This is the first report of ACC as a pharmacological target for nematodes and has broad implications for applied and basic science.

2.3 Introduction

Spirotetramat (SPT) belongs to the class of cyclic keto enols and is used as a systemic insecticide for crop protection. Within the plant, SPT is hydrolyzed to the corresponding SPT-enol. SPT-enol is thought to be the active form of SPT. SPT-enol is transported via the plant assimilate transport system, the phloem, and therefore distributed throughout the plant. In insects and mites SPT-enol suppresses fatty acid biosynthesis by inhibiting the action of acetyl-CoA carboxylase (ACC) (Nauen *et al.,* 2008; Brück *et al.,* 2009).Target identification in insects and mites is further supported by studies

on the structurally related keto -enol Spiromesifen (Karatolos *et al.,* 2012) suggesting a common mode of action. Recent studies show, that SPT-enol binds exclusively to the ACC-ATP (Enzyme-Substrate) complex (Lümmen *et al.,* 2014) indicating, that ATP bound to ACC is required for SPT-enol to act.

ACC catalyzes the initial and rate-limiting step in fatty acid *de novo* synthesis providing malonyl-CoA from the carboxylation of acetyl-CoA. Due to its importance in central metabolism, complex regulation of ACC is not surprising. ACC activity is highly responsive to the overall energy status of a cell which is in turn regulated by nutritional signals. The signal transduction depends on AMP-activated protein kinase (AMPK) that in times of fasting (high AMP, low ATP, low insulin) keeps ACC phosphorylated and thus inactive. In times of plenty and a high energy charge (low AMP, high ATP, high insulin) AMPK becomes inactive and the proportion of dephosphorylated ACC increases and more malonyl-CoA is synthesized (Berg *et al.,* 2002). Malonyl-CoA is used as a substrate by *de novo* fatty acid synthase (FAS) supplying fatty acids. These fatty acids may be further elongated by the ER-localized acyl-CoA elongation enzymes that also require malonyl-CoA. Fatty acids may be desaturated and eventually assembled into complex lipids. Lipids are vital constituents of life being involved in membrane biogenesis (e.g. phospholipds), energy storage (triacylglycerol) and modulation of growth and development (e.g. glycosylceramides) (Watts *et al.,* 2002; Barber *et al.,* 2005; Zhang *et al.,* 2011; Zhu *et al.,* 2013).

It has been shown previously, that ACC activity is crucial for embryonic and post-embryonic development of various organisms highlighting the importance of fatty acid *de novo* synthesis for development. In line with these findings, symptoms induced by SPT-enol in insects and mites include incomplete ecdysis and molting (=arrest in development) and reduced fecundity (=disturbed development). Interestingly, feeding experiments suggested that oral uptake is required (Nauen *et al.,* 2008; Brück *et al.,* 2009). Because SPT-enol was described to interfere with the development of insects and side-effect were described for nematodes, we were interested to test the effect of SPT-enol on developing as well as non-developing stages of nematodes.

Nematodes are ubiquitous organisms with diverse lifestyles including free-living microbe feeders, predators, and obligate parasites of plants and animals. Although they have fundamentally different life-history strategies some features are shared. All nematodes develop through consecutive molts until becoming fertile adults, which in turn can produce the next generation.

Control of plant-parasitic nematodes is difficult and often requires complex agricultural management. Conventional nematicidal chemical compounds often affect non-target organisms adversely.

To study the effect of spirotetramat on nematodes we used *Caenorhabditis elegans* and the plant parasitic nematode *Heterodera schachtii* on *Arabidopsis thaliana* as experimental systems. *C. elegans*

is a ubiquitous free-living soil inhabitant and is actively taking up xenobiotics while feeding bacteria from the surrounding medium. Additionally, the vast amount of available resources and protocols facilitates concept development and testing.

H. schachtii is a economically important biotrophic parasite of sugar beet, other Chenopodiaceae and Brassicaceae including the model plant *A. thaliana*. Following root invasion *H. schachtii* induces a feeding site containing a syncytium. *H. schachtii* actively feeds from the syncytium that supplies the nematode with all nutrients required for its development. While the nematode develops and grows, also the feeding site becomes larger. (Sijmons *et al.,* 1991; Golinowski *et al.,* 1996). After hatching from an egg, L1 of *C. elegans* and J2 of *H. schachtii*, will not develop unless they are supplied with a food source.In *C. elegans*, nutrients derived from feeding on *E. coli* activate a signaling cascade that promotes development (Ashraf, 2007). Similar, in *H. schachtii* the development of J2s is initiated after feeding from the previously induced syncytium. This indicates that in both nematodes, developmental initiation is linked to nutrient derived signalling. This study aims to characterize the mode of action of SPT on free-living and parasitic nematodes under developing and non-developing conditions.

2.4   Results

SPT-enol is not acutely toxic for nematodes

To test for an acute toxic effect of SPT-enol we examined its effect on non-developing stages of nematodes, *C. elegans* starved L1 and *H. schachtii* J2. When incubated with SPT-enol, both nematodes were not differently inactivated during the following time course analysis as compared to the solvent control (Figure 2-1 Figure 2-2). When the SPT-enol was removed by washing and nematodes were allowed to feed and grow, larval fate was unaffected (Figure 2-3, Figure 2-4).
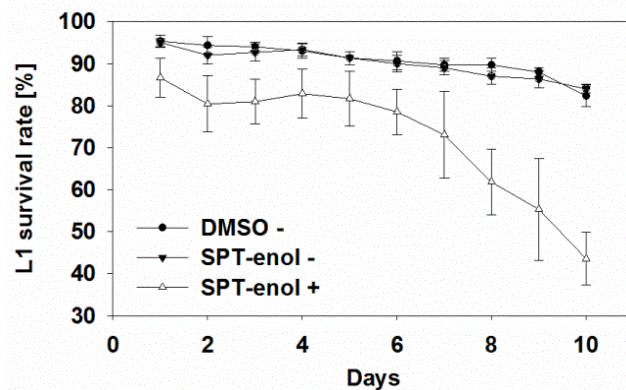
Figure 2-1   Long term survival of non-developing nematodes in liquid culture.

Synchronized L1 larvae were incubated in liquid S-medium with and without E. coli OP50 (+ and -) and with 200 µM SPT-enol or DMSO. Survival rates were determined daily. DMSO plus food was excluded from the analysis since larvae develop into adults under these conditions. In the absence of food L1 survival is unaffected by SPT-enol. When food is present, SPT-enol arrested L1 larvae are inactivated over time. For each time point T test SPT-enol+ vs control and SPT-enol+ vs SPT-enol– P = <0.001, n=3. Average +/-Standard deviation n=3.
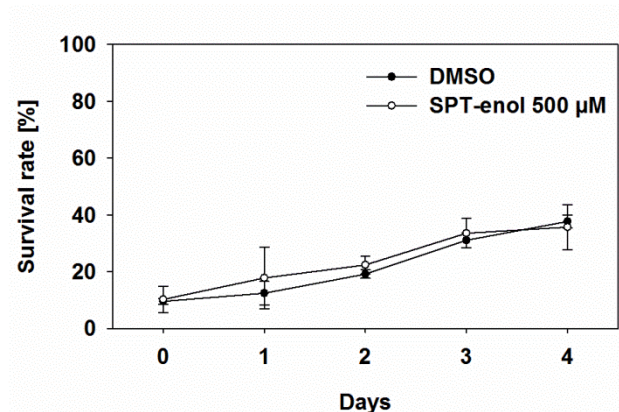
Figure 2-2   Time course analysis of *H. schachtii* J2 survival after incubation with SPT-enol

J2s larvae were incubated in M9 buffer containing 500 µM SPT-enol or DMSO. The number of moving nematodes was counted daily. SPT-enol does not significantly increase the number of inactive J2s over time. Average +/- standard deviation not significant n=5.
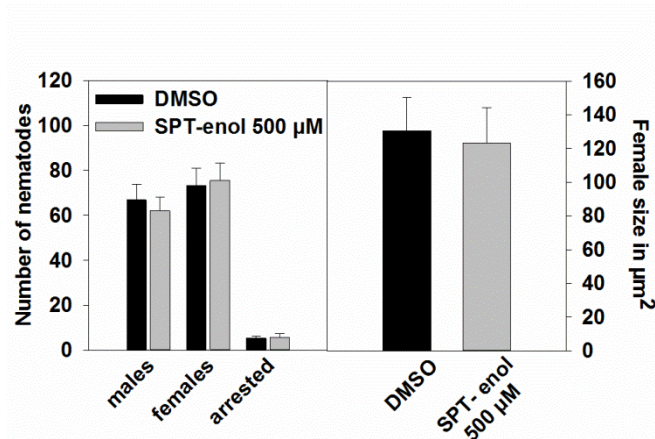
Figure 2-3   Effect of pre-incubation of *H. schachtii* J2 with SPT-enol on number and size.

J2s larvae were incubated in M9 buffer containing 500 µM SPT-enol or DMSO for 48h. J2s were washed and transferred to plates containing 10 day old *A. thaliana*. The numbers of males, females and arrested nematodes were counted at 10 DPI. Female sizes were determined at 14 DPI. No significant differences were found. Average +/- STD Not significant, n= 10, n=30.
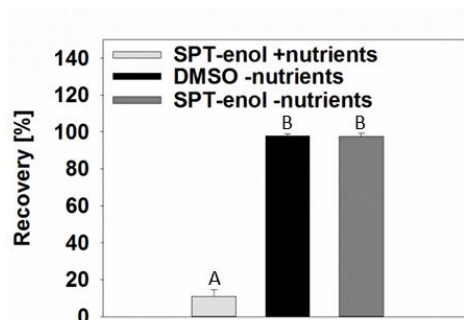
Figure 2-4   Recovery of non-developing and arrested worms.

Synchronized L1 larvae were incubated in liquid S-medium with and without E. coli OP50 (+ and -) and with 200 µM SPT-enol or DMSO for 48 hours. Afterwards the nematodes were washed and transferred to seeded ngm plates. Recovery was determined after 3 days. Nematodes that developed into adults were counted as successfully recovered. Essentially all L1s that were incubated without a food source were able to develop into adults. However only 10% of SPT-enol arrested L1s were able to recover. Incubation with DMSO plus food was excluded from the analysis since larvae developed into adults under these conditions. Average +/- STD Anova P = <0.001; Tukey Test, n=3.

To further provoke SPT-enol uptake by *H. schachtii* J2 we stimulated them with octopamine, which has been shown to enhance xenobiotic transfer from the surrounding medium into the nematode (Urwin *et al.,* 2002). Similar to our previous observation, this did not have a significant effect on nematode development (Figure 2-5). The findings that SPT-enol neither inactivated nematodes over time nor altered their developmental fate after incubation suggest that SPT-enol is not acutely toxic for larval stages that have not yet initiated development.
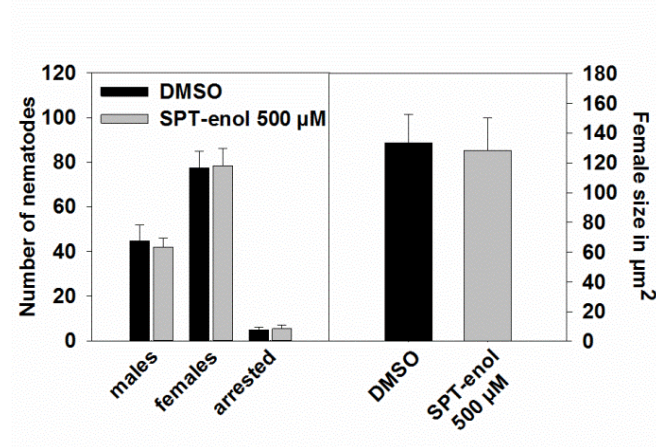
Figure 2-5 **Effect of stimulated pre-incubation of *H. schachtii* J2 with SPT-enol on number and size.**

J2 larvaes were incubated in soaking buffer (containing octopamine) containing 500 μM SPT-enol or DMSO for 24h. J2s were washed and transferred to plates containing 10 day old *A. thaliana*. The numbers of males, females and arrested nematodes were counted at 10 DPI. Female sizes were determined at 14 DPI. No significant differences were found. Average +/- STD Not significant, n= 10, n=30.

SPT and SPT-enol inhibit nematode development

Next, we tested if SPT and SPT-enol would affect the two nematode species during their development, which starts as soon as nutrients are available. SPT-enol inhibited *C. elegans* development in a concentration dependent manner, causing early arrest at stage L1. At a concentration of 200 μM or above L1 arrest occurs uniformly (Figure 2-6, Video1).
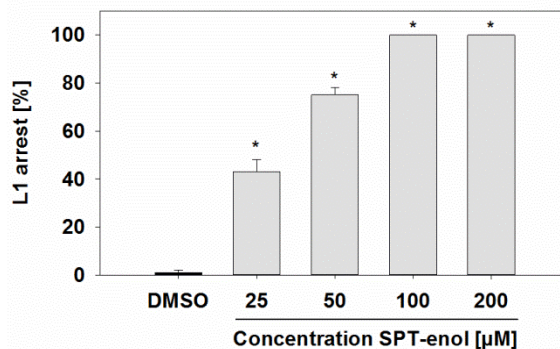
Figure 2-6 **Concentration-dependent effect of SPT-enol on *C. elegans*.**

Synchronized L1 nematodes were incubated in liquid culture with SPT-enol at different concentrations. Nematodes that showed developmental arrest at the L1 stage were counted after 3 days. With an increasing concentration of SPT-enol the number of L1 arrested larvae increases. At concentrations of 100-200 μM, L1 arrest occurs uniformly. Data show averages and standard deviation, n=3, T test vs control P = <0.001.

In a time course experiment, those early arrested larvae became inactive over time (Figure 2-1). To asses if the arrest was reversible, arrested L1 were washed after 2 days and transferred to nematode growth medium (NGM) plates without SPT-enol. Approximately 10% of those arrested L1 were able to recover and became adults whereas essentially all starved L1 (nematodes incubated without food source) were able to develop irrespective of the presence of SPT-enol (Figure 2-4).

To study the effect of SPT on the developing of *H. schachtii* a host plant, in this case *A. thaliana* is required. Therefore, we first analyzed if SPT is hydrolyzed to SPT-enol and transported basipetally from the leaf to the root. In addition, we addressed the question if this treatment would affect the growth of *A. thaliana*, to exclude phytotoxcity as a possible cause of disturbed nematode development.

After SPT had been applied onto leaves of *A. thaliana,* SPT-enol was detected in the roots and the growth medium of the plants (Figure 2-7). SPT at a concentration of 200 mM did not affect shoot and root weight of the treated plants (Figure 2-8). Taken together the results suggested that SPT is hydrolyzed to SPT-enol *in planta* and translocated basipetally (leaf-to-base) without causing an apparent harm to the plant.
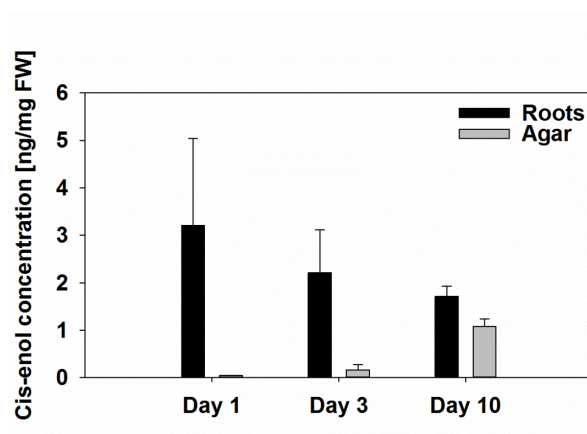
Figure 2-7   SPT-enol quantification in roots and medium after foliar SPT application.

3 µL of SPT-enol (200 mM) was foliar applied onto the largest green leaf of 10 days old *A. thaliana*. SPT-enol was quantified after 1, 3 and 10 days. SPT-enol is detected in the roots and the growth medium after foliar application. Whereas the SPT-enol concentration in roots tends to decrease over time, SPT-enol concentration in the medium increases. Average +/- standard deviation n=4.
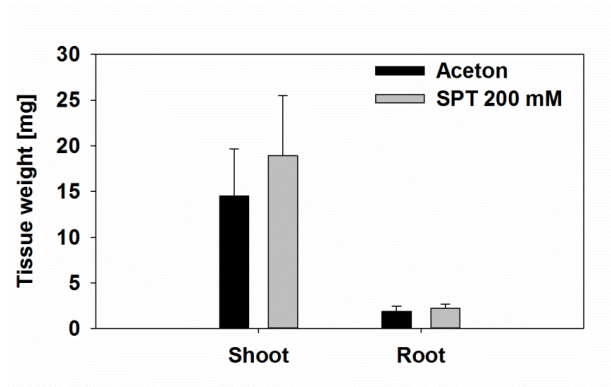
Figure 2-8 **Effect of foliar applied SPT on *A. thaliana* growth.**

3 µL of SPT at the indicated concentration was foliar applied onto the largest green leaf of 10 days old *A. thaliana*. 10 days later, root and shoot weights were recorded. No significant differences were found for root and shoot weight as compared to the control. Average +/- STD, n=5.

Similar to the effect of SPT-enol on *C. elegans*, development of the majority of *H. schachtii* juveniles infecting and feeding from SPT-treated (or SPT-enol) plants was arrested in early stages in a concentration-dependent manner. In addition, females that did develop on SPT-treated plants were significantly smaller than control nematodes (Figure 2-9). Interestingly, the feeding sites of these suppressed females were not significantly smaller than those of control nematodes, indicating appropriate plant responses to the infection (Figure 2-10). In contrast, feeding sites of nematodes arrested at an earlier stage were significantly smaller (Figure 2-10, Figure 2-11). Since the feeding sites were induced and development was initiated, SPT-enol seems to act only after feeding site induction and feeding suggesting, that SPT-enol might be taken up orally by the nematode.
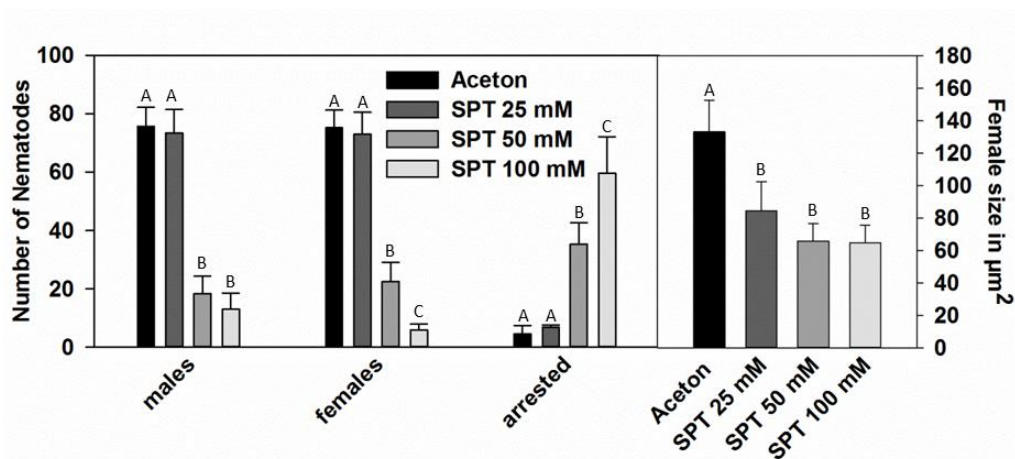
Figure 2-9 **Concentration-dependent effect of SPT on the development of *H. schachtii*.**

3 µL of SPT at the indicated concentrations was foliar applied onto the largest green leaf of 10 days old *A. thaliana*. The numbers of males, females and arrested nematodes were counted at 10 DPI. Female and sizes were determined at 14 DPI. At a concentration of 25 mM SPT the numbers of males, females and arrested nematodes are not significantly different as compared to the control. With an increasing concentration of SPT the number of males and females decreases while the number of arrested nematodes increases. At all tested SPT concentrations females are significantly smaller as compared to the control. Average +/- STD Anova Male P = 0.002, Tukey Test n=10, Average +/- STD Anova Female P = <0.001; Tukey Test n=10, Average +/- STD Anova Arrested P = 0.001, Tukey Test n=10, Average +/- STD Anova Sizes P= <0.001, Tukey Test n=30.
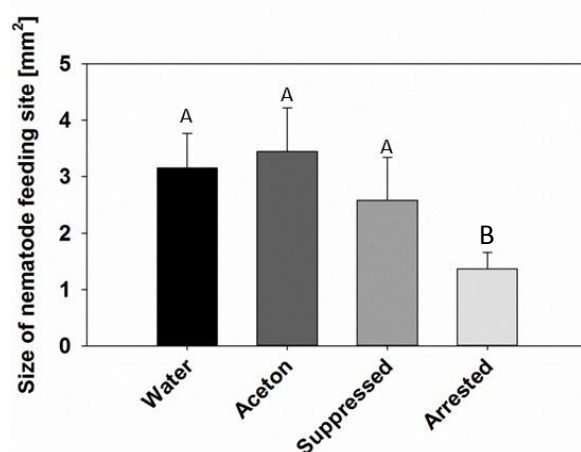
Figure 2-10 **Concentration-dependent effect of SPT on development of nematode feeding site.**

Feeding site sizes were determined 14DPI. The sizes of smaller/suppressed females are not significantly different as compared to the controls. Feeding sites of arrested nematodes are significantly smaller. Average +/- STD Anova P = <0.001; Tukey Test.
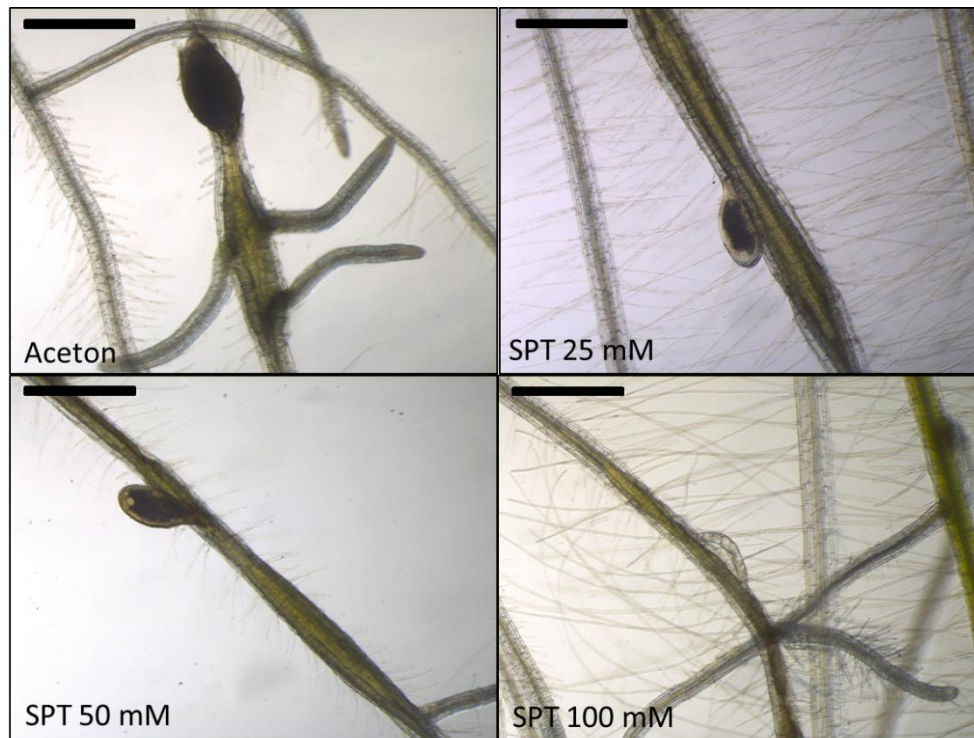
Figure 2-11   Concentration-dependent effect of SPT on the development of *H. schachtii* and associated feeding site.

3 µL of SPT at the indicated concentration was foliar applied onto the biggest green leaf of 10 days old *A. thaliana*. At a concentration of 25 mM SPT the numbers of males, females and arrested nematodes are not significantly different as compared to the control. With an increasing concentration of SPT the numbers of males and females decrease while the number of arrested nematodes increases. At all tested SPT concentrations females are significantly smaller as compared to the control. Bar represents 500 µm.

The data suggest that SPT-enol acts as a developmental inhibitor with a partly reversible effect, but requires feeding and developmental initiation. Additionally, the juvenile arrest seen in *H. schachtii* seems to be a direct effect on the nematodes rather than acting indirectly through phytotoxicity.

SPT-enol interferes with lipid metabolism by inhibiting Acetyl-CoA Carboxylase.

SPT-enol was shown to interfere with the lipid metabolism of insects and mites (Nauen *et al.,* 2008; Brück *et al.,* 2009). Given the importance of lipids for development we investigated the effect of SPT-enol on *C. elegans* storage lipids. When treated with SPT-enol, early arrested L1 larvae were found to be devoid of intestinal lipid droplets as shown by nile red staining. In contrast, lipid droplets of starved L1 incubated for the same time showed lipid droplet staining irrespective if SPT-enol was present or not (Figure 2-12).
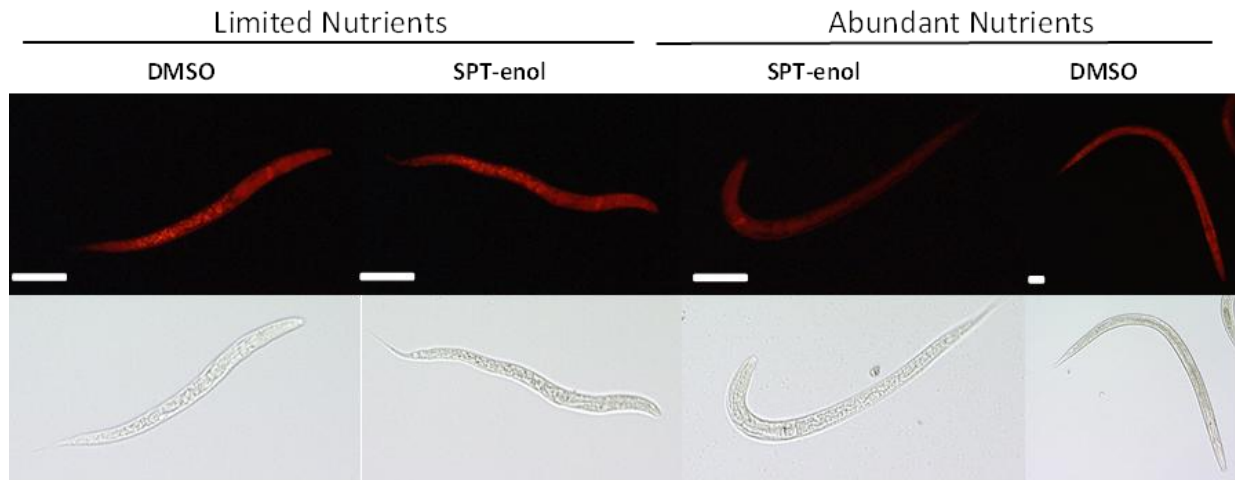
Figure 2-12   Nile red staining of *C. elegans*.

Synchronized L1 larvae were incubated in liquid S-medium with 200 µM SPT-enol or DMSO with and without nutrient source. After 48 hours worms were fixed and stained with nile red. The number of lipid depleted nematodes was counted. Only nematodes that were incubated with SPT-enol in the presence of E. coli OP50 show intestinal droplet depletion (92.3% +/-5.5%). Worms supplied with food and DMSO start to develop. Bar represents 50 µm.

Absence of intestinal lipid droplets maybe associated with an altered fatty acid metabolism. Therefore we analyzed total fatty acids in SPT-enol treated nematodes. We found a reduction of C18 fatty acids while C16 fatty acids are relatively increased. To get a more detailed view on storage lipid changes, we determined the triacylglycerol content and composition using direct infusion Q-TOF mass spectrometry. We found that SPT-enol treated nematodes had a significantly lower total TAG content as compared to the solvent control. The reduction of C18 observed in total fatty acid composition is reflected in changes in TAG composition. When analyzing the most abundant TAG molecular species (Schwudtke *et al.,* 2006) it became apparent that 18:1-containing TAG molecules were reduced while TAGs containing 16:1- and 17:0cyclo-fatty acids were unaffected (Figure 2-13). Together this indicates that SPT-enol affects the nematode's storage lipids, by specifically reducing C18-containing TAGs, linking developmental inhibition to fatty acid metabolism.
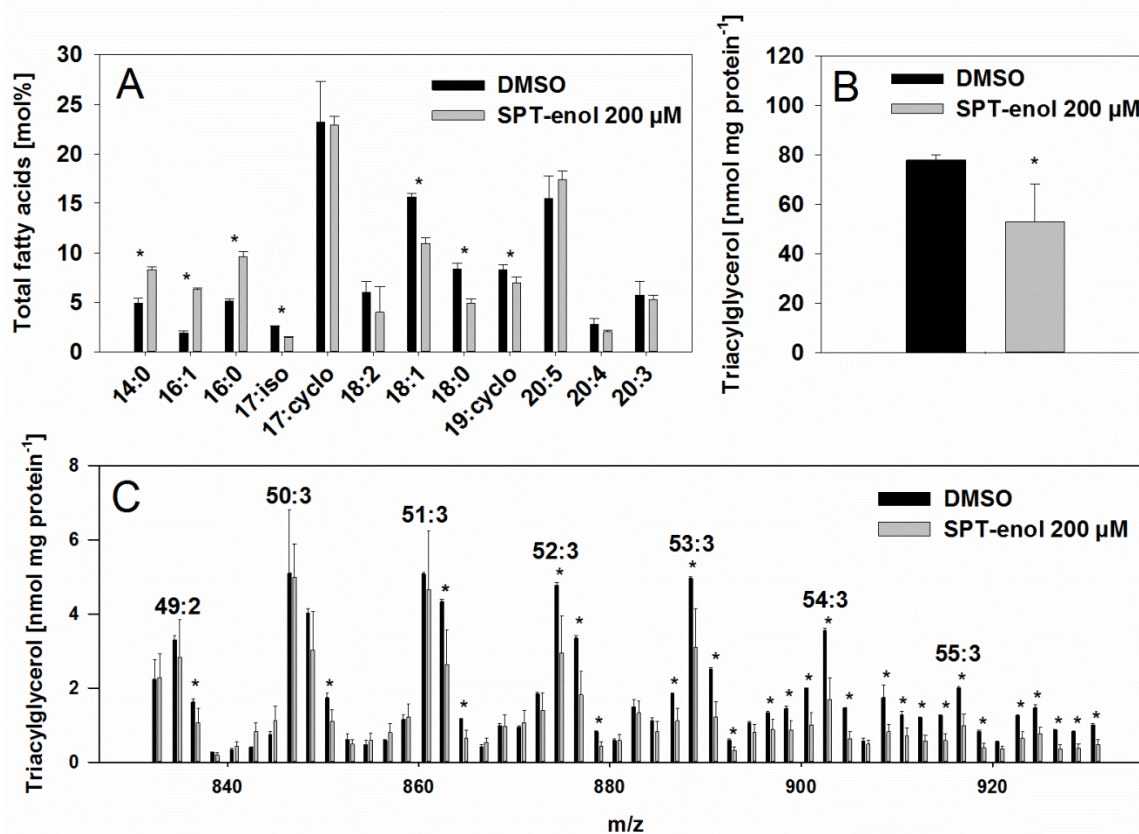
Figure 2-13 **TAG profiling of SPT-enol treated *C. elegans*.**

Mixed stage worms were incubated in liquid culture with 200 µM SPT-enol or DMSO with a nutrient source. After 24 hours worms were washed, lipids extracted and quantified by GC-MS or direct infusion Q-TOF MS. The total TAG content is significantly reduced in SPT-enol treated worms. The main molecular species of TAG were identified using Q-TOF MS/MS and are shown in Supplemental Figure X. Average +/-STD T test P= <0.05 n=3.

In the nematodes, 18:1 is produced via elongation and desaturation from 16:0, while 16:0 can be either taken up by the diet or produced by *de novo* fatty acid synthesis. Both fatty acid elongation and *de novo* synthesis require malonyl-CoA. The sole route for malonyl-CoA production is carboxylation of acetyl-CoA by ACC. Therefore, we tested if SPT-enol is able to inhibit ACC activity *in vitro*. The enzyme assays using radiolabeled sodium bicarbonate showed that *C. elegans* ACC activity is inhibited by SPT-enol with an IC50 of 50 µM (Figure 2-14). This supports the conclusion that the altered fatty acid metabolism in *C. elegans* is linked to ACC inhibition.

Our data show that developmental inhibition observed in both nematode species after SPT-enol treatment is the result of dysfunctional lipid metabolism due to specific inhibition of ACC by SPT-enol.
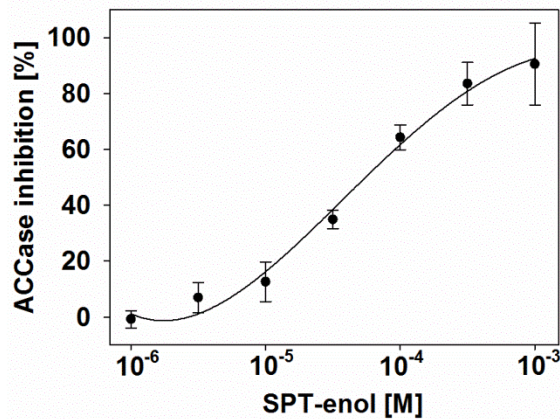
Figure 2-14   ACC inhibition assay.

C. elegans protein was extracted and used for ACC activity assays. The incorporation of 14C from sodium bicarbonate was used as a measure for ACCase activity. Average +/-STD n=5.

RNAi against nematode acetyl-CoA carboxylase mimics the effect of spirotetramat

To elucidate whether the two nematode species are subject to a common mode of action of SPT-enol on ACC, we searched for the ACC sequence in *H. schachtii*. Using a *H. schachtii* transcriptome data base and the *C. elegans* ACC (POD-2) sequence as query, we retrieved a single contig with a translated ORF of app. 2000 amino acids. The predicted protein contains all functional domains required for ACC activity and is similar to other nematode ACCs (Figure 2-15). Hence, this implies that we found the *H. schachtii* ortholog of the *C. elegans* ACC (POD-2) and therefore named it *Hs*ACC (HsPOD-2).
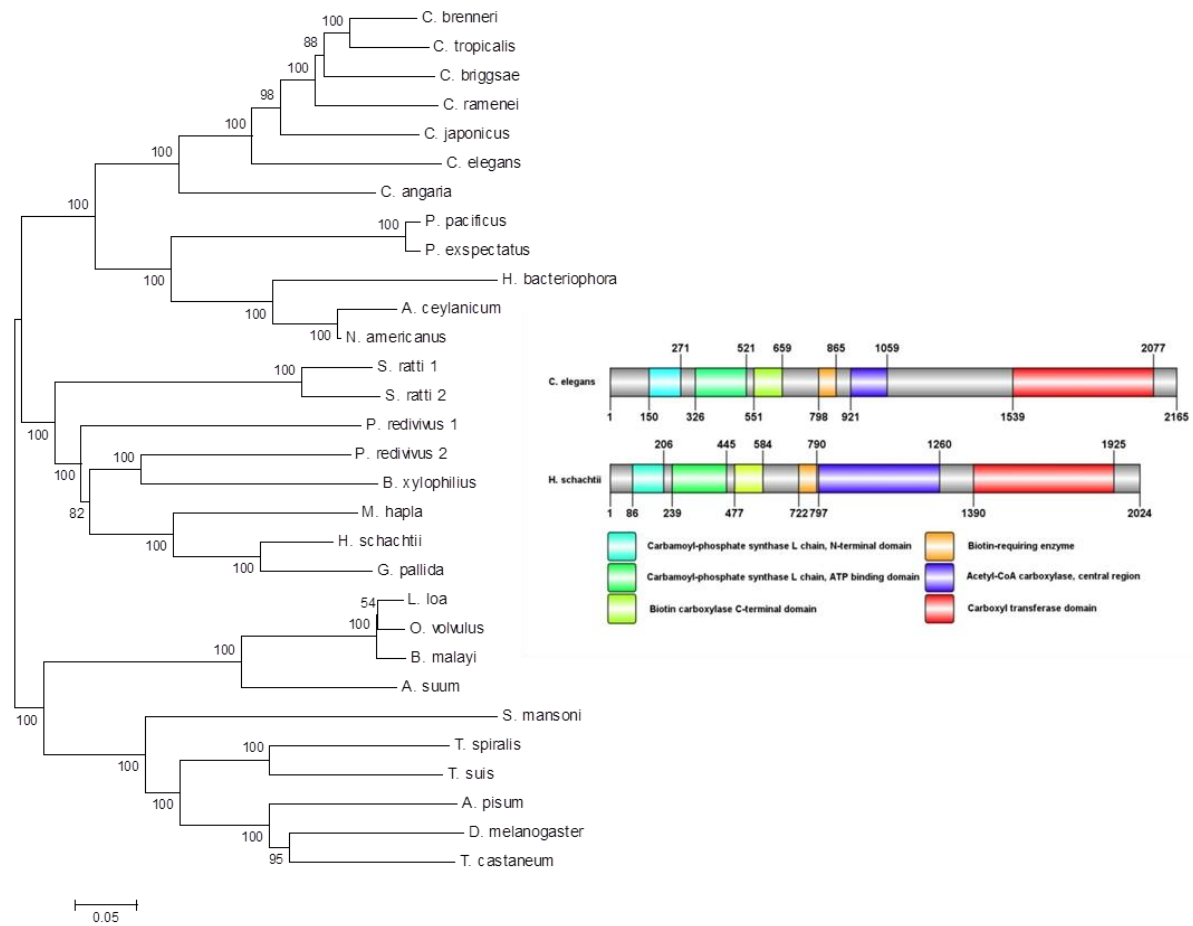
Figure 2-15   Sequence analysis of *H. schachtii* ACC.

The mRNA sequence of the *C. elegans* POD-2 isoform C was used as query to find the corresponding *H. schachtii* ortholog. We found a mRNA contiq that contains an ORF coding for a putative protein with 2024 amino acids. This protein contains all functional domains required for ACC activity and is similar to other ACC sequences found in public databases. Protein allignemt shows that Hs.ACC clusters together with other nematode ACCs and is most similar to a putative ACC of the cyst nematode *Globodera pallida*. Domain structures were illustrated using DOG 2.0 (Ren et al. 2009). The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 4.80671519 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The analysis involved 30 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 3405 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.,* 2011).

To test if *Hs*ACC is relevant for the development of *H. schachtii* we investigated the effect of its knock down upon RNAi. Soaking of J2 in a solution of dsRNA targeted against HsAcc reduced *Hs*Acc transcript levels by 90% (Figure 2-16). When allowed to develop on *A. thaliana* the numbers of males and females were not significantly different, but the developed females were significantly smaller as compared to the control (Figure 2-17, Figure 2-18). To check if transcript reduction was persistent we quantified HsACC transcript levels in the developed females 10 days after dsRNA treatment.

However, at this stage, HsACC transcript levels were no longer significantly different between control and treated females (Figure 2-16).

This suggests that the HsACC transcript is involved in *H. schachtii* development. Moreover, its transient knock-down phenocopies the suppressive effect of SPT-enol observed at low concentrations. Taken together, we conclude that SPT-enol acts on a common nematode target, which is ACC.
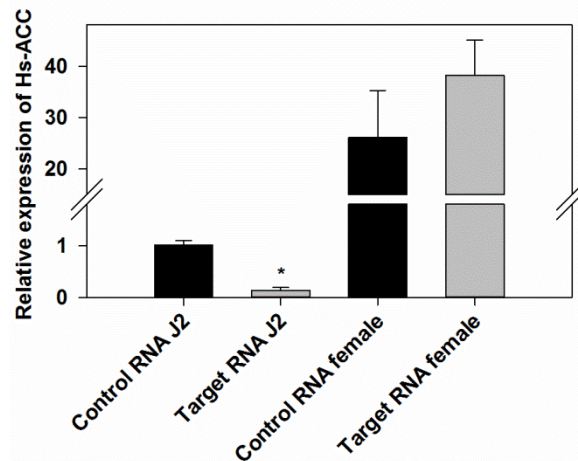
Figure 2-16 **RNAi against *H. schachtii* ACC (HsACC) – transcript changes.**

J2s larvae were incubated for 24h in soaking buffer containing control or target dsRNA (1µg/µL). ACC transcript levels were determined either from aliquots of soaked J2s or from developed females picked from the plate after 10 days of development. Average +/- STD T test vs Control RNA P = <0.001, n=3.
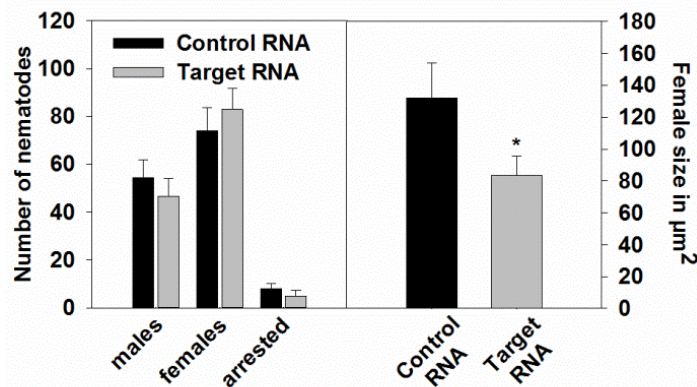
Figure 2-17 **RNAi against *H. schachtii* ACC (HsACC) affects numbers and sizes of nematodes.**

J2s larvae were incubated for 24h in soaking buffer containing control or target dsRNA (1 µg/µL). Soaked J2´s were transferred to 10 day old *A. thaliana*. ´The numbers of males, females and arrested nematodes were counted at 10 DPI. Female sizes were determined at 14 DPI. The numbers of nematodes are not significantly different between control and target RNA treated nematodes. Sizes of female nematodes treated with target RNA are significantly smaller as compared to control RNA treated nematodes. Average +/-STD T Test P = <0.001 n=30.
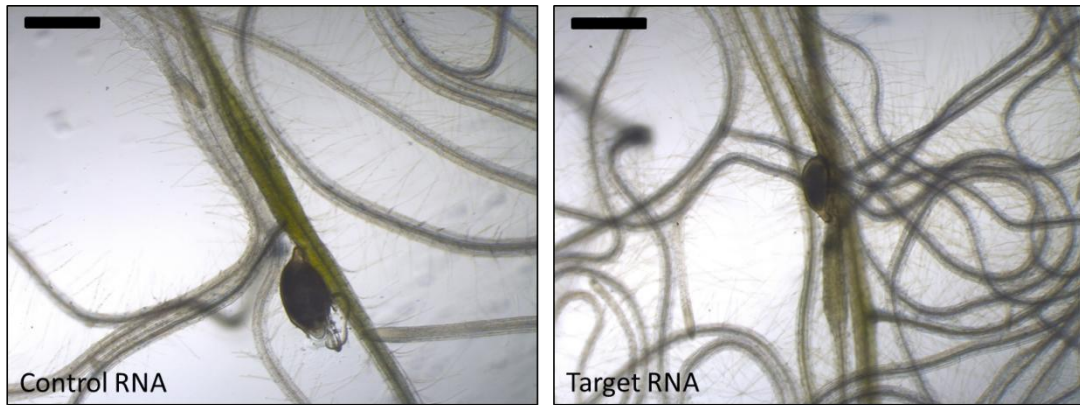
Figure 2-18   **RNAi against *H. schachtii* ACC (HsACC) affects sizes of female nematodes.**

J2s larvae were incubated for 24 h in soaking buffer containing control or target dsRNA (1 μg/μL). Soaked J2s were transferred to 10 day old *A. thaliana*. The number of males, females and arrested nematodes were counted at 10 DPI. Female sizes were determined at 14 DPI. The numbers of nematodes are not significantly different between control and target dsRNA treated nematodes. Female sizes treated with target RNA are significantly smaller as compared to control RNA treated nematodes. Bar represents 500 μm.

2.5 Discussion

In this article we show that SPT-enol interferes with the development of nematodes by inhibiting ACC. SPT-enol inhibits the development of two distantly related nematode species, causes lipid droplet depletion and has a specific effect on fatty acids that require malonyl-CoA for elongation. Subsequent radiolabelling experiments confirmed ACC as the target. Further, it was found that both nematode species are transcribing an ACC and its knockdown by RNAi can mimic the effect of SPT-enol at least in *C. elegans* and *H. schachtii*. Together, this strongly supports ACC as the target of SPT-enol in nematodes.

SPT-enol interferes with fatty acid biosynthesis causing an arrest of nematode development

ACC catalyzes the initial step in fatty acid biosynthesis converting acetyl-CoA to malonyl-CoA. Malonyl-CoA serves multiple purposes. It supplies C2 units for fatty acid *de novo* synthesis by fatty acid synthase (FAS), is required for fatty acid elongation and regulates mitochondrial β-oxidation (Watts, 2009).

Fatty acids are constituents of structural components of membranes (e.g. phospholipids), energy stores (triacylglycerol) and are important modulators of development (e.g. glycosylceramides) (Watts 2008; Zhu *et al.,* 2014).

In agreement with these important functions of fatty acids, ACC inactivation was found to be embryo lethal for various organisms including nematodes, flies, mice and yeast (Hasslacher *et al.,* 1993; Takawa *et al.,* 2001; Abu-Elheiga *et al.,* 2005; Parvy *et al.,* 2012). Given its importance for core metabolism, ACC requires stringent regulation. ACC regulation is achieved through reversible phosphorylation, whereas the proportion of phosphorylated versus dephosphorylated ACC determines its activity. Phosphorylation by AMP-activated protein kinase deactivates whereas dephosphorylation by protein phosphatase A2 activates ACC. Since AMPK is activated by AMP and repressed by ATP and insulin, AMPK relays the signal for energy status and downregulates ACC activity when the energy charge is low. When energy charge is high, ATP and insulin are abundant, AMPK becomes repressed and the proportion of dephosphorylated ACC increases (Witters *et al.,* 1992; Berg *et al.,* 2002). This suggests that ACC activation depends on nutritional cues that trigger development which is mediated by the synthesis and action of lipids.

In line with these findings polyunsaturated fatty acid (PUFA) synthesis is crucial for development beyond the L1 stage but can be complemented by dietary fatty acids (Brock *et al.,* 2007). However, until now we were not able to rescue the SPT-enol induced developmental arrest by dietary supplementation. Similar, ACC defective yeast could also not be rescued by exogenous supply of lipids (Hasslacher *et al.,* 1993). The inability to rescue the phenotype pinpointed to a defect in

supplying malonyl-CoA for very long chain fatty acid (VLCFA) production which is in turn required for the synthesis of sphingolipids (Tehlivets *et al.,* 2007). Indeed, for *C. elegans* glycosylceramide synthesis is essential for the establishment of cell-polarity and is also required for the activation of mTORC signaling, a key activator of development (Zhang *et al.,* 2011; Zhu *et al.,* 2013). The inability to synthesize glycosylceramides has been linked to L1 arrest in *C. elegans (*Marza *et al., 2009)*.

SPT-enol promotes lipolysis

We found that SPT-enol-treated *C. elegans* lacked intestinal lipid droplets only when nutrients were present. Besides its important role in straight chain fatty acid synthesis, malonyl-CoA inhibits the action of carnitine palmitoyl transferase (CPT) thereby preventing mitochondrial β-oxidation (Stryer *et al.,* 2002). The absence of malonyl-CoA due to ACC inhibition may explain lipid droplet depletion under high nutrient conditions. In contrast, lipid droplets in starved L1 appeared similar irrespective of the presence of SPT-enol.

Interestingly, lowering ACC expression levels by RNAi increases hepatic fat oxidation specifically in the fed state (Savage *et al.,* 2006), suggesting, that β-oxidation of fatty acids affects storage lipids only when the energy charge is high and ACC active. Similar, post embryonic knock-down (starting from L1) of *C. elegans* POD-2 encoding ACC led to larval arrest and lower storage lipid (TAG) content (Li *et al.,* 2011).

SPT-enol inhibits nematode acetyl-CoA carboxylase

Additional support for target identification comes from our studies on *H. schachtii*. SPT is apparently not harming *A. thaliana*, SPT-enol is phloem mobile and the nematodes are arrested in development after feeding site induction. This suggests that SPT acts directly on *H. schachtii* after uptake rather than through a phytotoxic effect. Although being phylogenetically separated, both nematode species transcribe an ACC mRNA. The identification of the Hs-ACC transcript sequence supports the presence of a similar enzyme, and its inhibition by RNAi phenocopies the suppressive effect of SPT-enol on female development. This effect indicates a role for Hs-ACC also in *H. schachtii* development and further supports target identification for nematodes.

However, the IC50 of SPT-enol for insect and mite ACCs *in vitro* is in the nM range and thus approximately 1000-fold lower than for nematode ACC (Nauen *et al.,* 2008; Brück *et al.,* 2009). Additionally, *in vivo* inhibition of nematodes requires relative high concentration of SPT-enol as compared to insects and mites. This may suggest that the target affinity of SPT-enol for nematode ACC is low. This may not be surprising since SPT was developed as an insecticide and the

phylogenetic distance and hence ACC sequence differences between nematodes and insects are large. This suggests that SPT may have limited use as foliar applied nematicide. Nevertheless, Movento, a formulation of SPT, has been reported to reduce cyst nematode populations in wheat (Smiley *et al.,* 2011). This may suggest that factors other than the target affinity e.g. uptake and distribution through the plant and into the feeding site, ultimately determine the efficacy.

We found that SPT-enol is not acutely toxic for nematodes but suppresses their development. Non-developing larval stages of *H. schachtii* and *C. elegans* are not inactivated by SPT-enol and their developmental fate is unaffected following SPT-enol removal. This suggests that ACC activation may be required for SPT-enol action. Interestingly, SPT-enol inhibition of ACC was shown to be non-competitive with respect to ATP suggesting that it binds exclusively to the enzyme-substrate (ES) complex (Lümmen *et al.,* 2014).

Since ATP content is presumed to be low under starved conditions SPT-enol may not be able to bind to the complex. The absence of nutrients would signal a low energy charge, may prevent ACC activation and thus may explain why SPT does not affect the nematodes under these conditions. However, decreased uptake and/or increased detoxification may also contribute to the absence of an effect on non-developing stages of nematodes.

Intriguingly, L1 arrest is controlled by AMPK that also regulates ACC activity, suggesting that AMPK dependent L1 arrest is mediated through ACC regulation (Narbonne *et al.,* 2009; Mihaylova *et al.,* 2011; Fukuyama *et al.,* 2012; Lee *et al.,* 2012). Thus, ACC activation seems to be a hallmark of developmental initiation and is likely required for SPT-enol to act. Additionally this suggests that ACC regulation provides the metabolic switch between development (lipid synthesis) and survival (lipid degradation). In conclusion, this is the first report of acetyl-CoA carboxylase as pharmacological target for nematodes. The findings reported here have important implications for applied science in the field of agriculture and medicine. Additionally, the discovery of small molecule inhibitors is an important complement for basic research.

2.6   Materials and Methods

Strains

C. elegans Bristol N-2 and *E. coli* OP50 were a kind gift from Prof. Schierenberg, University of Cologne. *C. elegans* was maintained at 20°C using standard methods and synchronous L1 stages were obtained using alkaline hypochlorite treatment and hatching in M9 (Stirnagle *et al.,* 2006).

Drug preparation

For experiment with nematodes in solution, SPT-enol was dissolved in DMSO. For foliar application, SPT was dissolved in acetone.

Liquid culture

Liquid cultures were set up in 24 well plates using 890 µL S-complete, 10 µL active ingredient in DMSO (1% final concentration) and 100 µL M9 containing 250-300 L1s. For starvation experiments bacteria were omitted from the S-complete medium. Liquid cultures were maintained on a shaker with 180 rpm at 20°C.

Drug recovery assay

Nematodes were collected from liquid cultures, washed twice with M9 and transferred to ngm plates containing *E.coli* OP50. Plates were checked after 3 days and developed nematodes were counted.

Nematode fixation and Nile red staining

C. elegans were fixed and stained as described in Barros et al. (2012).

Lipid quantification

Lipids were extracted from 50 µL mixed-stage worm pellet cultured in S-complete medium with 200 µM SPT-enol or DMSO as control. Triacylglycerol was purified and analyzed as described previously (vom Dorp *et al.,* 2013).

Acetyl-CoA Carboxylase assay/Radiolabel incorporation

Total proteins were extracted from 3 mL of dense *C. elegans* pellet using a protein isolation buffer containing 20 mM HEPES, 150 mM NaCl, 1 mM EDTA, , 1 mM DTT, 10% glycerol, 1% protease inhibitor cocktail „Halt" (Thermo Scientific). Determination of ACC activity and inhibition by SPT-enol was performed according to Lümmen et al. (2014). The assay was performed twice.

Residue Analysis

10 days old *A. thaliana* Col-0 plants cultured on KNOP plates were treated with 3 µL 200 mM SPT as described above. After one, three and 10 days roots and agar were sampled. Roots were thoroughly rinsed with H_2O, blotted dry, weighed, frozen in liquid N_2 and crushed in a Precelllys homogenzer (Peqlab Biotechnology). Residues were extracted with 1 mL acetonitrile/H_2O (9:1). Quantification was performed according to Schöning (2008). Measurements were performed twice.

Toxicology tests for *H. schachtii*

H. schachtii was incubated in a solution containing M9 buffer and up to 500 µM spirotetramat in DMSO. None moving nematodes were counted daily. After 48 h, nematodes in J2stage were washed with M9 buffer and transferred to KNOP plates containing *A. thaliana*.

***H. schachtii* infection assay**

Infection assays were performed on KNOP medium as described in Simmons et al. 1991. 5 plants per petri dish were used. The total numbers of female and male nematodes per plate were counted at 10 DPI and females and feeding site sizes were determined at 14 DPI. Just before nematode inoculation 3 µL spirotetramat in acetone with the indicated concentrations were applied foliar onto the largest green leaf. Acetone was allowed to evaporate.

Acetyl-CoA carboxylase transcript identification

A transcript sequence containing a flull-length ORF of *H. schachtii* Acetyl-CoA carboxylase was identified in a transcriptome database based on sequence similarity to *C. elegans* POD-2 that encodes acetyl-CoA carboxylase. Functional domains were predicted using Pfam (pfam.xfam.org). In order to compare the positions of the predicted functional domains, the HsACC protein sequence was aligned against the *C. elegans* POD-2 sequence (CLC genomics workbench version 6.0).

Sequence analysis

Protein sequences corresponding to full ORF orthologs of *C. elegans* POD-2 were collected from wormbase (wormbase.org), TreeFam (treefam.org) and BLAST (ncbi.nlm.nih.gov/blast). Sequences were aligned using ClustalW. The evolutionary history was inferred using the Neighbor-Joining method (Saitou et al 1987). The optimal tree with the sum of branch length = 4.67784699 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei et al. 2000) and are in the units of the number of amino acid differences per site. The analysis involved 29 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 3309 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011)

dsRNA synthesis

dsRNA was synthesized *in vitro* with T7 overhang primers listed below using MEGAscript® T7 Transcription Kit (Life technologies) according to the manufacturer protocol.

RNAi of J2s

Induction of RNAi was performed in soaking buffer with 1 µg/µL dsRNA as described by Urwin et. al (2002) with modifications from Patel et al. (2010) for transcript quantification by qPCR. dsRNA of a GFP fragment was used as a control.

Nematode RNA extraction and cDNA synthesis

RNA from nematodes was extracted using the the NucleoSpin RNA Plant Kit (Macherey Nagel) according to the supplier's recommendations for low amounts of RNA. Residual DNA was digested using Turbo DNase (Life Technologies) according to the manufacturer's protocol. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

qPCR

qPCR was performed using the Fast SYBR Green Master Mix (Life Technologies) on a StepOnePlus System (Life Technologies). qPCR primers for ACC quantification were validated for efficiency and specificity. Actin was used as the reference gene (Patel *et al.,* 2010). Relative transcript levels were

calculated by the comparative CT method (Schmittgen *et al.,* 2008) using the StepOnePlus System Software (Life technologies).

Primer used in this study

Table 1 Oligonucleotides used in this study.

Oligonucleotides and sequences (5´-3´) are shown.

ACC-GW F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTTTTTGCGAAAGGAATGACA
ACC-GW R	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAATGTGCGGTCCCATAA
ACC-T7 F	TAATACGACTCACTATAGGGAGACTTTTTGCGAAAGGAATGACA
ACC-T7 R	TAATACGACTCACTATAGGGAGAACTAATGTGCGGTCCCATAA
GFP-GW F	GGGGACAAGTTTGTACAAAAAAGCAGGCTGATCCTGTTGACGAGGGTGT
GFP-GW R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGTGGAGAGGGTGAAGGT
GFP-T7 F	TAATACGACTCACTATAGGGAGAGATCCTGTTGACGAGGGTGT
GFP-T7 R	TAATACGACTCACTATAGGGAGATCAGTGGAGAGGGTGAAGGT
Actin-qPCR F	CGTAGCACAACTTCTCCTTG
Actin-qPCR R	CGTGACCTCACTGACTACCT
ACC-qPCR F	CCAACTCCAAATCGCCAT
ACC-qPCR R	TCGCAGGACGGAAGCCTT

Molecular Species Composition of TAGs in *C. elegans*.

Table 2 Molecular composition of TAGs in *C. elegans*.

The molecular species composition of TAGs was determined by scanning Q-TOF MS/MS spectra for neutral loss of fatty acyl-NH_3. The fatty acid composition of the most abundant molecular species is shown below.

Triacylglycerol	$[M+NH_4]^+$	Molecular Species
49:2	834.7551	15:0-17:1-17:1
50:3	846.6612	16:1-17:1-17:1
51:3	860.6768	17:1-17:1-17:1
52:3	874.6925	17:1-17:1-18:1
53:3	888.7081	17:1-18:1-18:1, 17:1-17:1-19:1
54:3	902.7238	18:1-18:1-18:1, 17:1-18:1-19:1
55:3	916.7394	18:1-18:1-19:1, 17:1-19:1-19:1

Statistics

Statistics were performed with SigmaPlot 12.5. Unless stated otherwise, at least three independent experiments were performed.

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3 The lipidome of nematode feeding sites reveals the importance of plant-derived storage lipids for cyst nematode nutrition

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3.1 Abstract

Sedentary cyst nematodes are obligate biotrophic plant parasites with sophisticated feeding behaviors. They exclusively feed from a syncytium that has been induced in the roots of a host plant. However, the nutrients synthesized within the syncytium and subsequently taken up by the nematode are unknown. Fatty acids function in important biological processes such as membrane biogenesis and energy storage. Nematodes can synthesize fatty acids *de novo*, but also take up fatty acids with their diet.

In this study, the glycerolipid metabolism of the nematode induced feeding site using *A. thaliana* and *Heterodera schachtii* was investigated. In line with ultrastructural data, it could be shown that glycerolipids accumulate in the nematode feeding site compared to control roots. However, the individual glycerolipid classes differentially contribute to the overall lipid increase. The increased glycerolipid content is accompanied by a distinct transcriptomic signature which allowed identification of candidate genes relevant for changes in lipid metabolism. Finally mutations in triacylglycerol metabolism in the plant have a pronounced effect on nematode infection and fecundity possibly by an altered nutritional value of the syncytium. The results suggest that cyst nematodes induce glycerolipid accumulation in the syncytium which may increase the nutritional/caloric output of this structure.

3.2   Introduction

Glycerolipids are composed of fatty acids that are esterified to a glycerol backbone. They are essential to cells because they take part in membrane biogenesis, energy storage and signal transduction. Glycerolipids are found in all organisms such as bacteria, plants and animals including nematodes.

Nematodes are ubiquitously present round worms that have diverse lifestyles including free-living as well as animal and plant parasitic species. Lipid metabolism in nematodes in general is poorly understood but fairly well studied in *Caenorhabditis elegans*. *C. elegans* is a free-living nematode that uses *de novo* synthesized fatty acids as well as dietary fatty acids for lipid biosynthesis. However, the majority of fatty acids used for lipid biosynthesis are derived from bacterial lipids taken up with the diet (Perez and Van Gilst, 2008). Albeit contributing only to a lesser extent to the total lipid pool, fatty acid *de novo* synthesis is essential for the development of *C. elegans* (Brock *et al.,* 2007). However, resorption of dietary lipids has energetic advantages as compared to their *de novo* synthesis. Additionally, β-oxidation of fatty acids fuels into ATP synthesis (Lodish *et al.,* 2000). Similarly, migratory plant parasitic nematodes are also able to obtain fatty acids from their host and in addition possibly synthesize specific fatty acids *de novo* (Krusberg, 1967).

Cyst nematodes are sedentary plant parasites that have specialized feeding behaviors. They hatch from eggs as second-stage juveniles (J2) and search for a host plant. During this stage, the J2 neither feed nor develop, and stored lipids serve as a source of energy which is required for their locomotion. Accordingly, stored lipid content is proportional to the infectivity of the J2 (Story, 1984). Thus, plant parasitic nematodes can metabolize lipids. The lipids of the J2 are supplied maternally from the previous nematode generation. This generation has been exclusively nourished by plant-derived nutrients. Consequently, the question arises: What are the routes of fatty acid supply for lipid biosynthesis in cyst nematodes?

It is not known if feeding cyst nematodes take up host-derived lipids or metabolize them. Besides, none of the nutrients supplied by the feeding site has been defined yet. Cyst nematodes exclusively feed from a syncytium that forms the core of the feeding site that provides all nutrients required for the nematodes development. Structure and function of cyst nematode feeding sites induced under mono-axenic conditions using *A. thaliana* and in the field have been shown to be identical (Sijmons *et al.,* 1991; Golinowski *et al.,* 1996). Thus, studying cyst nematode nutrition and feeding is possible using this system and informative for the field condition.

The syncytium develops after the nematode has selected an initial cell through progressive cell wall dissolution and protoplasmic fusion of neighboring cells. The syncytium shows a high metabolic activity and specific ultra-structural changes, including breakdown of the central vacuole, plastid and

mitochondria replication as well as proliferating endomembrane system and lipid droplet accumulation (Sobczak and Golinowski, 2011; de Almeida Engler and Gheysen, 2013). There is a distinct temporal shift from a rough ER to a smooth ER and the formation of cell wall ingrowths during syncytium development (Golinowski *et al.,* 1996; Sobczak and Golinowski, 2011). Therefore, the syncytium shares characteristics with transfer cells (Jones and Northcote 1972; Rodiuc *et al.,* 2014).

The syncytium is fueled by phloem derived sugars leading to a high metabolic activity (Böckenhoff *et al.,* 1996; Hofmann *et al.,* 2007). This high metabolic activity is further characterized by a distinct transcriptomic signature, an increase of amino acids and organic phosphates (e.g. glucose-6-phosphate, phosphoenolpyruvic acid) and the accumulation of starch in syncytial plastids (Hofmann *et al.,* 2008; Szakasits *et al.,* 2009; Hofmann *et al.,* 2010;). Although the transcriptomic signature of syncytia is different as compared to other plant tissues, similarities with seeds and pollen have been emphasized (Szakasits *et al.,* 2009). These organs synthesize characteristic lipids *de novo* such as triacylglycerol that is essential for their normal development (Zhang *et al.,* 2009).

Feeding in *H. schachtii* occurs in cycles which contain a phase without nutrient uptake (Wyss and Grundler, 1992; Böckenhoff *et al.,* 1996). During this time the syncytium is supposed to be "refilled" with phloem-derived solutes leading to its expansion (Bohlmann and Sobczak, 2014). As such, the syncytium requires a flexible cell wall architecture to cope with changes in internal pressure (Davies *et al.,* 2012). During the feeding cycle, *H. schachtii* uses its stylet to deliver gland secretions. Part of these secretions form the feeding tube that is connected to the stylet orifice. The feeding tube is used to withdraw nutrients from a zone of modified cytoplasm that is free of larger organelles (Sijmons *et al.,* 1991; Wyss and Grundler, 1992). Both, secretions and the feeding tube associate with membranous structures (especially ER) within the syncytium and lipid uptake was suggested to take place at these sites (Grundler *et al.,* 1999).

In plants including *A. thaliana*, fatty acid *de novo* synthesis occurs in the plastids. However, non-plastidial pathways are also involved in lipid biosynthesis. The most abundant lipid classes are the glycerolipids that form important components of plasma membranes, endomembranes, organelles and energy stores. Extraplastidial membranes contain relative large amounts of phospholipids, but also sterol lipids and sphingolipids. These lipids are mainly synthesized in the ER. Plastids contain specific galactolipids that are exclusively made by the plastid. Triacylglycerol (TAG) represents the main form of lipid-bound energy reserves containing three fatty acids esterified to a glycerol backbone. TAG is made in the ER and accumulates in lipid droplets that bud off from the ER membrane. Fatty acids from TAG may be liberated from lipid droplets by lipolysis and the resulting free fatty acids are broken down by β-oxidation in the peroxisome (Li-Beisson *et al.,* 2010). The fate

of peroxisomes in nematode induced syncytia is not characterized. Nonetheless, modulation of plant peroxisomes in giant cells by sedentary root-knot nematodes has been described (Dinh *et al.,* 2014).

In general, specific lipid biosynthetic reactions occur in distinct subcellular compartments. Furthermore, the different organelles and subcellular structures contain specific lipids. This allows quantitative correlation between specific lipids and ultra-structural features. As described above, the ultrastructure of feeding sites induced by *H. schachtii* in *A. thaliana* is well established.

In the present study, the glycerolipidome of feeding sites was characterized for the first time. Moreover, differentially expressed genes were identified that correspond to the syncytial glycerolipid pathways involved. Finally, candidate genes were selected and their role in the interaction between *H. schachtii* and *A. thaliana* was studied.

3.3   Results

Fatty acid profile of J2

The fatty acid profile of infective J2 of *H. schachtii* was determined to get an overview of the fatty acids present in this nematode. The most abundant fatty acid in both fractions was 18:1(cis-n-7) followed by 18:0, 16:0 and minor amounts of other C16- and C20 fatty acids (Figure 3-1). Identical fatty acid profiles have been reported for other plant parasitic nematodes although the relative amounts may vary (Krusberg 1967).
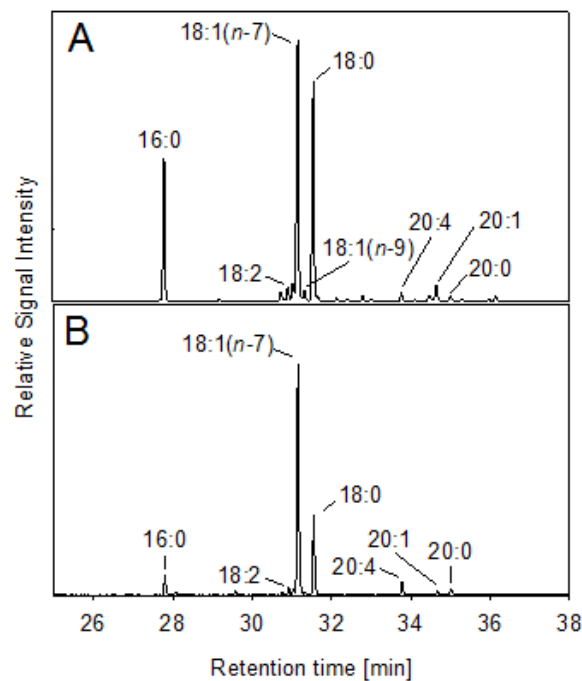
Figure 3-1   Fatty acid analysis of *H. schachtii* J2.

Total lipids were isolated from J2, fractionated into non-polar (A) and polar (B) lipids by solid phase extraction on silica columns, and trans methylated. The resulting fatty acid methyl esters were analyzed by GC-MS.

The lipidome of nematode feeding sites

To get a first overview about quantitative changes of glycerolipids in the syncytium, excised nematode feeding sites were compared with root tissue in a lipidomics approach. As seen in Figure 3-2A, the total glycerolipid content was significantly increased in root sections enriched in nematode feeding sites as compared to the control tissue. The individual lipid classes differentially contributed to the total lipid increase (Figure 3-2B). Plastidial galactolipids (MGDG and DGDG) had approximately 4-fold higher levels in feeding sites than in control roots. Phospholipids and diacylglycerol (DAG)

were about three-fold higher in nematode feedings sites than in control roots. The strongest relative change was observed for TAG with an increase of approximately 5-fold in nematode feeding sites compared to control roots. Interestingly, the fatty acid composition of the non-polar lipids TAG and DAG was altered in nematode feeding sites when compared to control roots: the DAG composition showed a shift towards saturated fatty acids (C16:0 and C18:0) whereas the TAG composition showed a shift towards monounsaturated fatty acids (C18:1) (Figure 3-3).
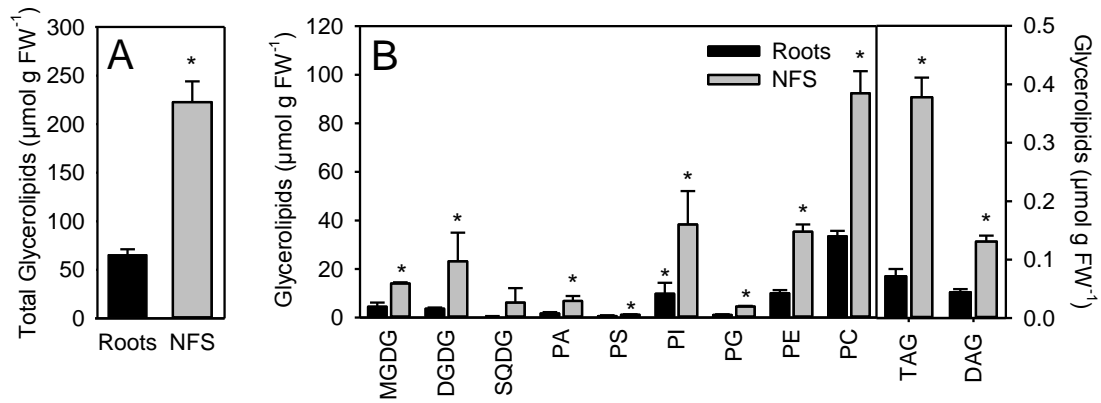
Figure 3-2   **Glycerolipid content and composition of nematode feeding sites (NFS) in *A. thaliana* roots.**

(**A**) Total glycerolipid content of 10 days old female feeding sites compared to total root as determined by direct infusion Q-TOF MS/MS. (**B**) Glycerolipid composition of 10 days old female feeding sites compared to control roots. The data represent averages and standard deviations (n=3). *Significantly different from control, $p \leq 0.05$.
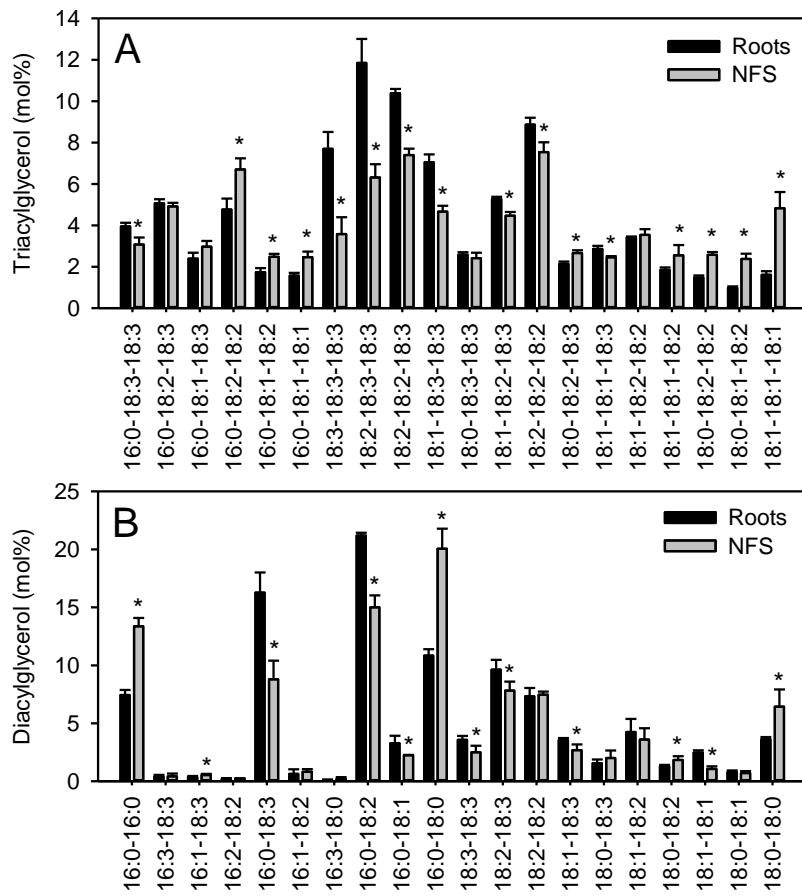
Figure 3-3   **Molecular species composition of non-polar lipids of the nematode feeding site (NFS) in *A. thaliana* roots.**

(**A**) Molecular species composition of TAG isolated from nematode feeding sites and control roots. (**B**) Molecular species composition of DAG isolated from nematode feeding sites and control roots. The data represent averages and standard deviation (n=3). *Significantly different from control, p ≤ 0.05.

Transcriptome analysis of nematode feeding sites

Since the most prominent changes among the profiled lipid classes were observed for TAG content and composition, the role of TAG metabolism in the *A. thaliana-H. schachtii* interaction was further investigated. To identify candidate genes involved in TAG metabolism in nematode feeding sites, annotated acyl lipid pathways (Li-Beisson *et al.,* 2010) were correlated with the syncytial transcriptome (Szakasits *et al.,* 2009) (Figures 3-4 to 3-10). Pathways and gene annotations are based on Li-Beisson et al. (2010) and transcriptomic data were published previously by Szakasits et al. (2009). Transcriptional changes are in log2-fold difference. Green color indicates up-regulation whereas blue indicates down-regulation.

Expression of genes involved in plastidial fatty acid *de novo* and plastid lipid synthesis are up-regulated in syncytia, while expression of genes involved in fatty acid elongation located in the cytosol and the ER are down-regulated (Figures 3-4 to 3-7). Transcripts for plastidial and ER localized fatty acid desaturases are induced (Figure 3-5, Figure 3-10).
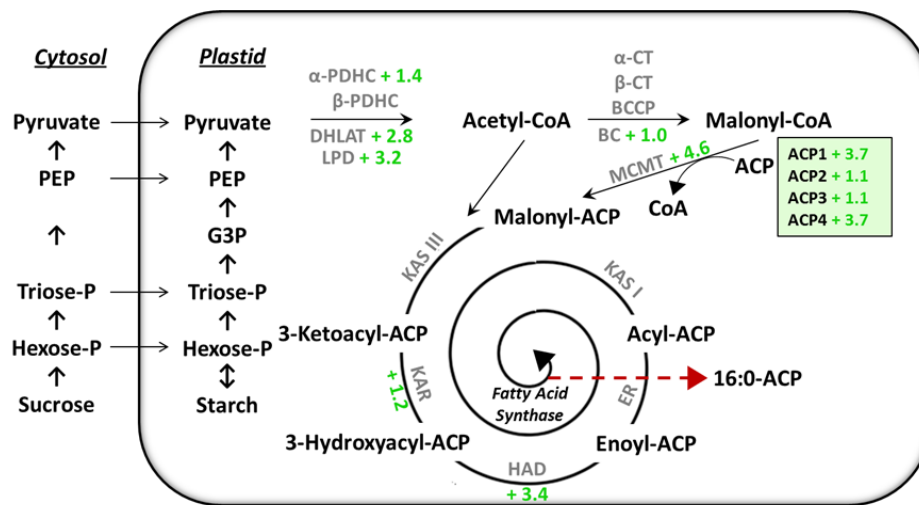
Figure 3-4 **Fatty acid *de novo* synthesis A, Import of carbon units into the plastid and synthesis of C16 acyl groups.**

Fatty acid *de novo* synthesis requires acetyl-CoA supplied by pyruvate dehydrogenase complex. Multimeric ACC carboxylates acetyl-CoA to form malonyl-CoA. Malonyl-CoA is transferred to ACP and used as the chain extender for fatty acid *de novo* synthesis by FAS. ACP, acyl carrier protein; BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; CT, carboxyltransferase; DHLAT, dihydrolipoamide acetyltransferase; ER, enoyl-ACP reductase; HAD, hydroxyacyl-ACP dehydrase; KAR, ketoacyl-ACP reductase; KAS, ketoacyl-ACP synthase; LPD, dihydrolipoamide dehydrogenase; MCMT, malonyl-CoA: ACP malonyltransferase; PDHC, pyruvate dehydrogenase complex; G3P, glycerol-3-phosphate; PEP, phosphoenolpyruvate.

Figure 3-5 **Fatty acid *de novo* synthesis B, C18 synthesis and desaturation.**

16:0-ACP resulting from fatty acid *de novo* synthesis is elongated, desaturated and esterfied to glycerol.
ACP, acyl carrier protein; FAS, fatty acid synthase; FATB (B), fatty acyl thioesterase A (B); KAS, ketoacyl-ACP synthase; SAD, stearoyl-ACP desaturase; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; ATS/ACT, glycerol-3-phosphate acyltransferase.

Figure 3-6 **Plastid lipid synthesis.**

Synthesis of plastidial lipids within plastids requires addition of a head group to DAG and desaturation of esterified fatty acids. . Glc-1-P, glucose-1-phosphate; UGP, UDP-glucose pyrophosphorylase; SQD, UDP-sulfoquinovose synthase; SQDG, sulfoquinovosyldiacylglycerol, MGD, monogalactosyldiacylglycerol transferase; MGDG, monogalactosyldiacylglycerol; DGD, digalactosyldiacylglycerol synthase; DGDG digalactosyldiacylglycerol; FAD, fatty acid desaturase.
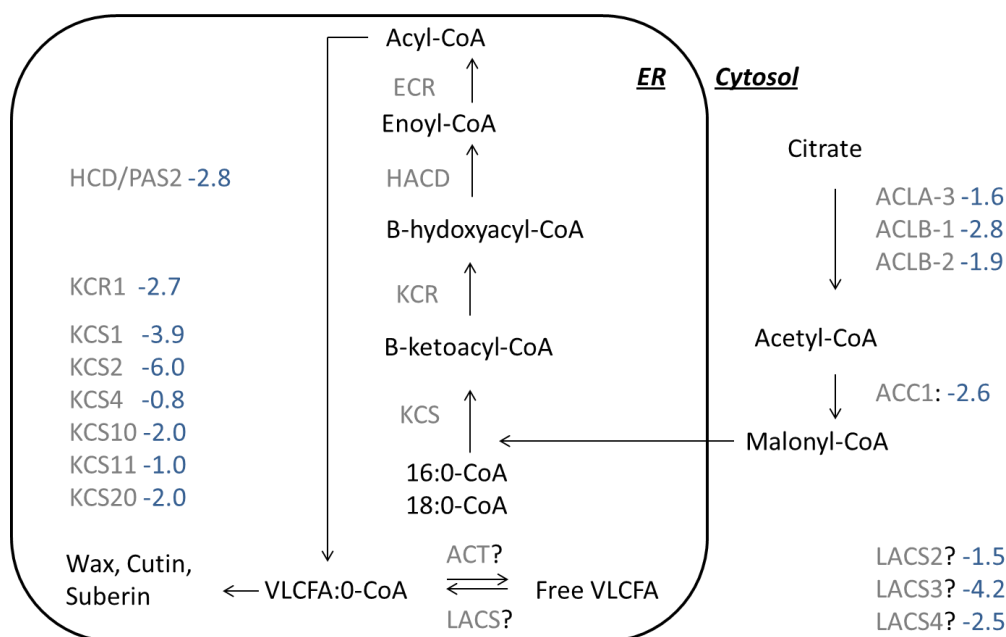
Figure 3-7   Fatty acid elongation.

Fatty acid elonagation of acyl-CoA´s within the ER requires cytosolic malonyl-CoA. Elongated Acyl CoA´s are used for the synthesis of wax, cutin and suberin. ACL, ATP citrate lyase ACC, acetyl-CoA carboxylase; LACS, long-chain acyl-CoA synthetase; KCS, ketoacyl-CoA synthase; KCR, ketoacyl-CoA reductase; HACD, hydroxyacyl-CoA dehydrase; ECR, enoyl-CoA reductase; VLCFA, very long-chain fatty acid.
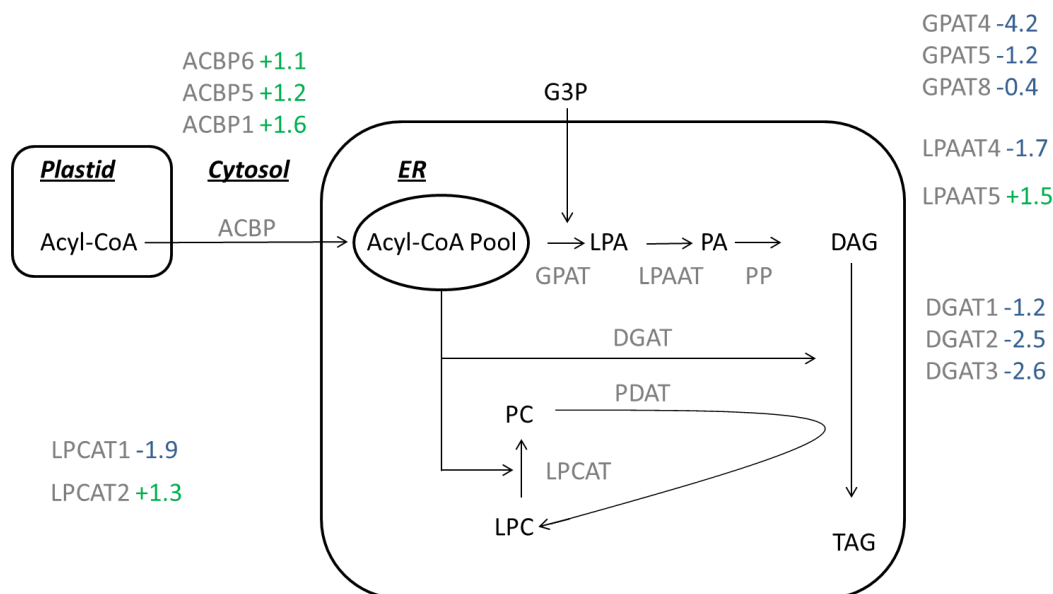
Figure 3-8   Triacylglycerol synthesis.

TAG synthesis from DAG is catalyzed by DGAT using Acyl-CoA or PDAT using PC as acyl donor. The resulting LPC is re-acylated by LPCAT. ACBP, acyl-CoA binding protein, DGAT, acyl-CoA: diacylglycerol acyltransferas; G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LPA, 2-lysophosphatidic acid; LPAAT, 2-lysophosphatidic acid acyltransferase; LPC, 2-lysophosphatidylcholine; LPCAT, 2-lysophosphatidylcholine acyltransferase; PA, phosphatidic acid; PDAT, phospholipid:diacylglycerol acyltransferase; DAG, diacylglycerol; TAG, triacylglycerol.
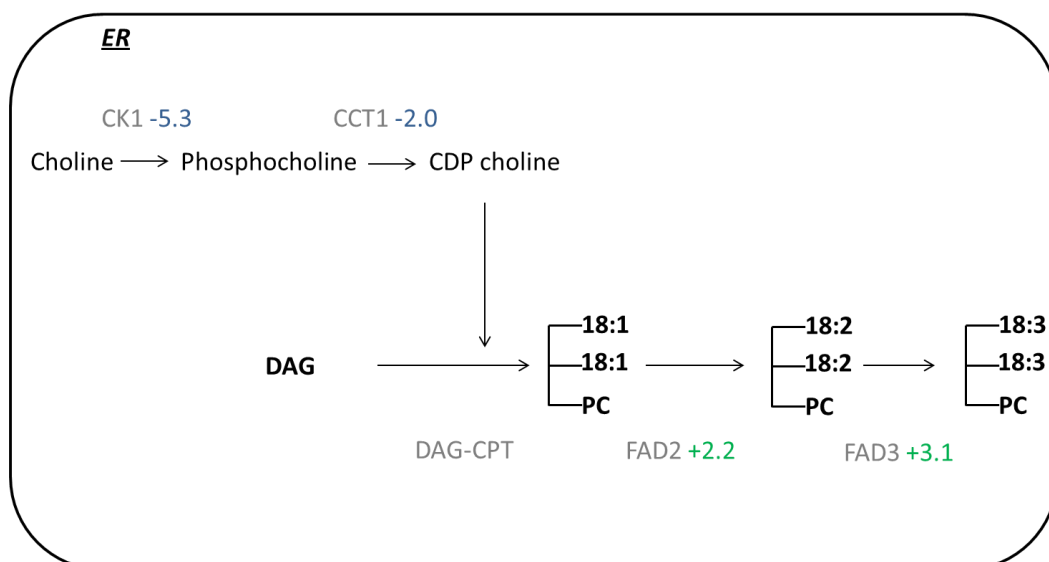
Figure 3-9 **Phosphatidylcholine synthesis and desaturation.**

PC synthesis involves CDP choline synthesis and transfer to DAG. PC may be further desaturated. CCT, choline-phosphate cytidylyltransferase; CK, choline kinase; DAG, diacylglycerol; DAG-CPT, diacylglycerol cholinephosphotransferase; FAD, fatty acid desaturase.

Figure 3-10 **Lipolysis.**

TAG stored in lipid droplets is consecutively broken down by lipases resulting in free fatty acids and glycerol. TAG, triacylglycerol; TAGL, triacylglycerol lipase; DAG, diacylglycerol; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase; FFA, free fatty acid; SDP1, sugar-dependent1; CGI-58 H, comparative gene identifier 58 homolog.

In *Arabidopsis*, TAG is mainly produced by diacylglycerol acyltransferases (DGAT) using acyl-CoA or by phosphatidylcholine: diacylglycerol acyltransferase (PDAT) using PC as acyl donor (Zhang *et al.,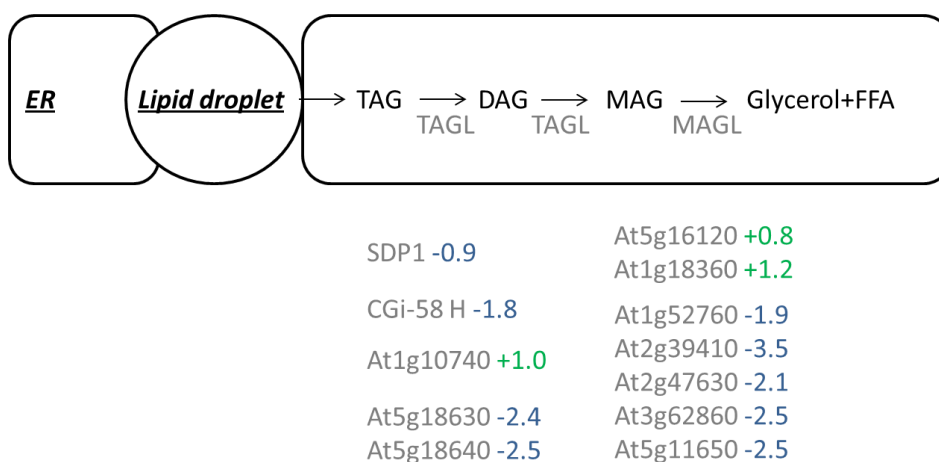* 2009). DGAT1/DGAT2/DGAT3 transcripts were down-regulated whereas PDAT expression was unchanged. However, PDAT function depends on re-acylation of PC by LPCAT2 that is transcriptionally induced (Figure 3-8). PC head group synthesis is down-regulated (Figure 3-9).

In addition, key genes involved in lipolysis (TAG breakdown) are down-regulated (Figure 3-10). Among these, CGI-58 (α/β hydrolase-type protein "Comparative Gene Identifier-58") and SDP1 ("sugar-dependent" lipase 1) have been shown to affect TAG levels in vegetative tissue without causing a drastic plant phenotype on artificial media (James *et al.,* 2010; Kelly *et al.,* 2013). It is known that TAG accumulation in the *sdp1* mutant is at least partly depending on DGAT1 activity (Kelly *et al.,* 2013). Therefore, these loss-of-function mutants (*cgi-58*, *sdp1* and *dgat1*) were selected for further analysis in nematode infection assays.
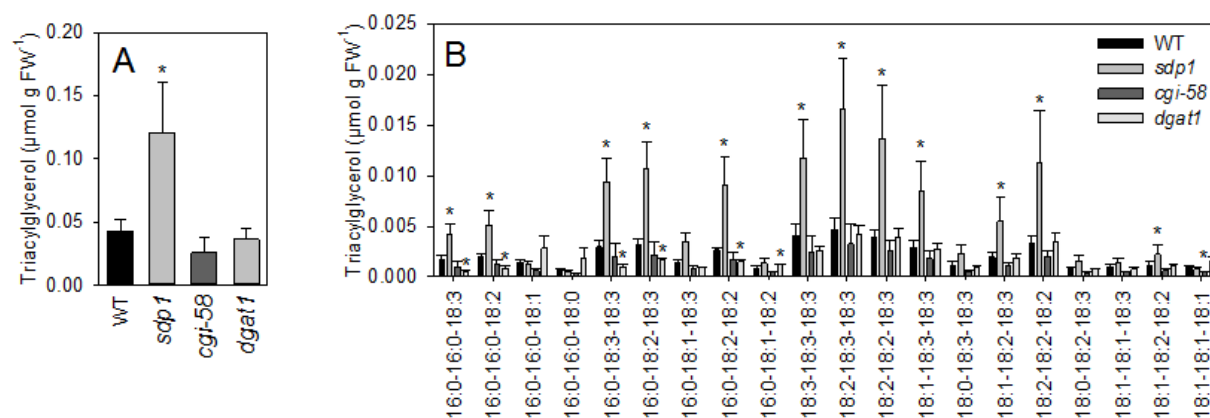
Figure 3-11   TAG content in roots of TAG metabolic mutants.

TAGs were isolated from roots of WT, the mutants *sdp1* and *cgi-58* affected in TAG lipolytic activity and the TAG synthesis mutant *dgat1,* and measured by Q-TOF MS/MS. (**A**) TAG content. (**B**) Molecular species composition of TAG. The data represent averages and standard deviations (n=3). *Significantly different from control, p ≤ 0.05.

Analysis of Nematode Infection on TAG Metabolic Mutants

TAG levels were determined in roots of WT, *cgi-58*, *sdp1* and *dgat1* plants by direct infusion Q-TOF MS/MS. As shown in Figure 3-11A, the total TAG content was significantly increased in roots of *sdp1*, but not in roots of *cgi-58* and *dgat1* plants. The TAG composition in roots was altered in *sdp1* and *dgat1*, whereas *cgi-58* did not show a different TAG profile as compared to WT roots (Figure 3-11B). Roots of *sdp1* are enriched in polyunsaturated fatty acids (C18:2 and C18:3) whereas *dgat1* showed minor reductions in some molecular species of TAG containing 16:0.

When challenged in infection assays, *sdp1* and *cgi-58* showed a significant increase in the number of developed females compared to WT plants (Figure 3-12A). Furthermore, these females were significantly bigger and contained more eggs as compared to the control (Figure 3-12B, C). In contrast, the number of developed females on *dgat1* was significantly lower. However, female sizes and the number of eggs per female in *dgat1* were not significantly different from the control (Figure 3-12C).

It has been previously shown that an altered nematode infection rate correlates with increased defense gene expression (Wubben *et al.,* 2008). To rule out the possibility that an altered defense response is responsible for the observed changes in infectivity, expression levels of plant defense related genes before and after nematode infection were determined using qPCR. No differences in expression levels for PR-1 (salicylate-dependent defense) and PDF1.2 (jasmonate-ethylene-dependent defense) were observed between the mutants and the control at the two time points (Figure 3-13).
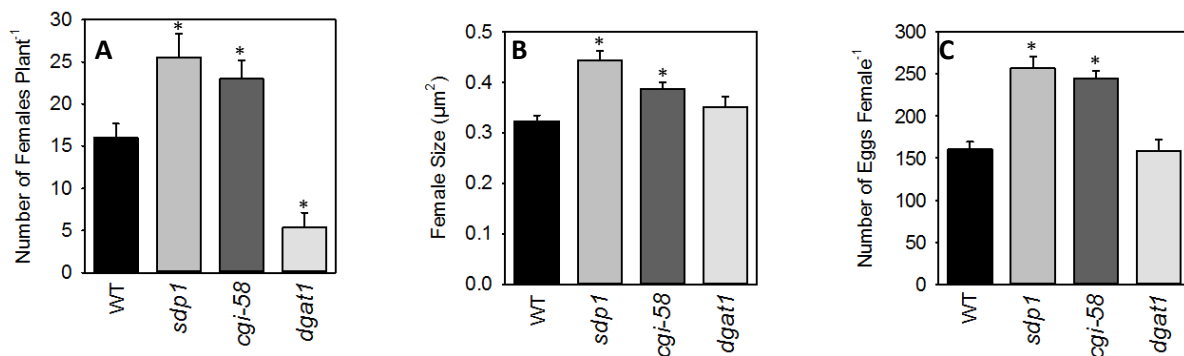
Figure 3-12   **Infection assay of *H. schachtii* with *A. thaliana* TAG metabolic mutants.**

Roots of 10 days old *A. thaliana* plants (WT, *sdp1*, *cgi-58* and *dgat1*) were inoculated with 60 J2. The number of females was counted after 14 days (**A**). The sizes of the developed females (area measured by microscopy) (**B**) and the number of eggs per female (**C**) were determined after 35 days. The data represent average and standard error (n=10, 30 and 30, respectively). *Significantly different from control, p ≤ 0.05.
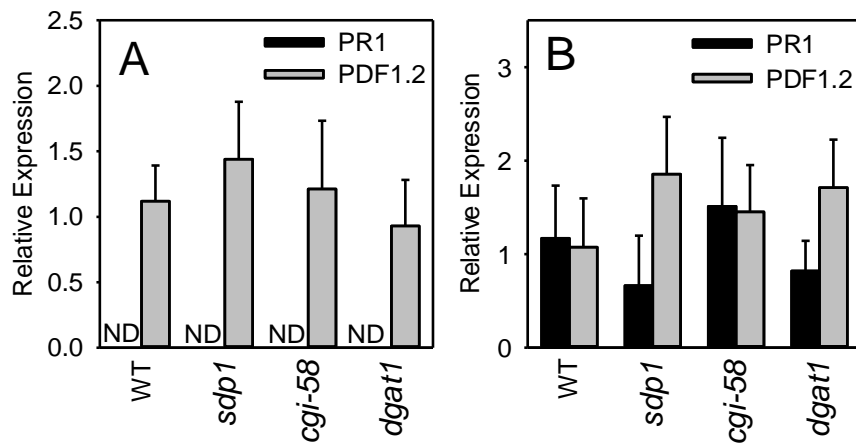
Figure 3-13   Expression analysis of defense genes in TAG metabolic mutants.

(**A**) Basal expression of defense genes (0 DPI). (**B**) Defense gene expression after nematode attack (1 DPI). The data represent average and standard deviation (n=3). *Significantly different from control, $p \le 0.05$. ND = not detected. PR1, pathogen-related gene 1 (salicylate dependent); PDF1.2, plant defensin 1 (jasmonate and ethylene dependent.

3.4   Discussion

In this study, the first glycerolipid profile of nematode induced feeding sites is presented. In line with ultrastructural data, we show that increased levels of glycerolipids are found in the nematode feeding site compared to control roots. However, the individual glycerolipid classes differentially contribute to the overall lipid increase. The increased glycerolipid content is accompanied by a distinct glycerolipid transcriptomic signature which allowed identification of candidate genes for further analysis. Lastly, we demonstrate that mutant plants affected in TAG metabolism show alterations in nematode infection and fecundity, possibly due to an altered nutritional value of the syncytium. These results suggest that cyst nematodes induce glycerolipid accumulation in the syncytium of the nematode feeding site which may increase the nutritional/caloric output of this structure.

The nematode feeding site contains a distinct glycerolipidome

The syncytium shows a high metabolic activity and specific ultra-structural changes including central vacuole breakdown, plastid and mitochondria replication as well as proliferation of the endomembrane system and lipid droplet accumulation. In agreement with the ultra-structural changes, we found that the nematode feeding site is enriched in glycerolipids. However, the individual lipid classes differently contribute to the total lipid increase.

Glycolipids like MGDG and DGDG are vital components of plastids and make up about 75 % of total plastid membranes in green leaves, and they are increased in nematode feeding sites compared to control root tissue. The endoreplication of chloroplast-like plastids has been reported in syncytia of nematode feeding sites and thus our measurements likely reflect the increase and change in lipid composition (Ji *et al.,* 2013; de Almeida Engler and Gheysen, 2013).

Phospholipids are important constituents of the plasma membrane and the ER. The proliferation of the endomembrane system with a shift from rough ER to smooth ER is regarded as a hallmark of syncytium development (Grundler *et al.,* 1999). Interestingly, functions of the smooth ER include the assembly of phospholipids and TAG (Staehelin, 1997). Indeed, we find both phospholipids and non-polar lipids (TAG and DAG) at higher levels in nematode feeding sites than in control roots.

Together this suggests that lipidomic data can be linked to ultra-structural characteristics of the feeding site. This finding may be further exploited to quantify changes in the syncytia as they may occur during feeding site development or between different plant lines/genotypes.

The glycerolipidome of feeding sides is accompanied by a specific transcriptomic signature of the syncytium

To identify the genes that are involved in syncytial glycerolipid biosynthesis we analyzed transcriptome data on nematode feeding sites that have been isolated by microaspiration (Szakasits *et al.,* 2009). We identified several key lipid biosynthetic processes that were differentially regulated in nematode induced syncytia.

Expression of genes encoding enzymes of plastidial fatty acid *de novo* synthesis were upregulated. The finding that the levels of essentially all measured glycerolipids were increased, suggests that this increase is due to an induction of plastidial fatty acid *de novo* synthesis fueling glycerolipid synthesis. The molecular species compositions of DAG and TAG were characterized by a relative decrease in polyunsaturated fatty acids suggesting that the fatty acids that were incorporated into these lipid classes originated from increased *de novo* synthesis. Interestingly, transcriptional activation of fatty acid synthesis has also been observed in feeding sites induced by *Heterodera glycines* in soybean. This activation was linked to the accumulation of lipid droplets and smooth ER within the induced syncytium (Matthews *et al.,* 2013).

Fatty acid *de novo* synthesis requires carbon supplied from acetyl-CoA. The sink character of syncytia is well established and large amounts of carbohydrates are delivered into the syncytium and metabolized (Hofmann *et al.,* 2007; Hofmann *et al.,* 2010). Interestingly, starch synthesis and breakdown are both transcriptionally induced in the syncytium and it was suggested that starch acts as a buffer for fluctuating sugar requirements of the feeding site or the nematode (Hofmann *et al.,* 2008). This suggests that the substrates and precursors required for fatty acid *de novo* synthesis are abundantly present.

Genes involved in plastid lipid (MGDG, DGDG) biosynthesis were upregulated. Since the plastids of syncytia are endoreplicating, this upregulation may fuel the extra need for lipids required for plastid biogenesis and functioning (de Almeida Engler and Gheysen, 2013).

Fatty acid elongation occurring in the ER with cytosolic malonyl-CoA supply is downregulated. In roots, fatty acid elongation is required for suberin biosynthesis. Interestingly, induction of syncytial suberin biosynthesis in soybean has been associated with a resistance response against *Heterodera glycines* (Klink *et al.*, 2010). Thus, syncytial fatty acid flux into specific lipid may be blocked while they might be preferentially channeled into other lipid classes.

Surprisingly, expression of DGAT1/DGAT2/DGAT3 that encode the acyltransferases performing the committed step in TAG biosynthesis, were down-regulated. Thus, the increased syncytial TAG level may not be explained by an increased synthesis by DGAT. Additionally, a considerable amount of TAG is produced by PDAT using PC as the acyl donor that is subsequently re-acylated by LPCAT using acyl-CoA (Xu *et al.,* 2012). PDAT expression was un-changed in syncytia whereas LPCAT1 is down-

regulated and LPCAT2 is up-regulated. Furthermore, PC head group synthesis is down-regulated whereas PC desaturation is up regulated. This suggests the involvement of acyl editing and head group limitation in syncytial glycerolipid metabolism (Bates *et al.,* 2012). Thus, a shear increase in TAG synthesis alone could not account for its accumulation within the syncytium. However, lipolysis required for fatty acid liberation from TAG was repressed as well indicating that syncytial TAG may accumulate because of its repressed breakdown. Since lipolysis is the initial step in TAG breakdown and competing reactions are lacking, its repression may allow TAG to accumulate as a syncytial end product during "refilling".

Collectively, the transcriptome changes in nematode induced syncytia serve to explain alterations in glycerolipid metabolism but then again highlight the complexity due to the involvement of multiple intertwined pathways. However, the pathways discussed here may serve as an outline for future research on lipid metabolism in nematode feeding sites.

Mutations affecting TAG metabolism have an influence on nematode infection and fecundity

To elucidate the role of TAG in the interaction of *H. schachtii* with *A. thaliana* we investigated plant mutants with altered TAG metabolism with emphasis on lipolysis. TAG profiling of roots of WT, *cgi-58*, *sdp1* and *dgat1* showed, that the quantitative changes described for leaf or seed TAG content are not reflected in the roots (Zhang *et al.,* 2009; James *et al.,* 2010). However, *sdp1* shows an increase in TAG content (Kelly *et al.,* 2013). In addition, the mutant plants did not show altered transcription of genes involved in the defense against the nematodes invasion.

When challenged in nematode infection assays, the two lipolytic mutants (*cgi-58* and *sdp1*) showed higher infection rates and increased female fecundity. However, the increased infection rate does not correlate with the root TAG content at the time point of infection. This suggests that regulation of TAG metabolism during establishment or within the syncytium are likely more important than the initial root TAG content.

In agreement with this finding, *dgat1* plants have similar root TAG levels as control plants but show a reduction in the infection rate suggesting an important role for TAG biosynthesis during the establishment or induction of the syncytium. However, fecundity of females developing on *dgat1* is not affected. It is known that loss of *dgat1* can be compensated by the activity of PDAT (Zhang *et al.,* 2009). This indicates multiple functions for plant TAG metabolism during the different stages of syncytium induction and development.

Nevertheless the increase in fecundity of females developing on *cgi-58* and *sdp1* roots indicates that a block in plant lipolysis is beneficial for nematode development possibly through increased syncytial TAG availability. In line with this result, Spodoptera exigua larvae gained more weight when they were feeding on plants with increased leaf TAG content due to ectopic expression of DGTT2. The

authors concluded that an increased nutritional value and/or energy density of the transgenic lines is responsible for increased S. exigua performance (Sanjaya *et al.,* 2013).

Similarly, reduced lipolysis may increase the amount of TAG in the syncytium which increases its nutritional output. Uptake of plant lipids by migratory plant parasitic nematodes has been suggested (Krusberg, 1967). For cyst nematodes, physical interaction between their feeding tube and syncytial lipids would be required. Actually, structures of smooth ER associate with nematode secretions and the formed feeding tube that is used for nutrient withdrawal. More importantly, the same structures are visible within the lumen of the feeding tube, showing the ability of the cyst nematode to withdraw lipids from the syncytium (Grundler *et al.,* 1999).

Thus, induction of syncytial lipid accumulation with subsequent uptake may be an efficient strategy employed by cyst nematodes to boost their development and fecundity. Moreover, interfering with this strategy may represent a promising tool for the control of cyst nematodes. Furthermore, understanding molecular mechanisms underlying the accumulation of TAG in plant vegetative tissue is of great economic importance for the development of future food and fuel production systems (Durrett *et al.,* 2008; Dyer *et al.,* 2008).

In conclusion, research on lipid metabolism in plant-nematode interactions prospers functional tools and advances our understanding on cyst nematode as well as plant biology that may be translated into novel approaches to improve crop plants.

3.5   Materials and Methods

Plant Material

Arabidopsis thaliana Col-0 was used a control. Seeds of the mutant lines *cgi-58* as well as *sdp1-5* and *dgat1-1* were kindly provided by John Dyer (US Arid-Land Agricultural Research Center, Maricopa, USA) and Peter Eastmond (Rothamsted Reasearch, Harpenden, UK), respectively.

Fatty acid analysis of *H. schachtii* J2

Fatty acids were extracted from approximately 5000 J2 using chloroform:methanol (2:1, v/v) and phase separation was achieved by addition of 1 M KCl/0.2 M H_3PO_4. The lipids were extracted from the remaining cells for a second time using 100% chloroform. The organic phases were combined, the solvent was evaporated and the lipids were re-dissolved in 100% chloroform. Lipids were separated on a silica column (Strata Si-1, 100 mg, Phenomenex) as described (Wewer *et al.,* 2011). Briefly, the silica column was equilibrated using 100% chloroform, the sample was loaded onto the column and non-polar lipids were eluted with 3 column volumes of 100% chloroform. Afterwards, polar lipids were eluted from the column using 3 column volumes of 100% methanol.

Fatty acid methyl esters (FAMEs) were generated from each fraction by trans-methylation of the fatty acid moiety of glycerolipids using 1 N HCl in methanol. FAMEs were dissolved in n-hexane and analyzed by gas chromatography coupled to mass spectrometric detection (GC-MS) (Agilent HP6890) as previously described (Devers *et al.,* 2011).

Glycerolipidome analysis of nematode feeding sites

Two *Arabidopsis* seeds were sown on petri dishes containing modified KNOP medium (Sijmons et al. 1991). After 10 days of growth, the seedlings were inoculated with 60 J2 of *H. schachtii*. At 10 days post infection (DPI), nematode feeding sites of females were excised and immediately frozen in liquid nitrogen as described in Hofmann et al. (2010). Excised feeding sites were pooled to achieve a total sample weight of approximately 50 mg. The frozen tissue was crushed to a fine powder. Total root tissue was used as the control. Plant glycerolipids were isolated and measured by direct infusion quadrupole time-of-flight mass spectrometry (Q-TOF MS/MS) as previously described (Lippold *et al.,* 2012; vom Dorp *et al.,* 2013; Gasulla *et al.,* 2013).

Transcriptome analysis of nematode feeding sites

Annotated acyl lipid pathways were obtained from Li-Beisson et al. (2010) and transcriptome data were taken from Szakasits *et al*. (2009).

Defense gene expression in TAG metabolic mutants

Two *Arabidopsis* seeds were sown on petri dishes containing KNOP medium. After 10 days of growth, plant roots were harvested. A second batch of plants was inoculated with 60 nematodes and harvested after 1 DPI. Expression of plant defense marker genes responsive to either salicylic acid (PR1) or jasmonic acid-ethylene (PDF1.2) was analyzed in whole roots using quantitative polymerase chain reaction (qPCR) as previously described (Siddique *et al.,* 2014).

Nematode infection assay

Two *Arabidopsis* seeds were sown on petri dishes containing KNOP medium. After 10 days of growth, each plant was inoculated with 60 freshly hatched J2 of *H. schachtii*. The number of female nematodes per plant was determined after 14 days of inoculation (DPI). The sizes of female nematodes and the number of eggs per female were determined after 35 DPI.

Statistics

Data were analyzed for significance using SigmaPlot 12. Lipid quantifications and defense gene expression studies were performed twice, nematode infection assays were performed three times. All experiments showed consistent results.

3.6 Literature

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4   Conclusion and Outlook

In the course of these studies the mode of action of SPT in nematodes was identified and glycerolipid metabolism in cyst nematode induced syncytia was studied. This allows to draw conclusions on general aspects of plant and nematode lipid metabolism and provides a framework for future research.

SPT-enol inhibits acetyl co a carboxylase (ACC) activity of *C. elegans* allowing it to be used as a tool to further dissect fatty acid *de novo* synthesis and lipid metabolism in this organism. To this point, ACC in *C. elegans* may represent the metabolic switch translating nutritional signals into growth and development through the synthesis of complex lipids. As complex lipids are involved in distinct biological processes SPT-enol may be used as tool to further characterize the specific involvement of fatty acid *de novo* within. Furthermore, it may be used to discover additional functions of ACC and co-regulated genes involved in nematode metabolism.

As further studies showed, ACC activity is also essential for cyst nematode development. This suggests conserved pathway function between *C. elegans* and *H. schachtii*. Therefore, concepts in lipid biosynthesis of the model *C. elegans* can be transferred to other nematode species and tested using SPT. Thus SPT may be used as a general tool to investigate lipid metabolism in nematodes including animal and plant-parasitic. Furthermore, RNAi based inactivation of ACC suggests that ACC as a target against parasitic nematodes is generally suitable which may be translated into novel control strategies. Additionally, *C. elegans* is used as a universal animal model which proposes that inhibition of ACC by SPT may also be possible in higher animals. This has important implications since human ACC is regarded as a drug target against symptoms in diseases such as diabetes, obesity, cancer, and microbial infections (Shi and Burn, 2004; Tong and Harwood, 2006).

Importantly, the IC50 of SPT-enol for nematode ACC seems relatively high. The fact that SPT was developed against insect ACC may explain the low specificity. Thus SPT may only function as a lead structure within the group of keto-enols to discover effective inhibitors for specific ACC´s. Noticeably, the inhibition is linked to the metabolic status that regulates the activation of ACC and needs to be considered when designing ACC-inhibitor screens.

Regarding cyst nematodes, nutrients may cause ACC activation and deliver acetyl-CoA for conversion into malonyl-CoA. Malonyl-CoA may then function in fatty acid *de novo* synthesis as well as in the modification and regulation of the lipid pool. The coordinated function of these processes is required for correct development and lack of malonyl-CoA due to ACC inhibition by SPT-enol causes it to arrest. However, it remains to be determined if *H. schachtii* expresses a functional fatty acid synthase (FAS) required for fatty acid *de novo* synthesis.
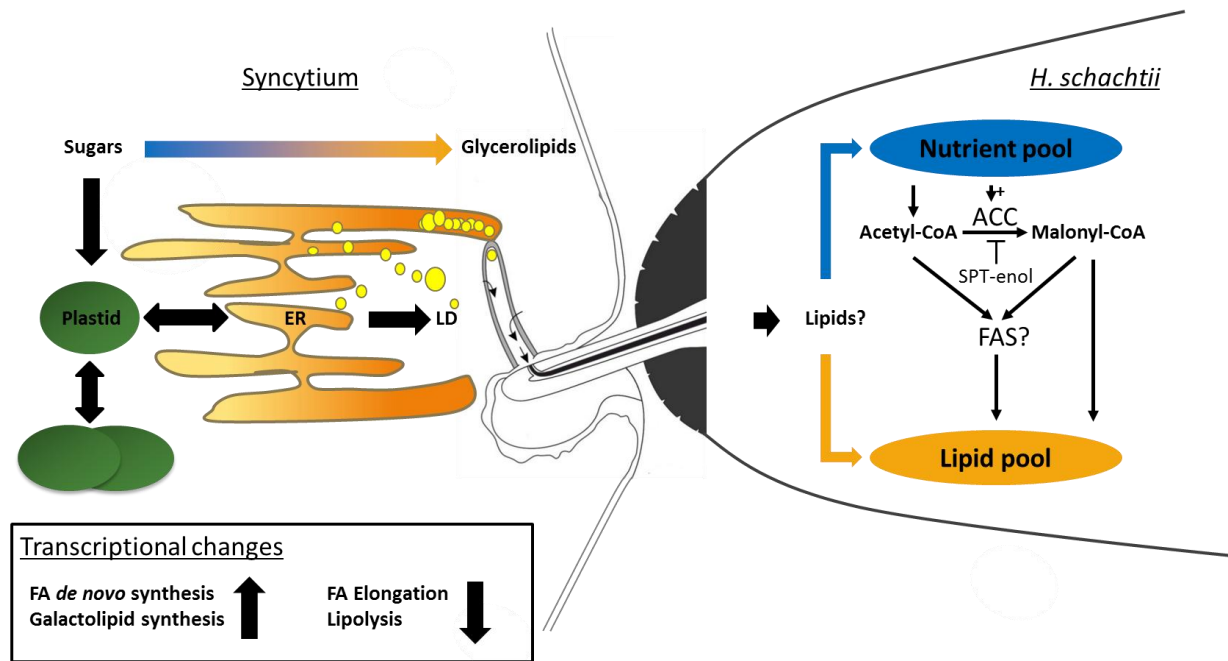
et al.,

Figure 4-1 **Fatty acid *de novo* synthesis and acyl lipid metabolism in cyst nematodes and their syncytial feeding sites.**

Cyst nematodes induce syncytial glycerlipid accumulation through modulation of plant acyl lipid metabolism. Glycerolipids may be resorbed by the nematode and either fuel the nematodes nutrient pool or lipid pool. The nutrient pool is required to produce acetyl-CoA and also to activate ACC. Activated ACC carboxylates acetyl-CoA to yield malonyl-CoA that is required for fatty acid *de novo* synthesis and elongation. SPT-enol likely acts on activated ACC and inhibits malonyl-CoA synthesis resulting in developmental inhibition of nematodes. Nematode head and stylet were adopted from (Eves-van den Akker *et al.,* 2014).

In this study we can also show that the *A. thaliana-H. schachtii* system is suitable to test small molecule inhibitors and allows mode of action studies in combination with *C. elegans*. Thus, the *A. thaliana-H. schachtii* system may be used as a model platform to screen for and elucidate the functions of nematicides. Additionally SPT conversion and phloem translocation suggest that this system is suitable to screen for phloem mobile pesticides by simple foliar application. This may also facilitate the development of adjuvants and formulations used to improve penetration and systemic distribution of active ingredients.

Using the *A. thaliana-H. schachtii* system, we also found that following foliar SPT application SPT-enol is detected in the plant growth medium. This "leakiness" is unwanted as it may reduce the concentration of the active ingredient within the plant and thereby the availability for the target organism. As multiple mechanisms could be responsible (e.g. diffusion, active export, etc.) it may be important to establish the mechanisms behind this effect and *Arabidopsis* can be employed to do so. In the field, SPT-enol that escaped into the soil may influence the ubiquitous nematode community

(Nematome) and possibly other organisms. Consequently, further studies using soil-based culture systems with focus on the plant-associated nematome are warranted.

Nonetheless, SPT-enol that has "leaked" from the phloem may provide additional protection for the plant as it may become also available for plant ecto-parasitic nematodes e.g. that are feeding on overlaying cortex cells. However, this study did not consider all possible metabolites that are known to be formed during SPT metabolism in plants. Particular SPT metabolites may be more prone to this effect as others, as some may also be preferentially retained *in planta* or possibly loaded into the syncytium (e.g. SPT-enol-glucoside). However, this would require a better understanding of SPT metabolism and transport dynamics in plants at the tissue or even cellular level. *Arabidopsis* provides the opportunity to study these processes but protocols for small sample analysis of SPT metabolites are likely required to be developed.

Again using the *A. thaliana-H. schachtii* system, we also establish lipidomic profiling as a suitable tool to describe the cyst nematode feeding site. Hence, this technique may also be applied to describe quantitative lipid changes occurring in syncytia of e.g. different developmental stages, between sexes, plant mutants etc. It may be also applied to study these changes in other plant species such as sugar beet. So far, we identified the availability of sample tissue as the main bottle neck for feeding site analysis – around 300 syncytial cut sections amount to 1 sample of 50 mg. However, lipidomics using Q-TOF mass spectrometry offers the theoretical sensitivity to quantify lipids in single feeding sites (e.g. 1 cm cut section) or nematodes greatly facilitating high through put analysis.

Based on the lipidomic profiling we found that cyst nematodes induce local glycerolipid accumulation in syncytia and further found that altered plant lipid metabolism influences cyst nematode development. As depicted, glycerolipid accumulation in syncytia is accompanied by specific transcriptional changes that may be required to channel increased fatty acids made in the plastids through the ER into lipid droplets. These changes may govern nematode nutrition by supplying lipids to the cyst nematode that may fuel into their nutrient and/or lipid pool. Fueling the nematodes lipid pool may be particular adventitious due to the reduced energetic costs as compared to lipid *de novo* synthesis. In the case of fatty acids, this will depend on their grade of modification (e.g length, position and degree of desaturation) completed by the plant.

Nevertheless, the depicted concept may be exploited to develop new ways for cyst nematode control. Modifying plant TAG metabolism seems particular interesting and the knowledge gained from *A. thaliana* may be already transferred to crop plants like sugar beet. However, as TAG metabolism is also important for seed development, the manipulation of the plants TAG content through any means will require an understanding of how TAG metabolism is regulated in the different organs. For sugar beet this understanding may be particular interesting as vegetative TAG synthesis may compete with carbohydrate metabolism.

For other plants an increased TAG content may be preferred as they are used as a source of fuel and food. Understanding how cyst nematodes are manipulating the plant to facilitate syncytial TAG accumulation may be turned into new approaches to boost TAG content in oil-producing plants. In this context it may be interesting to investigate the protein-effector repertoire of *H. schachtii* for the ability to manipulate plant lipid metabolism and in particular promote neutral lipid accumulation.

Together, the studies on fatty acid *de novo* synthesis and metabolism in free-living and plant parasitic nematodes and their feeding sites highlight the importance of acyl lipid metabolism in plant-nematode interactions. And yet the details of the involved processes remain elusive and necessitate further research. However, the model systems *C. elegans* and *A. thaliana* supply the required pathways and concepts to establish a framework for acyl lipid metabolism in plant parasitic nematodes. In proper sequence, the *H. schachtii - A. thaliana* system provides the necessary accessibility to study and test these pathways in host-parasite interactions. In the long run, advancing our understanding on the biology of cyst nematodes and their feeding sites may be translated into new strategies to improve crop plants and the well-being of human kind.

4.1 Literature

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5   Summary

Lipids are essential constituents of prokaryotic and eukaryotic cells being involved in membrane biogenesis, signaling and energy storage. Furthermore, organisms have evolved different ways to obtain and synthesize these molecules which is reflected in the various life styles. Whereas plants are capable to synthesize all their required lipids *de novo,* some animals like nematodes depend at least partly on dietary lipid supply. Nematodes are microscopic, ubiquitously present roundworms comprising free-living as well as plant- and animal-parasitic species. Among the plant parasitic nematodes, the sedentary cyst nematodes such as *Heterodera sp.* are economically important as they infect crops like soy bean and sugar beet. After invading a host plant, cyst nematodes establish a feeding site that acts as a sink tissue for plant metabolites supplying nutrients to the nematode.

In this work, I was interested to probe the importance of fatty acid *de novo* synthesis and dietary supply of fatty acids in the interaction between *Heterodera schachtii* and *Arabidopsis thaliana.*

To study fatty acid *de novo* synthesis, I determined the mode of action of spirotetramat (SPT) on the free living nematode *C. elegans*. Using a series of *in vivo*, biochemical, imaging and enzyme assays I show that SPT inhibits *C. elegans* acetyl-CoA carboxylase (ACC) causing developmental arrest. Follow-up studies using *H. schachtii* and *A. thaliana* show that foliar applied SPT also causes developmental arrest in cyst nematodes. Bioinformatics revealed the presence of a *H. schachtii* ACC similar to other nematode ACC´s and its knock down by RNAi phenocopied the effect of foliar applied SPT. Together this suggest that cyst nematodes are depending on ACC activity for fatty acid *de novo* synthesis that is essential for development.

To study the involvement of dietary fatty acid supply, I first determined the lipidome of the nematode feeding sites which supply all required nutrients for the nematodes' development. Most lipids were found at higher levels with triacylglycerol (TAG) showing the strongest fold-increase. Transcriptomic analysis suggested that increased syncytial fatty acid *de novo* synthesis and inhibition of lipolysis might be involved in TAG accumulation. This allowed candidate genes to be identified. *Arabidopsis* insertional lines for these candidate genes with impaired lipolytic capability favored nematode development. These findings support a concept of increased nutritional value of the feeding site due to impaired lipolysis and possibly increased TAG availability.

In conclusion, fatty acid *de novo* synthesis and dietary lipids are involved in cyst nematode development. Moreover, understanding the biology of these pathways can be translated into improved crop plants and novel approaches for nematode control.

6   Zusammenfassung

Lipide sind wesentliche Bestandteile von prokaryotischenund eukaryotischen Zellen und sind beteiligt in Membranbiogenese, Signal- und Energiespeicherung. Darüber hinaus entwickelten Organismen unterschiedliche Strategien um diese Moleküle aufzunehmen und zu synthetisieren. Diese verschiedenen Strategien spiegeln sich in den Lebensweisen der verschiedenen Organismen wider. Während Pflanzen in der Lage sind, alle ihre benötigten Lipide *de novo* zu synthetisieren sind einige Tiere wie Nematoden zumindest teilweise auf Lipide angewiesen, die mit der Nahrung aufgenommen werden. Nematoden sind mikroskopisch kleine, ubiquitär vorkommende Fadenwürmer, zu denen freilebende, sowie Pflanzen- und Tierparasitäre Arten zählen. Bei den pflanzenparasitären Nematoden, sind die sedentären Zystennematoden, wie *Heterodera sp.*, landwirtschaftlich relevant, da sie Kulturpflanzen wie Sojabohnen und Zuckerrüben infizieren. Nach der Invasion einer geeigneten Wirtspflanze induziert der Nematod ein Nährzellsystem, das als Sink-Gewebe für Pflanzenmetabolite wirkt und für den Nematoden Nährstoffe produziert.

In dieser Arbeit war ich daran interessiert, die Bedeutung der Fettsäure *de novo* Synthese und Nahrungsversorgung durch Fettsäuren in der Wechselwirkung zwischen *Heterodera schachtii* und *Arabidopsis thaliana* zu untersuchen.

Um die Fettsäure *de novo* Synthese zu studieren war ich erst daran interessiert die Wirkungsweise von Spirotetramat (SPT) auf den frei lebenden Fadenwurm *Caenorhabditis elegans* zu bestimmen. Mit einer Reihe von *in vivo*, biochemischen, bildgebenden und Enzymaktivitätstests zeige ich, dass SPT die Aktivität der Acetyl-CoA-Carboxylase (ACC) von *C. elegans* hemmt und einen Entwicklungsstillstand verursacht. Studien mit *H. schachtii* und *A. thaliana* zeigen, dass foliar-appliziertes SPT auch bei Zystennematoden einen Entwicklungsstillstand hervorruft. Bioinformatisch kann die Anwesenheit einer *H. schachtii* ACC, ähnlich der von anderen Nematoden gezeigt werden und ihr Knockdown mittels RNAi gleicht der Wirkung von SPT. Zusammen legt dies nah, dass Zystennematoden auf die Aktivität der ACC angewiesen sind, da sie für die Synthese von Fettsäuren, die für die Entwicklung der Nematoden gebraucht werden, essentiell ist.

Um die Beteiligung der Nematoden Nahrungsversorgung durch Fettsäuren zu studieren bestimmte ich das Lipidom des Nährzellsystem, das alle erforderlichen Nährstoffe für die Nematoden Entwicklung liefert. Obwohl die meisten quantifizierten Lipidklassen einen Anstieg zeigten war Triacylglycerol (TAG) am stärksten angereichert. Transkriptom-Analysen isolierter Nährzellsysteme, zeigte eine gesteigerte Fettsäure *de novo* Synthese und die Hemmung der Lipolyse als mögliche Ursachen der TAG Akkumulation und erlaubte mir die Identifizierung von Kandidatengenen. *Arabidopsis* Insertionslinien für diese Kandidatengene mit eingeschränkter lipolytischer Fähigkeit wiesen eine begünstigte Nematodenentwicklung auf. Diese Ergebnisse unterstützen ein Konzept eines erhöhten Nährwerts des Nährzellsystems aufgrund eingeschränkter Lipolyse und möglicherweise erhöhter TAG-Verfügbarkeit für den Nematoden.

Zusammenfassend wirken sich Fettsäure *de novo* Synthese und Nahrungs-Lipide auf die Zystennematoden Entwicklung aus. Ein Verständnis dieser Biologie kann in die Verbesserung von Kulturpflanzen und neuartige Ansätze zur Nematodenkontrolle übersetzt werden.

7 Erklärung

Ich versichere persönlich, dass ich die vorstehende Arbeit selbständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus Veröffentlichungen entnommen sind, wurden als solche kenntlich gemacht.

Die Arbeit hat in gleicher oder ähnlicher Form in keinem anderen Prüfungsamt vorgelegen.

Bonn, den   _____   _____

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