Institut für Nutzpflanzenwissenschaften und Ressourcenschutz
Molekulare Phytomedizin

Identification and characterization of effector proteins of the beet cyst nematode *Heterodera schachtii*

Dissertation

zur

Erlangung des Grades

Doktor der Agrarwissenschaften (Dr. agr.)

der

Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Samer Samir Habash

aus

Jordanien

Bonn 2017

Referent: Prof. Dr. Florian M.W. Grundler

Gutachter: Prof. Andreas Meyer

Gutachter: Assoc.Prof. Luma Al Banna

Tag der mündlichen Prüfung: 09.03.2017

Angefertigt mit Genehmigung der Landwirtschaftlichen Fakultäte der Universitäte Bonn

“This work is dedicated to my beloved family”

Table of contents

Abstract

Plant parasitic nematodes (PPN) are considered as economically important pests of a wide range of plants including ornamentals, vegetables, and fruit trees. The beet cyst nematode *Heterodera schachtii* causes massive yield loss in sugar beet production. *H. schachtii* is a biotrophic sedentary endoparasite which depends on a specific hypermetabolic syncytial nurse cell structure in the host root. To induce and maintain the feeding site, *H. schachtii* uses a specific set of effector proteins that are secreted mostly from the oesophageal glands. Identification and functional analysis of these proteins are crucial steps to understand the nature of nematode parasitism. In our study, we sequenced the *H. schachtii* transcriptome via Illumina MiSeq. We compared the assembled *H. schachtii* transcriptome with the available nematode ESTs from NEMBASE4 and transcripts from available nematode transcriptomes in order to identify new effectors. We found 484 putative secretory proteins specific to plant-parasitic nematodes (PSP). Further comparison with known *H. schachtii* ESTs resulted in the identification of so far unknown PSPs. The annotation of the identified PSPs showed enrichment in certain gene ontologies such as metabolic and catalytic activities in addition to growth regulation function. We selected two genes for a detailed functional analysis. Through Pfam domain analysis, we identified "*HsPDI"* encoding a protein disulfide-isomerase domain and "*Hs-Tyr"* encoding a tyrosinase functional domain. To our knowledge, these two domains have not yet been described in the context of nematode effector proteins.

Transcripts of both genes were localized in the esophageal gland of pre-parasitic juveniles, and their expression was found to be up-regulated during the parasitic developmental stages. Silencing of both genes by RNAi affected nematode development and syncytium formation: both females and syncytia were significantly smaller than the controls. On the contrary, ectopic expression of the effectors in Arabidopsis increased plant susceptibility to *H. schachtii.*

Silencing of *HsPDI* led to syncytia with distinct ultrastructural changes such as less dense cytoplasm with distorted and degraded organelles. Treating *HsPDI*-expressing Arabidopsis plants with the defense inducing peptide flg22 triggered ROS burst, but the measured H_2O_2 level was lower compared with control plants. Furthermore, treating pre-infective nematode juveniles with H_2O_2caused up-regulation of *HsPDI* expression. Silencing *HsPDI* in pre-infective nematode juveniles induced higher sensitivity to H_2O_2 stress compared with untreated nematodes. Fluorescence microscopy of *Nicotiana benthamiana* leaves transiently expressing *HsPDI*::GFP showed that it is specifically located in the apoplastic space. Thus, our results demonstrate the importance of the *HsPDI* for the interaction between nematode and host as an apoplastic effector, and indicates the possible function of *HsPDI* as a scavenger of plant ROS.

Ectopic expression of *Hs-Tyr* in Arabidopsis has a clear impact on plant growth: shoot growth was promoted and root architecture was changed. No changes where observed in the root length or weight. Additionally, the presence of *Hs-Tyr* in the plant caused changes in the homeostasis of several plant hormones especially auxin, jasmonate precursor *cis*OPDA and the ethylene precursor ACC. No significant changes of jasmonic acid and salicylic acid levels were observed. The transgenic plants were more susceptible to *H. schachtii*, but not to the root-knot nematode *Meloidogyne incognita.* This indicates that this effector is of specific importance for the parasitism of the cyst nematode *H. schachtii.* The results suggest that *Hs-Tyr* interferes with the orchestration of plant hormones in a still unknown way.

The presented results show that the analyzed PSPs have specific effects on nematode-plant interaction and therefore serve as effectors facilitating parasitism.

Zusammenfassung

Pflanzenparasitäre Nematoden sind wirtschaftlich bedeutsame Schaderreger an vielen landwirtschaftlichen Kulturen wie auch Zierpflanzen, Gemüse- und Obstarten. Der Rübenzystennematode *Heterodera schachtii* verursacht massive Ertragsverluste in der Zuckerrübenproduktion. *H. schachtii* ist ein biotropher, sedentärer Endoparasit, dessen Entwicklung von der Bildung eines hypermetabolischen Nährzellensystems in der Wurzel abhängt. Um dieses Nährzellensystem zu induzieren und auf Dauer zu erhalten, verfügt *H. schachtii* über Effektorproteine, die überwiegend in den Ösophagusdrüsen gebildet werden. Die Identifizierung und funktionelle Analyse dieser Effektoren sind wichtige Schritte auf dem Weg zu einem tieferen Verständnis des Parasitismus des Nematoden. Für unsere Analysen sequenzierten wir das Transkriptom von *H. schachtii* mit einem Illumina MiSeq Gerät. Anschließend wurde das zusammengesetzte Transkriptom mit den verfügbaren ESTs aus der Datenbank NEMABASE4 und Transkriptomen weiterer Nematoden verglichen, um neue Effektorseqzenzen zu identifizieren. Auf diese Weise wurden zunächst 484 mutmaßlich sekretierte Proteine (PSP) identifiziert. Eine weiterer Sequenzvergleich mit bekannten ESTs von *H. schachtii* führte zur Identifizierung bisher unbekannter PSPs. Die Annotation dieser neuen PSPs ergab eine Anreicherung bestimmter Genontologien wie z.B. metabolischen und katalytischen Aktivitäten und wachstumsregulierenden Funktionen. Es wurden zwei Gene für eine detaillierte funktionelle Analyse ausgewählt. Mit Hilfe einer Pfam-Domänenanalyse konnten *"HsPDI"*, das für eine protein disulfide-isomerase Domäne, sowie "*Hs-Tyr*", das für eine Tyrosinase-Domäne kodiert, identifiziert werden. Beide Domänen waren bisher noch nicht in Zusammenhang mit Nematoden-Effektoren gebracht worden.

Die Transkripte beider Gene konnten in den Ösophagusdrüsen von prä-infektiösen Nematodenlarven lokalisiert werden, wobei die Genexpression während der parasitischen Entwicklungsstadien aufreguliert ist. Das Stilllegen der beiden Gene mit Hilfe von RNAi hatte Auswirkungen auf die Nematodenentwicklung wie auch auf die Bildung des Nährzellensystems: sowohl Weibchen als auch Nährzellen waren kleiner als in unbehandelten Kontrollen. Im Gegensatz dazu führte die ektopische Expression der Gene in Arabidopsispflanzen zu einer erhöhten Anfälligkeit gegenüber *H. schachtii*. Die Stilllegung von *HsPDI* wirkte sich auf die Ultrastruktur der gebildeten Nährzellen in Form eines weniger dichten Zytoplasmas mit veränderten und degradierten Organellen aus. Wurden transgene Arabidopsispflanzen, die *HsPDI* exprimierten, mit dem Abwehr induzierenden Peptid flg22 behandelt, so reagierten diese mit starker ROS-Produktion, die H_2O_2-Konzentration war jedoch geringer als in nicht transgenen Kontrollpflanzen. Die Stilllegung von *HsPDI* in prä-infektösen Nematodenlarven führte zu deren erhöhter Sensitivität gegenüber einer Behandlung mit H_2O_2 im Vergleich zu einer Kontrollgruppe. Die fluoreszenzmikroskopische Analyse von Blättern von *Nicotiana benthamiana*, die *HsPDI*::GFP transient exprimierten, zeigte, dass *HsPDI* im Apoplasten lokalisiert ist. Die Ergebnisse der durchgeführten Experimente zeigen, dass *HsPDI* ein Effektor ist, der eine wichtige Rolle in der Interaktion zwischen Nematode und Wirtspflanze spielt, wobei es vermutlich als Radikalfänger für ROS pflanzlicher Herkunft fungiert.

Die ektopische Expression von *Hs-Tyr* in Arabidopsis hatte eine deutliche Auswirkung auf das Pflanzenwachstum: Das Sprosswachtum war verstärkt, die Wurzelarchitektur war verändert, wobei die Wurzellänge und das Wurzelgewicht unverändert blieben. Die transgenen Pflanzen wiesen darüber hinaus deutliche Veränderung der Homöostase verschiedener Hormone, vor allem Auxin, dem Jasmonatvorläufer *cis*OPDA und dem Ethylenvorläufer ACC auf. Die Konzentrationen von Jasmonsäure und Salizylsäure waren dagegen gleich bleibend. Die transgenen Pflanzen waren gegenüber *H. schachtii* anfälliger, nicht jedoch gegenüber dem Wurzelgallennematoden *Meloidogyne incognita*. Daraus läßt sich schließen, dass der Effektor physiologische Prozesse in Gang setzt, die spezifisch für den Parasitismus des Zystennematoden *H. schachtii* von Bedeutung sind. Die bisherigen Ergebnisse weisen darauf hin, dass *Hs-Tyr* dabei in einer noch unbekannten Weise in den Hormonhaushalt der Pflanze eingreift.

Die gewonnenen Erkenntnisse zeigen, dass die untersuchten PSPs spezifische Effekte auf die Interaktion zwischen Nematode und Pflanze haben und somit als Effektoren den Parasitismus des Nematoden unterstützen.

List of figures

List of tables

Chapter 1: An introduction to nematodes

1.1 Nematodes and their economic value

Nematodes are the most numerous and probably most diverse multicellular animals. They are prevalent and highly distributed in almost every ecological habitat (Hodda, 2011). All nematodes live in wet or liquid environment and need a film of water for being active. Some of them are restricted to specific geographical environmental conditions while others can be found all over the world. The distribution of nematodes is correlated with their dispersal, which can be active and slow by active movement, or passively by the help of environmental elements, human activities and vectors. So far, over 25,000 nematode species have been described including free living, animal parasitic and plant parasitic nematodes (PPNs) inhabiting a very broad range of environments (Hodda, 2011; Zhang, 2013). Approximately 50% of the nematodes are marine nematodes (Figure 1.1); they represent the majority of the phylum nematodes and are abundant in all oceans from intertidal zones to several thousand meters depth. Approximately 15% are animal-parasitic which infect invertebrates and vertebrates, including humans and domesticated animals. PPNs and free-living nematodes represent 10% and 25% of nematodes, respectively (Ayoub, 1980; Maggenti, 1981).

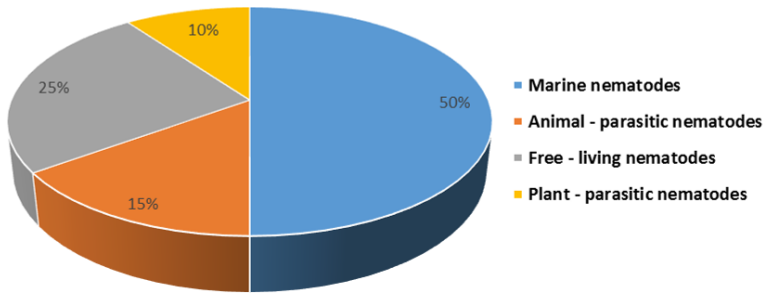

Figure 1.1 Nematodes distribution on different habitats (Ayoub, 1980; Maggenti, 1981)

Nematodes are pseudocoelomate cylindrical worms, which have a bilateral symmetric body containing simple organ systems including digestive, excretory, nervous and reproductive system (Figure 1.2). The nematode length varies from less than millimetres in some PPNs to several meters in some animal parasitic nematodes.

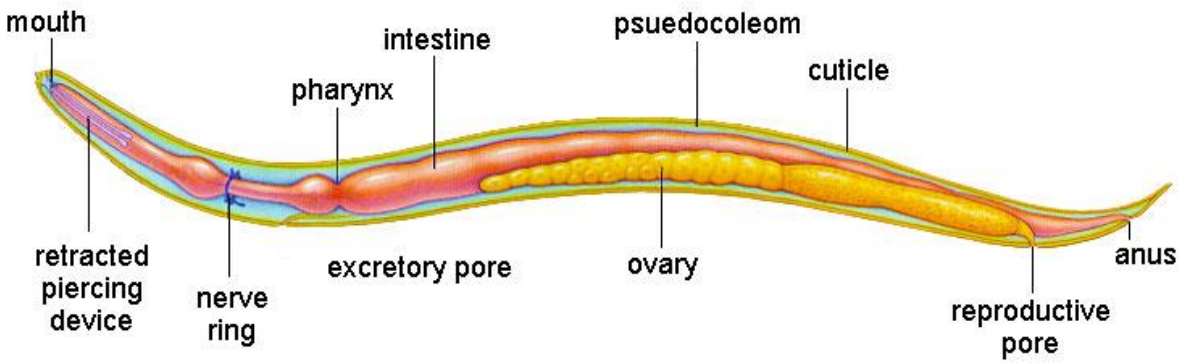

Figure 1.2 Nematode anatomy (Source: University of Illinois)

Nematodes come in the second place after arthropods, according to their direct or indirect impact on human's life (i.e. through affecting agriculture). Due to their damage and the economic impact, the majority of the identified and well-studied nematodes are animal- and plant-parasitic nematodes. Many studies were performed to estimate the socio-economic losses of the animal-parasitic nematodes and explained their damage either on animal's health or on the economy (McLeod, 1995; Murray & Lopez, 1996). Similarly for the PPNs, it has been estimated that the annual yield loss caused by them alone exceeds 100 Billion $, which makes them one of the most devastating group of agricultural pests (Koenning *et al.,* 1999). A study by Sasser and Freckman in 1987 showed the estimated percentage of losses for the most important crops affected by PPNs (Table 1.1). Losses reached up to 20 % in crops like okra and tomato.

Table 1.1 Worldwide estimated losses due to damage by plant parasitic nematodes (Sasser & Freckman, 1987)

Life crop sustaining	Annual loss (%)	Economically important crops	Annual loss (%)
Banana	19.7	Cacao	10.5
Barley	6.3	Citrus	14.2
Cassava	8.4	Coffee	15
Chickpea	13.7	Cotton	10.7
Coconut	17.1	Cowpea	15.1
Corn	10.2	Eggplant	16.9
Field bean	10.9	Forages	8.2
Millet	11.8	Grapes	12.5
Oat	4.2	Guava	10.8
Peanut	12	Melons	13.8
Pigeon pea	13.2	Misc. Other	17.3
Potato	12.2	Okra	20.4
Rice	10	Ornamentals	11.1
Rye	3.3	Papaya	15.1
Sorghum	6.9	Pepper	12.2
Soybean	10.6	Pineapple	14.9
Sugar beet	10.9	Tea	8.2
Sugar cane	15.3	Tobacco	14.7
Sweet potato	10.2	Tomato	20.6
Wheat	7	Yam	17.6
Average	**10.70%**	**Average**	**14.0%**
Overall average		**12.3 %**	

Most PPNs feed on plant roots, however, there are several species that target the vegetative part of the plant including stems, leaves, flowers and also seeds. Regarding the feeding habit, nematodes are classified to different groups. During feeding, PPNs either remain outside of the host roots (ectoparasites) or enter the host roots and feed internally on the root cells (endoparasites). In terms of mobility, nematodes are mobile during feeding and move from one cell to the other (migratory) or immobile during feeding, where they select specific cells and initiate morphological and physiological modifications and spend the rest of their life cycle feeding on these feeding structures (sedentary). The most devastating and economically damaging group of PPNs are sedentary endoparasites (Sasser & Freckman, 1987; Koenning *et al*., 1999; Chitwood, 2003; Jones *et al*., 2013). Cyst nematodes, as a group of endoparasitic nematodes, cause high yield losses and therefore have been studied intensively. Many studies focus on the interaction between cyst nematodes and their host plant and try to elucidate their biology. Cyst nematodes including the genera *Heterodera* spp. and *Globodera* spp. infect a wide range of host plants like vegetables, legumes and cereals. *H. schachtii* is a pest on sugar beet, but parasitizes more than 200 plant species in 23 different plant families. Most hosts belong to *Chenopodiaceae* and *Cruciferae* including some economically important crops.

Cyst nematode control is a challenging process. Many different management approaches were used to control *H. schachtii*. Crop rotation with non-host crop and trap crops for 3-5 years in addition to remove the host weeds is one of the most effective means to decrease the cyst nematode population in the soil. The usage of nematicides also effectively controls nematodes but is often restricted due to environmental concerns. Modern studies aim to find tolerant and resistant cultivars that can be integrated in the management programs. One way is the traditional screening of these cultivars, but novel technologies like TILLING (Targeting Induced Local Lesion In Genomes) and ecoTILLING are established and used increasingly. For successful selection, understanding cyst nematode biology and the interaction with the host plant is needed.

1.2 Nematode biology and life cycle of *H. schachtii*

The hibernating cysts in the soil, which contain the eggs, are considered as the first inoculum. In presence of the host plant, J2s start to hatch from eggs and move in the soil towards the plant roots (Masamune *et al.,* 1982). Once the J2s reach the roots, they

tend to enter them at the elongation region with continuous piercing of the spear-like stylet and the help of specific secretions. After penetration, the J2s migrate through the root, destroy the cells and cause severe damage to the host. Then they select a procambial cell in the vascular cylinder as Initial Syncytial Cell (ISC) (Wyss & Zunke, 1986; Wyss, 1992). The migratory J2s use their hollow stylet to inject a cocktail of secretions into the ISC. Secretions initiate cellular changes including cell wall degradation, fusion of the neighbouring cells with the ISC, hypertrophy of the affected cells and an increased activity of the cytoplasm. The parasitic J2s become immobile, feed on the syncytia, and undergo a series of three molts until reaching the adulthood as females or males. Females continue feeding, increase in size and rupture the root tissues to be exposed to males for mating. Vermiform males leave the roots, search for females and copulate (Figure 1.3). Afterwards females start to accumulate the fertilized eggs inside their reproductive system and later die retaining the eggs within their bodies. The dead female´s cuticle hardens to form the cyst. The cyst acts as a capsule protecting the eggs inside against the harsh soil environment to proceed the life cycle. Some J2s will hatch in the same season to do more than one infection cycle, while the rest stay dormant for the next season or until the availability of the host again. Some reports mentioned that eggs within cysts can stay dormant and viable up to 10 years. This is one of the factors that make the eradication of the cyst nematodes from the soil hard (Lilley *et al.*, 2005).

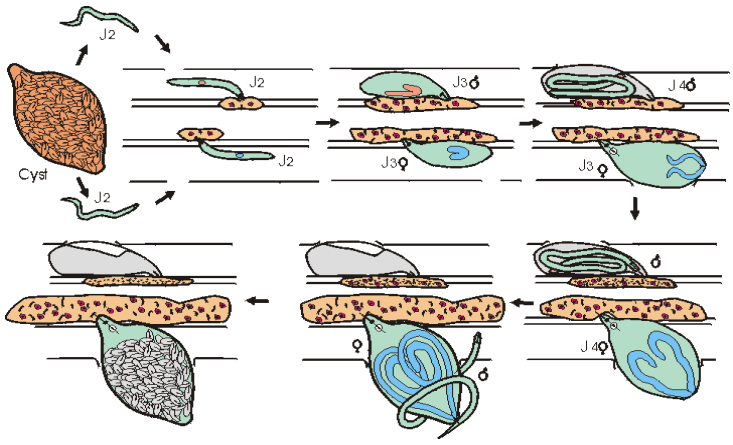

Figure 1.3 The life cycle of *H. schachtii*. Representing the eggs inside the cyst, the successive molts of the juveniles (J2 = second stage juvenile, J3 = third stage juvenile, and J4 = fourth stage juvenile) and the adult stages associated with the syncytium.

1.3 Syncytium morphology and physiology

The syncytium is the only food source for the nematode through the life cycle. For that reason, initiating and maintaining the syncytium are critical processes for nematode survival.

The J2s initiate the syncytia by injecting effectors into the ISC. As a result, the ISCs start to increase massively in size, local cell wall openings are formed to neighbouring cells and the cytoplasm of the cells fuse to form a complex of multinuclear syncytium. At the same time, the cell wall which surround the syncytia becomes thick in order to compensate the increased osmotic pressure (Jones & Northcote, 1972; Jones, 1981). In the advanced stages of syncytium formation, the cytoplasm becomes dense and metabolically active with increasing the size of the nuclei. At the same time the vacuoles break down to form small vesicles, mitochondria proliferate and smooth endoplasmic reticulum becomes prominent (Wyss & Grundler, 1992; Golinowski *et al.*, 1996; Sobczak *et al.*, 1997). The syncytium size increment is associated with nematode development. Once the female has completed its life cycle it dies and becomes a cyst. The associated syncytia remain functional as long as the nematodes feed. On *A. thaliana*, the *H. schachtii* life cycle takes around 6 weeks (Sobczak & Golinowski, 2011). The male's syncytium is smaller in size and is active only until the end of the J3 stage. J4 do not take up nutrients, but soon molt and develop to adult male. These males leave the root and search for females to mate with.

Several studies reported many changes at the molecular level of the infected compared with the uninfected roots showing that the syncytial formation process massively orchestrate the plant activities to serve nematode survival. The syncytial transcriptome gene ontology (GO) analysis of the five and 15 days after infection showed that most of the up-regulated genes are probably involved in the degradation of cell walls and belong to the pectate lyase and expansin families, whereas the down regulated genes belong to the peroxidase family (Szakasits *et al.*, 2009). They also showed that a high number of these differentially expressed genes are involved in metabolic activities and defence response. In another study, it was shown that nematodes are able to remodel plant metabolites to facilitate parasitism. GC–MS analysis of syncytia detect the accumulation of several amino acids and phosphorylated metabolites and other types of sugars such as 1-kestose that normally does not accumulate in the roots. This shows that nematode reorganize the plant metabolites and nutrients to their favour (Hofmann *et al.*, 2010).

1.4 Effectors secreted by the beet cyst nematode *H. schachtii*

Effectors are proteins expressed by plant pathogens including bacteria, fungi and nematodes to aid infection of specific plant species. These molecules can alter plant processes in favor of the pathogen to facilitate infection. In nematode, from the beginning of the parasitism until the end of the life cycle, they produce effectors proteins released into the host cells to facilitate parasitism. They are thought to be released through amphids, phasmids, rectal gland, hypoderm and the esophageal glands (Figure 1.4). It is believed that the majority of the effectors which are involved in the nematode-plant interaction are secreted in the three esophageal glands. These secretions then are injected into the host plant cells through the hollow mouth stylet in the cytoplasm to interact with plant proteins or translocate it to other cell compartments (Figure 1.5) (Jaouannet & Rosso, 2013)

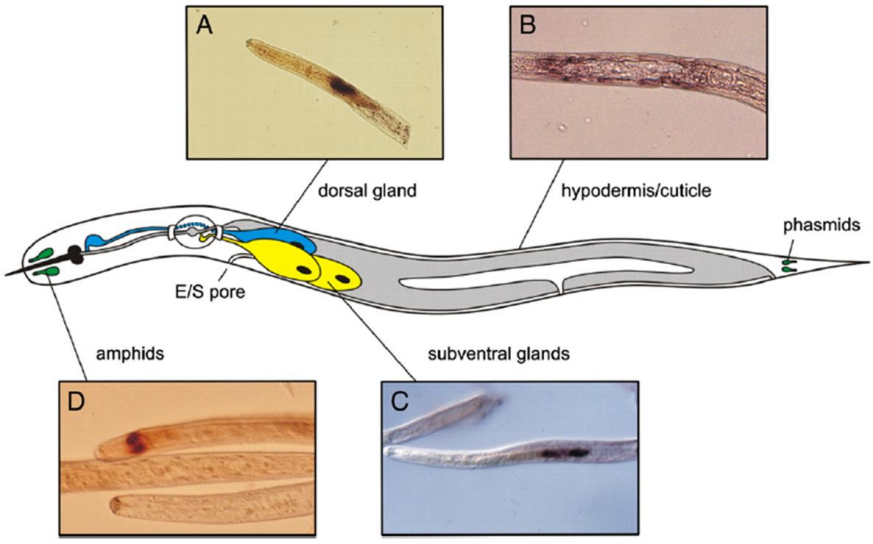

Figure 1.4 Schematic overview of a typical plant-parasitic nematode showing its most important secretory organs including: (A) Dorsal gland (B) Hypodermis (C) Amphids (D) Subventral glands (Haegeman *et al.*, 2012).

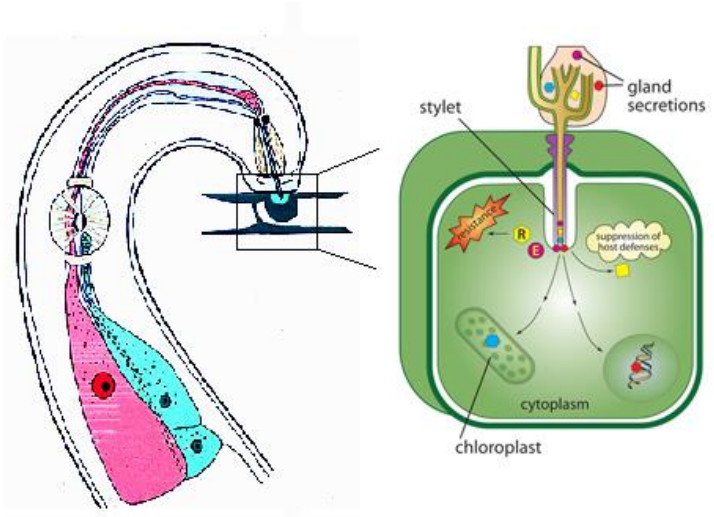

Figure 1.5 Schematic overview of the stylet and effector proteins injection in the plant cell. Adapted from Torto-Alalibo *et al.* (2009) and Wyss (Nemapix).

Currently, many studies were performed aiming to mine *H. schachtii* effectors. Several effectors were identified and structurally studied. However, these studies indicate that many effectors are still unknown. So far, the identified effectors can be grouped regarding to their function to the following categories: cell wall modifying, defense suppressing, and growth enhancing.

Cell wall-modifying effectors

The first stage of nematode parasitism is the plant roots penetration, followed by migration within the roots. During migration, nematode movement is restricted due to the presence of the strong cell wall. Plant cell walls consist of a cross-linked matrix of hemicelluloses and pectins, embedding a framework of cellulose fibres. To overcome this physical barrier, nematodes produce and release a cocktail of cell wall modifying enzymes with the help of the stylet to disintegrate and depolymerize its components. One of the first isolated cell wall modifying effectors belongs to the glycosyl hydrolase family 5 (GHF5) cellulases. These enzymes target and degrade the celluloses and hemicelluloses during the migratory stages (Haegeman *et al.*, 2012). Additionally, pectate lyase has been isolated from *H. schachtii* which cleaves the internal alpha-1,4-linked galacturonic acid glycosidic bond of pectate to facilitate the nematode movement (Vanholme *et al.*, 2007). Cell wall modifying effectors are well proven to be involved in the cell wall manipulation to form the syncytia. Cellulose binding protein (CBP) was identified from *H. schachtii*. It has been shown that Hs-CBP is expressed in the early stages of the syncytia formation but not in the migratory stages. It was also found that

expressing the Hs-CBP in Arabidopsis increasing the susceptibly to *H. schachtii* and the activity of the plant pectin methylesterase protein 3 (PME3).

PMEs was suggested to contribute to cell growth by regulating the mechanical and chemical properties of plant cell walls via demethylesterification of pectin (Micheli, 2001). This interaction seems to be important in the reduction of cell wall pectin methylesterification through CBP-mediated increased and targeted PME3 activity which allows improved access of other cell wall–modifying enzymes to cell wall polymers, thereby accelerating enzymatic activities, which is a requirement for syncytium development (Hewezi *et al.,* 2008).

Defense suppressing effectors

Plants and pathogens adopted many strategies to interact with each other. Since plants are surrounded by many organisms, they have evolved a multi-layer immune system to ensure recognition of pathogens and defense. The first line of defense is established by extracellular immune receptors that recognize pathogen associated molecular patterns (PAMPS) from diverse pathogens. The recognition of pathogens leads to PAMP-triggered immunity (PTI) which generate plant defense including cell modification and releasing reactive oxygen species (ROS). The second layer is activated once a pathogen overcomes the first layer. This may induce changes in molecular status of host resistance proteins (immune receptors) or in the host proteins that are being monitored by the immune receptors, so-called R proteins. This interaction usually activates a specific type of programmed cell death, known as hypersensitive response (HR) (Holbein *et al.,* 2016).

On the other hand, nematodes as successful biotrophic pathogens developed effectors that overcome or suppress plant immunity. In the last few years many nematode effectors involved in plant defense suppression were identified.

For example, Hs-10A06 binds to and activates the plant spermidine synthase (SPDS2), a key enzyme involved in polyamine biosynthesis. This interaction elevates the polyamine oxidase (PAO) activity, therefore stimulates the induction of the plant antioxidant machinery, which likely protects the nematode feeding structure and the nematode from ROS that are triggered during infection. Furthermore, the expression of the Hs-10A06 in Arabidopsis increased plant growth and susceptibility to *H. schachtii, Pseudomonas syringae* pv *tomato* (Pst DC300) and the yellow strain of Cucumber mosaic virus (Hewezi *et al.,* 2010).

The effector Hs-30C20 has been shown by yeast two hybrid to interact specifically and target the host plant β-1,3-endoglucanase (AT4G16260). It was also shown that this effector suppresses the β-1,3-endoglucanase activity to promote parasitism. Expression levels of the plant β-1,3-endoglucanase have a peak at 3-5 days after nematode infection. Hs-30C20 expression was found to be highest at the same time. The constitutive expression of the Hs-30C20 in Arabidopsis increased the plant suceptability to *H. schachtii* while overexpressing the β-1,3-endoglucanase decreased it (Hamamouch *et al.*, 2012).

As another example, *H. schachtii* Venom-Allergen like Protein (VAP1) was shown to be responsible for inhibiting plant immunity during nematode parasitism (Lozano-Torres *et al.*, 2014). Ectopically expressing the Hs-VAP1 in Arabidopsis increased plant susceptibility and showed loss of basal immunity to different unrelated pathogens including fungi, bacteria and nematode.

The *H. schachtii* effector (4F01) interacts specifically with the plant oxidoreductase a member of 2OG-Fe(II) oxygenase family and complement the Arabidopsis annexin. This interaction suggests that Hs-4F01 mimicks the plant annexin function in regulating plant defense and stress responses during infection (Patel *et al.*, 2010).

Growth enhancing effectors

Nematode established a cascade of effectors, which orchestrate plant gene expression and thus interfere with the plant growth to support the nematode development. These effectors either mimic plant hormones or interfere in hormone signaling pathways. Recently, cytokinins were revealed to be important signaling elements in nematode parasitism. It was presented that cytokinin signaling is activated not only in the syncytium but also in neighboring cells to be incorporated into the syncytium. It was shown that plant mutants deficient in cytokinin or cytokinin signaling resulted in less infection and smaller size of the females compared with control. In fact, Siddique *et al.* (2015) showed that juveniles of *H. schachtii* are able to synthesize a functional cytokinin in their esophageal glands and inject it into the plant in order to activate the cell cycle of affected root cells. It was also shown that silencing the key gene of cytokinine synthesis in the nematode affects syncytium formation and nematode performance (Siddique *et al.*, 2015).

H. schachtii is also shown to produce CLE-like effectors HsCLE1 and HsCLE2 which show high similarity to the Arabidopsis CLEs 1–7. Plant CLEs have been shown to

bind to extracellular receptors and activate signaling cascades regulating plant growth and development, including shoot and floral meristem maintenance (Brand *et al*., 2000; Clark *et al.,* 1995; Rojo *et al*., 2002). Secreting these peptides into the plant has been shown to functionally mimick plant CLE and to enable nematodes to manipulate root growth in favour of syncytium formation (Wang *et al.*, 2011).

1.5 Identification of nematode effectors

The identification and characterization of nematode effectors and their function is the key factor to understand plant-nematode interactions. A number of different techniques have been used to identify nematode effectors. The production of monoclonal antibodies (MAbs) directed against nematode secretions or fractionated homogenate of nematodes was a great method for nematode effectors identification. Several nematode β-1-4-endoglucanases were identified in *Globodera rostochiensis* by using MAbs raised against fractionated homogenate of pre-parasitic J2s (Smant *et al.,* 1998; Rehman *et al*., 2009). Additionally, mRNA finger-printing by complementary DNA- amplified fragment length polymorphism (cDNA-AFLP) allowed a comprehensive analysis of differentially expressed mRNAs isolated from various stages of *G. rostochiensis* (Qin *et al.*, 2000). In the last decades a major step was the availability of Expressed Sequence Tags (ESTs). ESTs are short sub-sequences of a cloned cDNA library either for the whole nematode mRNA or specific organs. The presence of other useful tools like NemaGene, NemaBlast, NemaBrowse, NemaSNP and NemaPath supported the annotation of the resulted ESTs and accelerated the selection and identification of effector proteins. The number of available *H. schachtii* ESTs reached 2,182 as reviewed by Rehman *et al.* (2016). Vanholme *et al*. (2006) generated a first set of ESTs of *H. schachtii* to identify the putative secretory proteins (PSP). By using different bioinformatic filters they ended up identifying 50 PSPs. They also showed that many of these ESTs match with different identified homologs of effector proteins from other nematodes. These results show that using the ESTs is a useful tool to identify the nematode PSPs. On the other hand, next generation sequencing technology (NGS) enhanced the coverage of the extracted sequences and facilitated the PSP mining procedure. The sequencing of nematode transcriptomes including *H. schachtii* led to the identification of further novel effectors (Maier *et al.*, 2013; Bauters *et al.*, 2014; Haegemann *et al.*, 2013; Fosu-Nyarko *et al.*, 2016). As a principal basis of effector identification, the target sequences should meet specific criteria. The first feature is the

presence of N-terminal sequence representing a signal peptide and the lack of any transmembrane domain. This feature indicates that the associated protein can be secreted. This feature is still used in many studies as a first step of candidate gene selection. Furthermore, the localization of the candidate genes in the nematode secretory organs increases their probability of being an effector. For that reason, most of the effectors studies are targeting those genes, which were mainly localized in the nematode esophageal glands. Due to that reason, the *in situ* hybridization of the candidate genes with digoxygenin labeled antisense riboprobes was used to localize the associated mRNA in the nematode organs (Vanholme *et al.,* 2002). Additionally, the increase of the expression level of the candidate genes in a pattern associated with the parasitic stages leading to suggest that the function(s) of these candidate genes could be important for parasitism. Moreover, hindering nematode infection, parasitism, and survival on the host plant by gene-silencing through RNA interference is another approach to study the importance of the gene for the nematode-plant interaction. RNAi has been used in many studies aiming to identify effectors and functionally studying their role in parasitism (Lozano-Torres *et al.,* 2014; Lin *et al.,* 2016). These collective positive results are confirming the function of a candidate gene as an effector and were used successfully in effector identification.

1.6 *Arabidopsis thaliana* as a model plant to study effectors

Arabidopsis belongs to the family *Brassicaceae* and has a relatively short life cycle of approximately six weeks (Figure 1.6). It has a small sequenced genome of 135 MB and 5 chromosomes (Bennett *et al.*, 2003). It was the first plant species to be fully sequenced (The Arabidopsis Genome Initiative, 2000). The genome of *A. thaliana* is maintained, continuously curated and updated, and available for downloading by the Arabidopsis Information Resource (TAIR). Around 27,000 genes were identified which encode 35,000 proteins (The Arabidopsis Genome Initiative, 2000). These traits had nominated Arabidopsis to be used as ideal model plant to study plants on different levels including genetic evolution, population genetics, and plant development. Furthermore, Arabidopsis has been widely used to study plant–pathogen interactions. The fact that Arabidopsis is a host for many pathogens including insects, bacteria, fungi and nematodes facilitate and speed the understanding of the plant-pathogen interaction. Furthermore, the fast and simple generation of mutation and transgenic plant enable straight forward gene function studies. In 1991, Arabidopsis was established as a model

host plant for *H. schachtii* in order to study the molecular basis of the host-pathogen interaction (Sijmons *et al.*, 1991).

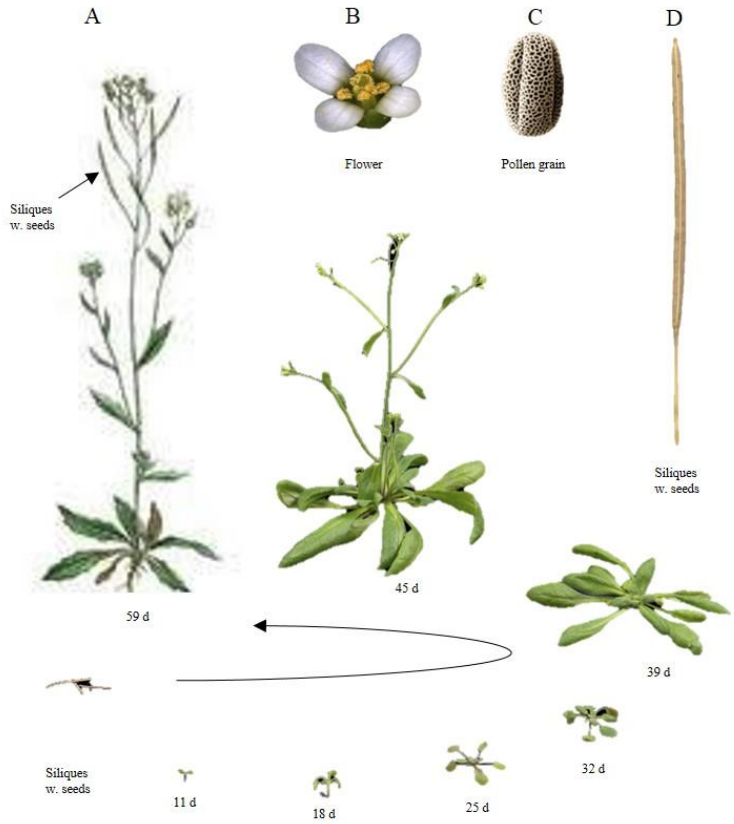

Figure 1.6 The life cycle of the model plant *Arabidopsis thaliana*. (A) Different stages of its life cycle, from seed (bottom left) to seedling (11 days), to vegetative growth (39 days), and to reproductive growth (45 days). (B) Flower, (C) Pollen grain and (D) mature siliques. Image credits: Modified from B and C, Maria Bernal and Peter Huijser; other photographs, Ines Kubigsteltig and Klaus Hagemann. Doi: http://dx.doi.org/10.7554/eLife.0600.002.

1.7 Transcriptome analysis and PSP selection

The total RNA from *H. schachtii* second stage juveniles was extracted using the Mirvana Kit following the manufacturer instructions (Applied Biosystems). Two independent RNA extractions, with RIN (RNA Integrity Number) value greater than 9.0, were used, independently, for 100 paired-end sequencing. The RNA was fragmented using divalent cations under elevated temperature. The cDNA preparation was performed for each run separately, fragmented with an average length of 330 bp

and sequenced via Illumina MiSeq. Sequencing resulted in a collection of 148,125,233 of 100bp paired-end reads.

Reads were trimmed for quality, ambiguity and adapter sequences, then duplicates were removed and transcript assembly was performed using CLC genomic workbench (version 5.1). Trimming for quality was performed excluding low quality bases (>0.05). Ambiguous nucleotides (>2n) and adapter sequences were removed. The *de novo* assembly of all reads that passed quality filtering was computed with following parameters: word size 24, similarity = 0.8, length fraction = 0.5, insertion cost = 3, deletion cost = 2, and mismatch = 2. In total, 115,027,834 reads with an average length of 96.8bp were assembled to form the transcriptome. It was built from 66,886 contigs with an average length of 427bp and a total size of 28 MB.

All contigs were translated based on their longest open reading frame ORF and the orientation of their best hit using BLAST2GO and EMBOSS Transeq tools. All contigs were annotated by using Swissprot (BBH), TrEMBL (BBH), and *C. elegans* (BBH) (Boeckmann et al. 2003) and the results were used as an input for the Automated Human Readable tool (AHRD). Furthermore, BLAST2GO (version 2.6.0) was used to assign gene ontology (GO) terms and for Gene Set Enrichment Analysis (GSEA). Gene ontology analysis and function enrichment showed the number of sequences and their associated activities (Figure 1.7). Proteins with signal peptide (SignalP) and transmembrane domains (TMHMM) were selected as *H. schachtii* secretome. In this way, 1081 putative secretory proteins (PSP) were identified. The PSPs were compared with all ESTs available in Nembase4 database. ESTs were categorized to 4 groups, plant-, animal-parasitic nematodes, free-living nematodes and entomopathogenic nematodes. The comparison resulted in the identification of 484 plant specific PSPs. A BLAST was performed on the P-PSP with 64 sequences from *H. schachtii* and 81 sequences from *H. glycines* which are predicted to be PSPs (e-value <e-10). This comparison showed a subset of sequences predicted to be pioneer effectors. Figure 1.8 shows the filtering work flow of the transcriptome to identify the candidate PSPs.

Figure 1.7 Gene ontology annotation of *H. schachtii* transcriptome. (A) Molecular functions, (B) Biological processes and (C) Cellular components.

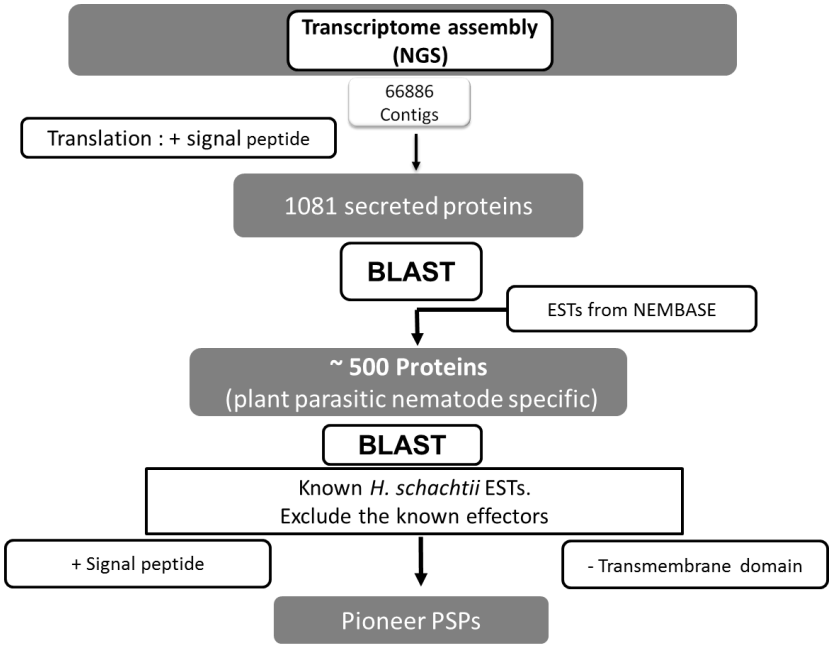

Figure 1.8 Schematic overview of the PSPs selection procedure.

Since the field of nematode effectors is relatively new, not much is known about the *H. schachtii* effectors. In this studies we use the well-established aseptic growing system of Arabidopsis on Knop medium, to perform detailed analyses. We used the resulted data set and performed functional analysis to identify novel effector proteins involved in nematode parasitism.

1.8 References

Ayoub SM. 1980. Plant Nematology An Agricultural Training Aid. NemaAid Publication, Sacramento, CA. 195 pages.

Bauters L, Haegeman A, Kyndt T, Gheysen G. 2014. Analysis of the transcriptome of *Hirschmanniella oryzae* to explore potential survival strategies and host-nematode interactions. *Molecular Plant Pathology* **15**: 352-363.

Bennett MD, Leitch IJ, Price HJ, Johnston JS. 2003. Comparisons with Caenorhabditis (100 Mb) and Drosophila (175 Mb) Using Flow Cytometry Show Genome Size in Arabidopsis to be 157 Mb and thus 25% Larger than the Arabidopsis Genome Initiative Estimate of 125 Mb. *Annals of Botany* **91**(5): 547-557. doi: 10.1093/aob/mcg057. PMID 1264649.

Boeckmann B, Bairoch A, Apweiler R, Blatter MC, Estreicher A, Gasteiger E, Martin MJ, Michoud K, O'Donovan C, Phan I, Pilbout S, Schneider M. 2003. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. *Nucleic Acids Research* **31**(1): 365-370.

Brand U, Fletcher JC, Hobe M, Meyerowitz EM, Simon R. 2000. Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. *Science* **289**: 617-619.

Chitwood DJ. 2003. Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture - Agricultural Research Service. *Pest Management Science* **59**(6-7): 748-753.

Clark SE, Running MP, Meyerowitz EM. 1995. CLAVATA3 is a specific regulator of shoot and floral meristem development affecting the same processes as CLAVATA1. *Development* **121**: 2057-2067.

Fosu-Nyarko J, Nicol P, Naz F, Gill R, Jones MGK. 2016. Analysis of the Transcriptome of the Infective Stage of the Beet Cyst Nematode, *H. schachtii*. *PLoS ONE* **11**(1): e0147511. doi:10.1371/journal.pone.0147511.

Golinowski W, Grundler FMW, Sobczak M. 1996. Changes in the structure of *Arabidopsis thaliana* during female development of the plant-parasitic nematode *Heterodera schachtii. Protoplasma* **194**: 103-116.

Haegeman A, Bauters L, Kyndt T, Rahman MM, Gheysen G. 2013. Identification of candidate effector genes in the transcriptome of the rice root knot nematode *Meloidogyne graminicola*. *Molecular Plant Pathology* **14**(4): 379-390.

Haegeman A, Mantelin S, Jones JT, Gheysen G. 2012. Functional roles of effectors of plant –parasitic nematodes. *Gene* **492**: 19-31.

Hamamouch N, Li C, Hewezi T, Baum TJ, Mitchum MG, Hussey RS, Vodkin LO, Davis EL. 2012. The interaction of the novel 30C02 cyst nematode effector protein with a plant β-1,3-endoglucanase may suppress host defence to promote parasitism. *Journal of Experimental Botany* **63**: 3683-3696.

Hewezi T, Howe P, Maier TR, Hussey RS, Mitchum MG, Davis EL, Baum TJ.

2008. Cellulose Binding Protein from the Parasitic Nematode *Heterodera schachtii* Interacts with Arabidopsis Pectin Methylesterase: Cooperative Cell Wall Modification during Parasitism. *Plant Cell* **20**: 3080-3093.

Hewezi T, Howe P, Maier TR, Hussey RS, Mitchum MG, Davis EL, Baum TJ. 2010. Arabidopsis spermidine synthase is targeted by an effector protein of the cyst nematode *Heterodera schachtii*. *Plant Physiology* **152**: 968-984.

Hodda M. 2011. "Phylum Nematoda Cobb, 1932. In: Zhang, Z.-Q. (Ed.) Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness". *Zootaxa* **3148**: 63-95.

Hofmann J, El Ashry A, Anwar S, Erban A, Kopka J, Grundler FMW. 2010. Metabolic profiling reveals local and systemic responses of host plants to nematode parasitism. *Plant Journal* **62**(6):1058-1071.

Holbein J, Grundler FMW, Siddique S. 2016. Plant basal resistance to nematodes: An update. *Journal of Experimental Botany.* doi: 10.1093/jxb/erw005.

Jaouannet M, Rosso MN. 2013. Effectors of root sedentary nematodes target diverse plant cell compartments to manipulate plant functions and promote infection. *Plant Signaling & Behavior* **8**: 9, e25507, DOI: 10.4161/psb.25507.

Jones JT, Haegeman A, Danchin EG, Gaur HS, Helder J, Jones MG, Kikuchi T, Manzanilla-Lopez R, Palomares-Rius JE, Wesemael WM, Perry RN. 2013. Top 10 plant-parasitic nematodes in molecular plant pathology. *Molecular Plant Pathology* **14**: 946-96.

Jones MG, Northcote DH. 1972. Nematode-induced syncytium a multinucleate transfer cell. *Journal of Cell Science* **10**(3): 789-809.

Jones MGK. 1981. Host-cell responses to endo-parasitic nematode attack - structure and function of giant-cells and syncytia. *Annals of Applied Biology* **97**(3): 353-372.

Koenning SR, Overstreet C, Noling JW, Donald PA, Becker JO, Fortnum BA. 1999. Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994. *Journal of Nematology* **31**(4): 587-618.

Lilley CJ, Atkinson HJ, Urwin PE. 2005. Molecular aspects of cyst nematodes. *Molecular Plant Pathology* **6**(6): 577-588.

Lin B, Zhuo K, Chen S, Hu L, Sun L, Wang X, Zhang L, Liao J. 2016. A novel nematode effector suppresses plant immunity by activating host reactive oxygen species-scavenging system. *New Phytologist* **209**(3): 1159-1173.

Lozano-Torres JL, Wilbers RHP, Warmerdam S, Finkers-Tomczak A, Diaz-Granados A, van Schaik CC, Helder J, Bakker J, Goverse A, Schots A, Smant G. 2014. Apoplastic Venom Allergen-like Proteins of Cyst Nematodes Modulate the Activation of Basal Plant Innate Immunity by Cell Surface Receptors. *PLoS Pathogens* **10**: e1004569.

Maggenti AR. 1981. General Nematology. Springer-Verlag, New York. 372 pages.

Maier TR, Hewezi T, Peng J, Baum TJ. 2013. Isolation of Whole Esophageal Gland Cells from Plant-Parasitic Nematodes for Transcriptome Analyses and Effector Identification. *Molecular Plant-Microbe Interaction* **26**: 31-35.

Masamune T, Anetai A, Mitsuo T, Nobukatsu K. 1982. Isolation of a natural hatching stimulus, glycinoeclepin-a, for the soybean cyst nematode. *Nature* **297**(5866): 495-496.

McLeod RS. 1995. Costs of major parasites to the Australian livestock industries. *International Journal for Parasitology* **25**: 1363-1367.

Micheli F. 2001. Pectin methylesterases: Cell wall enzymes with important roles in plant physiology. *Trends in Plant Science* **6**: 414-419.

Murray CJL, Lopez AD. 1996. Global Burden of Disease. Harvard University Press, Boston, USA.

Patel N, Hamamouch N, Li C, Hewezi T, Hussey RS, Baum TJ, Mitchum MG, Davis EL. 2010. A nematode effector protein similar to annexins in host plants. *Journal of Expermintal Botany* **61**: 235-248.

Qin L, Overmars B, Helder J, Popeijus H, van der Voort J, Groenink W, van Koert P, Schots A, Bakker J, Smant G. 2000. An efficient cDNA-AFLP-based strategy for the identification of putative pathogenicity factors from the potato cyst

nematode *Globodera rostochiensis*. *Molecular Plant-Microbe Interaction* **13**: 830-836.

Rehman S, Butterbach P, Popeijus H, Overmars H, Davis EL, Jones JT, Goverse A, Bakker J, Smant G. 2009. Identification and Characterization of the Most Abundant Cellulases in Stylet Secretions from *Globodera rostochiensis***.** *Phytopathology* **99**(2): 194-202.

Rehman S, Gupta VK, Goyal AK. 2016. Identification and functional analysis of secreted effectors from phytoparasitic nematodes. . *BMC Microbiology*. DOI 10.1186/s12866-016-0632-8.

Rojo E, Sharma VK, Kovaleva V, Raikhel NV, Fletcher JC. 2002. CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem cell signaling pathway. *Plant Cell* **14**: 969-977.

Sasser JN, Freckman DW. 1987. A world perspective on nematology: the role of the society. Pp 7-14 in J.A. Veech and D.W. Dickson (eds) Vistas on Nematology. Society of Nematologists, Hyattsville, Maryland. 509p

Siddique S, Radakovic ZS, De La Torre CM, Chronis D, Novák O, Ramireddy E, Holbein J, Matera C, Hütten M, Gutbrod P, Anjam MS, Rozanska E, Habash S, Elashry A, Sobczak M, Kakimoto T, Strnad M, Schmülling T, Mitchum MG, Grundler FM. 2015. A parasitic nematode releases cytokinin that controls cell division and orchestrates feeding site formation in host plants. *Proceedings of the National Academy of Sciences* **112**(41) : 12669-12674.

Sijmons PC, Grundler FMW, von Mende N, Burrows PR, Wyss U. 1991. *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *Plant Journal* **1**: 245-254.

Smant G, Stokkermans JPWG, Yan Y, de Boer JM, Baum TJ, Wang X, Hussey RS, Gommers FJ, Henrissat B, Davis EL, Helder J, Schots A, Bakker J. 1998. Endogenous cellulases in animals: Isolation of β-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes. *Proceedings of the National Academy of Sciences* **95**(9): 4906-4911.

Sobczak M, Golinowski W. 2011. Cyst nematodes and syncytia. In: Jones J,Gheysen G, Fenoll C, eds. Genomics and molecular genetics of plant–nematode interactions.

Berlin, Germany: Springer, 61-82.

Sobczak M, Golinowski W, Grundler FMW. 1997. Changes in the structure of *Arabidopsis thaliana* roots induced during development of males of the plant parasitic nematode *Heterodera schachtii*. *European Journal of Plant Pathology* **103**: 113-124.

Szakasits D, Heinen P, Wieczorek K, Hofmann J, Wagner F, Kreil DP, Sykacek P, Grundler FMW, Bohlmann H. 2009. The transcriptome of syncytia induced by the cyst nematode *Heterodera schachtii* in Arabidopsis roots. *Journal of Experimental Botany* **60:** 771-784.

The Arabidopsis Genome Inatiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**: 796-815.

Torto-Alalibo T, Collmer CW, Lindeberg M, Bird D, Collmer A, Tyler BM. 2009. Common and contrasting themes in host cell-targeted effectors from bacterial, fungal, oomycete and nematode plant symbionts described using the Gene Ontology. *BMC Microbiology* **9** (Suppl 1):S3. doi:10.1186/1471-2180-9-S1-S3.

Vanholme B, De Meutter J, Tytgat T, Gheysen GD, Vanhoutte I, Gheysen GD. 2002. An improved method for whole-mount in situ hybridization of *Heterodera schachtii* juveniles. *Parasitology Reserch* **88**(8): 731-733.

Vanholme B, Mitreva M, Van Criekinge W, Logghe M, Bird D, McCarter JP, Gheysen G. 2006. Detection of putative secreted proteins in the plant parasitic nematode *Heterodera schachtii*. *Parasitology Research* **98**(5): 414-424.

Vanholme B, Van Thuyne, Vanhouteghem K, De Meutter J, Cannoot B, Gheysen G. 2007. Molecular characterization and functional importance of pectate lyase secreted by the cyst nematode *Heterodora schachtii*. *Molecular Plant pathology* **8**: 267-278.

Wang J, Replogle A, Hussey RS, Baum TJ, Wang X, Davis EL, Mitchum MG. 2011. Identification of potential host plant mimics of CLAVATA3/ESR (CLE)-like peptides from the plant-parasitic nematode *Heterodera schachtii*. *Molecular Plant Pathology* **12:** 177-186.

Wyss U. **1992**. Observations on the feeding behaviour of *Heterodera schachtii* throughout development, including events during moulting. *Fundamental and Applied*

Nematology **15**: 75-89.

Wyss U, Grundler FMW. 1992. Feeding behaviour of sedentary plant parasitic nematodes. *Netherlands journal of plant pathology* **2**: 165-173.

Wyss U, Zunke U. 1986. Observations on the behaviour of second stage juveniles of *Heterodera schachtii* inside host roots. *Revue Nematol* **9**: 153-166.

Zhang Z. 2013. "Animal biodiversity: An update of classification and diversity in 2013. In: Zhang, Z.-Q. (Ed.) Animal Biodiversity: An Outline of Higher-level Classification and Survey of Taxonomic Richness (Addenda 2013)". *Zootaxa* **3703**(1):5–11.

Chapter 2: Identification and characterization of a putative protein disulfide isomerase (HsPDI) as a novel effector of *Heterodera schachtii*

Samer S. Habash, Miroslaw Sobczak, Shahid Siddique, Boris Voigt, Florian M.W. Grundler, Abdelnaser Elashry

2.1 Summary

- The plant-parasitic cyst nematode *Heterodera schachtii* is an obligate biotroph that induces long-term syncytial feeding sites in roots of its host plants. The nematodes produce effector proteins that are secreted into the host and facilitate infection process. Here we identified *H. schachtii* protein disulphide isomerase (HsPDI) as an effector that interferes with the host's redox status.
- *In situ* hybridization showed that HsPDI is specifically localized within esophageal glands of pre-parasitic second stage juveniles (J2). HsPDI is up-regulated in the early parasitic J2s. Silencing of HsPDI expression by RNA interference in the J2s hampers their development and leads to structural malfunctions in associated feeding sites induced in Arabidopsis roots.
- Expression of HsPDI in Arabidopsis increases plant's susceptibility towards *H. schachtii*. HsPDI expression is up-regulated in the presence of exogenous H_2O_2, whereas HsPDI silencing results in increased mortality under H_2O_2 stress.
- Stable expression of HsPDI in Arabidopsis plants decreases ROS burst induced by flg22. Transiently expressed HsPDI in *N. benthamiana* leaves is localized in the apoplast.
- HsPDI plays an important role in the interaction between nematode and plant, probably through inducing local changes in the redox status of infected host tisse. It also contributes to protecting the nematode from exogenous H_2O_2 stress.

Key words: *Arabidopsis thaliana*, *Heterodera schachtii*, effector, Protein disulphide isomerase (PDI), Reactive oxygen species (ROS), Plant-nematode interaction.

2.2 Introduction

Plant-parasitic cyst nematodes are obligate biotrophs that induce and maintain intimate and long-term feeding relationships with their host plants. Second stage juveniles (J2s) of cyst nematodes hatch from eggs and invade the roots primarily in the elongation zone. After entering the roots, nematodes pierce single cells with their stylet, penetrate them and migrate through various tissue layers until they reach the vascular cylinder. Nematode migration inside the root is aided by releasing cell wall-degrading enzymes via the mouth stylet. Reaching the vascular cylinder, nematodes select a suitable cell to establish an initial syncytial cell (ISC). Once an ISC is established, cell walls of neighbouring cells are partially dissolved and the protoplasts of these cells fuse. This process continues so that a multinucleate, hypertrophied and metabolically hyperactive syncytium is formed. Syncytium formation is accompanied by massive transcriptomic and metabolomics changes, which have been previously reported (Wyss & Zunke, 1986; Sijmons *et al*., 1991; Wyss, 1992; Golinowski *et al*., 1996; Szakasits et al., 2009; Hofmann *et al*., 2010). After establishment of the ISC, J2s pursue their life cycle, increase in size and moult three times (J3, J4, and adult) until reaching adult stages. Adult males leave the roots to search for females to mate, whereas the lemon-shaped adult females remain attached to the roots. After mating, females lay eggs inside their bodies, then die and turn into cysts protecting the eggs from the surrounding hostile environment (Wyss & Zunke, 1986; Wyss, 1992). For successful parasitism, *H. schachtii* releases various effectors into the plant that help the nematodes successfully invade the roots, suppress the plant's defence mechanisms, induce and maintain the syncytium (Williamson & Gleason, 2003; Mitchum *et al*., 2013; Holbein *et al*., 2016).

Plants are hosts to a wide range of pathogens, including bacteria, fungi, viruses, insects and nematodes. During evolution, both pathogens and plants have developed various strategies to facilitate their efforts, resembling a continuous battle of actions and counteractions (Mittler *et al*., 2004; Quentin *et al*., 2013; Goverse & Smant, 2014; Holbein *et al*., 2016). One of the responses by which plants defend themselves against pathogens is the production of reactive oxygen species (ROS). Because ROS are highly toxic and reactive, they can restrict pathogen growth and development. In addition to their role in plant defence, ROS have also been shown to act as signalling molecules and regulate a variety of key biological processes, such as, growth, differentiation, proliferation and apoptosis (Shetty *et al*., 2007; Slesak *et al*., 2007; Veal *et al*., 2007;

Forman *et al*., 2010). A number of studies have demonstrated and clarified the positive role of ROS in environmental stresses other than plant defence. ROS with prominent biological significance include superoxide anion (O^{-2}), hydroxyl radical (OH-) and hydrogen peroxide (H_2O_2). Among these, H_2O_2 is less reactive and can freely diffuse through lipid membrane, thus making it an ideal candidate for signalling processes. A number of studies have shown the correlation between ROS levels and intensity of pathogen infections (Torres *et al*., 2002; Mittler *et al*., 2004). In the context of plant-nematode interactions, the presence of ROS-generated signals and their spatiotemporal expression in the interaction of tomato with root-knot nematodes have been studied in detail (Melillo *et al*., 2006). In Arabidopsis, invasion and parasitism by *H. glycines* were shown to induce H_2O_2 production not only in the infected cells but also in the cells which are not in direct contact with nematodes (Waetzig *et al*., 1999). Similarly, it has recently been shown that infection of Arabidopsis *by H. schachtii* activates the plasma membrane-localised NADPH oxidase (RbohD and RbohF) to produce ROS, which, however, is required for proper infection and syncytium development (Siddique *et al*., 2014). These observations on parasitic nematodes together with other previously published literature led to the suggestion that redox homeostasis is crucial for both effective plant defence and successful parasitism (Foley *et al*., 2013; Siddique *et al*., 2014).

Because ROS are highly toxic, the development of an efficient scavenging system is crucial for both plants and pathogens. This is especially important in case of biotrophic pathogens, who require living cells for successful infection (Jones *et al*., 2004; Molina & Kahmann, 2007; Blackman & Hardham, 2008; Flores-Cruz & Allen, 2009; Dubreuil *et al*., 2011; Li *et al*., 2011). Animal parasitic nematodes have developed several ROS-scavenging mechanisms to protect themselves against the oxidative defence mechanisms of their hosts (Henkle-Dührsen & Kampkötter, 2001; Sotirchos *et al*., 2009). Two animal parasitic nematodes species, *Brugia malayi* and *Haemonchus contortus*, possess different thioredoxins that have been shown to increase nematode immunity against host ROS production (Kunchithapautham *et al*., 2003; Sotirchos *et al*., 2009).

Plant parasitic nematodes have also been shown to be capable of manipulating the redox status of their hosts (Lin *et al*., 2016). The root-knot nematode *Meloidogyne incognita* secretes peroxiredoxins to successfully develop within its tomato host (Dubreuil *et al*.,

2011). *Meloidogyne javanica* produce a transthyretin-like protein, MjTTL5, which has been shown to manipulate the host immune system by interacting with the Arabidopsis ferredoxin-thioredoxin reductase catalytic subunit (AtFTRc), that plays an important role in the ferredoxin/thioredoxin regulatory chain and decrease ROS burst (Lin *et al*., 2016). The potato cyst nematode (*Globodera rostochiensis*) produces peroxiredoxins (PXN) and glutathione peroxidases (GXP), which are likely responsible for regulation of ROS level at nematode infection sites (Robertson *et al*., 2000; Jones *et al*., 2004).

Protein disulfide isomerase (PDI) family includes PDI and PDI-like proteins with thioredoxin domains, also called thioredoxin superfamily. They vary in size, expression, localization and enzymatic function. Typical PDI consists of four thioredoxin-like domains, the domains (a and a′) with catalytic domains are separated by two non-catalytic domains (b and b´). In addition to this, an ER retention signal is located at the small C-terminal domain (c), and also PDI has an N-terminal signal sequence. The two catalytic domains containing characteristic CGHC active-site motif are essential for PDI enzymatic activity (Appenzeller-Herzog & Ellgaard, 2008). PDIs are very versatile enzymes as they are able to catalyze *in vitro* thiol oxidation reactions and disulfide reduction or isomerisation, depending on their redox states (Ali Khan & Mutus, 2014). PDIs that are found in an oxidized form most likely function as thiol oxidases, whereas PDIs functioning as isomerases need to be in a reduced state (Frand & Kaiser, 1999). In addition, it was found that PDI play an important role in the regulation of ROS. Oxidized PDI stimulates ROS production whereas reduced PDI inhibited the production of ROS (De *et al*., 2011).

PDIs play an important role during host-pathogen interactions (Stolf *et al*., 2011). It was shown that the expression of *Leishmania* PDI (LmPDI) is higher in virulent parasitic strains of *Leishmania,* suggesting that PDI protein is a virulence factor (Ben Achour *et al*., 2002). The use of PDI inhibitors affected parasite growth (Hong & Soong, 2008). Studying the expression pattern of the PfPDI-8 from *Plasmodium falciparum* showed that it is associated with all parasitic stages (Mahajan *et al*., 2006). Recently, a PDI from the oomycete plant parasitic *Phytophthora parasitica* (PpPDI1) was identified as a virulence factor. It was shown that expressing PpPDI1 induce strong cell death in *Nicotiana benthamiana* leaves while mutating the gene decreased the effect (Meng *et al*., 2015). To date, nothing is known about the role played by PDIs in *H. schachtii*. Here, we describe a novel effector of *H. schachtii* which belongs to the

PDI family. We show that it is involved in the interaction with the nematode´s host plant and in protecting the parasite against plant-released ROS.

2.3 Materials and methods

Plant growth and nematode culture

Arabidopsis thaliana L. Heyn. ecotype Col-0 plants and two transgenic lines expressing HsPDI were grown aseptically on agar medium supplemented with modified 0.2 Knop's nutrient solutions for 16h light and 8h dark at 25ºC as described previously (Sijmons *et al.*, 1991).

Heterodera schachtii Schmidt used in the experiments was reared on white mustard (*Sinapis alba* L. cv. Albatros) plants which were grown aseptically on agar containing 0.2% Knop medium. Mature cysts were collected in funnels and hatched in 3 mM $ZnCl_2$ (Sijmons *et al.*, 1991). The hatched pre-parasitic J2s were collected and used in the experiments.

Infection assay

Nematode infection assays on Arabidopsis plants either for the nematode RNA interference (RNAi) experiments or on HsPDI-expressing lines were performed as described previously (Siddique *et al.*, 2014). Briefly, sterilised seeds were placed on 0.2% Knop medium. After 10 days, roots were inoculated with 60-70 J2s per plant. For each experiment, 12 plants per treatment were used. Numbers of adult males and females were counted per plant at 12 days after inoculation (DAI). Furthermore, sizes of females and associated syncytia were measured after 13 DAI, sizes of cysts and numbers of eggs per cyst were examined at 45 DAI using Leica M165C Binocular (Leica Microsystems, Wetzlar, Germany) and Leica Application Suite software. Experiments were repeated three times and analysed using Student's *t*-test.

Sequence analysis

HsPDI (KU948160) was identified by performing a BLASTn of the esophageal gland cells putative secretory hsp3 (AF273730.1) which was isolated from *Heterodera glycines* (Wang *et al*., 2001) against draft transcriptomic data of *H. schachtii*. The draft transcriptome was generated by using next generation sequencing (Illumina, 100bp paired end reads) of pre-parasitic J2s. The assembly was done using CLC genomics workbench after trimming the reads for adapter sequence and low quality nucleotides

(less than 0.05) and ambiguity nucleotides (more than 2 adjacent ambiguous nucleotides).

The deduced protein was analysed to predict its functional domain(s). The conserved domains search was performed using the National Center for Biotechnology Information NCBI CD search program (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), signal peptide was identified using signalP4 server (Petersen *et al*., 2011), and transmembrane domains using TMHMM algorithm (Krogh *et al*., 2001).

The PDI protein sequences from different organisms were obtained from NCBI (http://www.ncbi.nlm.nih.gov/), see supporting information **Table S1**. All sequences were aligned using CLC Main Workbench (V7.7.3).

In situ hybridization

Digoxigenin (DIG)-labelled probes complementary to identified HsPDI fragments were amplified in asymmetric PCR with single sense (negative control) or antisense primer and DIG-labelled deoxynucleoside triphosphates (dNTPs) (Roche) in the reaction mixture (see supporting information **Table S2**). The hybridization was performed on the pre-parasitic J2s of *H. schachtii* at temperature of 47 °C as performed in previous study (de Boer *et al.*1998). The hybridized nematodes were examined using Leica DMI2000 compound microscope.

Developmental expression pattern analysis

Transcription of HsPDI was analysed in different developmental stages (eggs, pre-parasitic J2, parasitic juveniles and females) by quantitative PCR (qRT-PCR) using specific primers (supporting information **Table S2**). Around 3,000 eggs and 3,000 pre-parasitic J2s were collected directly from hatching funnels (Sijmons *et al.*, 1991). Around 500-600 nematodes were collected manually from nematode infected roots of *A. thaliana* at 5, 10, 15 DAI, what corresponds to J3, J4, and young female stages.

Total RNA was extracted using NucleoSpin RNA kit (MACHEREY-NAGEL) following manufacturer's protocol. Quality and quantity of the extracted RNA samples were tested using the Agilent 2100 Bioanalyzer system (Agilent Technologies). The RNA with RNA integrity number (RIN) value higher than 8.5 was used for first strand cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and oligo-dT primer. The resulted cDNAs were tested for the expression

changes using the Stepone Plus Real-Time PCR System (Applied Biosystems) with 95 ºC for 15 s and 60 ºC for 30 s (40 cycles) for amplification. Each sample contained 10 µl of Fast SYBR Green qPCR Master Mix (Invitrogen), 9 µl of the specific primer mixture with final concentration 1 µM for each primer, 1 µl of cDNA. The amplified data were analysed by using the Stepone Plus Real-Time PCR software to create Ct values. The data were analysed and relative expression was calculated following Pfaffl (2001). Actin was used as internal control for all experiments (supporting information Table S2). Three biological replicates from each stage in three technical replicates for each biological replicate were used.

Double stranded RNA (dsRNA) and gene silencing in nematodes

HsPDI dsRNA was synthesized using RiboMAX RNA Large-Scale Production System (Promega) according to the manufacturer's instruction. The forward and reverse primers (supporting information Table S2) were supplemented with the SP6 and T7 promoter sequences and used for dsRNA synthesis. The *GFP* template was used for synthesis of a dsRNA construct that was used as a negative control.

About 10,000 freshly hatched J2s of *H. schachtii* were soaked in 50 µL of dsRNA incubation mixture consisting of 25 µL HsPDI-dsRNA or *GFP*-dsRNA (2 mg*mL^{-1}) in 5 µL of 10x M9 buffer(55mM KH_2PO_4, 21mM NaCl, 47mM NH_4Cl) supplemented with 100 mM spermidine (1.5 µL), 500 mM octopamine (5.0 µL) and 13.5 µL (nematode suspension) for one day. Incubated J2s were washed three times in H_2O, sterilized in 0.05 M $HgCL_2$ for 2 minutes and washed again three times with sterile water. Afterwards, the batch of juveniles was divided equally and one part was used for plant infection assay whereas the second part was used to evaluate the level of HsPDI silencing using qRT-PCR as described above.

Ten days old *A. thaliana* Col-0 plants were inoculated with 60-70 J2s incubated in HsPDI or *GFP* dsRNA. Numbers of developed females and males were counted after 12 DAI, sizes of females and syncytia associated with females were measured at 13 DAI, whereas sizes of cysts and numbers of eggs per cyst were examined at 45 DAI. All measurements were conducted under a Leica M165C stereo microscope using manufacturer's software. Experiments were repeated three times with 12 plants per treatment in each experiment. The obtained data were merged and analysed using the Student's *t*-test.

Syncytium anatomy and ultrastructure

Segments of roots containing nematode induced syncytia were dissected from Arabidopsis Col-0 plants inoculated with HsPDI or *GFP* dsRNA-treated juveniles at 5 and 10 DAI. They were processed for light and transmission electron microscopy as described by (Daneshkhah *et al.*, 2013). Semi-thin sections (3 µm thick) taken on a Leica RM2165 microtome (Leica) were stained with hot 1% (w/v) aqueous solution of Crystal violet (Sigma, St. Louis, MI, USA) for 1 min at 65 °C. They were examined with an Olympus AX70 'Provis' (Olympus, Tokyo, Japan) light microscope equipped with an Olympus DP50 digital camera. Ultra-thin sections (70–80 nm thick) taken on a Leica UCT ultramicrotome (Leica Microsystems) were collected and on formvar-coated (Fluka, Buchs, Switzerland) single-slot copper grids and stained with uranyl acetate (Fluka) and lead citrate (Sigma) (Golinowski *et al.*, 1996). They were examined with an FEI 268D 'Morgagni' transmission electron microscope (FEI, Hillsboro, OR, USA) operating at 80 kV. The images were recorded with an SIS 'Morada' digital camera (Olympus SIS, Münster, Germany) at 10 Mpix resolution. The images were equalized for similar contrast and brightness, resized and cropped using Adobe Photoshop graphic software.

Survival of *H. schachtii* and HsPDI expression check under the H_2O_2 stress

Around 100-150 freshly hatched J2s were incubated in 0, 5, 10, 25 mM of H_2O_2. Dead nematodes were counted after 15, 30, 45 and 60 min and percentage of survival was calculated. Treatments were replicated three times and experiment was repeated three times. To check HsPDI gene expression under H_2O_2 stress, J2s were incubated for 30 min in 5 or 10 mM H_2O_2, and washed in sterile tap water. J2s incubated in sterile tap water were used as a control. RNA was extracted, cDNA was generated and expression of HsPDI was quantified by qRT-PCR as described above. Three biological replicates were carried out and each one was replicated three times.

Effect of HsPDI depletion on nematode survival under the H_2O_2 stress

Around 150 freshly hatched J2s were incubated overnight in solution of HsPDI dsRNA and in *GFP* dsRNA as described above and then washed in tap water. Afterwards, the J2s were incubated in 5 mM H_2O_2 or in tap water as a control for 30 min. The numbers of dead J2s were counted and their percentage was calculated and analysed using Student's *t*-test. Each treatment consisted of 4 replicates and the experiment was repeated 4 times.

Construct generation and production of transgenic HsPDI expressing plants

The ORF of the HsPDI with no signal peptide was cloned into the binary Gateway over-expression vector pMDC83 to obtain a C-terminal fusion with GFP (Curtis & Grossniklaus, 2003) using the primers listed in **Table S2**. The construct was transferred to *Agrobacterium tumefaciens GV3101::pMP90* strain (Sparkes *et al.*, 2006) and transformed into *A. thaliana* Col-0 using the floral dip method (Clough & Bent, 1998). The transformed plants were selected for hygromycin resistance on modified 0.2 Knop medium and grown for 3 generations to obtain homozygous lines for infection assays. The expression of HsPDI in homozygous lines was confirmed using qRT-PCR as described previously (Pfaffl, 2001).

The homozygous lines were grown on Murashige and Skoog media (MS) plates for 10 days. The number of lateral roots, main root length, fresh root weight and fresh shoot weight were measured and compared with Col-0. The experiment was repeated 3 times and each experiment consists of 10 plants for each line. After phenotyping, plants were infected with *H. schachtii* as described above.

Agroinfiltration and subcellular localization of HsPDI

A. tumefaciens transformed with HsPDI*::pMDC83* construct was grown overnight in 50 ml of YEB liquid medium (Sparkes *et al.*, 2006) supplemented with 10 mg*mL^{-1} gentamycin, 50 mg*mL^{-1} kanamycin and 50 mg*mL^{-1} rifampicin to an OD600 of 0.8 in an incubator/shaker at 28 ºC. Bacteria were pelleted then they were re-suspended in an infiltration buffer (Sparkes *et al.*, 2006). Bacterial suspensions were diluted with the infiltration buffer to get the required OD600 of 1. After incubation for 4h at RT, the transformed bacteria were injected in the leaves abaxial side of 6 week-old *Nicotiana benthamiana* plant using 1 mL hypodermic syringe without needle. For co-infiltration of RNA silencing inhibitor P19 and the apoplastic marker, equal volumes of a bacterial suspensions harbouring *pBin61::P19* (Voinnet *et al.*, 2003), HsPDI*::pMDC83* and apoplastic marker constructs were mixed and injected. To perform co-localization experiments in the apoplast, the N-terminal region of a membrane-localized receptor-like-kinase (At4g31250) was amplified and cloned in frame with mCherry under the control of the 35S CaMV promoter and terminator. The cloning of only N-terminal region assured the delivery of fusion protein into extracellular region. The complete expression cassette was further cloned with restriction enzyme SdaI into the binary

vector pGreenII (Hellens *et al*., 2000) resulting in a pGreen-apo-mCherry marker. The primers sequences are given in **table S2**. Infiltrated plants were incubated in the growth chamber (16hrs light, 8hrs dark and at 25ºC) for 6 days. Slides were made from the infiltrated leaves and examined for the presence of fluorescence signal using Zeiss CLSM 710 with and without plasmolysis with 1M $NaCl_2$.

ROS measurement in HsPDI over-expressing plants

ROS production in Col-0 and transgenic HsPDI over-expressing plants after treatment with bacterial peptide flg22 was measured on leaf disc samples using luminol-based assay and 96 wells plate luminometer Mithras LB 940 (Berthold Technologies) as described by Prince *et al.* (2014). Light emission was measured in relative units over 120 min long incubation period and analysed using instrument software. Data was tabulated and analysed statistically using Student's *t*-test. Experiments were repeated three times by using 4 leaf discs in each time as technical replicates. Total ROS production was calculated and presented.

2.4 Results

Sequence analysis of HsPDI

BLAST results revealed 100 % similarity of hsp3 (AF273730.1) with one of the contigs in our draft transcriptome. The contig was extracted and further analysed. A detailed sequence analysis showed that the resulted contig contains a signal peptide of 22 amino acid residues and lacks a transmembrane domain. Further domain analysis with NCBI CD search program revealed four conserved TRX domains (a,b,b´,a´) with two catalytic domains (Cys-Gly-His-Cys), as outlined in **Figure 1A**. This arrangement of the domains is a classical feature of the protein disulfide isomerase family members. For that reason, the described sequence was annotated as HsPDI.

Alignment results of HsPDI with other PDIs from different organisms showed high conserved active domains (CGHG) in eukaryotic organisms including some protozoans (supporting information **Figure S1**). However, overall the highest level of sequence similarity among PDIs was within nematodes (identity > 70%). In comparison, similarity was less to the other organisms PDIs (supporting information **Table S1)**

HsPDI is expressed in oesophageal glands during early parasitic stages

We used *in situ* hybridization to localise the expression of the HsPDI in the pre-parasitic juveniles of *H. schachtii*. The labelled antisense riboprobe of HsPDI gave a clear signal in the dorsal gland cell of the pre-parasitic J2s (**Figure 1B**). To further investigate the expression pattern of HsPDI during different developmental stages of *H. schachtii,* we used the qRT-PCR using cDNA generated from nematode RNA isolated at different pre-parasitic (eggs and freshly hatched J2s) and parasitic developmental stages (J3s, J4s and young females). HsPDI expression level increased during the sedentary stages of nematode development, reaching its maximum in J3 with 15-fold increase compared with unhatched J2 in eggs. In J4s and young females, expression decreased, respectively, to 2.3 and 1.6-fold compared with unhatched J2s in eggs and hatched pre-parasitic J2s (**Figure 1C**).

HsPDI is involved in parasitism

To analyse whether HsPDI plays a role in parasitism, we performed *in vitro* RNAi targeting HsPDI (see methods for details). Our results showed that RNAi caused a significant decrease in the transcript abundance of HsPDI in J2s of *H. schachtii* (see supporting information **Figure S2**). Next we infected the roots of Arabidopsis wild-type plants with nematodes soaked in dsRNA targeted against HsPDI or *GFP* and counted the number of females and males at 12 DAI. We also measured the average sizes of females, associated syncytia and cysts and counted the average number of eggs per cyst. Our analysis showed no significant difference in number of infecting nematodes found on plants infected with J2s treated with dsRNA targeting HsPDI compared to those treated with dsRNA targeting *GFP* (**Figure 2A**). However, the average size of syncytia, average size of females, average size of cysts and average number of eggs per cyst were reduced significantly in plants infected with juveniles treated with dsRNA against HsPDI compared with *GFP* (**Figure 2B-3E**).

To characterise further these differences, we examined the anatomical and ultrastructural features of syncytia induced by HsPDI or *GFP* dsRNA-treated juveniles, we performed a detailed time-course analysis via light and transmission electron microscopy. To obtain comparable materials for these analyses, we took sections in the middle region of syncytia. At 5 DAI, syncytia induced by HsPDI dsRNA-treated juveniles were composed of a similar number of elements as syncytia induced by the *GFP* dsRNA-treated control juveniles (**Figure 3A and 3B**). Nevertheless, the hypertrophy of a single syncytial element were lower in HsPDI silenced nematodes.

The openings were also observed to be narrower than those found in syncytia induced by *GFP* dsRNA-treated juveniles. At 10 DAI, the differences between syncytia induced by HsPDI and *GFP* dsRNA-treated juveniles were less obvious (**Figure 3C and 3D**), but the number and extent of cell wall openings were lower and the regions with confluent cytoplasm were smaller in syncytia induced by HsPDI dsRNA-treated juveniles.

Parallel examinations of the ultrastructure of syncytial elements revealed developmental abnormalities in syncytia induced by HsPDI dsRNA-treated juveniles. They showed differences in the electron density of the cytoplasm, the organisation and composition of endoplasmic reticulum (ER) and vacuole formation. First of all, cytoplasm electron density was lower in syncytia induced by HsPDI dsRNA-treated juveniles at 5 DAI (syncytia associated with sedentary J2) and 10 DAI (syncytia associated with young females) than in syncytia induced by *GFP* dsRNA-treated juveniles (**Figure 4A, B, C vs 4E, F**). However, this difference was less pronounced when comparing syncytia at 10 DAI (**Figure 4C, D vs. 4G, H**). In addition, large organelle-free regions were present in syncytia induced by HsPDI dsRNA-treated juveniles at 5 DAI (**Figure 4A, B**). At the interface to the organelle-containing cytoplasm, small vesicles or dilated cisterns of accumulated ER appeared (**Figure 4A, B**). Secondly, the organization and composition of the ER differed strongly between syncytia induced by both groups of juveniles. In control syncytia, numerous cisternae of ER were present (**Figure 4F**), whereas they were almost absent in syncytia induced by HsPDI dsRNA-treated juveniles at 5 DAI (**Figure 4C**). Interestingly the total number of ER cisternae decreased during syncytia development in both groups. At 10 DAI they were still quite numerous in syncytia induced by *GFP* dsRNA-treated juveniles (**Figure 4G, H**), but completely absent in syncytia induced by HsPDI dsRNA-treated juveniles (**Figure 4C, D**). The ER system in syncytia induced by HsPDI dsRNA-treated juveniles consisted predominantly of tubular ER that occupied large regions of syncytial cytoplasm (**Figure 4A, C**). Tubular ER was present also in syncytia induced by *GFP* dsRNA-treated juveniles, but never appeared alone in any syncytial element (**Figure 4E-H**). Thirdly, syncytia induced by HsPDI dsRNA-treated juveniles were strongly vacuolated at 10 DAI (**Figure 4C, D**). These vesicles were apparently formed from dilating cisternae of ER that accumulated at the interface between the organelle-free and organelle-containing regions of syncytial cytoplasm observed at 5

DAI (**Figure 4A-H**). Other organelles such as nuclei, plastids or cell walls, displayed no structural changes (**Figure 4A-H**), and their ultrastructure was typical as described for syncytial elements (Golinowski *et al*., 1996; Sobczak *et al*., 1997).

To analyse the function of HsPDI in more detail, we produced transgenic Arabidopsis plants over-expressing HsPDI (35S::HsPDI-GFP). The relative expression of HsPDI in transgenic lines was measured using qRT-PCR (supporting information **Figure S3)**. A detailed phenotypic analysis did not reveal any significant differences in plant growth as indicated by number of lateral roots, length of main root, fresh root weight and fresh shoot weight between HsPDI expressing plants and Col-0 (supporting information **Figure S4**). Next, we analysed these lines for susceptibility via nematode infection assay and found that at least one of the HsPDI expressing lines was more susceptible to *H. schachtii* infection as compared with wild-type control (**Figure 5A**). However, considering significantly higher average female, cyst, and syncytium sizes, both HsPDI expressing lines appear to be more susceptible than the wild type Col-0 plants (**Figure 5B-5C**). Furthermore, the average number of eggs were also increased significantly compared with the control (**Figure 5D)**.

HsPDI expression is triggered by H_2O_2 and increases H_2O_2 tolerance

We tested whether treating J2s with H_2O_2 influence the survival of nematodes. To test vitality, pre-parasitic J2s were soaked in 5, 10 or 25 mM H_2O_2 and the percentage of dead juveniles was counted after 15, 30, 45, and 60 min. We found that J2s can survive up to 30 min in 10 mM H_2O_2 without significantly increased mortality rate (**Figure 6A**), but prolonged treatment substantially increased mortality of J2s. Next, we used qRT-PCR to analyse the expression of HsPDI in response to H_2O_2. By analysing the juveniles that were exposed to H_2O_2 (5 or 10 mM) for 30 min, we found a significant increase in transcript abundance of the HsPDI when compared with water-treated control J2s (**Figure 6B**).

We examined the mortality rate of HsPDI or *GFP* dsRNA-treated J2s after soaking them in 5mM H_2O_2 for 30 min. As a control, we incubated dsRNA-treated J2s in water **(Figure 6C**). We observed a significantly lower percentage of J2s that survived in H_2O_2 in case of HsPDI dsRNA-treated nematodes, indicating that HsPDI plays a role in protecting J2 from the impact of the exogenous H_2O_2. Following up on these results, we investigated whether HsPDI could modulate the plant endogenous ROS burst. We

incubated the plants leaf discs in bacterial peptide flg22 and total ROS burst was measured in HsPDI expressing transgenic plants and wild type Col-0. We observed a significant decrease in total ROS in both transgenic plant lines expressing the HsPDI to half of the total amount of ROS produced by Col-0 plants (**Figure 6D,** supporting information **Figure S5**).

HsPDI is localised in the plant apoplastic space

To determine the sub-cellular localization of the HsPDI protein inside the host cells, we transiently expressed HsPDI*::GFP* in *Nicotiana benthamiana* epidermal cells with the constitutive cauliflower mosaic virus (CaMV) 35S promoter and assessed its localization by co-infiltrate it with an apoplastic marker under the confocal microscope (**Figure 7A-D**). Our observations clearly showed that GFP signal was localized in the cell periphery and co-localized with the mCherry signal. To further investigate the specificity of subcellular localization, we induced plasmolysis by adding 1 M NaCl to the leaf tissue. Upon dissociation of plasma membrane from the cell wall, the signal was observed in the apoplastic space indicating the localization of HsPDI in outer cell periphery (**Figure 7E-H**).

2.5 Discussion

Plant endo-parasitic nematodes spend most of their life cycle inside host tissue. To establish parasitism, nematodes have evolved a repertoire of physical and chemical tools including secretions of proteinaceous and non-proteinaceous effectors into the host tissues (Hewezi *et al.*, 2010; Hamamouch *et al.*, 2012; Lozano-Torres *et al.*, 2014). In the current work, we have cloned a full-length HsPDI from *H. schachtii* based on information from nematode transcriptome and characterised its role in facilitating parasitism.

HsPDI encodes a 22-amino acid, signal peptide at its N-terminal and lacks a transmembrane domain. Moreover, four thioredoxin domains and two active catalytic motifs were also detected (CGHC and CGHC) (**Figure 1A**). Being a typical PDI protein, HsPDI contains the main structural building block. It also contains a-type domains containing two cysteines in a CXXC active-site motif with an intervening GH sequence, which is the most common CGHC motif in the PDIs (Kozlov *et al.*, 2010). Presence of a signal peptide and lack of a transmembrane domain supported the role of HsPDI as a putative effector (Wang *et al.*, 2001; Jones *et al.*, 2009). Previous studies

have shown that the active catalytic motifs are important for the protein activity as mutations in the active site domains result in the loss of protein functions (Kim & Mayfield, 2002). It was also shown that, as a virulent factor, mutating the active motifs of the PpPDI1 abolished necrosis-inducing activity of the oomycete plant parasitic *Phytophthora parasitica*, indicating that the cell death-inducing function might be related to the catalytic properties (Meng *et al.*, 2015). Here we showed that the active catalytic motifs are highly conserved in the tested sequences from various eukaryotes (supporting information **Figure S1**).

The observation that transcript for HsPDI was localized in oesophageal gland cells supports the hypothesis that the HsPDI protein is secreted into the host tissues to facilitate parasitism (Maier *et al.*, 2013; Mitchum *et al.*, 2013). Furthermore, our expression analysis found that transcript abundance for HsPDI was increased significantly during early stages of infection reaching its maximum at 5 DAI, which coincides with rapid enlargement of the nematode induced syncytium (Golinowski *et al*., 1996). This particular expression pattern points towards the importance of HsPDI in the early stages of infection including syncytium formation and maintenance. This hypothesis is further supported by results from infection assays where silencing HsPDI expression via RNAi led to impaired nematode development and ultrastructural and anatomical abnormalities in associated syncytium. Additionally, the described set of ultrastructural features strongly resembles the ultrastructure of syncytia associated with developing male juveniles (Sobczak *et al.*, 1997). This suggests that syncytia induced by HsPDI depleted-nematodes may suffer from a shortage of nutrients, which can lead to smaller females with a lower numbers of eggs. Earlier studies suggest that in *H. schachtii* (i) sex determination is regulated epigenetically by the composition and amount of nutrients withdrawn from syncytia (Müller *et al*. 1982), (ii) sex differentiation occurs during the sedentary late J2 stage (Wyss, 1992). Therefore we conclude that HsPDI dsRNA-treated juveniles can induce fully functional syncytia that support their development into females during J2 sedentary stage. Afterwards these syncytia start to develop structural abnormalities. Theses abnormalities are similar to ultrastructural features of syncytium senescence typically occurring in degrading syncytia associated male J4 and adult males which had ceased feeding. The impaired function of their syncytia makes associated females developing smaller and producing fewer eggs.

It has been shown previously that ROS oxidize DNA, proteins, and lipids, which causes damage to the cellular organelles and inhibits cell functions (Baker & Orlandi, 1995). Plant-parasitic nematodes encodes various antioxidant enzymes, such as superoxide dismutase (SOD), catalase, ascorbate, p-phenylenediamine-pyrocatechol (PPD-PC), o-dianisidine, guaiacol isoperoxidases, peroxiredoxins and glutathione peroxidases, which are important for parasitism and could have protective function against ROS (Molinari & Miacola, 1997; Robertson *et al*., 2000; Jones *et al*., 2004; Dubreuil *et al.*, 2011). The fact that HsPDI expression was elevated in presence of exogenous H_2O_2 and that silencing the expression of HsPDI using dsRNA decrease the tolerance of nematodes to 5 mM H_2O_2 points to the role of HsPDI to protect the nematode. These observations are supported by previous studies where similar expression pattern was observed for *M incognita* peroxiredoxins. Silencing peroxiredoxins expression of *M incognita* impaired the nematode infectivity on tomato and their tolerance to exogenous H_2O_2 (Dubreuil *et al.*, 2011).

Our data for subcellular localization showed that HsPDI (without signal peptide) is localized in apoplast; however, this observation also raises the question of how an effector that is putatively secreted into the cytoplasm of infected tissues is ultimately translocated to the apoplastic space. Although the exact mechanism is currently unknown, it is plausible that host trafficking machinery is manipulated to deliver effectors into the apoplastic space (Wang *et al*., 2010; Ali *et al*., 2015).

In plants, apoplastic ROS are actively produced through the action of NADPH oxidases and class III peroxidases, but the biological significance and the mechanism by which these ROS are scavenged during the plant–nematode interaction are not well understood. Recently, it was shown that *H. schachtii* infection of Arabidopsis plants stimulates ROS burst via host NADPH oxidases (RbohD and RbohF). Surprisingly, knocking out RbohD and RbohF restricted nematode development and nurse cell formation and triggered massive cell death upon nematode infection (Siddique *et al*., 2014). Combining the previous studies with our result we conclude that although ROS are needed for successful nematode infection, they are harmful for the plants and nematodes when exceeding a certain level. Therefore, nematode not only induce ROS burst, but they also manipulate their levels for optimal infection and less plant damage. HsPDI is proposed as one of the effectors which may serve this purpose. Alternatively, it is possible that Rbohs are guarded by a nucleotide-binding and leucine-rich repeat

protein (NLR), leading to a strong immune response in mutants deficient in Rboh genes upon infection (Kadota *et al.*, 2014; Holbein *et al.*, 2016).

In conclusion, our results strongly indicate that HsPDI is a nematode effector that is secreted into host tissues and become a part of the host antioxidant mechanisms as plant ROS scavenger not only during invasion but also during sedentary parasitism. Clarifying further details of the interplay between various ROS-producing and ROS-scavenging systems during plant-nematode interaction will provide exciting information on nematode parasitism.

2.6 Figures

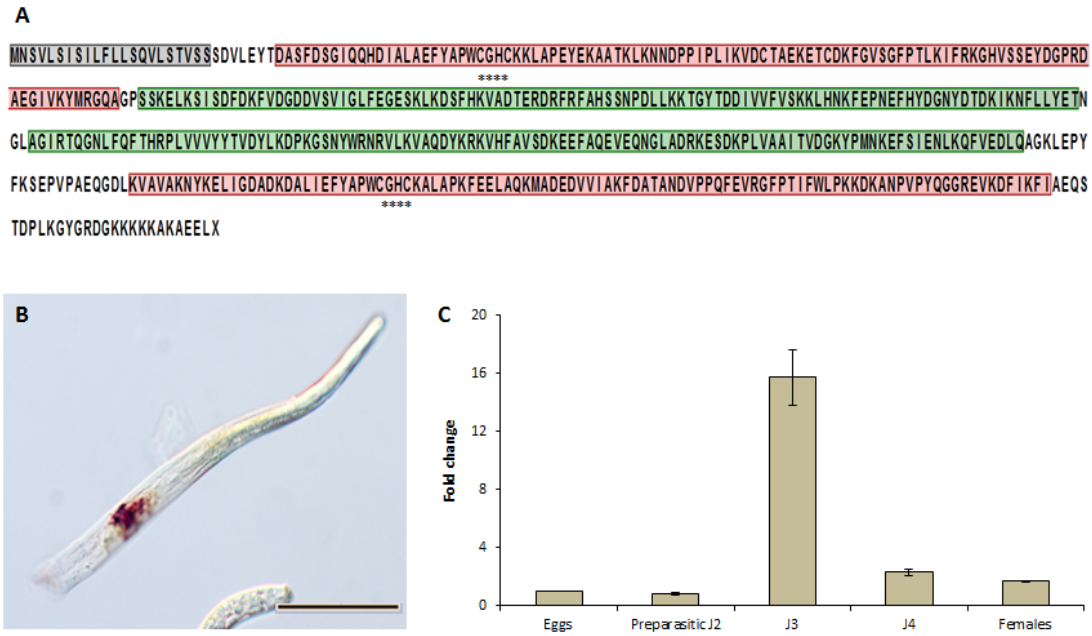

Figure 2.1 Structure and functional annotation of HsPDI and expression pattern of HsPDI gene. (A) Detailed amino acid sequence of HsPDI protein with predicted signal peptide (grey), thioredoxin domains (a and a´in red, b and b´ in green) and functional catalytic active site (asterisks). (B) *In situ* hybridization of DIG-labelled antisense HsPDI probe to pre-parasitic J2s showed transcripts localized inside the oesophageal gland Bars: 50 µm. (C) The relative expression levels of HsPDI mRNA quantified using qRT-PCR. The fold change values of changes in HsPDI mRNA abundance in pre-parasitic J2, J3, J4, and female relative its abundancy in eggs. Data are averages of three biologically independent experiments, each consisting of three technical replicates. Bars represent standard errors of the mean values. *H. schachtii* actin gene was used as an internal control to normalize gene expression level.

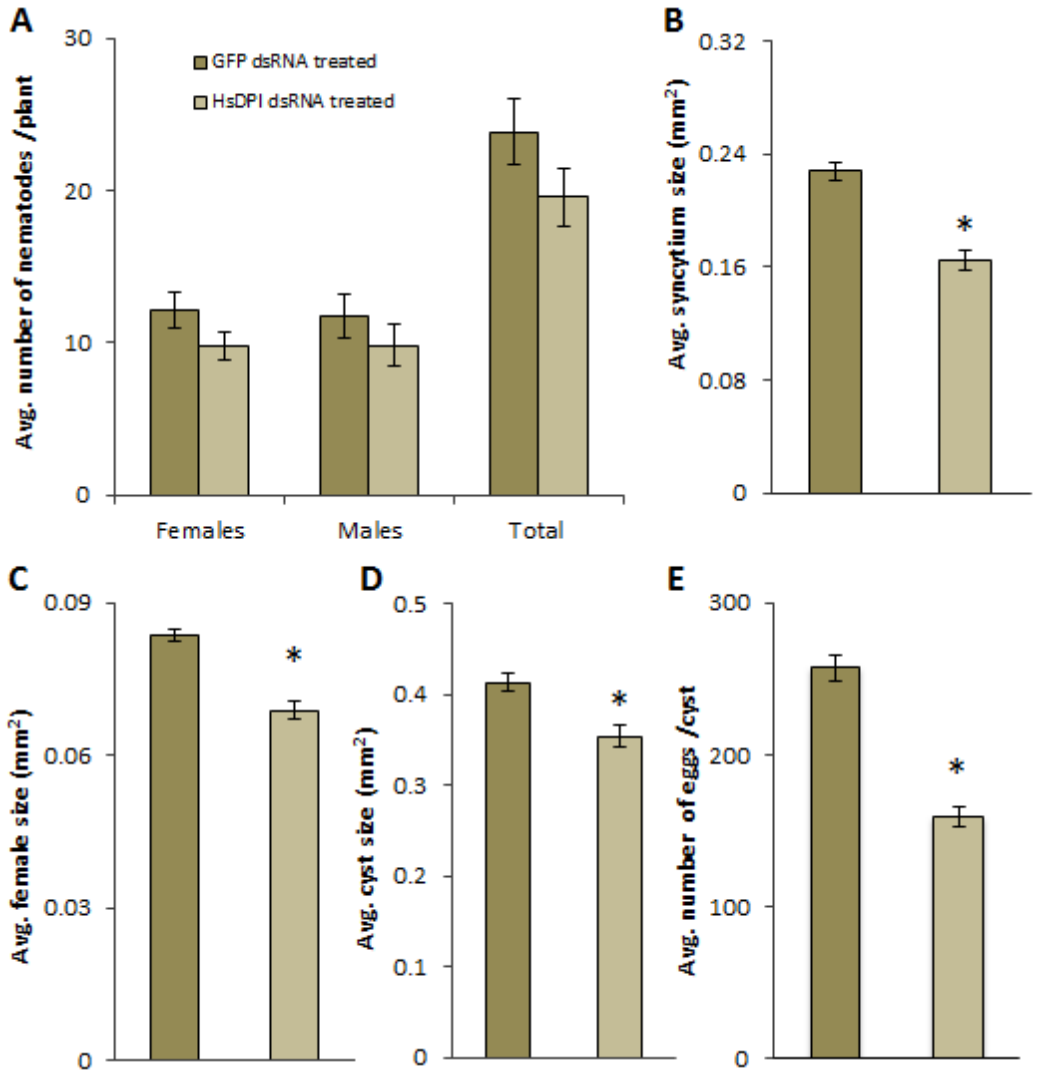

Figure 2.2 Effect of HsPDI silencing on *H. schachtii* parasitism. Figures show susceptibility parameters representing the parasitism of the nematodes that were soaked in the HsPDI dsRNA compared with those nematodes which were soaked in *GFP* dsRNA as a negative control (**A**) Average number of males, females and total nematodes developed on Arabidopsis Col-0 plant. (**B**) Average sizes of syncytia at 13 DAI (C) Average sizes of females at 13 DAI. (**D**) Average sizes of cysts at 45 DAI. (**E**) Average numbers of eggs inside cysts. Data points represent mean of three independent experiments. Bars represent standard errors of the mean values. Asterisks indicate significance according to Student's *t*-test ($P<0.05$).

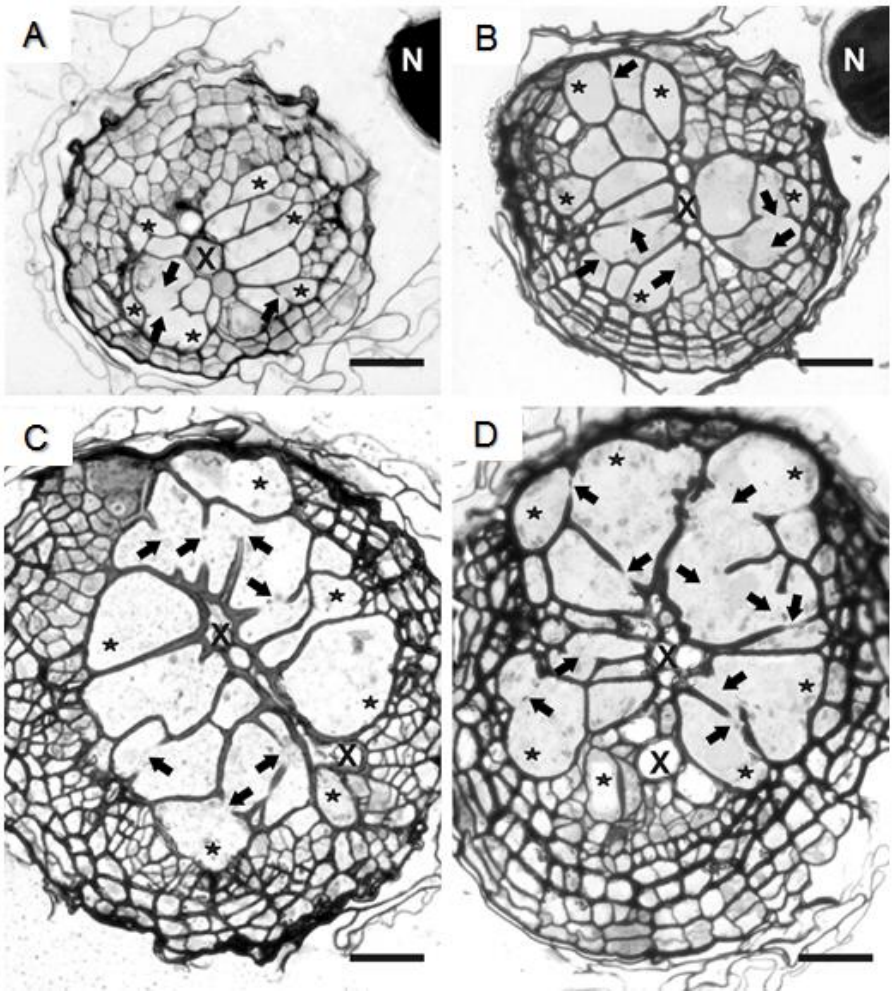

Figure 2.3 Anatomy of nematode-induced syncytia. Light microscopy images of cross sections of syncytia at (**A**,**B**) 5 DAI and (**C**,**D**) 10 DAI induced in Arabidopsis Col-0 roots upon infection with J2s treated with HsPDI dsRNA (**A**,**C**) or *GFP* dsRNA (**B**,**D**). Selected syncytial elements are marked with asterisks and cell wall openings are pointed with arrows. Abbreviations: N, nematode; X, xylem. Bars: 20 µm.

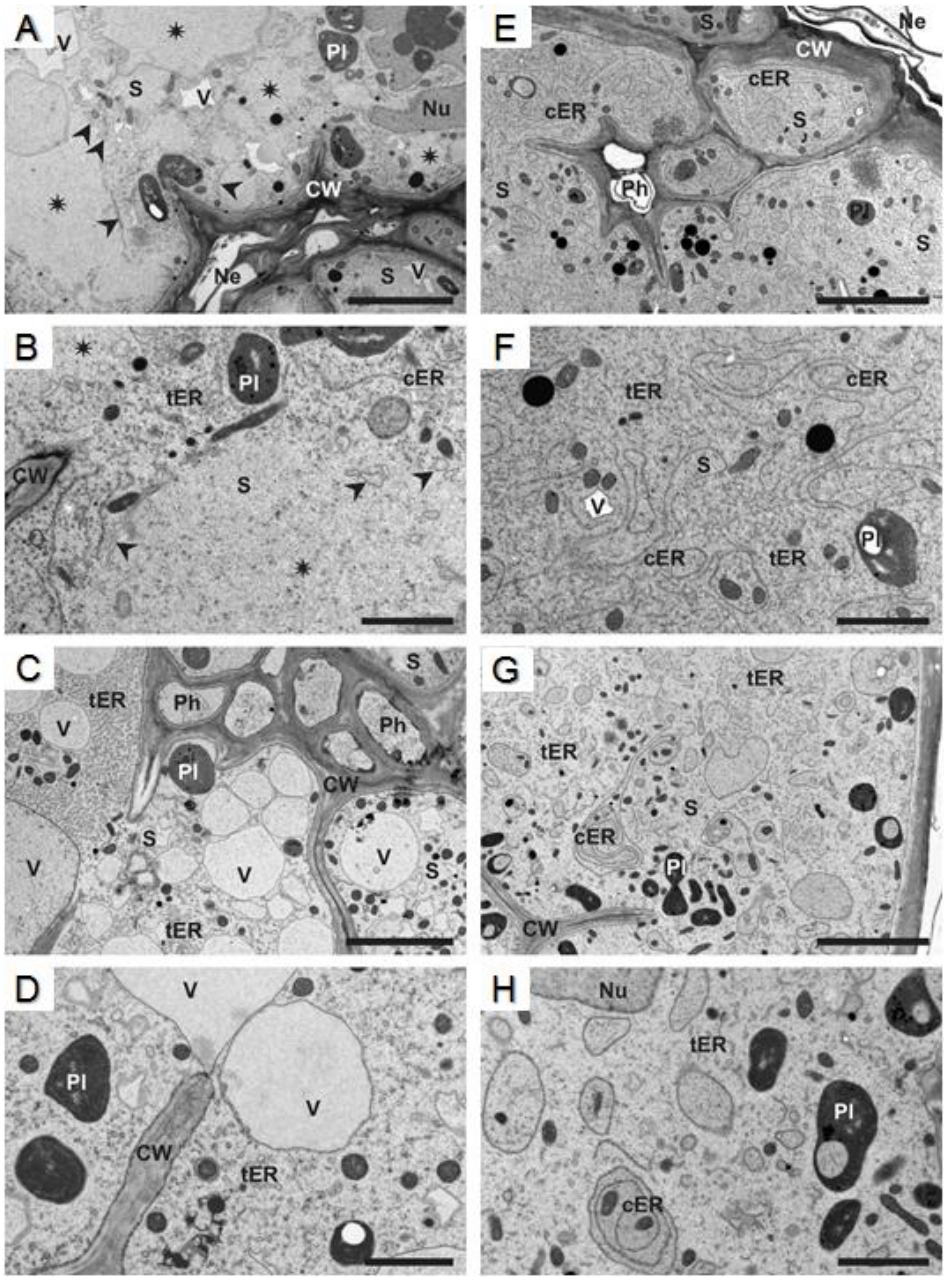

Figure 2.4 Ultrastructure of nematode-induced syncytia. Transmission electron microscopy images of cross sections of syncytia at 5 (**A**, **B**, **E**, **F**) and 10 DAI (**C**, **D**, **G**, **H**) induced in Arabidopsis Col-0 roots upon infection with J2s treated with HsPDI dsRNA (**A-D**) or *GFP* dsRNA (**E-H**). Asterisks indicate organelle-free regions in syncytial cytoplasm, arrow-heads point to dilated cisternae at the interface of organelle-free region and regular syncytial cytoplasm. Abbreviations: CW, cell wall; cER, cisternal endoplasmic reticulum; tER, tubular endoplasmic reticulum; Ne, necrosis; Nu, nucleus; Ph, phloem; Pl, plastid; S, syncytium; V, vacuole/vesicle. Bars: 5 µm (**A**, **C**, **E**, **G**) and 2 µm (**B**, **D**, **F**, **H**).

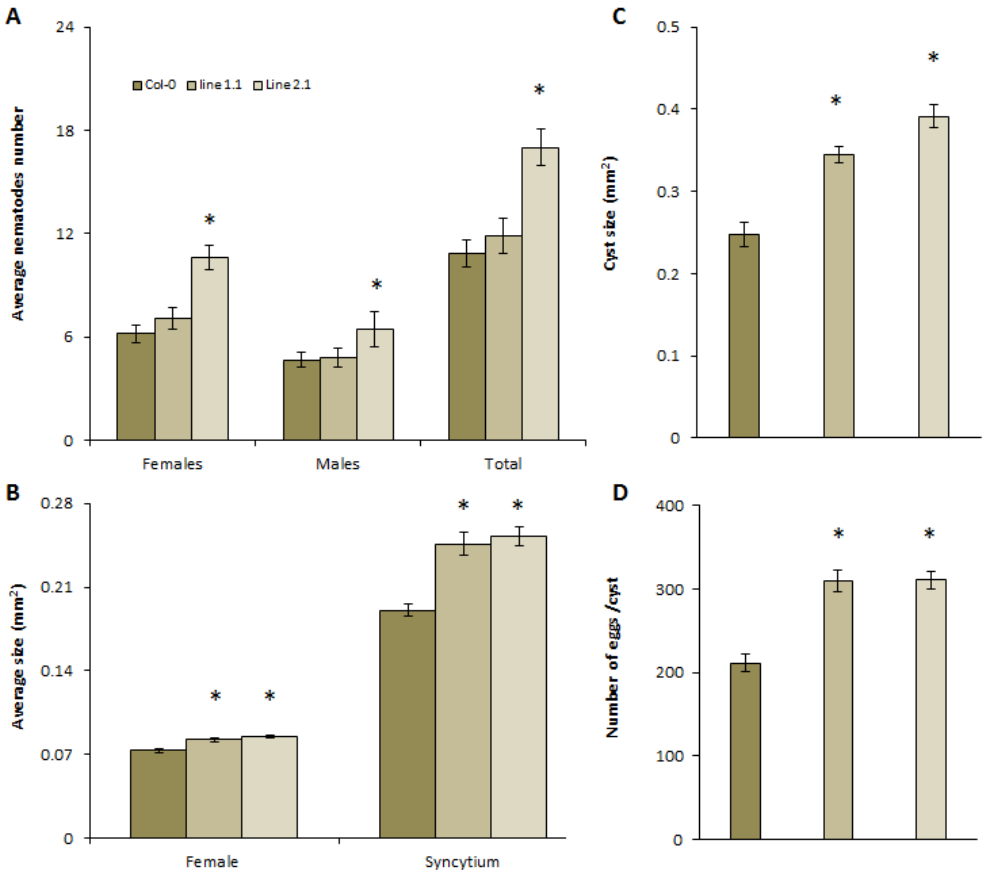

Figure 2.5 Effect of HsPDI expression on Arabidopsis susceptibility to *H. schachtii* infection. Stable transformed Arabidopsis plants expressing HsPDI gene (Line 1.1 and 2.1) were infected with J2s of *H. schachtii*. Susceptibility parameters were. (**A**) Average numbers of females, males and total nematodes per plant. (**B**) Average sizes of females and syncytia at 13DAI. (**C**) Average sizes of cysts at 45 DAI. (**D**) Average numbers of eggs inside cysts. Data represent three independent experiments. Bars indicate standard errors of the mean values. Asterisks indicate significance compared with control according to Student's *t*-test (with $P<0.05$).

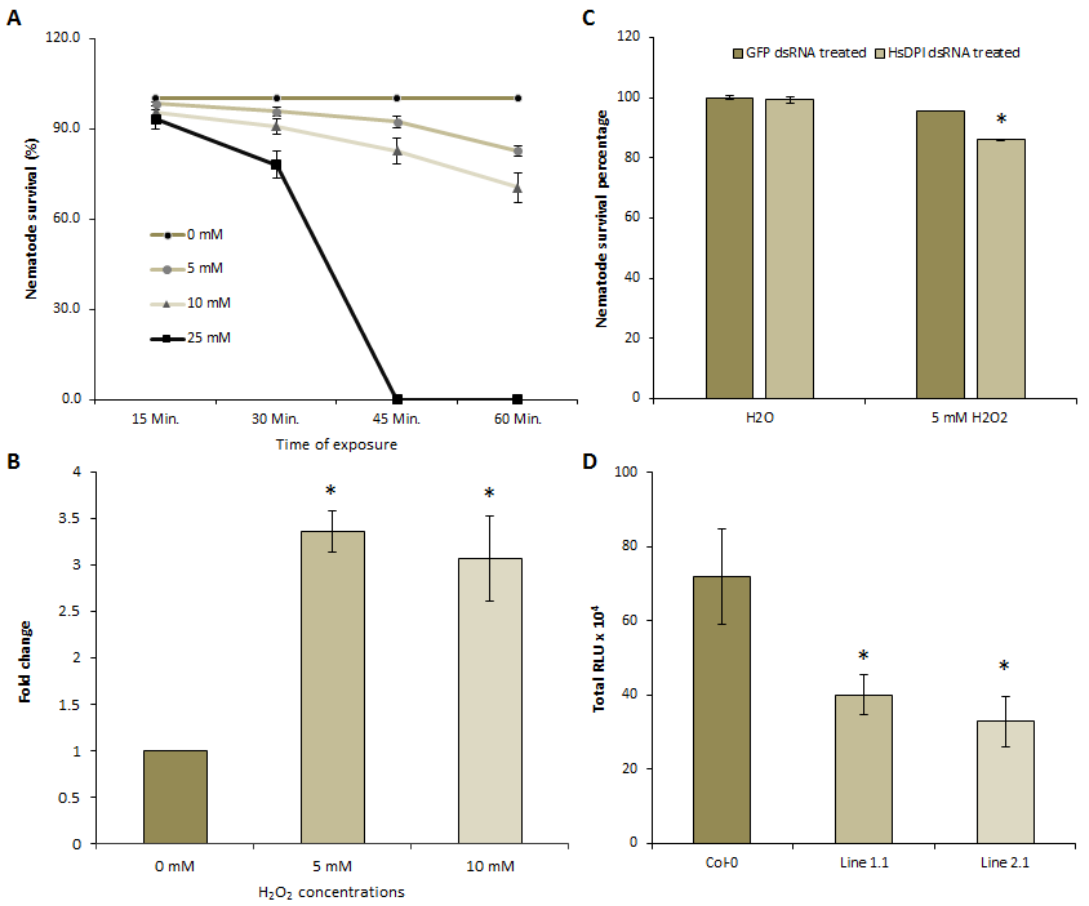

Figure 2.6 HsPDI expression is triggered by H_2O_2 and increases H_2O_2 tolerance. (**A**) Mortality rate of freshly-hatched J2s in H_2O_2. (**B**) The relative HsPDI mRNA expression levels in freshly hatched J2s quantified using qRT-PCR after soaking for 30 min in 5 and 10 mM H_2O_2. The fold change values were calculated in H_2O_2 incubated nematodes relative to J2s soaked in sterile distilled water (0mM H_2O_2). (**C**) Effect of HsPDI silencing on H_2O_2 stress tolerance**,** freshly hatched J2s of *H. schachtii* were soaked in HsPDI dsRNA or *GFP* dsRNA as a control. dsRNA-treated nematodes were soaked in 5mM H_2O_2 or in sterile water and alive nematodes were counted after 30 min. (**D**) ROS bursts in response to the bacterial elicitor peptide flg22 was measured in relative light units (RLU) in plants expressing HsPDI and compared with Col-0 using luminol-based assay after 120 min-long incubation. Data are averages from three biologically independent experiments, each consisting of three technical replicates. Bars represent standard errors of mean values. Asterisk indicates significance according Student's *t*-test (with P <0.05).

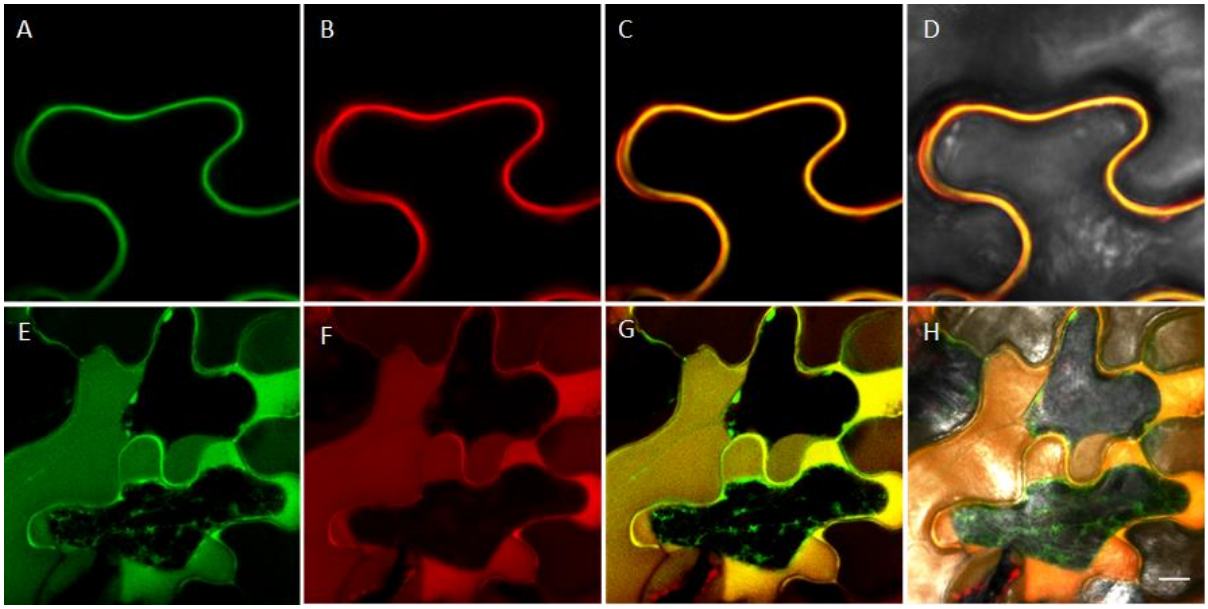

Figure 2.7 Subcellular localization of HsPDI:GFP within *Nicotiana benthamiana* leaf epidermal cell. Observation was done at 6 days post inoculation with Agrobacterium infiltrated at OD600nm = 1 **(A-D)** Florescent signal in the outer cell periphery. **(E-H)** The signal localized in the outer cell periphery after plasmolysis in 1 M NaCl for 10 min. (**A, E**) Green fluorescence originated from HsPDI:GFP fusion protein. **(B, F)** Red fluorescence originated from apoplastic marker::mCherry fusion protein. **(C, G)** Merged image shows the red and the green signal in orange. (**D, H)** Merged image shows the florescent signal in bright field. Bars = 10μm.

Supporting information

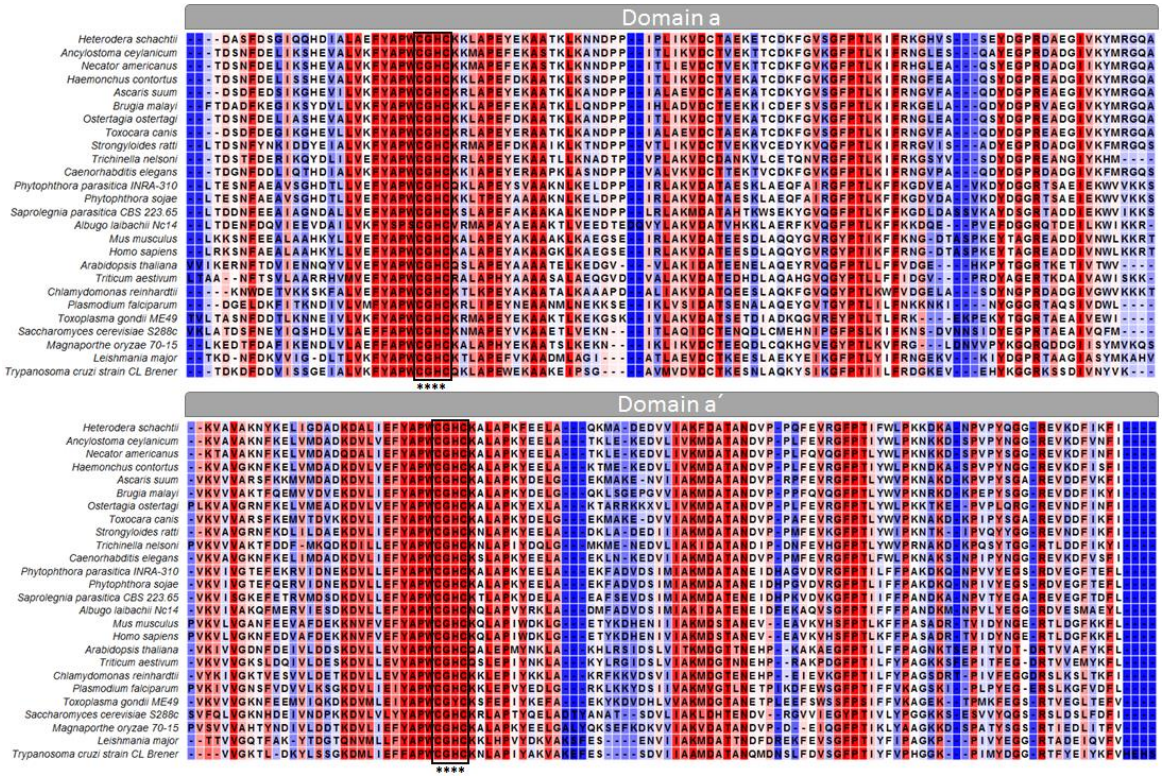

Figure 2.S1 Multiple sequence alignment of the a and a´ thioredoxin damains from HsPDI and PDIs from other organisms. The regions with red colour background show the conserved sequences with high similarity. The blue coloured background represent the non-conserved sequences. The sequence in the black box shows the active catalytic motifs (CGHC). Alignment was performed using CLC Main Workbench (V7.7.3).

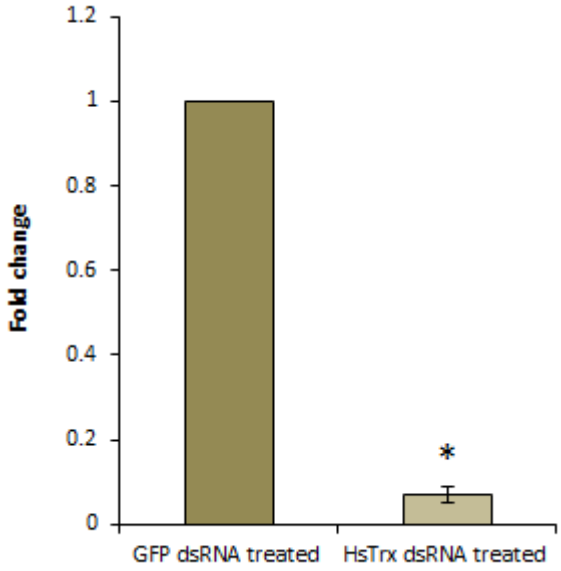

Figure 2.S2 Silencing of HsPDI expression in J2s via RNA interference. Freshly hatched J2s were incubated in HsPDI dsRNA or GFP dsRNA as negative control. Bars represent standard errors of mean values. Experiment was repeated three times. Asterisk indicates significance according to Student's *t*-test (with $P < 0.05$).

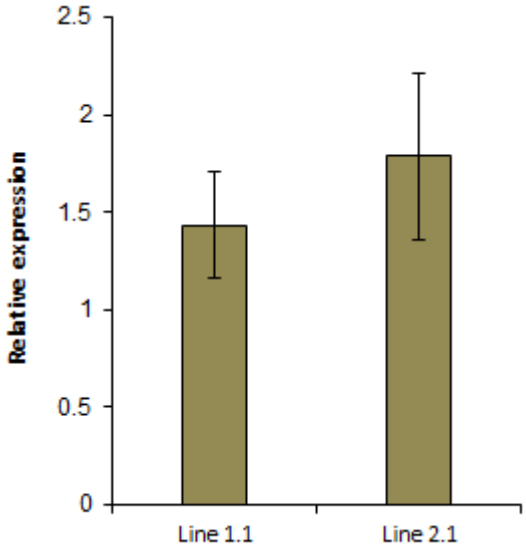

Figure 2.S3 Expression of HsPDI transcripts in transgenic Arabidopsis confirmed by qRT-PCR. The qRT-PCR was performed on two independent T3, homozygous Arabidopsis lines. The expression of the transgene was determined in relation to the Arabidopsis housekeeping gene Actin. Bars represent standard errors of mean values.

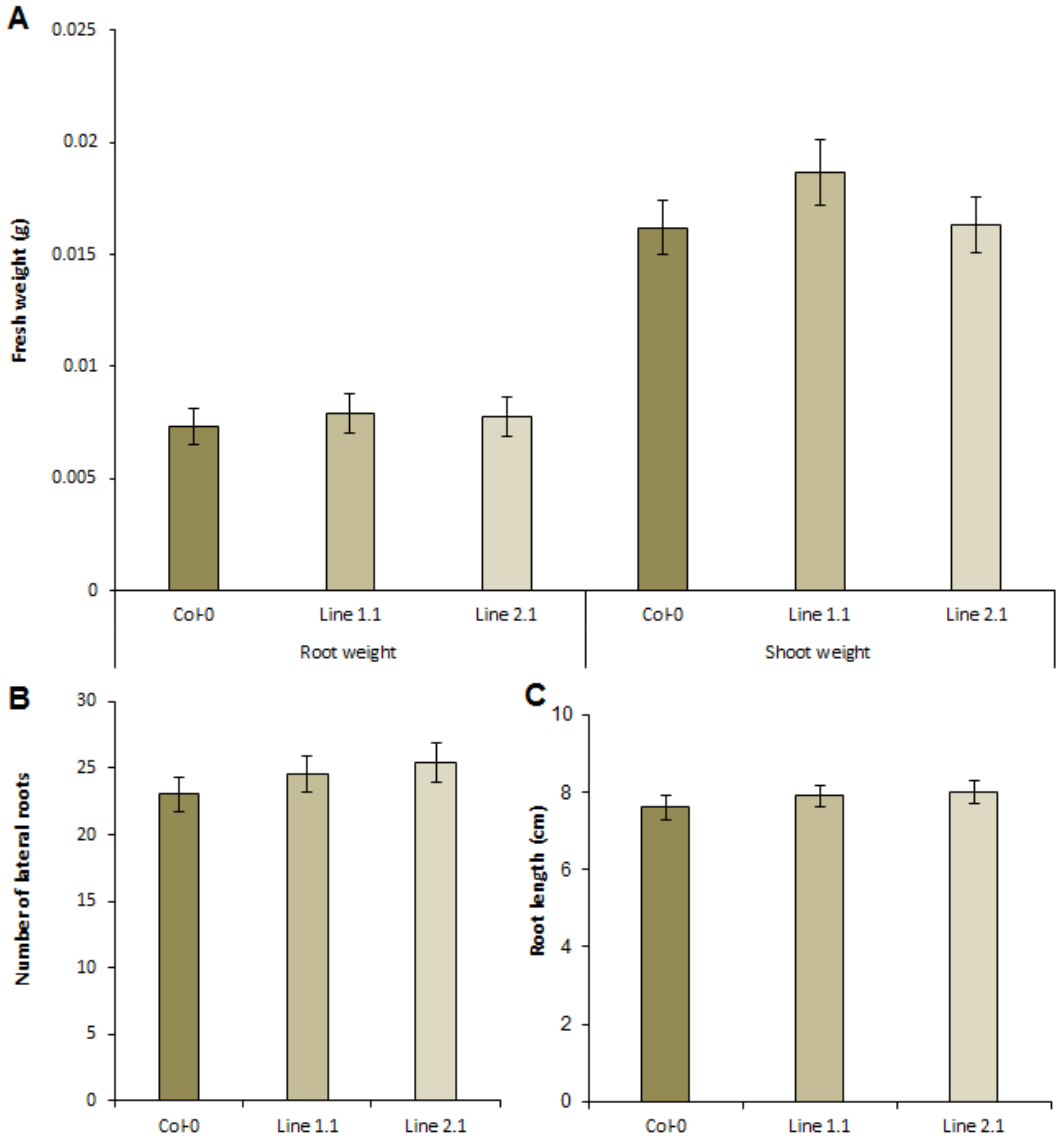

Figure 2.S4 Effect of ectopic expression of HsPDI on Arabidopsis growth. The following parameters were analysed: (A) root and shoot fresh weight (B) number of lateral roots per plant (C) length of main root compared with Col-0. Data represent the average of three independent experiments with 10 plants per each. Bars represent standard error of the mean. Asterisk marks indicates significance in a student's *t*-test (with $P < 0.05$).

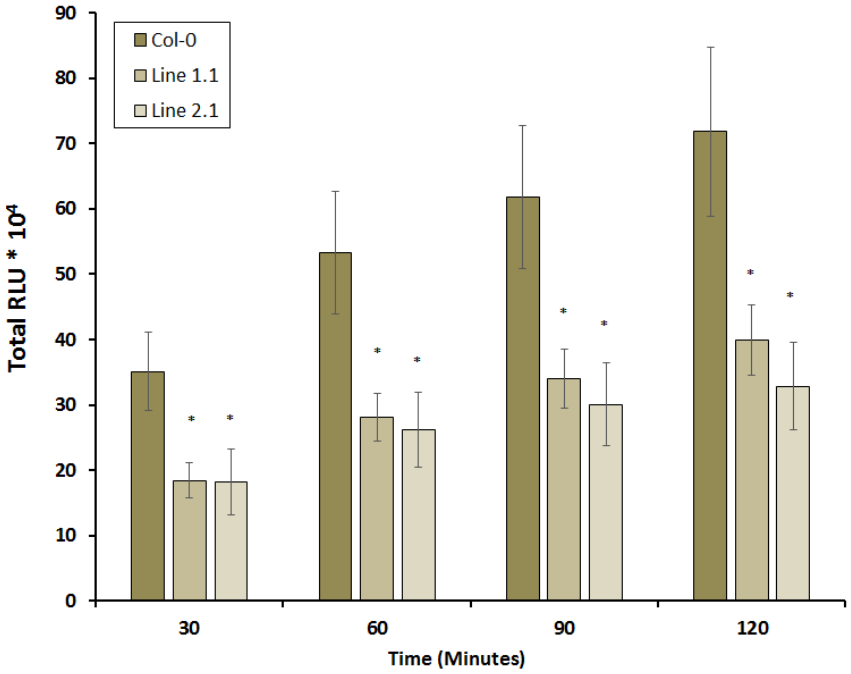

Figure 2.S5 ROS bursts in response to the bacterial elicitor peptide flg22 in transgenic lines. ROS burst was measured in relative light units (RLU) in plants expressing HsPDI and compared with Col-0 using luminol-based assay after 30, 60, 90 and 120 min-long incubation. Data are averages from three biologically independent experiments, each consisting of three technical replicates. Bars represent standard errors of mean values. Asterisk indicates significance according Student's *t*-test (with $P < 0.05$).

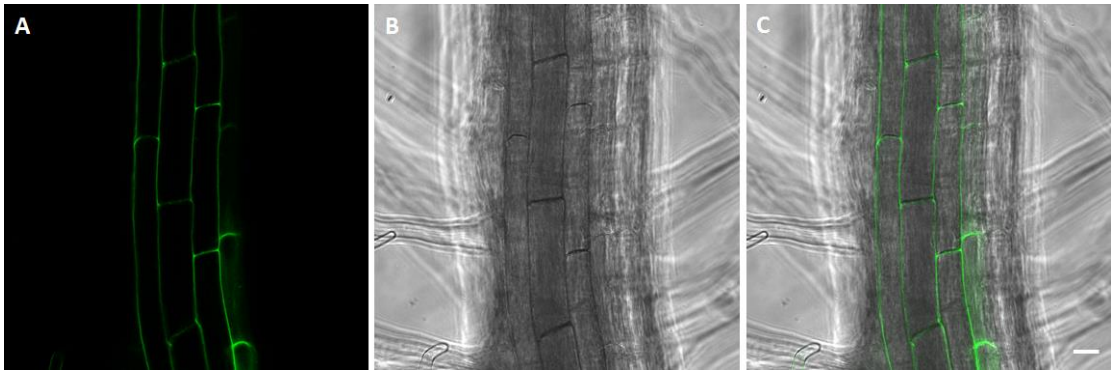

Figure 2.S6 Subcellular localization of HsPDI:GFP within transgenic *Arabidopsis thaliana* roots. Florescent signal of HsPDI::GFP was observed under confocal microscope in the apoplast of the root cells. (A) Dark filed (B) bright field (C) Merged photo. Bars = 10µm.

Table 2.S1 Details of sequences used for phylogeny analysis

Species	**Contig name/ Accession number**	**Identity %**
Ancylostoma ceylanicum	EYC45980	76.6
Necator americanus	XP_013298836	74.8
Haemonchus contortus	CDJ86767	76.5
Ascaris suum	ERG84937	71.3
Brugia malayi	XP_001897232	36.9
Ostertagia ostertagi	CAD29445	71.5
Toxocara canis	KHN78570	72.9
Strongyloides ratti	CEF66381	70.5
Trichinella nelsoni	KRX16041	56.8
Caenorhabditis elegans	NP_491995	73.3
Phytophthora parasitica	XP_008914616	36.9
Phytophthora sojae	XP_009520350	36.9
Saprolegnia parasitica	KDO30563	37.7
Albugo laibachii	CCA26649	32.8
Mus musculus	NP_035162	33.8
Homo sapiens	NP_000909	34
Arabidopsis thaliana	NP_851234	31
Triticum aestivum	BAO79451	31.7
Chlamydomonas reinhardtii	XP_001701755	33.3
Plasmodium falciparum	CAC15387	30.2
Toxoplasma gondii	XP_002371293	32.3
Saccharomyces cerevisiae	NP_009887	31.9
Magnaporthe oryzae	XP_003710672	37.8
Leishmania major	AAN75008	28.9
Trypanosoma cruzi	XP_821173	31

Table 2.S2 Primer labels and sequences used in the study

Primer label	**Primer sequence**
HsPDI-In situ-F	GAAGGAGAAAGCAAGCTG
HsPDI -In situ-R	TGCACTTTGCGCTTGTAA
HsPDI -qRT-PCR- F	CGAACAATCCACCGACCCTC
HsPDI -qRT-PCR- R	ACATTAGGGGAGAAGGAG
Hs-Actin-F	CGTGACCTCACTGACTACCT
Hs-Actin-R	CGTAGCACAACTTCTCCTTG
RNAi-F	TAATACGACTCACTATAGGGAGA GAAGGAGAAAGCAAGCTG
RNAi-R	CATACGATTTAGGTGACACTATAG TGCACTTTGCGCTTGTAA
HsPDI Localization Fw	ATGAATAGTGTTTTATCGATC
HsPDI Localization Rw	GAGTTCCTCAGCCTTTGC
At-Actin-F	ACAGCAGAGCGGGAAATTGT
At-Actin-R	AGCAGCTTCCATTCCCACAA
Rlk Fw	gcCCATGGCAATGACCCGTGATGACAAATTC
RlkNter Rw	gcCCATGGGCGGACGAGTGTATCTGCACGG

2.7 References

Ali Khan H, Mutus B. 2014. Protein disulfide isomerase a multifunctional protein with multiple physiological roles. *Frontiers in Chemistry* **2**:70**.** doi:10.3389/fchem.2014.00070.

Ali S, Magne M, Chen S, Côté O, Stare BG, Obradovic N, Jamshaid L, Wang X, Bélair G, Moffett P. 2015. Analysis of putative apoplastic effectors from the nematode, *Globodera rostochiensis*, and identification of an expansin-like protein that can induce and suppress host defences. *PLoS One* **10**: e0115042.

Appenzeller-Herzog C, Ellgaard L. 2008. The human PDI family: versatility packed into a single fold**.** *Biochimica et Biophysica Acta* **1783**: 535-548.

Baker CJ, Orlandi EW. 1995. Active oxygen in plant pathogenesis. *Annual Review of Phytopathology* **33:** 299-321.

Ben Achour Y, Chenik M, Louzir H, Dellagi K. 2002. Identification of a disulfide isomerase protein of *Leishmania* major as a putative virulence factor. *Infection and Immunity* **70**: 3576-3585.

Blackman LM, Hardham AR. 2008. Regulation of catalase activity and gene expression during *Phytophthora nicotianae* development and infection of tobacco. *Molecular Plant Pathology* **9**: 495-510.

Clough SJ, Bent AF. 1998. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16**: 735-743.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiology* **133**: 462-469.

Daneshkhah R, Cabello S, Rozanska E, Sobczak M, Grundler FMW, Wieczorek K, Hofmann J. 2013. *Piriformospora indica* antagonizes cyst nematode infection and development in Arabidopsis roots. *Journal of Experimental Botany* **64**: 3763-3774.

De APAM, Verissimo-Filho S, Guimaraes LL, Silva AC, Takiuti JT, Lopes LR. 2011. Protein disulfide isomerase redox-dependent association with p47(phox): evidence for an organizer role in leukocyte NADPH oxidase activation**.** *Journal of Leukocyte Biology* **90**: 799-810.

de Boer JM, Yan Y, Smant G, Davis EL, Baum TJ. 1998. *In-situ* hybridization to messenger RNA in *Heterodera glycines*. *Journal of Nematology* **30**: 309-312.

Dubreuil G, Deleury E, Magliano M, Jaouannet M, Abad P, Rosso M. 2011. Peroxiredoxins from the plant parasitic root-knot nematode, *Meloidogyne incognita*, are required for successful development within the host. *International Journal of Parasitology* **41**: 385-396.

Flores-Cruz Z, Allen C. 2009. *Ralstonia solanacearum* encounters an oxidative environment during tomato infection. *Molecular Plant-Microbe Interactions* **22**: 773-782.

Foley RC, Gleason CA, Anderson JP, Hamann T, Singh KB. 2013. Genetic and genomic analysis of *Rhizoctonia solani* interactions with Arabidopsis; Evidence of resistance mediated through NADPH oxidases. *PLoS One* **8**: e56814.

Forman HJ, Maiorino M, Ursini F. 2010. Signaling functions of reactive oxygen species. *Biochemistry* **49**: 835-842.

Frand AR, Kaiser CA. 1999. Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. *Molecular cell* **4**: 469-477.

Golinowski W, Grundler FMW, Sobczak M. 1996. Changes in the structure of *Arabidopsis thaliana* during female development of the plant-parasitic nematode *Heterodera schachtii*. *Protoplasma* **194**: 103-116.

Goverse A, Smant G. 2014. The activation and suppression of plant innate immunity by parasitic nematodes. *Annual Review of Phytopathology* **52**: 243-265.

Hamamouch N, Li C, Hewezi T, Baum TJ, Mitchum MG, Hussey RS, Vodkin LO, Davis EL. 2012. The interaction of the novel 30C02 cyst nematode effector protein with a plant β-1,3-endoglucanase may suppress host defence to promote parasitism. *Journal of Experimental Botany* **63**: 3683-3696.

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM. 2000. pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. *Plant molecular biology* **42**(6): 819-832.

Henkle-Dührsen K, Kampkötter A. 2001. Antioxidant enzyme families in parasitic nematodes. *Molecular and Biochemical Parasitology* **114**: 129-142.

Hewezi T, Howe PJ, Maier TR, Hussey RS, Mitchum MG, Davis EL, Baum TJ. 2010. Arabidopsis spermidine synthase is targeted by an effector protein of the cyst

nematode *Heterodera schachtii*. *Plant Physiology* **152**: 968-984.

Hofmann J, El Ashry A, Anwar S, Erban A, Kopka J, Grundler FMW. 2010. Metabolic profiling reveals local and systemic responses of host plants to nematode parasitism. *Plant Journal* **62**(6): 1058-1071.

Holbein J, Grundler FM, Siddique S. 2016. Plant basal resistance to nematodes: an update. *Journal of Experimental Botany* **67**(7): 2049-2061.

Hong BX, Soong L. 2008. Identification and enzymatic activities of four protein disulfide isomerase (PDI) isoforms of *Leishmania amazonensis*. *Parasitology Research* **102**: 437-446.

Jones JT, Kumar A, Pylypenko L, Thirugnanasambandam A, Castelli L, Chapman S, Cock PJ, Grenier E, Lilley CJ, Phillips MS, *et al.* 2009. Identification and functional characterization of effectors in expressed sequence tags from various life cycle stages of the potato cyst nematode *Globodera pallida*. *Molecular Plant Pathology* **10**: 815-828.

Jones JT, Reavy B, Smant G, Prior AE. 2004. Glutathione peroxidases of the potato cyst nematode *Globodera rostochiensis*. *Gene* **324**: 47-54.

Kadota Y, Sklenar J, Derbyshire P, Stransfeld L, Asai S, Ntoukakis V, Jones JD, Shirasu K, Menke F, Jones A, Zipfel C. 2014. Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity. *Molecular Cell* **54**(1): 43-55.

Kim J, Mayfield SP. 2002. The active site of the thioredoxin-like domain of chloroplast protein disulfide isomerase, RB60, catalyzes the redox-regulated binding of chloroplast poly(A)-binding protein, RB47, to the 5' untranslated region of psbA mRNA. *Plant & Cell Physiology* **43**: 1238-1243.

Kozlov G, Maattanen P, Thomas DY, Gehring K. 2010. A structural overview of the PDI family of proteins. *Federation of European Biochemical Societies Journal* **277**: 3924-3936**.**

Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of Molecular Biology* **305**: 567-580.

Kunchithapautham K, Padmavathi B, Narayanan RB, Kaliraj P, Scott AL. 2003. Thioredoxin from *Brugia malayi* : Defining a 16-kiloDalton class of thioredoxins from nematodes. *Infection and Immunity* **71**: 4119-4126.

Li Z, Liu X, Chu Y, Wang Y, Zhang Q, Zhou X. 2011. Cloning and characterization of a 2-Cys peroxiredoxin in the pine wood nematode, *Bursaphelenchus xylophilus*, a putative genetic factor facilitating the infestation. *International Journal of Biological Sciences* **7**: 823-836.

Lin B, Zhuo K, Chen S, Hu L, Sun L, Wang X, Zhang L, Liao J. 2016. A novel nematode effector suppresses plant immunity by activating host reactive oxygen species-scavenging system. *New Phytologist* **209**: 1159-1173.

Lozano-Torres JL, Wilbers RHP, Warmerdam S, Finkers-Tomczak A, Diaz-Granados A, van Schaik CC, Helder J, Bakker J, Goverse A, Schots A, *et al.* 2014. Apoplastic venom allergen-like proteins of cyst nematodes modulate the activation of basal plant innate immunity by cell surface receptors. *PLoS Pathogens* **10**: e1004569.

Mahajan B, Noiva R, Yadava A, Zheng H, Majam V, Mohan KVK, *et al*. 2006. Protein disulfide isomerase assisted protein folding in malaria parasites**.** *International Journal for Parasitology* **36**: 1037-1048**.**

Maier TR, Hewezi T, Peng J, Baum TJ. 2013. Isolation of whole esophageal gland cells from plant-parasitic nematodes for transcriptome analyses and effector identification. *Molecular Plant-Microbe Interactions* **26**: 31-35.

Meng Y, Zhang Q, Zhang M, *et al*. 2015. The protein disulfide isomerase 1 of *Phytophthora parasitica* (PpPDI1) is associated with the haustoria-like structures and contributes to plant infection**.** *Frontiers in Plant Science* **6**: 632. doi:10.3389/fpls.2015.00632.

Mitchum MG, Hussey RS, Baum TJ, Wang X, Elling A, Wubben M, Davis EL. 2013. Nematode effector proteins: An emerging paradigm of parasitism. *New Phytologist* **199**: 879-894.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. 2004. Reactive oxygen gene network of plants. *Trends in Plant Science* **9**: 490-498.

Molina L, Kahmann R. 2007. An *Ustilago maydis* gene involved in H_2O_2

detoxification is required for virulence. *Plant Cell* **19**: 2293-2309.

Molinari S, Miacola C. 1997. Antioxidant enzymes in phytoparasitic nematodes. *Journal of Nematology* **29**(2): 153-159.

Melillo MT, Leonetti P, Bongiovanni M, Castagnone-Sereno P, Bleve-Zacheo T. 2006. Modulation of reactive oxygen species activities and H2O2 accumulation during compatible and incompatible tomato-root-knot nematode interactions. *New Phytologist* **170**(3): 501-512.

Müller J, Rehbock K, Wyss U. 1982. Growth of *Heterodera schachtii* with remarks on amounts of food consumed. *Revue de Nématologie* **4**: 227-234.

Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods* **8**: 785-786.

Pfaffl MW. **2001**. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**: e45.

Prince DC, Drurey C, Zipfel C, Hogenhout S. 2014. The leucine-rich repeat receptor-like kinase BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 and the cytochrome P450 PHYTOALEXIN DEFICIENT3 contribute to innate immunity to aphids in Arabidopsis. *Plant Physiology* **164**: 2207-2219.

Quentin M, Abad P, Favery B. 2013. Plant parasitic nematode effectors target host defense and nuclear functions to establish feeding cells. *Frontiers in Plant Science* **4**: 53. doi: 10.3389/fpls.2013.00053

Robertson L, Robertson WM, Sobczak M, Helder J, Tetaud E, Ariyanayagam MR, Ferguson MAJ, Fairlamb A, Jones JT. 2000. Cloning, expression and functional characterisation of a peroxiredoxin from the potato cyst nematode *Globodera rostochiensis*. *Molecular and Biochemical Parasitology* **111**: 41-49.

Shetty NP, Mehrabi R, Lütken H, Haldrup A, Kema GHJ, Collinge DB, Jørgensen HJL. 2007. Role of hydrogen peroxide during the interaction between the hemibiotrophic fungal pathogen *Septoria tritici* and wheat. *New Phytologist* **174**: 637-647.

Siddique S, Matera C, Radakovic ZS, Shamim Hasan M, Gutbrod P, Rozanska E, Sobczak M, Angel Torres M, Grundler FMW. 2014. Parasitic worms stimulate

host NADPH oxidases to produce reactive oxygen species that limit plant cell death and promote infection. *Science Signaling* **7**: ra33.

Sijmons PC, Grundler FMW, von Mende N, Burrows PR, Wyss U. 1991. *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *Plant Journal* **1**: 245-254.

Slesak I, Libik M, Karpinska B, Karpinski S, Miszalski Z. 2007. The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. *Acta Biochimica Polonica* **54**: 39-50.

Sobczak M, Golinowski W, Grundler FMW. 1997. Changes in the structure of *Arabidopsis thaliana* roots induced during development of males of the plant parasitic nematode *Heterodera schachtii*. *European Journal of Plant Pathology* **103**: 113-124.

Sotirchos IM, Hudson AL, Ellis J, Davey MW. 2009. A unique thioredoxin of the parasitic nematode *Haemonchus contortus* with glutaredoxin activity. *Free Radical Biology and Medicine* **46**: 579-585.

Sparkes IA, Runions J, Kearns A, Hawes C. 2006. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nature Protocols* **1**: 2019-2025.

Stolf BS, Smyrnias I, Lopes LR, Vendramin A, Goto H, Laurindo FRM, *et al.* 2011. Protein disulfide isomerase and host-pathogen interaction. *Scientific World Journal* **11**: 1749-1761.

Szakasits D, Heinen P, Wieczorek K, Hofmann J, Wagner F, Kreil DP, Sykacek P, Grundler FMW, Bohlmann H. 2009. The transcriptome of syncytia induced by the cyst nematode *Heterodera schachtii* in Arabidopsis roots. *Journal of Experimental Botany* **60**: 771-784.

Torres MA, Dangl JL, Jones JD. 2002. Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proceedings of the National Academy of Sciences* **99**: 517-522

Veal EA, Day AM, Morgan BA. 2007. Hydrogen peroxide sensing and signalling. *Molecular Cell* **26**: 1-14.

Voinnet O, Rivas S, Mestre P, Baulcombe D. 2003. An enhanced transient

expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant Journal* **33**: 949-956.

Waetzig G, Grundler F, Sobczak M. 1999. Localization of hydrogen peroxide during the defence response of *Arabidopsis thaliana* against the plant-parasitic nematode *Heterodera glycines*. *Nematology* **1**: 681-686.

Wang J, Lee C, Replogle A, Joshi S, Korkin D, *et al.* 2010. Dual roles for the variable domain in protein trafficking and host-specific recognition of *Heterodera glycines* CLE effector proteins. *New Phytologist* **187**: 1003-1017**.**

Wang X, Allen R, Ding X, Goellner M, Maier T, de Boer JM, Baum TJ, Hussey RS, Davis EL. 2001. Signal peptide-selection of cDNA cloned directly from the esophageal gland cells of the soybean cyst nematode *Heterodera glycines*. *Molecular Plant-Microbe Interactions* **14**: 536-544.

Williamson VM, Gleason CA. 2003. Plant-nematode interactions. *Current Opinion in Plant Biology* **6**: 327-333.

Wyss U. 1992. Observations on the feeding behaviour of *Heterodera schachtii* throughout development, including events during moulting. *Fundamental and Applied Nematology* **15**: 75-89.

Wyss U, Zunke U. 1986. Observations on the behaviour of second stage juveniles of *Heterodera schachtii* inside host roots. *Revue de Nematologie* **9**: 153-166.

Chapter 3: *Heterodera schachtii* Tyrosinase-like protein - a novel nematode effector

Samer S. Habash, Zoran S. Radakovic, Radomira Vankova, Shahid Siddique, Petre Dobrev, Cynthia Gleason, Florian M.W. Grundler, Abdelnaser Elashry

3.1 Summary

The beet cyst nematode *Heterodera schachtii* causes major yield losses in sugar beet. Understanding the interaction between *H. schachtii* and its host plant is important for developing a sustainable management system. Nematode effectors play a crucial role in initializing and sustaining successful parasitism. In our study, we identified a gene (*Hs-Tyr*) encoding a tyrosinase functional domain (PF00264). We describe *Hs-Tyr* as a novel nematode effector. *Hs-Tyr* is localized in the nematode esophageal gland. Up-regulation of its expression coincided with the parasitic developmental stages of the nematode. Silencing *Hs-Tyr* by RNA interference made the treated nematodes less virulent. When RNAi-treated nematodes succeeded in infecting the plant, developing females and their associated syncytia were significantly smaller than the controls. Ectopically expressing the *Hs-Tyr* effector in Arabidopsis increased plant susceptibility to *H. schachtii,* but not to the root-knot nematode *Meloidogyne incognita*. Interestingly, expressing *Hs-Tyr* in the plant promoted plant growth and changed the root architecture. Additionally, the presence of *Hs-Tyr* in the plant caused changes in the homeostasis of several plant hormones especially auxin and ethylene precursor the aminocyclopropane-carboxylic acid.

3.2 Introduction

Plant parasitic nematodes (PPNs) cause massive yield losses in many important crops and are therefore considered as a major problem in crop production [1]. The beet cyst nematode *H. schachtii* is an important sedentary parasite of sugar beet[2]. In the cysts, the eggs can hibernate for many years in the soil environment. When conditions are favorable, the infective second stage juveniles (J2s) hatch from the eggs and spread in the soil. When they reach a host root, they enter it and migrate through the cells into the vascular cylinder. There they search for a specific cell that is used to establish the initial syncytial cell (ISC)[3,4]. From ISC a highly active syncytial nurse cells systems develops through the fusion of neighboring cells[5]. The hypertrophic and hypermetabolic syncytium serves as the only source of nutrients of the developing juveniles. The nematodes undergo three molts until reaching the adult stage; males leave the root in a vermiform shape, whereas females grow to a lemon-like shape, rupture the root cortex, but continue feeding and remain sedentary. After mating, females produce eggs inside their bodies until they eventually die; their cuticles turn into brown-tanned cysts containing the eggs.

Since the nematodes become sedentary upon feeding, they rely on their syncytia as the sole source of nutrition throughout their life cycle. Therefore, initiating and maintaining the syncytium is the key factor for the nematodes successful parasitism. As a result, nematodes are equipped with effectors which support their parasitism by manipulating the host plant through suppression of plant defenses, and altering developmental and physiological processes[3,6,7].

Recent molecular studies have focused on the identification and characterization of effectors in order to understand their function in the plant-nematode interaction. Some effectors are able to modulate the plant defenses during the parasitism process, either by mimicking plant proteins or manipulating the plant defense. For example, a recent study found that the *H. schachtii* effector 4F01 is mimicking plant annexin and, by doing so, alters the host defenses against nematodes[8]. In another example, effector 10A06 has been shown to interact with spermidine synthase, and this interaction disturbs the host´s ability to produce defense-associated compounds such as salicylic acid[9]. According to a recent report, effector 30C02 binds and inhibits the pathogenesis related protein β-1,3-endoglucanase in the infected Arabidopsis plants and increases

host susceptibility to nematode infection[10]. Nematode effectors can also affect basal immune responses. Basal defenses may be triggered by cell wall fragments produced during the nematode's migration through the root or by unidentified PAMPs. However, *H. schachtii* venom-allergen like protein (VAP) acts as an apoplastic immune repressor which dampens the plant's immune responses. Overexpressing the VAP in Arabidopsis also increased the plant susceptibility to unrelated pathogens, suggesting that it interferes with defense responses to different biotic stresses[11].

In addition to effectors that seem to target plant defenses, nematodes are also able to produce molecules with hormone activity. These effectors enhance the plant's physiological activities to the benefit of the nematodes. Recently, *H. schachtii* juveniles were shown to secret cytokinins into the feeding site, which stimulate cell division and growth for feeding site formation[12]. Additionally, nematodes secrete peptides which mimic plant peptide hormones CLEs and allow the nematode to be able to developmentally reprogram the root cells in order to initiate and maintain its feeding site[13].

Based on the results of the analysis of the transcriptome of a *H. schachtii*, we identified a novel candidate effector that encodes a nematode protein with a Tyrosinase domain (*Hs-Tyr)*. Tyrosinases are copper monooxygenases that catalyze the hydroxylation of monophenols and the oxidation of o-diphenols to o-quinols. These proteins are polyphenol oxidases that are involved in formation of pigments such as melanin and other polyphenolic compounds. They exist in prokaryotes as well as in eukaryotes.

Within the *Hs-Tyr* sequence we found that it contains a signal peptide and lacks a transmembrane domain suggesting that it is a secreted protein. We showed that the *Hs-Tyr* is transcribed in the esophageal gland and is required for successful nematode parasitism. Moreover, expressing the *Hs-Tyr* in Arabidopsis increased the plants' susceptibility to *H. schachtti*, increased vegetative growth and induced hormonal changes, suggesting that *Hs-Tyr* affects plant growth and development to support nematode parasitism.

3.3 Results

Sequence domains prediction and phylogenetic analysis

The transcriptome analysis of *H. schachtii* J2s using next generation sequencing (Illumina) revealed a sequence that we designated *Hs-Tyr* (accession No. KU975565). It contains a tyrosinase domain (E-value: 3.2E-11), defensin_2 (E-value: 0.0043), and 4 SHK domains like (E-value: 2.2E-34) as predicted by pfam domain analysis. Further sequence analysis predicted that the protein contained a signal peptide of 19 amino acids and lacked any transmembrane domains. Aligning *Hs-Tyr* to other tyrosinases from several nematode species revealed a high level of sequence and structure similarity. In comparison to *Hs-Tyr*, Ce-tyr4 (*Caenorhabitis elegans*), Nab_25123_c0_seq1 (*Nacobus aberrans*), and GPLIN_000202700 (*Globodera pallida*) have the most similar organization of functional domains. The phylogenetic analysis of nematode tyrosinases showed that tyrosinases of plant parasites cluster separately from that of free living and animal parasitic nematodes. Furthermore, within the cluster of plant parasitic nematodes, the tyrosinases of the cyst- and root knot-nematodes cluster separately (Figure 3.1).

The *Hs-Tyr* localization and expression profile analysis

To localize *Hs-Tyr* expression in the nematode body we performed a whole mount in situ hybridization on the pre-infective J2s. The hybridized riboprobe was visualized within the esophageal gland cells of the J2s (Figure 3.2a), while no signal appeared in the negative control using the sense probe (Figure 3.2b). We further studied the expression profile of *Hs-Tyr* in correlation with the eggs, J2s, J3s, J4s, females and late females by qRT-PCR amplification using stage specific cDNA. Results showed that *Hs-Tyr* expression was the lowest in eggs compared with the infective stages. The expression of *Hs-Tyr* was not significantly changed in pre-parasitic J2s, but there was a massive increase in expression in the later life stages when the nematodes had started feeding. The highest expression was in J3s. Later, gene expression decreased in J4s and female stages. The high expression of *Hs-Tyr* in the parasitic stages compared to the pre-parasitic stages suggests that it plays a role in nematode parasitism (Figure 3.2c).

The effect of *Hs-Tyr* silencing and ectopic expression in the plant on nematode infection

In order to analyze the role of *Hs-Tyr* for *H. schachtii* parasitism, we silenced *Hs-Tyr* in the nematodes by RNA-interference. Soaking the pre-parasitic J2s in *Hs-Tyr* dsRNA

knocked down 80% of the endogenous *Hs-Tyr* transcript compared with nematodes treated with GFP dsRNA (Figure 3.S1). In a next experiment, plants were inoculated with the dsRNA treated nematodes. Soaked nematodes were clearly affected in development. The numbers of males and females per plant (12 days after inoculation (DAI)) decreased significantly compared with nematodes soaked in the GFP dsRNA control (Figure 3.3a). Total nematode infection decreased by 50% (Figure 3.3b). The average female size was also reduced significantly compared with the GFP-treated nematodes (Figure 3.3c). Syncytia associated with *Hs-Tyr* treated nematodes were significantly smaller, reaching a size of 0,2 mm^2 compared with 0,27 mm^2 in the control (Figure 3.3d).

In order to examine whether expressing *Hs-Tyr* in Arabidopsis affects susceptibility to *H. schachtii* infection, three separate transgenic Arabidopsis lines ectopically expressing 35s::*Hs-Tyr* were infected with J2s. The transgenic lines showed increased susceptibility to nematode infection. The total number of nematodes and the number of mature females increased significantly in lines 2.3 and 5.7 while no significant increase was found in line 14.1 (Figure 3.4a and 4b). Similarly, the size of the mature females and their associated syncytia was increased significantly in the overexpression lines 2.3 and 5.7 while line 14.1 showed no significant difference (Figure 3.4c and 4d). We then analyzed the susceptibility of the transgenic plant lines to other sedentary nematodes. Therefore they were infected with *M. incognita*, but the number and size of galls did not change compared with the wild type Col-0 (Figure 3.S2).

Ectopic expression of *Hs-Tyr* in Arabidopsis stimulates plant growth and modulates root architecture

Growth and development of the transgenic Arabidopsis plants ectopically expressing the *Hs-Tyr* were analyzed and compared with Col-0. The *Hs-Tyr* expressing lines did not show changes in root length and root weight compared with Col-0 (Figure 3.S3), but the root architecture was significantly changed. The number of lateral roots compared to the Col-0 plants was higher (Figure 3.5a) and the shoot weight and growth was significantly increased (Figure 3.5b, c and d).

Ectopic expression of *Hs-Tyr* triggers changes in plant hormone homeostasis

To explain the changes of the plant growth, endogenous hormones were measured in *Hs-Tyr* expressing Arabidopsis roots and compared with the wild type Col-0 using

HPLC-MS. Roots of the line 2.3, which showed the highest susceptibility were analyzed and compared with Col-0 roots. Results showed that level of auxins precursor indole-3-acetonitrile as well as content of IAA metabolites was significantly increased in *Hs-Tyr* expressing plants (Figure 3.5). Jasmonate precursor *cis*OPDA concentration was lower in the transgenic plants while no significant change of the jasmonic acid and jasmonate isoleucine was observed. Concentration of the immediate ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was higher in the roots of transgenic plants compared with Col-0. No significant changes were detected in the SA concentration (Figure 3.5).

Hs-Tyr localization in *Nicotiana benthamiana* leaves

To investigate the action site of the *Hs-Tyr* in the plant, a transient transformation of *N. benthamiana* leaves was performed. The leaves were infiltrated with the Agrobacterium expressing *Hs-Tyr::GFP* and checked for green fluorescence after 5 days. Results showed that the protein was translated and its fluorescent signal was localized in the cytoplasm of the *N. benthamiana* leaf cells (Figure 3.6).

3.4 Discussion

H. schachtii can dramatically decrease the yield of sugar beet. Understanding the mechanisms by which nematodes manipulate the plants may give clues in search for novel management strategies. Recent advances in techniques such as the RNAseq and next generation sequencing facilitate mining of plant parasitic nematode genes involved in the parasitism[14,15]. The ability of *H. schachtii* to infect the model host plant *A. thaliana*[16] opens additional perspectives in studying gene functions. Using available transcriptomic data of *H. schachtii* we identified a putative effector that may play a role in the nematode parasitism. The bioinformatic analysis of the predicted amino acid sequence showed the presence of a signal peptide and a lack of transmembrane domains, which is a strong indication that the protein is secreted. These criteria have been used as standards for screening secreted nematode effectors in other labs[17,18]. Furthermore, checking for the functional domain(s) showed the presence of a Tyrosinase like domain that is, up to our knowledge, the first time to be described in relation to nematode plant-parasitism. Phylogenetic analysis revealed *Hs-Tyr* homologues in plant parasitic, animal parasitic and free-living nematodes. The constructed phylogenetic tree displays a relation to other nematodes (Figure 3.1a). It shows that *Ascaris spp.* is clustering separately from *C. elegans* and plant parasitic

nematode species. Furthermore, cyst nematodes form a subcluster separate from root-knot nematodes within the plant parasitic nematode cluster. This separation may also hint to a functional divergence and could explain why the *Hs-Tyr* transgenic lines were more susceptible to cyst nematodes but not to root-knot nematodes.

Supported by several experimental approaches we show that *Hs-Tyr* contributes to successful parasitism of the cyst nematode. First, we found that *Hs-Tyr* expression is exclusively observed within the dorsal oesophageal gland by using whole mount *in situ* hybridization, suggesting that the protein is secreted from the dorsal gland into the plant. Secondly, we showed a dramatic increase in *Hs-Tyr* expression after Arabidopsis infection, which is further linking *Hs-Tyr* expression with a role in parasitism. Knocking down *Hs-Tyr* by RNAi resulted in approximately 80% decrease of *Hs-Tyr* expression. This result is in a range that was found in previous studies on gene silencing by RNAi[19]. The *Hs-Tyr*-silenced nematodes were suffering throughout their life stages as indicated by the small size of females and associated syncytia (Figure 3.2). This findings are similar to previous studies, where it was shown that reduced expression of nematode effectors is obstructing nematode development in plants[11,20]. Furthermore, we showed that expressing *Hs-Tyr* in the Arabidopsis plants increased the plant susceptibility to the cyst nematodes. Remarkably, this holds true only for *H. schachtii*, but not for the root-knot nematode *M. incognita*. We therefore conclude that *Hs-Tyr* functions specifically within a pathway supporting an efficient *H. schachtii* parasitism, while being redundant for *M. incognita.*

We did not observe any hypersensitive responses in the infiltrated *N. benthamiana* leaves and in the transgenic plant, suggesting that *Hs-Tyr* was not recognised by the plant immune system. This assumption is in agreement with the lack of significant changes in JA and SA levels. According to previous analyses some nematode effectors induced a hypersensitive response, however, *Hs-Tyr* obviously does not belong to this type of effectors[21].

Interestingly, *Hs-Tyr* expression in the plant caused changes in the plant growth represented by increasing the plant lateral roots and increasing the shoot weight, both features that may be related to the observed changes in hormone homeostasis. We showed that auxin biosynthesis was enhanced, as indicated by higher content of both auxin precursor and auxin metabolites in the transgenic plant. This may explain increased shoot growth as well as stimulation of lateral root formation. Auxin

homeostasis in roots is well known to determine lateral root formation[22]. Hyper-susceptibility of the *Hs-Tyr* overexpression plants to nematodes could be correlated to the hormonal changes in two ways. First, high concentration of the plant ACC/ET results in higher attraction of the infective J2s to the roots, which leads to the increase of the number of J2s infecting the plant. This explanation is supported by previous studies which showed that root exudates of ET-overexpressing mutants are more attractive to nematodes[23]. Furthermore, treating the plant with ethephon as a source of ET, increased the plant attraction to the pre-infective J2s. In contrast, the plant ACC inhibition was triggered by the aminooxyacetic acid treatment decreased the number of females and males developed on the plants[24]. Secondly, a lower concentration of the jasmonic acid increases susceptibility of the transgenic plants towards J2s. In fact, it was shown that JA is a main player during the early plant defence against nematode infection. On the other hand, nematodes were shown to trigger the up-regulation of plant genes which suppress JA-based defence during the infection[24]. Additionally, the Arabidopsis mutants DELAYED DEHISCENCE 2 (*dde2*) and LIPOXYGENASE 6 (l*ox6*), which are deficient in JA biosynthesis showed higher susceptibility towards *H. schachtii* and increased female development compared to wild-type plants[24]. These results indicate that *Hs-Tyr* interferes with plant growth pathways and triggers hormonal changes promoting nematode parasitism[12, 25, 26].

Our results clearly entitle *Hs-Tyr* as an esophageal gland effector protein playing an important role during parasitism of *H. schachtii*. Functional analysis of the proteins suggests a mode of action through changes in the homeostasis of plant hormones.

3.5 Methods

Plant growth and nematode culture

Transgenic lines and the Col-0 wild type *Arabidopsis thaliana* L. Heyn plants were grown aseptically on agar medium supplemented with modified Knop's nutrient solutions for *H. schachtii* infection and on MS medium for *M. incognita* under conditions described previously[16].

Mature cysts were collected from white mustard (*Sinapis alba* L.) cvar. Albatros plants in funnels and hatched in 3mM $ZnCl_2$[16]. The freshly hatched pre-parasitic second stage juveniles (J2s) were collected for direct RNA extraction, infecting the Arabidopsis plants for post infective stages collection and for infection assay.

The *M. incognita* J2s were collected from egg masses cultured on tomato grown in the greenhouse. Eggs were isolated from egg masses on tomato roots with 1.5% sodium hypochlorite and rinsed with water on a 25-µm sieve. Eggs were hatched in a solution of 2ml of gentamycin sulphate (22.5 $mg.ml^{-1}$) and 150 µl of nystatin (10.000 $U.ml^{-1}$) in 30 ml water for 4 days at room temperature in the dark. The hatched nematodes were collected and further surface sterilized as described previously [27]. Briefly, nematodes were surface sterilized by incubation for 20 min in 0.5% (w/v) streptomycin/ penicillin solution, for 20 min in 0.1% (w/v) ampicillin/gentamycin solution, for 5 min in sterile tap water, and for 3 min in 0.1% (v/v) chlorhexidine solution. The nematodes were subsequently washed three times in sterile tap water then used for infection.

Infection assay

Nematode infection assays on Arabidopsis plants either for the RNA interference (RNAi) experiments or on *Hs-Tyr* overexpression lines were performed for the *H. schachtii* as described before[28]. Briefly, seeds were plated for ten days on 0.2% Knop medium. Plants were inoculated with 60-70 J2 nematodes per plant. Various susceptibility parameters including, number of male and female nematodes per plant was counted after 12 DAI. On the following day, average size of female nematodes and associated syncytia were measured. Each experiment contained 12 plants per line.

For *M. incognita* infection, ten days old plants on MS media were infected with 100 nematodes per plant. Number and size of galls were collected after 22 days. Ten plants per line and wild type Col-0 were infected. All measures were taken using Leica M165C Binocular (Leica Microsystems, Wetzlar, Germany) and Leica Application Suite software. Experiments for both nematodes were repeated three times and statically analysed using the Student's t-test.

Sequence analysis and phylogeny

Hs-Tyr was determined as one of the predicted putative secreted protein (PSPs) in *H. schachtii* transcriptome assembly (Elashry et al. unpublished). The *Hs-Tyr* sequence was translated using CLC genomics workbench (V8.0) and analysed to predict the longest ORF and functional domain(s) by Pfam database (http://pfam.xfam.org/) and HMMER algorithm[29], signal peptide by signalP4 server[30], and transmembrane domain by TMHMM[31].

Hs-Tyr homologs in other nematode species were identified using BLASTP search against the nr database in the NCBI database. Further, we downloaded transcriptomes of *M. incognita*[32], *M. hapla*[33], *Nacobus aberrans*[14], *H. avenae*[34] and *G. pallida*[35]. All transcriptomes were examined by CLC genomics workbench (V8.0) to identify *Hs-Tyr* homologues. All *Hs-Tyr* homologues (Table S1) were analysed structurally to confirm similarities of their functional domains and aligned to each other to build a phylogenetic tree by UPGMA algorithm with distance measured by Jukes-Cantor and 1000 bootstraps (CLC genomics workbench V.8.0).

In situ hybridization

Subsequent PCR was performed on gene specific PCR product using primers in Table S2 with presence of DIG-labelled deoxynucleotide triphosphates (dNTPs) (Roche). Riboprobes were prepared using single sense primer (negative control) and the antisense primer. The riboprobes hybridized in pre-parasitic J2s as described previously[36]. The hybridized nematodes were visualized using the DMI2000 compound microscope (Leica Microsystems).

Developmental expression pattern analysis

Real time quantitative PCR was used to analyse the *Hs-Tyr* transcript at different developmental stages of *H. schachtii* using a gene specific primers (Table S2). Around 3000 eggs and 3000 pre-paparsitic J2s were collected directly from cysts. Around 500-600 nematodes were collected manually by separating them from the *A. thaliana* roots after 5, 10, 15, 20 DAI representing J3s, J4s, females and late females respectively.

RNA was extracted using NucleoSpin RNA kit (MACHEREY-NAGEL) following the manufacture´s protocol. The first strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in presence of the oligo-dT primer. The resulted cDNA were tested for the expression changes using the Stepone Plus Real-Time PCR System (Applied Biosystems) following the amplification conditions: 95ºC for 15 s and 60ºC for 30 s (40 cycles). Each sample contained 10 µl of Fast SYBR Green qPCR Master Mix (Invitrogen), 9 µl of the primer mix with final concentration 1µM for each primer, 1 µl of cDNA. The amplified data were analysed using one step system to create Ct values. The resulted data were analysed and relative expression was calculated[37]. Actin was used as internal control for all experiments. Three biological replicates from each stage were used with three technical replicates.

RNA interference and *Hs-Tyr* silencing in nematodes

Hs-Tyr specific dsRNA was generated following the manufacturer's instructions of MEGAscript T7 kit (Ambion, Life Technologies). The GFP DNA fragment was amplified to synthesise dsRNA as a negative control.

Freshly hatched nematode were soaked for one day in 50 µL soaking mix (1µg/µL dsRNA (25µL), 10x soaking buffer (5µL), 100mM spermidine (1.5µL), 500mM octapamine (5µL), nematodes in water (13.5µL)). After that, nematodes were washed three times with sterile water and sterilized using $HgCl_2$ for 4 min. Nematodes were washed three times with fresh water. After sterilization, nematodes were divided to two parts. One part was used to evaluate the gene expression after silencing by qPCR. While, the second part was used to infect the Arabidopsis plants as described above.

Construct generation and *N. benthamiana* agroinfiltration

The *Hs-Tyr* without signal peptide-encoding region was cloned in the binary expression vector pMDC83 using primers in Table S2. The pMDC83 vector contains C-terminal GFP fusion protein driven by 2x 35S promoter[38]. The *Hs-Tyr::GFP* construct was transformed in *Agrobacterium tumefaciens* strain GV3101::pMP90[39]. The transformed Agrobacterium were grown overnight in 50ml YEB liquid medium with 10 $mg.ml^{-1}$ gentamycin, 50 $mg.ml^{-1}$ kanamycin and 50 $mg.ml^{-1}$ rifampicin to an OD600 of 0.8 in an incubator/shaker at 28 ºC. Bacteria were harvested by centrifugation at 4000 rpm for 7 min at room temperature. The pellet was suspended in infiltration buffer[39]. Bacterial suspensions were diluted in the infiltration buffer to OD600= 1. After incubation for 2-4h at RT, Agrobacteria suspensions were infiltrated in the abaxial side of 6 weeks *N. benthamiana* leaves by using 1 ml syringe. For co-infiltration of RNA silencing inhibitor P19, an equal volume of a bacterial suspension harbouring pBin61-P19[40] was mixed and infiltrated. Infiltrated plants were incubated for 5 days. Slides were made from the infiltrated leaves and tested under the confocal microscope for detecting the green signal in the leaves cells.

Production of transgenic lines, phenotyping and infection assays

The *Hs-Tyr* ORF was cloned into the binary Gateway overexpression vector pB2GW7[41]. The construct was then transferred to the *A. tumefaciens* strain GV3101, and transformed into *Arabidopsis thaliana* Col-0 using the floral dip method[42]. The

seeds of the primary transformants were selected for BASTA resistance (Bayer CropScience, Wolfenbüttel, Germany). In the T2 generation, the lines segregating 3:1 (BASTA-resistant/BASTA-susceptible) were grown to the next generation. Three homozygous lines were selected on BASTA plates and used in the study.

The selected lines were grown on MS plates for 10 days, then several phenotypes were measured and compared with the wild type plants Col-0 including the number of the lateral roots, the main root length, the fresh root weight and the fresh shoot weight. The experiment was repeated 3 times and each experiment consists of 9 plants for each line. The selected lines were subjected as well to the infection of the nematodes *H. schachtii* and *M. incognita* as mentioned in the infection assay section.

Hormone analysis

Root samples were collected from ten-day *Hs-Tyr* plants and Col-0. Root samples were purified and analysed as mentioned previously[43,44]. Briefly, samples were homogenized with a ball mill (MM301, Retsch) and extracted in cold (-20 °C) methanol/water/formic acid (15/4/1 v/v/v). The following labelled internal standards (10 pmol/sample) were added: $^{13}C_6$-IAA (Cambridge Isotope Laboratories); $^{2}H_4$-SA (Sigma-Aldrich); $^{2}H_2$-OxIAA and $^{2}H_5$-JA(Olchemim). Extracts were purified using SPE-C18 column (SepPak-C18, Waters) and a mixed mode reverse phase–cation exchange SPE column (Oasis-MCX, Waters). Hormone metabolites were analysed using HPLC (Ultimate 3000, Dionex) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems). Quantification of hormones was done using the isotope dilution method with multilevel calibration curves ($r^2 > 0.99$). Data processing was carried out with Analyst 1.5 software (Applied Biosystems). Data are presented as mean ± standard error.

3.6 Figures

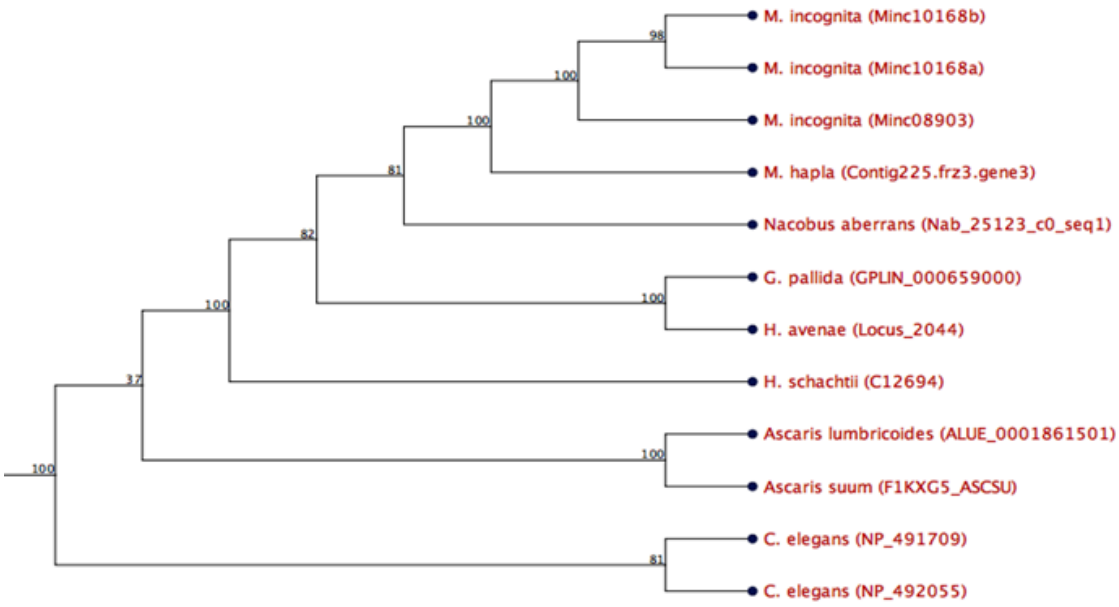

Figure 3.1 *Hs-Tyr* phylogenetic tree for tyrosinase-like genes of several nematode species. Tyrosinase from cyst nematodes clusters separately from root-knot nematodes among plant parasites, which, on the other hand, are separated from the animal parasitic and free–living nematodes. Numbers on branches are the percentage of bootstrap (100bootstrap).

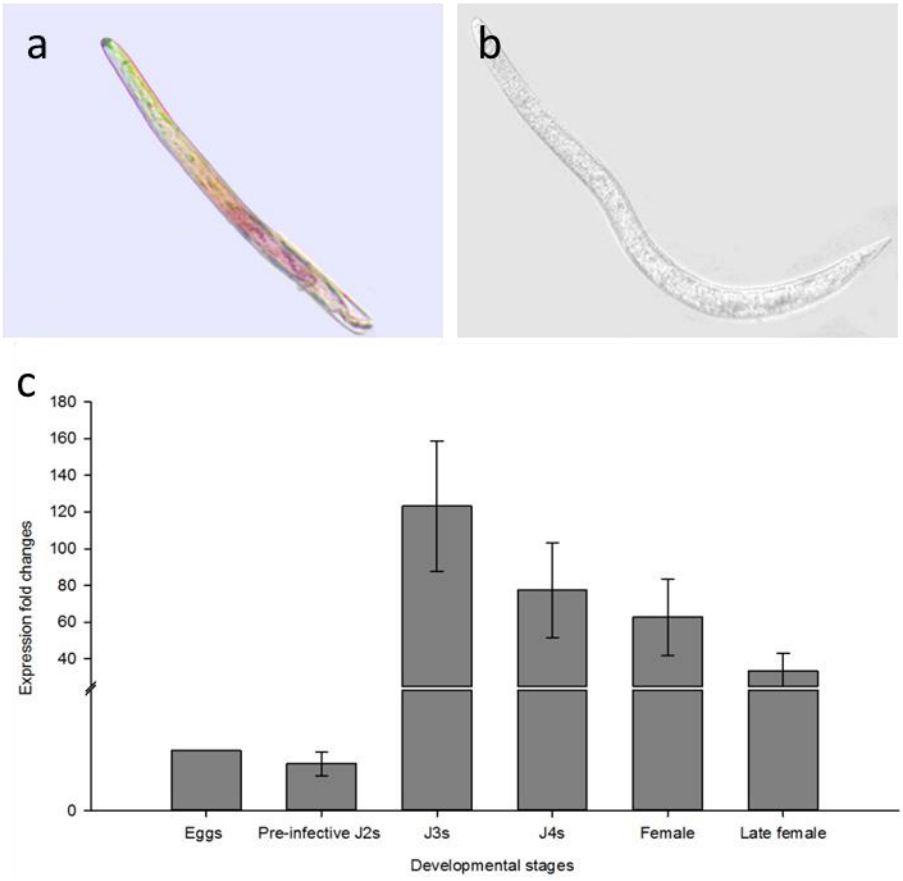

Figure 3.2 *Hs-Tyr* expression localization and profiling. (a) Localization of *Hs-Tyr* transcripts in the esophageal gland cells of *H. schachtii* J2s by whole mount *in situ* hybridization of digoxigenin-labeled antisense cDNA. (b) *In situ* hybridization negative control treated with digoxigenin-labeled sense probes showing no signals in the esophageal gland (Bar=100 μm). (c) Relative mRNA expression levels of *Hs-Tyr* quantified by qPCR in six different life stages of *H. schachtii*. The fold change values were calculated and represent changes in mRNA level in pre-infective J2s, J3, J4, females and late females relative to that of eggs. Data are averages of three biologically independent experiments, each consisting of three technical replicates. *H. schachtii*. Actin was used as an internal control to normalize gene expression level.

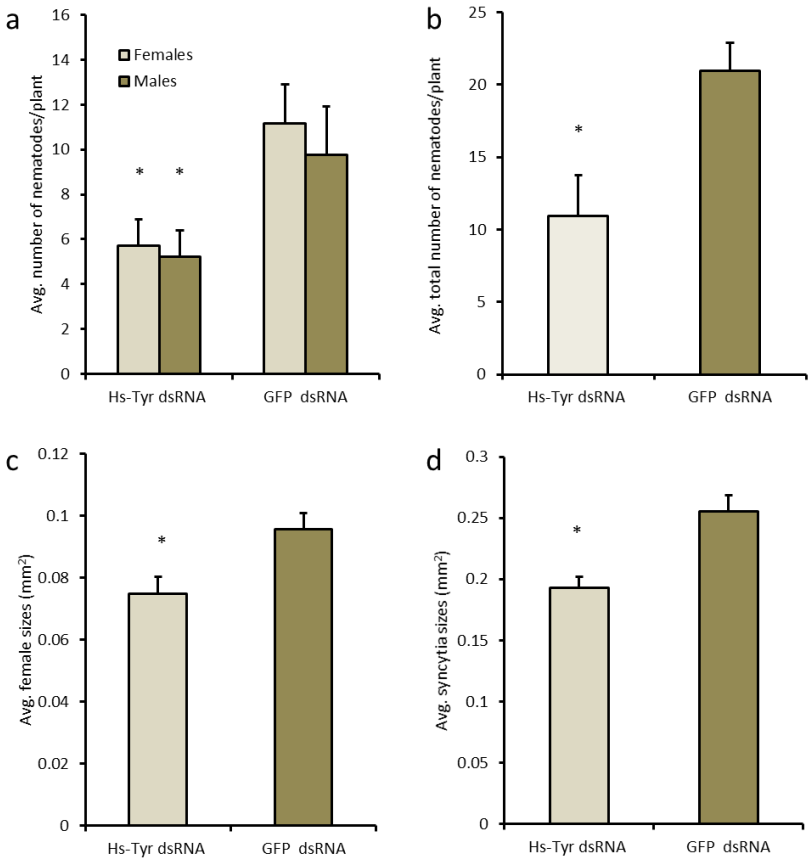

Figure 3.3 Effects of silencing *Hs-Tyr* on *H. schachtii* parasitism. The graphs show the results of bio-assays with J2 soaked in *Hs-Tyr* -specific dsRNA compared with J2 soaked in GFP dsRNA as a negative control. The following parameters were analysed: (a) number of males and females (b) total number of nematode infections (c) female size (d) size of syncytia. Data are based on three independent experiments (means ± SE). Bars represent standard error of the mean values mean. Asterisk marks significance in a Student's *t-test* (with p-value <0.05).

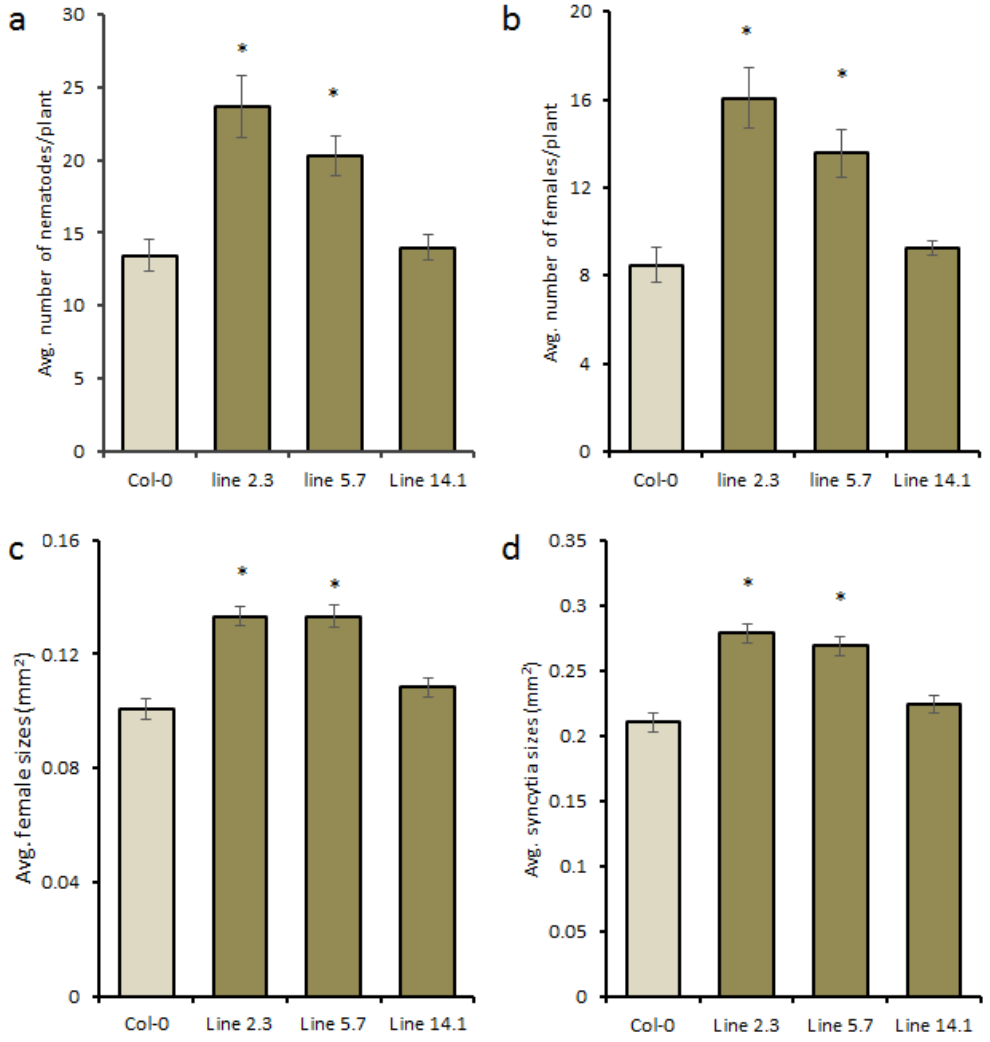

Figure 3.4 Effect of ectopic expression of *Hs-Tyr* on the development of *H. schachtii* in Arabidopsis. The following parameters were analysed: (a) total number of nematode infection per plant (b) number of females per plant (c) female size (d) syncytium size, compared with the wild type Col-0. Data represent average of three independent experiments (means ± SE). Bars represent standard error of the mean. Asterisk marks significance in a Student's *t-test* (with p-value <0.05).

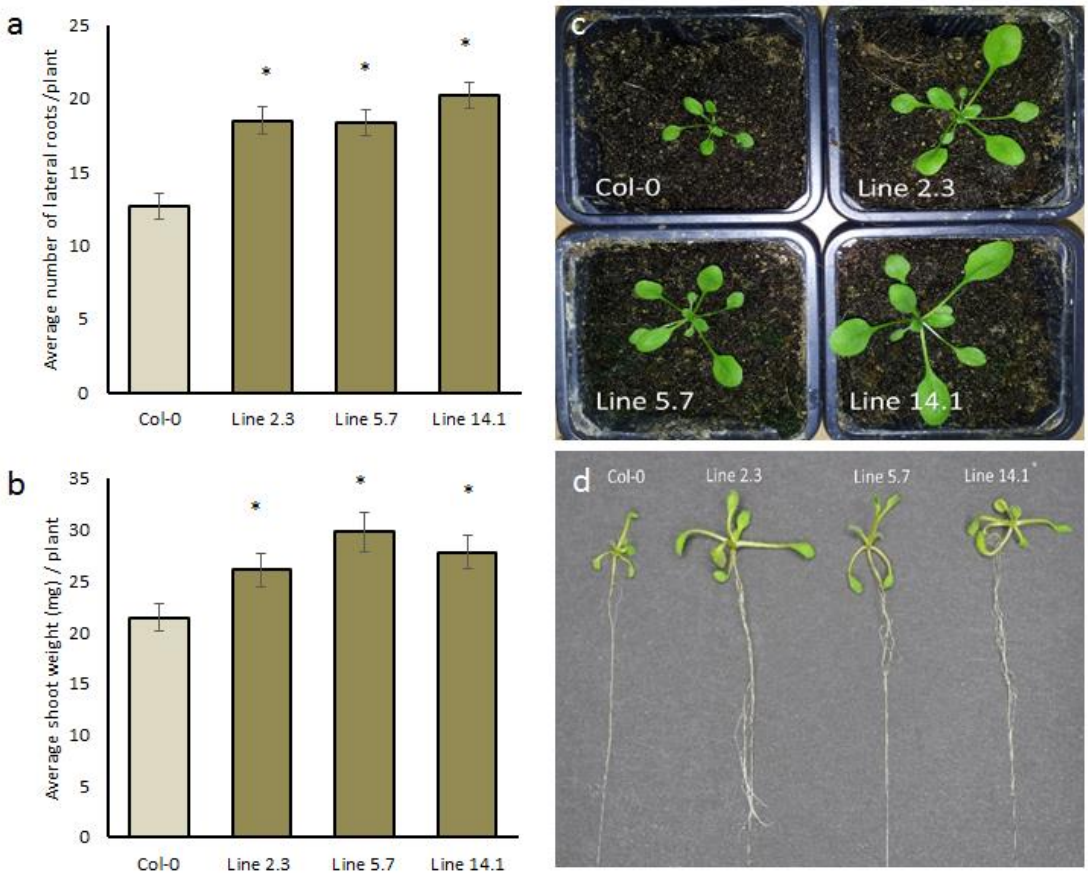

Figure 3.5 Effect of ectopic expression of *Hs-Tyr* on Arabidopsis growth. The following parameters were analysed: (a) number of lateral roots per plant (b) shoot weight and visible increase in the shoot growth. (c) and (d) comparison of the wild type Col-0 with the overexpression lines. Data represent the average of three independent experiments with nine plants per each. Bars represent standard error of the mean. Asterisk marks significance in a student's *t-test* (with p-value <0.05).

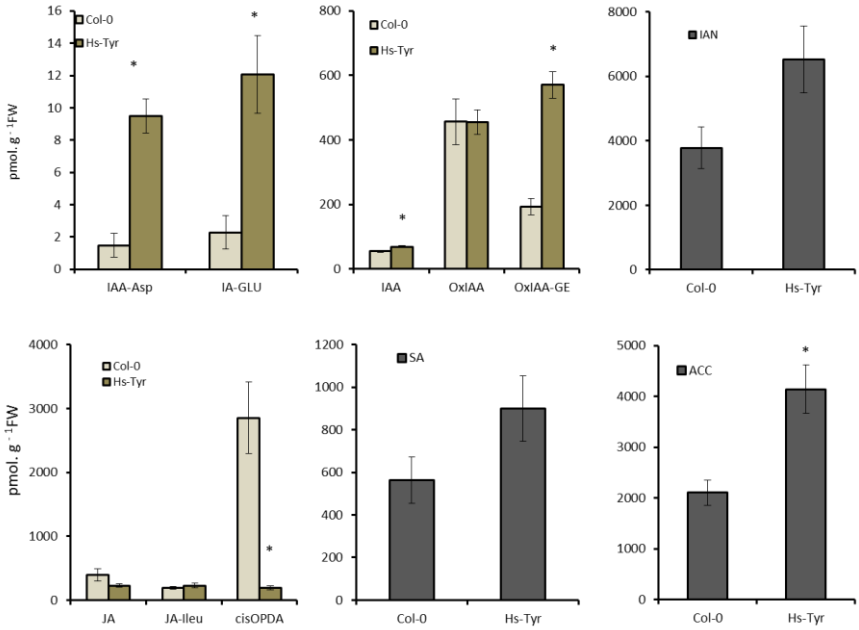

Figure 3.6 Concentration of various hormones (pmol.g^{-1}FW) in roots of transgenic plants ectopically expressing *Hs-Tyr* compared with Col-0. IAA, indole-3-acetic acid; IAA-Asp, IAA-aspartate; IAA-Glu, IAA-glutamate; OxIAA, oxo-IAA; OxIAA-GE = oxo-IAA-glucose ester; IAN, Indole-3-acetonitrile (IAA precursor); of jasmonic acid (JA), JA-isoleucine (JA-Ile), JA precursor *cis*-12-oxo-10,15-phytodienoic acid (cisOPDA), salicylic acid (SA) and 1-aminocyclopropane-1-carboxylic acid (ACC). Values are means ± SE, N=5; asterisks indicate significant differences (*, P<0.05).

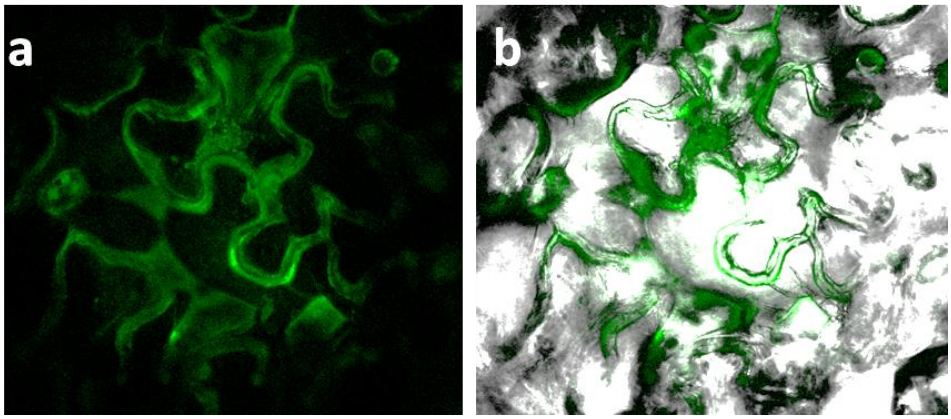

Figure 3.7 Subcellular localization of *Hs-Tyr*::GFP within *N. benthamiana* leaf epidermal cell. (a) The green fluorescence originates from *Hs-Tyr*::GFP fusion protein localized in the cytoplasm of the cells. (**b**) Merged image shows the GFP signal in bright field.

Supporting information

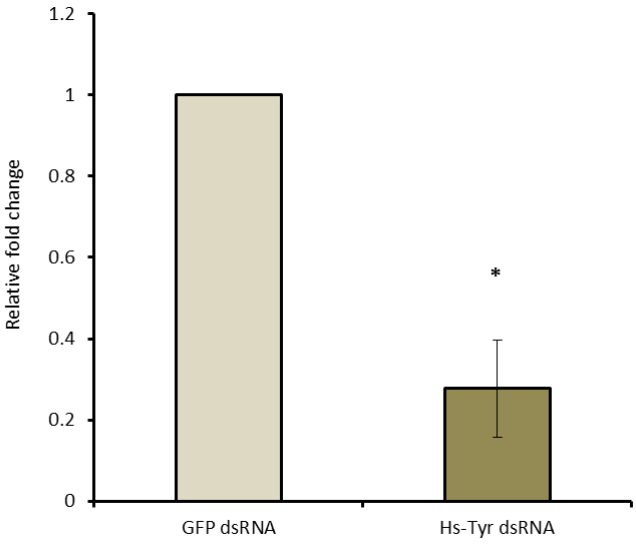

Figure 3.S1 Silencing of *Hs-Tyr* expression in J2s via RNA interference. *Hs-Tyr* expression was measured after incubating the J2s in *Hs-Tyr* dsRNA and compared with the J2s were incubated in GFP dsRNA as control.

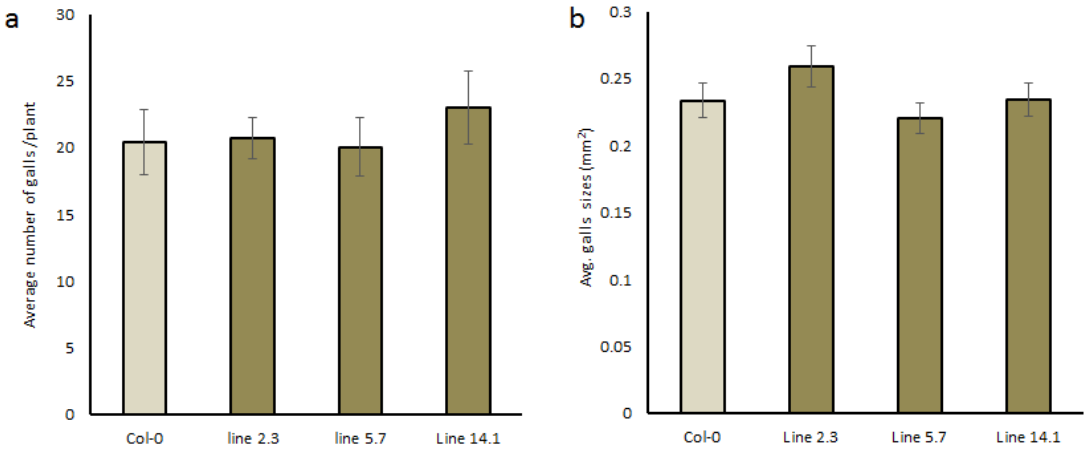

Figure 3.S2 Overexpressing the *Hs-Tyr* did not affect the *M. incognita* infection on Arabidopsis. Transgenic plants didn't show any susceptibility towards nematode parasitism compared with the wild type Col-0 plants represented by (a) Average number of galls per plant (b) Average size of nematode galls. Data points represent average of three independent experiments (means ± SE). Bars represent standard error of the values mean. Significance was tested by student's *t-test* (with p-value <0.05).

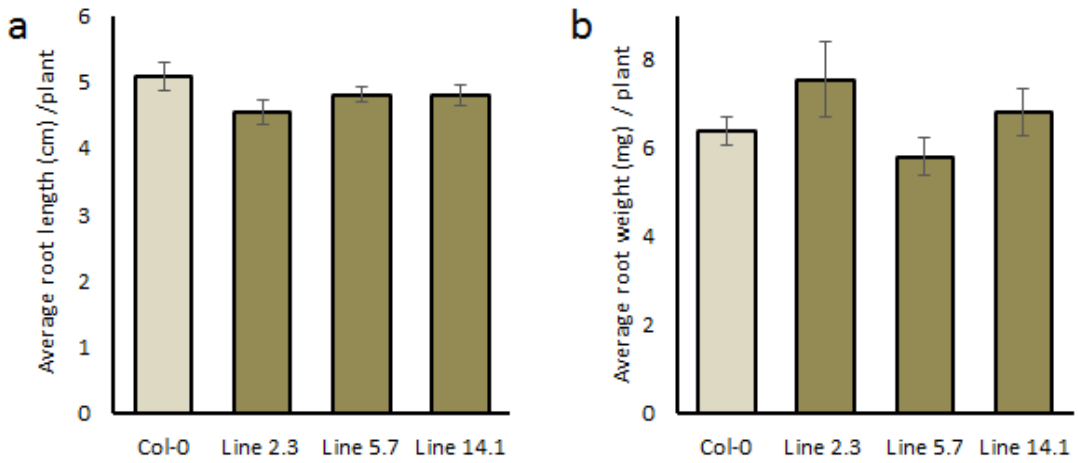

Figure 3.S3 Phenotype of the transgenic Arabidopsis plants root ectopically expressing *Hs-Tyr*. (a) root length per plant (b) root weight per plant, compared with the wild type Col-0. Data represent average of three independent experiments with nine plants per each. Bars represent standard error of the mean. Significance was tested by student's *t-test* (with p-value <0.05).

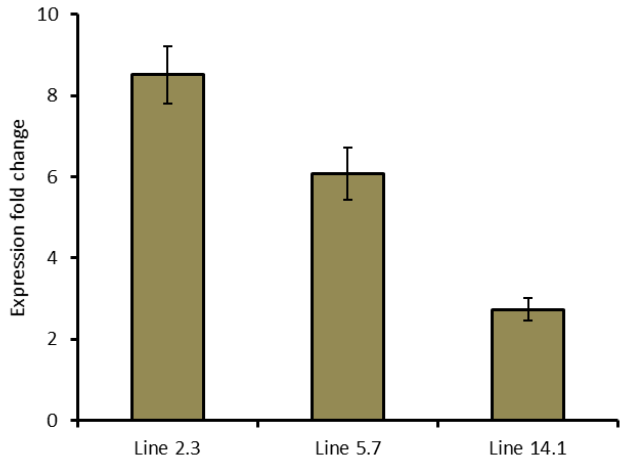

Figure 3.S4 Relative mRNA expression level of *Hs-Tyr* in the transgenic Arabidopsis lines. Data are averages of three biologically independent experiments, each consisting of three technical replicates. The plant actin was used as an internal control to normalize gene expression level.

Table 3.S1 Sequence names and accession numbers that were used in the phylogeny analysis

Species	Contig name/ Accession number	Source
Meloidogyne incognita	Minc10168b	Abad *et al.* 2008
Meloidogyne incognita	Minc10168a	Abad *et al.* 2008
Meloidogyne incognita	Minc08903	Abad *et al.* 2008
Meloidogyne hapla	Contig225.frtz3.gene3	Opperman *et al.* 2008
Nacobbus aberrans	Nab_25123_c0_seq1	Eves-van den Akker et al.2014
Heterodera avenae	Locus_2044	Kumar *et al.* 2014
Globodera pallida	GPLIN_000659000	Cotton *et al.* 2014
Heterodera schachtii	*Hs-Tyr* (C12694)	Elashry *et al.* Unpublished
Ascaris lumbricoides	ALUE_0001861501	NCBI
Ascaris suum	F1KXGS_ASCSU	NCBI
Caenorhabditis elegans	NP_491709	NCBI
Caenorhabditis elegans	NP_492055	NCBI

Table 3.S2 Primer names and sequences used in the study

Primer Lable	Primer sequence
Tyr-In situ-F	TCCGCCGACAACATTCCA
Tyr-In situ-R	TGATGCGCTGGTGGTTTT
Tyr-qPCR- F	ACAAGCATGCGGAAAGTG
Tyr-qPCR- R	TGATGCGCTGGTGGTTTT
Hs-Actin-F	CGTGACCTCACTGACTACCT
Hs-Actin-R	CGTAGCACAACTTCTCCTTG
RNAi-F	TAATACGACTCACTATAGGGAGA AGCGACGAAGAACGAATC
RNAi-R	TAATACGACTCACTATAGGGAGA GTGTCGCCCATGAAATCT
Loc Fw	GAAGAAAAGCATGAAAAATC
Loc Rw	TGTTGGCATTCCGTTACT
At-Actin-F	ACAGCAGAGCGGGAAATTGT
At-Actin-R	AGCAGCTTCCATTCCCACAA

3.7 References

1. Jones, J.T. *et al.* Top 10 plant-parasitic nematodes in molecular plant pathology. *Mol Plant Pathol* **14**, 946–961 (2013).
2. Müller, J. The economic importance of *Heterodera schachtii* in Europe. *Helminthologia* **36**, 205–213 (1999).
3. Wyss, U. & Grundler, F.M.W. Feeding-Behavior of Sedentary Plant Parasitic Nematodes. *Netherlands J Plant Pathol* **98**, 165–173 (1992).
4. Wyss, U. & Zunke, U. Observations on the behaviour of second stage juveniles of *Heterodera schachtii* inside host roots. *Rev Nematol* **9**, 153–166 (1986).
5. Grundler, F.M.W., Sobczak, M. & Golinowski, W. Formation of wall openings in root cells of *Arabidopsis thaliana* following infection by the plant-parasitic nematode *Heterodera schachtii*. *Eur. J. Plant Pathol* **104**, 545–551 (1998).
6. Davies, L.J., Lilley, C.J., Paul Knox, J. & Urwin, P.E. Syncytia formed by adult female *Heterodera schachtii* in Arabidopsis thaliana roots have a distinct cell wall molecular architecture. *New Phytol* **196**, 238–246 (2012).
7. Gheysen, G. & Mitchum, M.G. How nematodes manipulate plant development pathways for infection. *Curr Opin Plant Biol* **14**, 415–421 (2011).
8. Patel, N. *et al.* A nematode effector protein similar to annexins in host plants. *J Exp Bot* **61**, 235–248 (2010).
9. Hewezi, T. *et al*. Arabidopsis spermidine synthase is targeted by an effector protein of the cyst nematode *Heterodera schachtii*. *Plant Physiol* **152**, 968–984 (2010).
10. Hamamouch, N. *et al***.** The interaction of the novel 30C02 cyst nematode effector protein with a plant β-1,3-endoglucanase may suppress host defence to promote parasitism. *J Exp Bot* **63**, 3683–3696 (2012).
11. Lozano-Torres, J.L. *et al.* Apoplastic Venom Allergen-like Proteins of Cyst Nematodes Modulate the Activation of Basal Plant Innate Immunity by Cell Surface Receptors. *PLoS Pathog* **10**, e1004569 (2014).
12. Siddique, S. *et al.* A parasitic nematode releases cytokinin that controls cell division and orchestrates feeding site formation in host plants. *Proc Natl Acad Sci* **112**, 12669-12674 (2015).
13. Wang, J. *et al.* Identification of potential host plant mimics of

CLAVATA3/ESR (CLE)-like peptides from the plant-parasitic nematode *Heterodera schachtii*. *Mol Plant Pathol* **12**, 177–186 (2011).

14. Eves-van den Akker, S *et al***.** The transcriptome of *Nacobbus aberrans* reveals insights into the evolution of sedentary endoparasitism in plant-parasitic nematodes. *Genome Biol Evol* **6**, 2181–2194 (2014).
15. Haegeman, A., Bauters, L., Kyndt, T., Rahman, M.M. & Gheysen, G. Identification of candidate effector genes in the transcriptome of the rice root knot nematode *Meloidogyne graminicola*. *Mol Plant Pathol* **14**, 379-390 (2012).
16. Sijmons, P.C., Grundler, F.M.W., Mende, N. von, Burrows, P.R. & Wyss, U. *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *Plant J* **1**, 245–254 (1991).
17. Jones, J.T. *et al.* Identification and functional characterization of effectors in expressed sequence tags from various life cycle stages of the potato cyst nematode *Globodera pallida*. *Mol Plant Pathol* **10**, 815–828 (2009).
18. Wang, X. *et al*. Signal peptide-selection of cDNA cloned directly from the esophageal gland cells of the soybean cyst nematode *Heterodera glycines*. *Mol Plant Microbe Interact* **14**, 536–544 (2001).
19. Urwin, P.E., Lilley, C.J. & Atkinson, H.J. Ingestion of double-stranded RNA by preparasitic juvenile cyst nematodes leads to RNA interference. *Mol Plant Microbe Interact* **15**, 747–752 (2002).
20. Lin, B. *et al*. A novel nematode effector suppresses plant immunity by activating host reactive oxygen species-scavenging system. *New Phytol* **209**, 1159-1173 (2016).
21. Ali, S. *et al*. Analysis of putative apoplastic effectors from the nematode, *Globodera rostochiensis*, and identification of an expansin-like protein that can induce and suppress host defenses. *PLoS One* **10**, e0115042 (2015).
22. Overvoorde, P., Fukaki, H. & Beeckman, T. Auxin control of root development. *Cold Spring Harb Perspect Biol* **2**, a001537 (2010).
23. Wubben, M. J. II, Su, H., Rodermel, S. R. & Baum, T. J. Susceptibility to the sugar beet cyst nematode is modulated by ethylene signal transductionin *Arabidopsis thaliana*. *Mol Plant Microbe Interact* **14**, 1206-1212 (2001).
24. Kammerhofer, N. *et al.* Role of stress-related hormones in plant defence during early infection of the cyst nematode *Heterodera*

schachtii in *Arabidopsis***.** *New Phytol* **207**, 778–89 (2015).

25. Huang, G. *et al*. A root-knot nematode secretory peptide functions as a ligand for a plant transcription factor. *Mol Plant Microbe Interact* **19**, 463–470 (2006).
26. Wang, X. *et al.* A parasitism gene from a plant-parasitic nematode with function similar to CLAVATA3/ESR (CLE) of *Arabidopsis thaliana*. *Mol Plant Pathol* **6**, 187–191 (2005).
27. Postma, W.J. *et al*. The effector SPRYSEC-19 of *Globodera rostochiensis* suppresses CC-NB-LRR-mediated disease resistance in Plants. *Plant Physiol* **160**, 944–954 (2012).
28. Siddique, S. *et al.* Parasitic worms stimulate host NADPH oxidases to produce reactive oxygen species that limit plant cell death and promote infection. *Science Signal* **7**, ra33 (2014).
29. Finn, R.D., Clements, J. & Eddy, S.R. HMMER web server: interactive sequence similarity searching. *Nucleic Acids Res* **39**, W29–37 (2011).
30. Petersen, T.N., Brunak, S., Heijne, G. von & Nielsen, H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* **8**, 785–786 (2011).
31. Krogh, A., Larsson, B., Heijne, G. von & Sonnhammer, E.L. Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. *J Mol Biol* **305**, 567–580 (2001).
32. Abad, P. *et al.* Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nat Biotechnol* **26**, 909–915 (2008).
33. Opperman, C.H. *et al.* Sequence and genetic map of *Meloidogyne hapla* : A compact nematode genome for plant parasitism. *Proc Natl Acad Sci* **105**, 14802–14807 (2008).
34. Kumar, M. *et al*. De novo transcriptome sequencing and analysis of the cereal cyst nematode, *Heterodera avenae*. *PLoS One* **9**, e96311(2014).
35. Cotton, J.A *et al*. The genome and life-stage specific transcriptomes of *Globodera pallida* elucidate key aspects of plant parasitism by a cyst nematode. *Genome Biol* **15**, R43 (2014).
36. de Boer, J.M., Yan, Y., Smant, G., Davis, E.L. & Baum, T.J. In-situ Hybridization to Messenger RNA in *Heterodera glycines*. *J. Nematol* **30**, 309–312 (1998).

37. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45 (2001).
38. Curtis, M.D. & Grossniklaus, U. A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physio.* **133**, 462–469 (2003).
39. Sparkes, I.A., Runions, J., Kearns, A. & Hawes, C. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nat Protoc* **1**, 2019–2025 (2006).
40. Voinnet, O., Rivas, S., Mestre, P. & Baulcombe, D. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J* **33**, 949–956 (2003).
41. Karimi, M., Inzé, D. & Depicker, A. GATEWAY^{TM} vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sci* **7**, 193–195 (2002).
42. Clough, S.J. & Bent, A.F. Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J* **16**, 735–743 (1998).
43. Dobrev P.I. & Kaminek M. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *J Chrom A.* **950**, 21-29 (2002).
44. Dobrev, P. I. & Vanková R. Quantification of abscisic acid, cytokinin, and auxin content in salt-stressed plant tissues. *Methods Mol. Biol.* **913**, 251-261 (2012).

Chapter 4: General conclusion

Plant pathogens, including bacteria, fungi, oomycetes, and nematodes, secrete so called effectors to different cellular compartments of their hosts to establish parasitism, evade host defenses, promote pathogen propagation and gain access to target host tissues (Abramovitch *et al*., 2006; Birch *et al*., 2006; Block *et al*., 2008; Cambronne *et al.*, 2006; Chisholm *et al.*, 2006; Mitchum *et al*., 2013; Kamoun, 2006). Effectors of different chemical nature have been shown to alter host-cell structure and function. These alterations either facilitate infection (virulence factors and toxins) or trigger defense responses (avirulence factors and elicitors) or both (Hogenhout *et al*., 2009). The term effector became common with the discovery of the gram-negative bacteria type III secretion system (T3SS), which is used to deliver proteins inside host cells (Abramovitch *et al*., 2006; Block *et al.*, 2008; McCann & Guttman, 2008). These proteins trigger the hypersensitive response in resistant plants and are therefore considered as avirulence factors, whereas it was found later that they also contribute to virulence in susceptible plants. Hence, the term avirulence became restricted, since the same protein with an avirulence activity in incompatible interactions may display a positive virulence activity in compatible interactions (Hogenhout *et al*., 2009).

Plant pathogens have evolved various mechanisms to deliver effectors to the host cells. Gram-negative bacteria use specialized secretion systems, such as T3SS which consists of approximately 30 different proteins to deliver effectors inside host cells. The T3SS is a needle-like protein appendage used as a sensory probe to detect the presence of eukaryotic organisms and secrete effector proteins. In this way, they are secreted directly from the bacterial cell into the host (Abramovitch *et al*., 2006; Block *et al.*, 2008; Galan & Wolf-Watz 2006; McCann & Guttman 2008).

Fungi and oomycetes have different systems to deliver effectors. Effectors are delivered mostly via the infection structures. Some intercellularly-growing beneficial and pathogenic biotrophs form a special structure called haustoria. For haustorium formation, specialized fungal hyphae penetrate the plant cell wall and expand inside that cell. The haustorium is surrounded by an extrahaustorial membrane, which is a modified derivative of the plant cell plasma membrane. This membrane is a barrier where nutrients must pass sequentially through different layers reaching the haustorial cytoplasm (Szabo & Bushnell, 2001). Recently, evidence emerged that haustoria take

part in the secretion of particular classes of host-translocated fungal and oomycete effectors (Catanzariti *et al.* 2007; *Dodds et al*., 2004; Whisson *et al.,* 2007).

In plant parasitic nematodes, effectors are thought to be released through different organs with openings such as amphids, phasmids, rectal gland, hypoderm and the esophageal glands (Haegeman *et al.,* 2012). The majority of the identified effectors are believed to be injected inside the parasitized plant cell through the specialized stylet (Davis *et al.,* 2008). Stylet secretions are associated with nematodes penetration, migration, and initiation of long term feeding site in host roots.

In general, pathogens need to come in contact with the host in order to establish parasitism. Penetration and migration is associated with cell wall degrading enzymes to facilitate the parasites´ growth and expansion (Vanholme *et al.,* 2007; Haegeman *et al*., 2012; Kubicek *et al.,* 2014). After that, plant defense has to be suppressed by various effectors that target the host innate defense mechanisms (Mattoo *et al.,* 2007; Hewezi *et al.,* 2010; Lozano-Torres *et al.,* 2014).

In the last few years, several nematode effectors were identified and their function in the plant were elucidated and reviewed (Mitchum *et al.,* 2013). The transcriptomic and metabolic changes during syncytium formation were also investigated (Szakasits *et al*., 2009; Hofmann *et al.,* 2010). It was shown that syncytium formation was accompanied with high expression of cell wall degredation enzymes such as pectate lyase and expansin families. This suggests the role of these genes to serve formation of the syncytia. On the other hand, defense gene expression was repressed in syncytia compared with control (Szakasits *et al.,* 2009). The high accumulation of several plant metabolites and sugars in the syncytium indicates that nematodes orchestrate plant metabolic process to provide optimal nutrient supply. It is thought that these changes initiated and controlled by nematode effectors (Hofmann *et al.,* 2010).

So far, the identified effectors are involved in different stages of parasitism starting with penetration and migration where cell wall modifying enzymes are released to facilitate movement and initiate the feeding site. For example, cellulase is one of these enzymes that degrade the celluloses and hemicelluloses during migration, pectate lyase also cleaves pectate bonds to facilitate the nematode movement (Vanholme *et al.,* 2007; Haegeman *et al*., 2012). It has been shown that cellulose binding protein is expressed in the early stages of the syncytia formation but not in the migratory stages and interacts

with the plant pectin methylesterase protein 3. This interaction seems to be important in accelerating enzymatic activities, which is a requirement for syncytium development (Hewezi *et al.*, 2008).

Hormone like effectors and growth manipulating effectors were reported to be secreted by nematodes. It was shown that growth manipulating effectors were used to interfere with the plant growth and reprogram their system to establish feeding site. Such effector is the Hs19C07. It interacts with the auxin influx transporter LAX3. LAX3 expression activates and provides auxin signaling that triggers the expression of cell wall-modifying enzymes. LAX3-induced cell wall-modifying enzymes are expressed in the developing syncytium and in cells to be incorporated into the syncytium (Lee *et al*., 2011). Cytokinin is one of the hormones which is mimicked by the *H. schachtii*. Siddique *et al.* (2015) showed for the first time that *H. schachtii* is able to synthesize a functional cytokinin and inject it into the plant in order to activate the root cell cycle.

Since *H. schachtii* is a devastating pest on a wide range of economically important plants including sugar beet, there is an urgent need to develop sustainable control measures. Farmers often rely on multiple nematicide treatments, which, however, can have negative effects on the environment. Resistant crop varieties are also used. The continuous use of such crops decrease the nematode population but will promote the selection of populations that overcome the plant resistance. These reasons introduce the necessity to understand how nematodes are able to break resistance and introduce new resistance traits into crops by breeding approaches. One of the approaches is to identify nematode effectors and their interactome in the plant. This will help to find crucial genes for the nematode parasitism as a target for new nematicides, or find resistant and susceptible genes in the plant to be used in crop breeding.

In the presented studies, novel nematode effector proteins were identified from *H. schachtii* by using the available transcriptom data in comparison with other PPNs. Furthermore, a detailed functional analysis was performed to explain the role of these effectors during nematode parasitism.

The availablity of new technologies such as next generation sequencing massively contributed to predict larger number of candidate effectors. The abillity of *H. schachtii* to infect the model plant *A. thaliana* also facilitated the functional analysis of these candidate effectors. The short life cycle and the availiability of genetic data of *A.*

thaliana additionally accelerated these kind of studies. These available approaches effectivily contributed in the discovery of new effectors and elucidate the nature of their interaction with the plant.

Many criteria were used for effectors screening. The *in silico* screening of the available meta-data based on presence of signal peptide and lack of trancemembrance domains have been used in many labs, and helped to minimize the target genes from thousands to hundred of genes. Further more, presence of these genes in the exeretory organs and coinciding their expression with nematode parasitism decrease the number to couple of effectors to be studied in detail.

In the presented studies, we used the previous criteria to identify and prove the function of two candidate genes as an effector proteins. Furthermore, after expressing ectopically these effecctors in Arabidopsis we were able to analyze the resulted changes and set the basis for determining their role in the plant. Here we are suggesting two novel effectors, *HsPDI* which is involved in protecting the nematode against the plant ROS and *Hs-Tyr* that could be involved in the plant growth manipulation.

Evidence is rising that PPN are equipped with genes which manipulate plant ROS homeostasis. Lin *et al.,* (2016) showed that the root-knot nematode *Meloidogyne javanica* expresses the effector MjTTL5 to suppress plant defense by decreasing ROS burst. Also many of other plant parasitic nematode had some genes which are speculated to be involved in ROS detoxification like the *M. incognita* peroxiredoxin, and the *Globodera rostochiensis* peroxiredoxins (PXN) and glutathione peroxidases (GXP) (Robertson *et al*., 2000; Jones *et al*., 2004; Dubreuil *et al.,* 2011). In our study, we indicate that *H. schachtii* has one of these candidate genes termed by the *HsPDI*. We showed that presence of the *HsPDI* is important for the nematode viability under the presence of exogenous H_2O_2 as a ROS component. Furthermore, when *HsPDI* was expressed ectopically in the plant, the elicited ROS burst by presence of the bacterial peptide flg22 was reduced. This evidence supports our hypothesis that *HsPDI* is involved in the ROS pathway and also shows that *HsPDI* protects the nematode from H_2O_2 stress.

On the other hand, we showed that *H. schachtii* has another effector protein (*Hs-Tyr*) that promotes growth and rearranges the lateral roots and increases the vegetative growth of Arabidopsis plants that ectopically express *Hs-Tyr*. It also induces changes

in the hormonal homeostasis. The hormonal changes were in favour of nematode parasitism. These changes increased the plant susceptibility towards *H. schachtii*. It has been shown previously that nematodes are able to manipulate and reprogram plant growth to supports their parasitism and produce the feeding site (Siddique *et al.*, 2015; Pogorelko *et al.*, 2016).

The resulted data increase our knowledge and fill some gaps in the puzzle of the nematode effectors cocktail which is produced to facilitate parasitism. However, more investigation is needed concerning the effectors pathway in the plant and to find the plant gene(s) which are interacting with these effectors.

4.1 References

Abramovitch RB, Anderson JC, Martin GB. 2006. Bacterial elicitation and evasion of plant innate immunity. *Nature Reviews Molecular Cell Biology* **7**(8): 601-611.

Birch PR, Rehmany AP, Pritchard L, Kamoun S, Beynon JL. 2006. Trafficking arms: oomycete effectors enter host plant cells. *Trends in Microbiology* **14**(1): 8-11.

Block A, Li G, Fu ZQ, Alfano JR. 2008. Phytopathogen type III effector weaponry and their plant targets. *Current Opinion in Plant Biology* **11**(4): 396-403.

Cambronne ED, Roy CR. 2006. Recognition and delivery of effector proteins into eukaryotic cells by bacterial secretion systems**.** *Traffic* **7**: 929-939.

Catanzariti A, Dodds PN, Ellis JG. 2007. Avirulence proteins from haustoria-forming pathogens. *FEMS (Fed Eur Microbiol Soc) Microbiol Lett* **269**:181-188.

Chisholm ST, Coaker G, Day B, Staskawicz BJ. 2006. Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* **124**(4): 803-814.

Davis EL, Hussey RS, Mitchum MG, BaumTJ. 2008. Parasitism proteins in nematode-plant interactions**.** *Current Opinion in Plant Biology* **11**: 360-366.

Dodds PN, Lawrence GJ, Catanzariti A, Ayliffe MA, Ellis JG. 2004. The *Melampsora lini* AvrL567 avirulence genes are expressed in haustoria and their products are recognized inside plant cells. *Plant Cell* **16**: 755-768.

Dubreuil G, Deleury E, Magliano M, Jaouannet M, Abad P, Rosso M. 2011. Peroxiredoxins from the plant parasitic root-knot nematode, *Meloidogyne incognita*, are required for successful development within the host. *International Journal of Parasitology* **41**: 385-396.

Galán JE, Wolf-Watz H. 2006. Protein delivery into eukaryotic cells by type III secretion machines. *Nature* **444** (7119): 567-573.

Haegeman A, Bauters L, Kyndt T, Rahman MM, Gheysen G. 2012. Identification of candidate effector genes in the transcriptome of the rice root knot nematode *Meloidogyne graminicola*. *Molecular Plant Pathology* **14**: 379-390.

Hofmann J, El Ashry A, Anwar S, Erban A, Kopka J, Grundler FMW. 2010. Metabolic profiling reveals local and systemic responses of host plants to nematode parasitism. *Plant Journal* **62**(6): 1058-1071.

Hogenhout SA, Van der Hoorn RA, Terauchi R, Kamoun S. 2009. Emerging concepts in effector biology of plant-associated organisms. *Molecular Plant-Microbe Interaction*. **22**(2): 115-122.

Hewezi T, Howe P, Maier TR, Hussey RS, Mitchum MG, Davis EL, Baum TJ. 2010. Arabidopsis spermidine synthase is targeted by an effector protein of the cyst nematode *Heterodera schachtii*. *Plant Physiology* **152**: 968-984.

Jones JT, Reavy B, Smant G, Prior AE. 2004. Glutathione peroxidases of the potato cyst nematode *Globodera rostochiensis*. *Gene* **324**: 47-54.

Kamoun S. 2006. A catalogue of the effector secretome of plant pathogenic oomycetes. *Annual Review of Phytopathology* **44**: 41-60.

Kubicek CP, Starr TL, Glass, NL. 2014. Plant cell wall-degrading enzymes and their secretion in plant-pathogenic fungi. *Annual Review of Phytopathology* **52**:427-451.

Lee C, Chronis D, Kenning C, Peret B, Hewezi T, Davis EL, Baum TJ, Hussey RS, Bennett M, Mitchum MG. 2011. The novel cyst nematode effector protein 19C07 interacts with the Arabidopsis auxin influx transporter LAX3 to control feeding site development. *Plant Physiology* **155**: 866-880.

Lin B, Zhuo K, Chen S, Hu L, Sun L, Wang X, Zhang L, Liao J. 2016. A novel nematode effector suppresses plant immunity by activating host reactive oxygen species-scavenging system. *New Phytologist* **209**(3): 1159-1173.

Lozano-Torres JL, Wilbers RHP, Warmerdam S, Finkers-Tomczak A, Diaz-Granados A, van Schaik CC, Helder J, Bakker J, Goverse A, Schots A, Smant G. 2014. Apoplastic venom allergen-like proteins of cyst nematodes modulate the activation of basal plant innate immunity by cell surface receptors. *PLoS Pathogens* **10**: e1004569

McCann HC, Guttman DS. 2008. Evolution of the type III secretion system and its effectors in plant-microbe interactions. *New Phytology* **177**(1): 33-47.

Mitchum MG, Hussey RS, Baum TJ, Wang X, Elling AA, Wubben M, Davis EL. 2013. Nematode effector proteins: an emerging paradigm of parasitism. *New Phytologist* **199** (4):879-894.

Mattoo S, Lee YM, Dixon JE. 2007. Interactions of bacterial effector proteins with host proteins. *Current Opinion in Immunology* **19**(4): 392-401.

Pogorelko G, Juvale PS, Rutter WB, Hewezi T, Hussey R, Davis EL, Mitchum MG, Baum TJ. 2015. A cyst nematode effector binds to diverse plant proteins, increases nematode susceptibility and affects root morphology. *Molecular Plant Pathology*. doi: 10.1111/mpp.12330.

Robertson L, Robertson, WM, Sobczak M, Helder J, Tetaud E, Ariyanayagam MR, Ferguson MAJ, Fairlamb A, Jones JT. 2000. Cloning, expression and functional characterisation of a peroxiredoxin from the potato cyst nematode *Globodera rostochiensis*. *Molecular and Biochemical Parasitology* **111**: 41-49.

Siddique S, Radakovic ZS, De La Torre CM, Chronis D, Novák O, Ramireddy E, Holbein J, Matera C, Hütten M, Gutbrod P, Anjam MS, Rozanska E, Habash S, Elashry A, Sobczak M, Kakimoto T, Strnad M, Schmülling T, Mitchum MG, Grundler FM. 2015. A parasitic nematode releases cytokinin that controls cell division and orchestrates feeding site formation in host plants. *Proceedings of the National Academy of Sciences* **112**(41): 12669-12674.

Szabo LJ, Bushnell WR. 2001. Hidden robbers: the role of fungal haustoria in parasitism of plants. *Proceedings of the National Academy of Sciences* **98**(14): 7654-7655.

Szakasits D, Heinen P, Wieczorek K, Hofmann J, Wagner F, Kreil DP, Sykacek P, Grundler FMW, Bohlmann H. 2009. The transcriptome of syncytia induced by the cyst nematode *Heterodera schachtii* in Arabidopsis roots. *Journal of Experimental Botany* **60**: 771-784.

Vanholme B, Van Thuyne, Vanhouteghem K, De Meutter J, Cannoot B, Gheysen G. 2007. Molecular characterization and functional importance of pectate lyase secreted by the cyst nematode *Heterodora schachtii*. *Molecular Plant pathology* **8**: 267-278.

Whisson SC, Boevink PC, Moleleki L, Avrova AO, Morales JG, Gilroy EM, Armstrong MR, Grouffaud S, van West P, Chapman S, Hein I, Toth IK, Pritchard L, Birch PR. 2007. A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* **450**: 115-118.

Acknowledgment

Firstly, I would like to express my sincere gratitude to my advisor Prof. Dr. Florian Grundler for accepting me as a PhD student in his lab and providing me an opportunity to access the laboratory and research facilities to achieve this research. I am grateful for his enormous support during my study on both the scientific and the social levels. His guidance helped me in all the time of my research and writing of this thesis.

Also, I would like to thank Dr. Abdelnaser Elashry for his kind support and co-advising this work and for his personal and scientific support in the bioinformatics and sequences analysis.

Also, I would like to thank the thesis committee: Prof. Dr. Andreas Meyer, Assoc. Prof. Luma Al Banna, Prof. Dr. Walter Witke, and Prof. Dr. Mathias Wüst, for taking part at my thesis defence.

My sincere thanks also goes to Assoc. Prof. Luma Al Banna and Assoc. Prof. Dr. Nidaa Salem for the continues moral and spiritual support during my work. Special thanks to Dr. Shahid Siddique for his valuable remarks during my work and the manuscript preparation.

My sincere thanks also goes to the German Academic Exchange Service (DAAD) for the financial support as scholarship holder. Also for giving me this valuable chance to achieve my PhD and being a part of the Molecular Phytomedicine department in such nice country and environment, in Germany.

I would like to thank my fellow lab mates at the Molecular Phytomedicine for the stimulating discussions, for the sleepless nights we were working together in the lab, and for all the fun we have had in the last four years; many thanks also to Brigit Otte, Stefan Neumann, Ute Schlee, Gisela Sichtermann and Thomas Gerhardt for their excellent technical support.

Last but not the least, I would like to thank my beloved family: my parents, my brothers and sisters for their endless and unconditional love and support.

Sincere thanks for everyone who had a part in my life and cared about me.

Publications

- **Samer S. Habash**, Miroslaw Sobczak, Shahid Siddique, Florian M.W. Grundler, Abdelnaser Elashry Identification and characterization of a putative protein disulfide isomerase (HsPDI) as a novel effector of *Heterodera schachtii.* New Phytologist. Submitted.

- **Samer S. Habash**, Zoran S. Radakovic, Radomira Vankova, Shahid Siddique, Petre Dobrev, Cynthia Gleason, Florian M.W. Grundler, Abdelnaser Elashry. *Heterodera schachtii* Tyrosinase-like protein - a novel nematode effector. Scientific Reports. Submitted

- Boubacar Toukal Assoumana, **Samer S. Habash**, Mbaye Ndiaye, Grace Van Der Puije, Etienne Sarr, Haougui Adamou, Mamourou Diourte, Florian M. W. Grundler, Abdelnaser Elashry. Morphological and molecular identification of *Meloidogyne enterolobii* infesting sweet pepper in southern and eastern Niger. In process.

- Syed Jehangir Shah, Muhammad Shahzad Anjam, Muhammad Arslan Anwer, **Samer S. Habash**, Jose Lozano-Torres, Florian M. W. Grundler, and Shahid Siddique. Damage-associated responses of the host contributes to defence against cyst but not root-knot nematode infection. New Phytologist. Submitted.

- Luma S. Banna, Nida Salem, **Samer Habash**, Ayoup M Ghrair. Impact of silicon carbide nanoparticles on hatching and survival of soil nematodes. Plos One. Submitted.

- Cynthia Gleason, Frederik Polzin, **Samer S. Habash**, Lei Zhang, Jan Utermark, Florian M.W. Grundler, Abdelnaser Elashry.. Identification of two *Meloidogyne hapla* genes and an investigation of their roles in the plant-nematode interaction. MPMI. online published

- Shahid Siddique, Zoran S. Radakovic, Carola M. De La Torre, Demosthenis Chronis, Ondrej Novák, Eswarayya Ramireddy, Julia Holbein, Christiane Matera, Marion Hütten, Philipp Gutbrod, Muhammad Shahzad Anjam, Elzbieta Rozanska, **Samer Habash**, Abdelnaser Elashry, Miroslaw Sobczak, Tatsuo Kakimoto, Miroslav Strnad, Thomas Schmülling, Melissa G. Mitchum, Florian M. W. Grundler: A parasitic nematode releases cytokinin that controls cell division and orchestrates feeding site formation in host plants. Proceedings of the National Academy of Sciences 09/2015; DOI:10.1073/Pnas.1503657112.

- **Samer Samir Mohd Habash**, Mohammad Saleh Al-Bess, Ahmad Saleh Al-Bess and Luma Shareef AL Banna. Effect of Phosphonate Fertilizers on the Growth of Soil Fungi, Journal of Life Sciences 2014; 8(10): 835-840.

- **Samer Habash** and Luma Albanna**.** Phosphonate fertilizers suppressed root knot nematodes *Meloidogyne javanica* and *M. incognita*. Journal of Nematology 2011; 43(2): 95-100.