Institut für Nutzpflanzenwissenschaften und Ressourcenschutz

Identification and characterisation of *Heterodera schachtii* susceptibility genes AtPANB1 and HIPP27 in *Arabidopsis thaliana*

Dissertation

zur Erlangung des Grades

Doktor der Agrarwissenschaften (Dr. agr.)

der Landwirtschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

von

Zoran Radakovic

aus
Orahovica, Kroatien

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For Akary, Gordana, Sreten, Miroslav and Elizabeta

The most important people in my life

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Abstract

Sedentary plant-parasitic cyst nematodes are obligate biotrophs that infect the roots of their host plant. Their parasitism is based on modification of infected root cells to form a hypermetabolic syncytium from which the nematodes draw their nutrients. The aim of this study was to identify nematode susceptibility genes in *Arabidopsis thaliana* and to characterize their roles in supporting the parasitism of *Heterodera schachtii*. By selecting genes that were most strongly upregulated in response to cyst nematode infection, we identified HIPP27 (Heavy metal-associated Isoprenylated Plant Protein 27) and AtPANB1 (3-methyl-2-oxobutanoate hydroxymethyltransferase 1) as host susceptibility factors required for cyst nematode infection. The role of *HIPP27* and *PANB1* was then characterized in detail during cyst nematode infection.

In *A. thaliana*, HIPPs make up the largest metallochaperone family, comprising 45 members divided into seven distinct classes. HIPPs are present only in vascular plants and are involved in a variety of biological processes, including heavy metal homeostasis and detoxification, transcriptional responses to abiotic stresses such as drought and cold, and plant–pathogen interactions. However, the mechanistic details underlying the roles of HIPPs in these biological processes has remained mostly unknown. Our detailed expression analysis using *pHIPP27::GUS* and *pHIPP27:GFP* lines revealed that HIPP27 is a cytoplasmic protein and that *HIPP27* is strongly expressed in leaves, flowers, young roots and nematode-induced syncytia. Analysis of loss-of-function mutants and overexpression lines revealed that *HIPP27* positively regulates the cyst nematode infection of host plants. Moreover, we found no difference in the activation of the reactive oxygen species (ROS) burst or in the expression of defence genes between Col-0 and *hipp27* mutant lines. Based on these results, we hypothesized that expression of *HIPP27* is required for maintaining the optimal development or functioning of the syncytium. Notably, microscopic observations confirmed that lack of HIPP27 protein causes physiological or metabolic abnormalities leading to the accumulation of phloem-provided polysaccharides such as starch grains in peridermal and syncytial plastids.

Vitamin B5 (VB5) is an essential nutritional factor that is synthesized via a three-step process in plants. In Arabidopsis, *AtPANB1* and *AtPANB2* encode the enzyme for

the first step and *AtPANC* the enzyme for the last step of the pathway. In comparison to plants, multicellular animals absorb VB5 from their diet. Our analysis showed that expression of *AtPANB1* and *AtPANB2* is strongly induced upon infection, and this upregulation is essential for nematode development. In comparison to *AtPANB*, *AtPANC* is not upregulated and does not play a role in parasitism. Notably, we identified a nematode PANC gene (HsPANC) and showed that the nematodes are able to perform the last step of VB5 biosynthesis using HsPANC. A detailed analysis revealed that compartmentalization of VB5 biosynthesis between plants and nematodes might be needed to avoid feedback/feed-forward inhibition and ensures a continuous supply of VB5 to rapidly developing nematodes. Altogether, our results suggest that *HIPP27* and *PANB1* are susceptibility genes in Arabidopsis whose loss-of-function reduces plant susceptibility to cyst nematode infection without increasing susceptibility to other pathogens or negatively affecting plant phenotype. These results have potential to improve breeding strategies for modern agriculture.

Zusammenfassung

Sedentäre pflanzenparasitäre Zystennematoden sind obligate Biotrophen, welche die Wurzeln von Wirtspflanzen infizieren. Ihr Parasitismus basiert auf diversen Modifizierungen von befallenen Wurzelzellen, die ein hypermetabolisches Synzytium bilden, welchem der Nematode Nährstoffe entzieht. Das Ziel dieser Arbeit war, potentielle Suszeptibilitätsgene in *Arabidopsis thaliana* zu identifizieren und ihre Bedeutung für die Aufrechterhaltung der parasitären Lebensweise von *Heterodera schachtii* zu beschreiben. Unter den in Infektionsversuchen mit Zystennematoden am stärksten aufregulierten Genen haben sich im Zuge unserer weiteren Untersuchungen die beiden Gene *HIPP27* (Heavy metal-associated Isoprenylated Plant Protein 27) und AtPANB1 (3-methyl-2-oxobutanoate hydroxymethyltransferase 1) als relevante Faktoren für die Suszeptibilität gegenüber Zystennematoden herausgestellt. Daraufhin wurde die Rolle von *HIPP27* und *AtPANB1* im Verlauf der Infektion im Detail untersucht.

In *A. thaliana* bilden die HIPPs mit 45 Genen die größte Familie der Metallochaperone, die sich in sieben Klassen unterteilen lassen. HIPPs kommen nur in Gefäßpflanzen vor, wo sie an diversen biologischen Prozessen beteiligt sind, wie etwa der Metallhomöostase oder Detoxifikation, sowie an Transkriptionsantworten auf Stressfaktoren wie Trockenheit und Kälte, aber auch Pflanze-Pathogen Interaktionen. Allerdings sind die zugrundeliegenden mechanistischen Details für die Funktion der HIPPs in diesen biologischen Prozessen noch weitgehend unbekannt. Unsere ausführlichen Expressionsanalysen unter Verwendung von *pHIPP27::GUS* and *pHIPP27:GFP* Linien haben ergeben, dass HIPP27 ein zytoplasmatisches Protein ist, welches besonders stark in Blättern, Blüten und jungen Wurzeln sowie nematodeninduzierten Synzytien exprimiert wird. Analysen von Funktionsverlustmutanten und Überexpressionslinien zeigten, dass *HIPP27* den Verlauf einer Infektion mit Zystennematoden positiv beeinflusst. Zudem konnten wir keine Unterschiede zwischen der Akkumulation von Reaktiven Sauerstoffspezies (ROS) oder der Expression von Abwehrgenen in Col-0 und *hipp27* Mutanten gefunden werden. Diese Ergebnisse führten zu der Hypothese, dass die Expression von *HIPP27* für eine optimale Entwicklung oder Funktion eines Synzytiums erfoderlich ist. Mikroskopische Beobachtungenzeigten, dass ein Mangel des Proteins

HIPP27 physiologische und metabolische Abnormitäten verursacht, welche zu einer Anreicherung von Polysacchariden in Form von Stärkekörnern in den peridermalen und synzytialen Plastiden führt.

Vitamin B5 (VB5) ist ein essentieller Nahrungsbestandteil, welcher über einen dreistufigen Prozess in Pflanzen synthetisiert wird. In Arabidopsis codieren *AtPANB1* und *AtPANB2* das Enzym für die erste Stufe. *AtPANC* ist für den letzten Schritt des Synthesewegs verantwortlich. Im Unterschied zu Pflanzen absorbieren multizelluläre Tiere VB5 aus ihrer Nahrung. Unsere Analysen haben gezeigt, dass die Genexpression von *AtPANB1* und *AtPANB2* durch eine Infektion stark induziert wird und dass diese Aufregulierung entscheidend für die Nematodenentwicklung ist. Im Unterschied zu *AtPANB* wird *AtPANC* nicht aufreguliert und es spielt keine Rolle für den Parasitismus. Beachtenswerterweise konnten wir ein *PANC* Nematodengen (HsPANC) identifizieren und zeigen, dass Nematoden in der Lage sind, den letzten Schritt der Biosynthese von VB5 mithilfe von *HsPANC* selbst durchzuführen. Detaillierte Untersuchungen haben offengelegt, das bei der VB5 Biosynthese möglicherweise eine Kompartimentierung zwischen Pflanzen und Nematoden nötig ist, um eine feedback/feed-forward Inhibierung zu vermeiden, so dass eine kontinuierliche Versorgung mit VB5 und somit eine schnelle Entwicklung der Nematoden gewährleistet wird.

Zusammengefasst lässt sich sagen, dass *HIPP27* und *PANB1* Suszeptibilitätsgene für *H. schachtii* in Arabidopsis darstellen, deren Funktionsverlust die Pflanzenanfälligkeit gegenüber den Nematoden reduziert, ohne die Suszeptibilität anderen Pathogenen gegenüber zu erhöhen oder den Pflanzenphänotypen negativ zu beeinflussen. Auf dieser Grundlage lassen sich Möglichkeiten zur Verbesserung von Züchtungsstrategienin betroffenen Kulturpflanzen entwickeln.

List of Abbreviations

AtPANB1	Arabidopsis thaliana Pantothenate synthetase B1
dpi	Days post infection
HIPP27	Heavy metal associated isoprenylated plant protein 27
ISC	Initial syncytial cell
PPN	Plant-parasitic nematodes
R-gene	Resistance gene
RKN	Root-knot nematode
RLU	Relative light units
ROS	Reactive oxygen species
S-gene	Susceptiblity gene
siRNA	Small interfering RNA
VB5	Vitamin B5

CHAPTER 1

Introduction

1.1. Biology and ecology of the nematodes

Nematodes are among the most diverse types of animals, with only insects being more abundant. These soft-bodied, multicellular roundworms in the group Ecdysozoa—which they share with insects, arachnids and crustaceans—shed their cuticle between different life stages. They are vermiform in shape, except in some life stages of certain nematodes (**Figure 1**.). More than 15,000 species of nematode are known, and new ones are regularly discovered (Aguinaldo et al., 1997). The majority of nematodes live in the seas and oceans (50%), but other live in soil and fresh water or inside the plants or animals (Walia and Bajaj, 2003).

Figure 1: Examples of phylum Nematoda. All nematodes are worm-shaped with exception of root-knot and cyst nematodes whose shape varies in specific life stages. Pictures present examples of important from free-living and plant-parasitic nematodes. A) *Caenorhabditis elegans* B) *Mononchus ssp*. C) *Heterodera schachtii* D) *Aporcelaimellus ssp.* E) *Meloidogyne incognita* F) *Xiphinema ssp.*

Most nematodes are microscopic, but a few reach meters in length. These simple organisms, usually comprising up to 1,000 somatic cells, are part of almost every ecosystem on Earth, even in deserts and the arctic; so long as at least a film of water is present, they can establish an ecological niche for themselves. Nematodes are classified into five groups according to their mouth structure (**Figure 2**.). According to their feeding habits They can be plant-parasitic (15% of all described nematodes), animal parasitic (44%) or free-living, feeding on bacteria, fungi, protozoa and other nematodes (40%). The remaining 1% of described nematodes are omnivores, feeding on multiple food sources. Nematodes have been shown to evolve their ability to parasitize animals and plants several times during their evolution (Blaxter et al., 1998). Because nematodes do not have hard skeleton, fossils cannot be found. Accordingly, no one knows how ancestral nematodes looked, but probably they were microbial feeders in the primordial oceans.

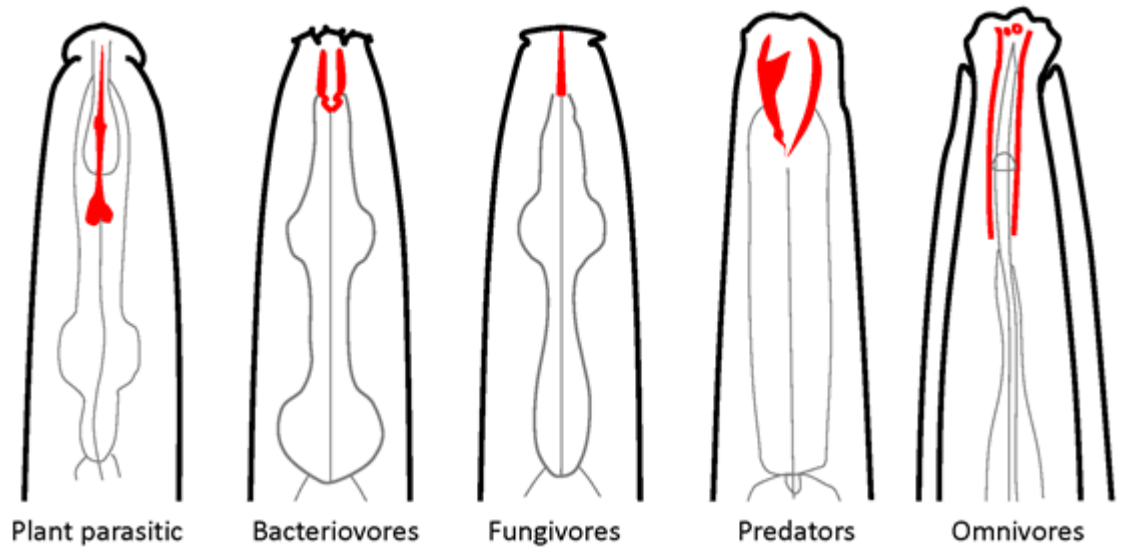

Figure 2: Five classes of nematodes according to their feeding habits and mouth structures. Plant parasitic nematodes possess a hollow spear called stylet. Bacteriovores possess tube-like structure while fungivores possess thin stylet for piercing the fungal cells. Predators have teeth like structure for catching prey and omnivores possess long tube for catching various microbes. Many animal-parasitizing nematodes also attack humans, causing diseases such as elephantiasis, river blindness, and trichinosis.

The oldest documented nematode is an entomopathogenic nematode found in an insect preserved in amber. Thus our knowledge about nematodes' evolution comes from comparative anatomy of existing nematodes, trophic factors and comparison between DNA sequences of different nematode species (Thomas et al., 1997). In agriculture, nematodes are one of the most significant parasites for many crops. Because this thesis focuses on plant-parasitic nematodes (PPN), I describethem in detail here.

1.2. History of PPNs and their importance in agriculture

Although there is little documented evidence of how ancient civilizations coped with PPNs, the earliest hint that these plant parasites were a problem for agriculture dates back to a 235 B.C. with a Chinese script showing the symbol of a root-infecting organism resembling in shape to the adult female soybean cyst nematode (Noel, 1992). The first documented PPN was the seed–gall nematode (*Anguia tritici*), which was described in wheat seeds by Needham in 1743. A century later, in 1855, Bakeley described the first root-knot nematode (*Meloidogyne incognita*) parasitizing cucumber roots, and only four years later, Schacht discovered the beet–cyst nematode (*Heterodera schachtii*) in the sugar beet fields. These were earliest detected PPNs in agriculture, but these were not recognized as a threat to food production until 1940, at which point soil fumigation was first used to reduce nematode populations and increase of yield. In the later years of the 20th century, many new PPNs become important threat worldwide. Since 1954, the soybean-cyst nematode (*Heterodera glycines*) has been the number one problem in the soybean fields of the United States, earning it the name as "the billion dollar nematode" because of the tremendous losses it causes in soybean production. In 1970 and 1975, the potato cyst nematodes *Globodera pallida* and *G. rostochiensis,* respectively, were characterized; and today seed potato can be commercially grown only on the cyst nematode free fields. In banana production, *Radopholus similis* cause severe damage by destroying the roots of the banana trees, resulting most to fall. *Bursaphelenchus xylophilous* is the world's foremost parasite of pine wood production today, and *Meloidogyne sp.*, the most important nematode in agriculture, is parasitizing a wide range of crops. Today, PPNs are major threats to crop production. So far, about 4100 species of PPN have been described (Jones et al., 2013) that collectively threaten food security the world over. Overall global crop

production losses due to nematodes are about 12.3%, causing estimated annual yield losses of $80 billion (Nicol et al, 2011) . This figure is probably underestimated, because many growers, especially in developing countries, are not aware of these parasites. **Table 1.** lists some crops that are significantly affected by nematodes.

Table 1. An international survey of crop losses due to nematodes (Nicol et al, 2011).

Crop	**Annual loss (%)**	**Most important nematode**
Tomato	20.6	*Meloidogyne incognita*
Banana	19.7	*Radopholus similis*
Eggplant	16.9	*Meloidogyne incognita*
Sugar cane	15.3	*Heterodera sacchari*
Potato	12.2	*Globodera rostochiensis*
Sugar beet	10.9	*Heterodera schachtii*
Soybean	10.6	*Heterodera glycines*

Many of these crops show very different symptoms of nematode attack ranging from reduced shoot growth, stunting of the root, falling of the stem or even virus symptoms as a secondary effect of nematode attack (**Figure 3.**).

Figure 3: Symptoms of nematode attack on agricultural crops. A) sugar beet roots infected with *H. schachtii*, B) Tomato root infected with *M. incognita*, C) symptom of the banana tree falling due to *Rodophilus similis* attack D) symptoms of grapwine fanleaf virus transmitted by borrowing nematode *Xiphinema sp*. (source for figures C and D: www.apsnet.org).

From an economical point of view, the most important PPNs are root–knot nematodes with cyst nematodes coming second (Jones et al., 2013). Many other PPNs are also important locally **(Table 2.)**, and modern agriculture is constantly threatened by arising of resistance-breaking population. Once they are detected in a field, eradicating them is almost impossible. Instead, their population density must be decreased and maintained at lower levels. Although plant breeders have bred crops for nematode resistance for decades, our knowledge of plant–nematode interactions was primarily based on visual and microscopic observation of nematode symptoms and behaviour. Basic research into plant–nematode interactions began with Sijmons et al., (1991), who discovered that *H. schachtii* and *M. incognita* are able to parasitize Arabidopsis, which is the model plant for research in molecular biology. This finding significantly increased the body of knowledge about plant-nematode interactions and deepened our understanding of the ways by which nematodes maintain infection sites in a host plant.

1.3. Biology of PPNs

Many diverse PPNs have very different feeding strategies and host plants, but all share a number of features **(Figure 4.)**.

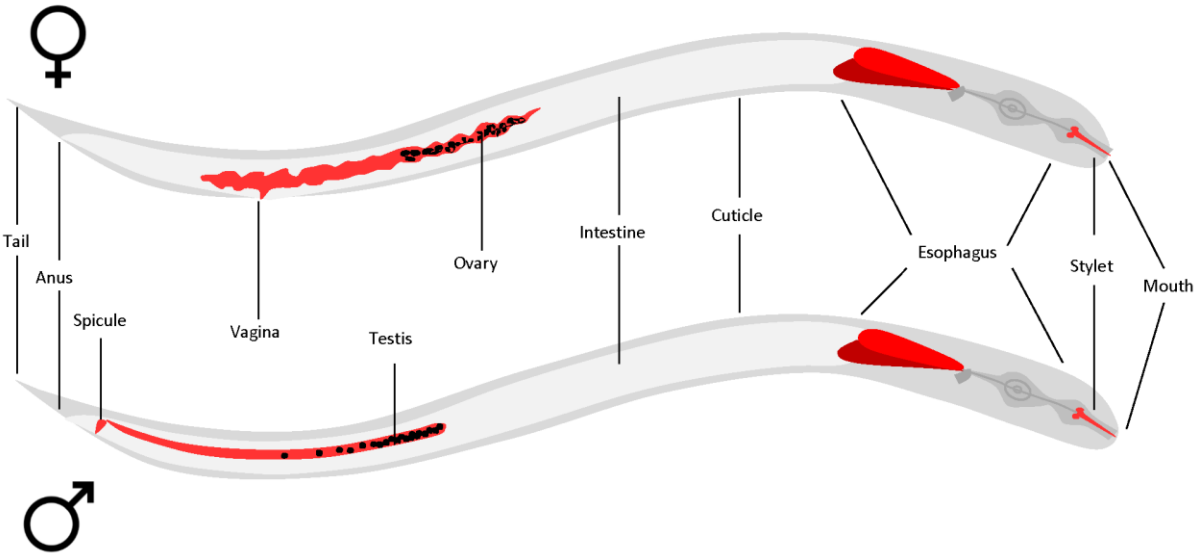

Figure 4: Basic body plan of female and male plant – parasitic nematodes. All PPNs are worm-shaped with stylet as a tool for plant penetration and feeding. Although they are sexually dimorphic, in some species males are often absent and arise only in stressed environmental conditions.

They are transparent and simple in structure, worm-shaped, unsegmented animals having a body size of 300 µm–12 mm long and 15–35 µm wide (Walia and Bajaj, 2003). The body wall of the PPN comprises three major layers: the outer layer (cuticle), the middle layer (hypodermis) and the inner layer (muscles). Because PPNs are soft-bodied animals, the cuticle is essential for maintaining body morphology and integrity and plays a critical role in locomotion, being attached to the body wall muscles. In addition, numerous annulations on the cuticle allow PPNs to bend without kinking. PPNs do not have a defined circulatory system and thus depend on diffusion of water, gasses and other substances through their semipermeable body walls. The internal mixing and transport of the body fluids and nutrients is passive, as a result of nematode movement. The digestive system is relatively simple: an internal tube beginning at the oral opening and ending in the ventral anus (females) or cloaca (males). The system is divided into three parts: the stomodeum, which is the stomata and esophagus; the mesenteron, or intestine; and the proctodeum, which is the rectum or anus (Walia and Bajaj, 2003). All PPNs use a hollow spear called a stylet for piercing and destroying cells and for uptake of host plant nutrients. **Table 2.** summarises the seven major PPN feeding strategies used by important nematodes within this group.

Near the stylet are found amphids, chemoreceptors used to assess the environment and detect host plants. Most PPNs are sexually dimorphic, although males are absent and parthenogenesis is a dominant form of reproduction. In both sexes, the reproductive organs consist of one or two tubules, called the gonads that can be derived from mesoderm or ectoderm. The mesodermal part of the gonad is responsible for nutrition and development. The ectodermal parts of the gonad are responsible for union between the sexes, transfer of sperm and deposition of eggs. The female reproductive organ is called the vulva; the male organ is called the spicules. Despite large numbers of commonalities among various species of PPNs, they are very diverse parasites with very specific and interesting life cycles and host ranges. Therefore, in the next chapters I will primarily focus on the description of sedentary endoparasitic nematodes, which consists of cyst nematodes and RKNs.

Table 2: Classification of PPNs according to their feeding strategies with most important parasites on agricultural crops (Lambert and Bekal, 2002).

Feeding strategy	Characteristics	Nematode
Ectoparasites	Remains outside the plant feeding on the plant roots	***Xiphinema ssp.*** ***Enoplea ssp.***
Semi – endoparasites	Usually head of the nematode is in the root with permanent feeding cell	***Rotylenchulus ssp.*** ***Tylenchulus ssp.***
Migratory endoparasites	Migrates through the root tissue destructively feeding on the cells	***Pratylenchus ssp.*** ***Hirschmaniella ssp.***
Sedentary endoparasites	Feeding on permanent feeding site inside the root tissue	***Meloidogyne ssp.*** ***Heterodera ssp.*** ***Globodera ssp.***
Stem and bulb nematodes	Migratory nematodes inside the stems and bulbs	***Ditylenchus ssp.***
Seed gall nematodes	Feeding on the leaf after which it migrates to the floral primordial where it forms seed gall called cockle	***Anguia ssp.***
Foliar nematodes	Migratory nematodes which enter the plants through natural openings like stomata	Genus ***Aphelenchoides***

1.4. Cyst nematodes

Cyst nematodes belong to the order Tylenchida and family *Heteroderiadae*. The two most important genera in this group are *Heterodera* and *Globodera*, which include the species *H. glycines*, *H. schachtii*, *G. pallida* and *G. rostochiensis*. Among various cyst nematodes, *H. glycines* causes the strongest economic losses (Jones et al., 2013). However, *H. schachtii* is important as it makes a compatible interaction with model plant *Arabidopsis thaliana* and this system has been intensively used to study molecular aspects of nematode parasitism and behaviour. Cyst nematodes have a relatively narrow host range (except some weeds, potato is the only plant that *G. pallida* can use as a host (Evans and Stone, 2009) they are all obligate sedentary endo-parasites, which have similar life cycle presented in **Figure 5**.

After embryogenesis, the first-stage juvenile (J1) is formed, still enclosed within the egg-shell. It moults within the egg itself and the second stage juvenile (J2) hatches out and moves towards the host plant, guided by plant volatiles, pH, amino acids,

temperature and CO_2 concentration. When J2s locate a host plant, they attack the plant roots near the root tip, predominantly in the zone of elongation (Wyss and Grundler, 1992). After piercing with their hollow, protrusive mouth spear (stylet), they migrate destructively and intracellularly through the root tissue towards the vascular cylinder.

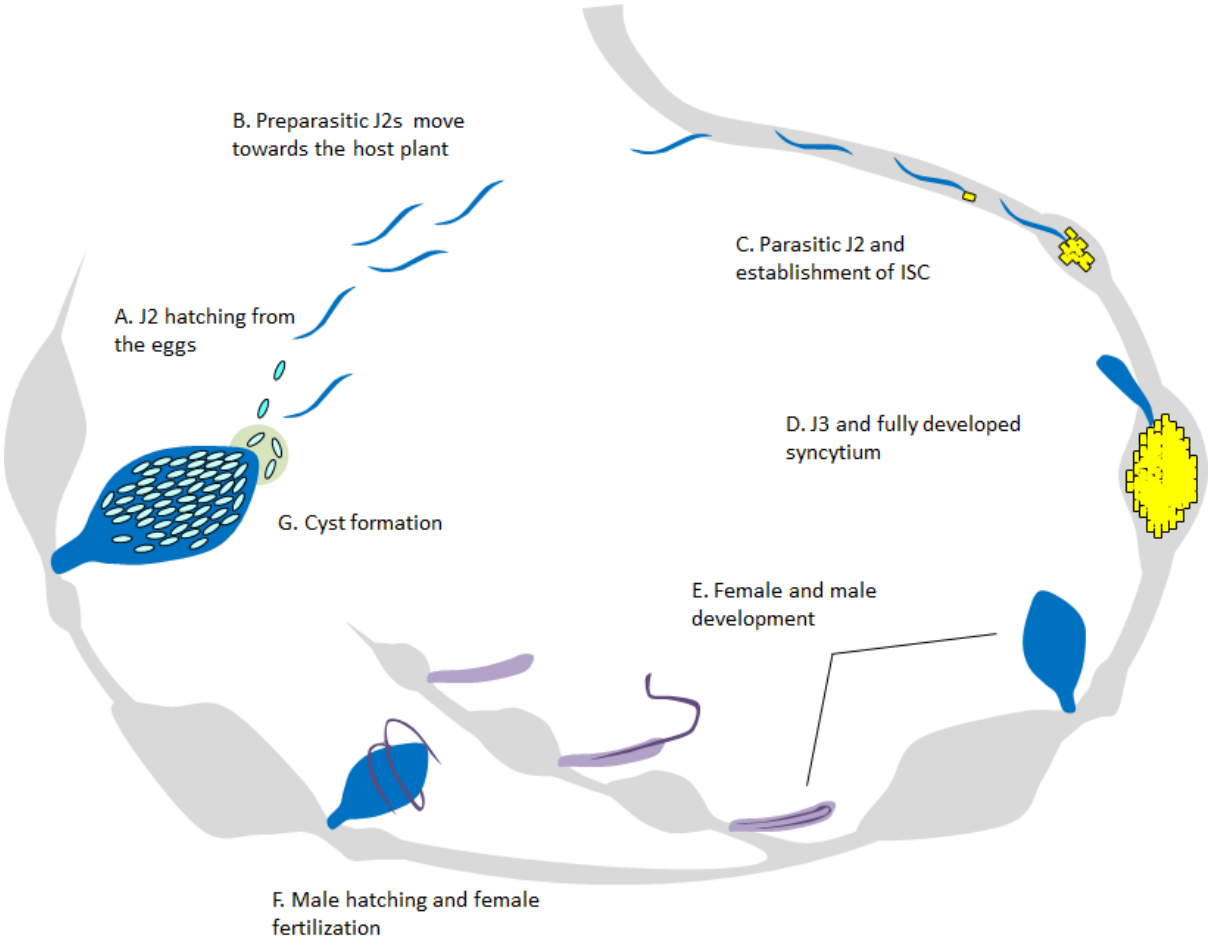

Figure 5: The life cycle of the cyst nematodes. Dormant J2s hatch from the dead cyst due to host plant exudates (A) after which J2s move to the root (B) and enter the host by piercing of the stylet. Parasitic J2s move to the vascular cylinder and establish ISC (C). After the development of syncytia (D), sexual differentiation of J3s begins and they become females or males depending on the tropic factors (E). After maturation, the motile male fertilizes sedentary female (F) after which females become dead cysts carrying 200-300 eggs inside (G).

There the J2s pierce a single cell with the stylet and wait for cell response. If the stylet becomes covered with a layer of callose-like material or the protoplast

collapses, then the stylet is retracted; the nematode repeats this behaviour until it finds a cell that does not negatively respond to the presence of nematode (Golinowski et al, 1996). This cell will become the initial syncytial cell (ISC) and nematode releases secretion (effectors) into the ISC to facilitate syncytium formation. Once the ISC is established, the nematode remains motionless for 6-8 hours and prepares to feed in accordance with its sedentary mode of life. In the following two weeks, nematodes feed from the syncytium and develop into females or males after three molts (J2, J3 and adult). Although *H. schachtii* is dimorphic, the mechanism of sex determination is not fully understood; it is known , however, that under favourable environmental conditions (susceptible plant, access to sugar and nutrients), more females develop (Trudgill, 1967). If conditions are adverse, such as in the case of resistant plant or a nutrient deficiency, the sex ratio favours males. This is because females need 29 times more food than males do (Müller, 1999). Nematode size, penetration rate, number of eggs and syncytium size are influenced by the host plant (Siddique et al., 2013). Also, J2s that establish their feeding sites on lateral roots are more likely to become males. Grundler et al,. (1991) concluded that under optimal conditions, more than 90% of infective J2 of *H. schachtii* will become females (Trudgill, 1967). Still, the molecular mechanisms underlying cyst nematode sex determination is not clearly known.

A female-associated syncytium is composed of around 200 cells and reaches its maximum size about 10 days post infection (Siddique and Grundler, 2015). Plant cells that form a syncytium undergo dramatic changes: DNA synthesis is induced and metabolism increases, providing a rich food source for the nematode. Their nuclei become hypertrophied and the cytoplasm condenses containing an increased numbers of mitochondria, plasmids, and ribosomes. The structures of the endoplasmic reticulum and central vacuole are replaced by several small vacuoles. The metabolomics and transcriptomic changes of the syncytium are dramatic and are well studied (Hofmann et al., 2010). Szakasits et al., (2008) measured changes in gene expression of 21,138 Arabidopsis genes in syncytium, of which 18. 4% were upregulated and 15.8% were downregulated. This finding leads to the conclusion that syncytium is transcriptionally different from roots and all other organs.

Mature females have swollen lemon-shaped bodies filled with 200–250 eggs. Whereas females are sedentary throughout their life, adult males, by contrast have a vermiform shape within the J3 cuticle; they become motile after the third molt and

migrate to the females for fertilization attracted by the female´s sexual pheromones. After fertilization, the female dies and become a dead brown cyst containing eggs that are ready to hatch as soon as suitable host is detected. If there is no chemical signal from the host plant, eggs within the cyst can survive in the soil for more than 10 years in their dormant stage. This makes control of cyst nematodes in the field very difficult. The optimal ways of reducing nematode populations in the field includes growing resistant plants, using trap crops or use non-host plants.

1.5. Root-knot nematodes

Root-knot nematodes (RKN) belong to the order *Tylenchoida* and the family *Meloidoginidae* and are the most important and most widespread crop-parasitic nematodes. They have a wide range of hosts that includes more than 2,000 plant species. About 5% of global annual crop losses are caused by root-knot nematodes and the most important species in this family are *Meloidogyne incognita, M. javanica, and M. arenaria* in the tropics and *M. hapla* in temperate regions.

RKNs primarily attack host plants' roots and tubers. They are obligate biotrophic sedentary endoparasites, causing the formation of characteristic galls in roots. Most RKN species reproduce by facultative meiotic parthenogenesis, an asexual form of reproduction in which the oocytes keep the original diploid or polyploidy chromosome number. Under stress conditions, males are also formed and sexual reproduction can occur to produce hybrid progeny. In the absence of males, diploidy is restored through fusion of sister haploid nuclei to generate diploid inbred progeny (Jones et al., 2013). The life cycle of RKNs (**Figure 6.**) is similar to that of cyst nematodes but has a few differences (Holbein et al, 2016). Because most RKN have a wide host range, hatching is primarily depends on optimal water and temperature conditions. J2s invade host plant rootlets; preferably at the root tips but they can infect any part of the root. After penetration, RKNs move intercellularly towards the root cap to make a so-called "U"-turn in the apical meristem region to avoid crossing the endodermal root barriers.

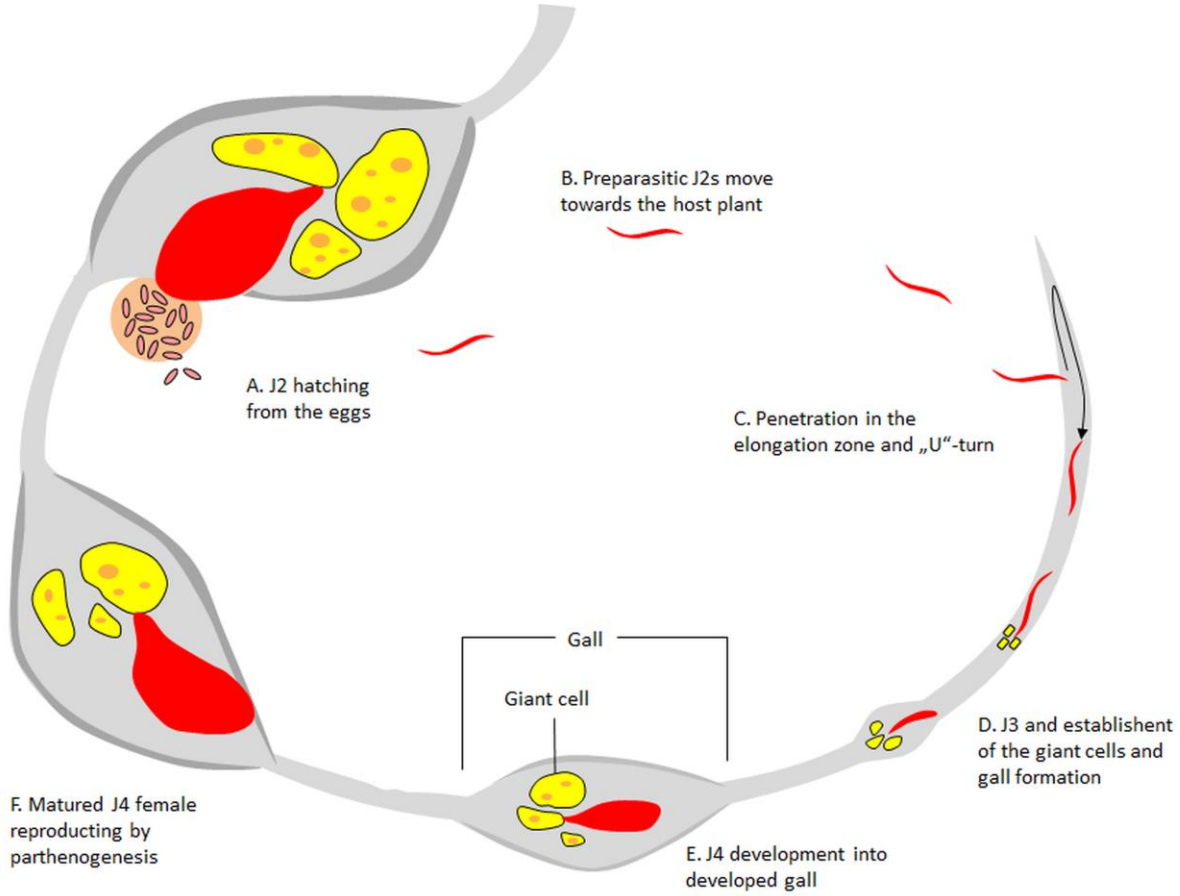

Figure 6: The life cycle of the root – knot nematodes. After hatching of the (A) occurs, pre-parasitic J2s move towards the host plant (B) where they penetrate the root and make the "U"-turn on the root tip (C). They move upwards to the inner cortex where they include the formation of several giant cells (D). With time J3 will molt into a J4 inside the developed gall (E). Matured females (F) lay eggs into a gelatinous matrix by which the life cycle is completed.

They then migrate upwards inside the root until reaching vascular tissues in the zone of differentiation, where they release effectors that induce reprogramming of selected cells by promoting mitosis and cell expansion. Giant cells are RKN-specialised nutrient sinks for the rest of the nematode life cycle. Usually, 5–7 cells are selected to form giant cells (Favery et al, 2015). Infected cells undergo structural and physiological modifications that increase the flow of nutrients and solutes into these cells. The nematode takes all the nutrients required for their development from the giant cells. After three molts in the root, adult females become spherical and lay eggs in a gelatinous egg mass.

The genomes of *M. incognita* and *M. hapla* were the first sequenced genomes of animals that parasitize plants (Abad et al., 2008). Comparison of RKN genomes with genomes of other nematodes reveal that RKNs have an abundance of genes involved in immunity, defence and detoxification, which hints why they are so adept at surviving soil and plant environments. Remarkably, about 3% of RKN genes are acquired by horizontal gene transfer from different bacteria and fungi (Paganini, 2012).

In general, control of RKN is challenging. They can cause quantitative and qualitative yield losses at an incredibly low damage threshold (the point beyond which yield losses are so high that crop production is no longer economical) of 1 egg per cm^3 of soil (Greco and Di Vito, 2009). *M. graminicola,* for example, parasitizes upland and lowland rice fields, causing yield losses of up to 87% (Lilley et al., 2011). In addition, RKN attacks are often followed by other soil-borne pathogens, such as *Fusarium* wilt, *Rhizoctonia solani*. The potential for crop rotation is often limited since the range of host plants is relatively large. The most effective approach to coping with them is breeding for resistant crop varieties.

1.6. Nematode survival strategies and nematode control

PPNs use several strategies to cope with biotic and abiotic stresses in their environment, whether soil or plant. The most important abiotic stresses for PPNs are a lack of water, (they must have at least a film of water to survive) and temperature (most cannot tolerate soil temperatures higher than 40°C). In addition, soil itself contains various parasites and predators of nematode including predatory nematodes that eat PPNs. Thus PPNs commonly avoid soil biotic and abiotic stresses by living inside the plant host tissue or at least limiting their mobility in the soil. Such an approach, of course, brings trade-offs. By spending less time in the soil, PPNs decrease their chances of encountering a pathogen or predator. However, these nematodes are more prone to challenges posed by host stresses, such as defence reactions. This is especially true for sedentary endoparasitic nematodes. By contrast, migratory nematodes, which move from plant to plant, are

relatively more tolerant to plant stress but they are increasing their chances of death in the soil environment. A very effective nematode survival strategy involves increasing host range, thereby decreasing the nematode's chance of - and indeed its opportunities for – dying in the soil. All these survival strategies make PPNs very difficult to control in agriculture, and several methods have been used. Although one particularly common method is crop rotation, growing non-host plants is often difficult: many nematodes have a wide host range and can survive by parasitizing weeds. Also, some nematodes, such as cyst nematodes, can survive in the soil for many years within dead cysts. Chemical control is an effective way of decreasing nematode populations, but because soil fumigation and most nematicides are forbidden in many countries, this method is not widely used. Another very effective metod, widely used for decreasing Heterodera schachtii populations is the use of trap crops such as nematode resistant mustard or oil seed radish. These crops are sawn as a fallow in the autumn. They induce *Heterodera schachtii* hatching after which J2s infect roots of the crop plants. In most cases females do not develop which result in rapid decline of nematode populations. Thus nematode populations are significantly decreased and, in addition, give rise to beneficial effects for the soil. Another common and effective method involve the use of nematode-resistant crops. Unfortunately, because nematodes are very adaptive, due to rapidly emerging new nematode populations which are able to overcome plant defence. Plant breeders are thus constantly developing new strategies and searching for resistant varieties for preserving a successful agricultural system.

1.7. Plant immunity against nematodes

Plants, as part of an ecosystem, are in constant contact with their environment, which contains various pests and diseases, such as viruses, bacteria, fungi, oomycetes, nematodes, insects and parasitic plants. To survive in this turbulent environment, plants have developed sophisticated and complex defence systems consisting of several layers of passive and active defence.

Passive defence against pathogen attack relies on constitutive plant characteristics such as production of phytochemicals, possession of fat wax layers, positioning of thorns or even growth and flowering during times of the year when pathogen are not present. However, If pathogens overcome this passive layer, then a second and

active layer of resistance is induced. The main goal of active plant defence is to recognize the presence of invading pathogen. There are two mechanisms of active plant defence (Couto and Zipfel, 2016):

(i) PTI (PAMP – triggered immunity). The first layer of active plant defence involves recognition of pathogen-associated molecular patterns (PAMPs); PAMPs are conserved molecules that are mostly present on the outer layers of the pathogen, such as flagellin in bacteria or chitin in fungi and insects. PAMPs are recognized by the plant cell surface proteins called PRRs (pathogen recognition receptors), which then activate complex cascades of plant cell defence responses.

ii) ETI (effector–triggered immunity). The second layer of active plant defense occurs once pathogens overcome PTI and they start to parasitize the plant by releasing secretory proteins called effectors. Plants have a range of so-called R-genes that are intracellular nucleotide binding-leucine rich repeats proteins (NLRs). In the resistant plant, pathogen effectors are directly or indirectly recognized by a specific R-protein after which ETI is induced. Nevertheless, the evolutionary battle between plants and pathogens has seen development of a mechanism called ETS (effector–triggered susceptibility): pathogens possess effectors that are able to inhibit or decay R-proteins in the susceptible plant. Even as plants are constantly developing new R-proteins, pathogens are constantly developing/modifying new effectors.

Both PTI and ETI activate a cascade of defence responses in which ETI is a usually stronger response to pathogen attack. The cascade of activated plant defences may well be mediated by salicylic acid (in biotrophic pathogens), jasmonic acid and ethylene (in necrotrophic pathogens), producing ROS (reactive oxygen species) and Ca2+ bursts, activation of MAPK and CDPK cascades, reprogramming of plant transcriptomes and reinforcements of the cell wall.

1.8. Nematode Resistance Genes

Breeding of crops for nematode resistance is well established and many resistance markers and loci are known (review by Williamson & Kumar, 2006). Also the functioning and mechanism of many nematode R-genes is relatively well characterized. One of the best-characterised nematode R-genes (and its protein product, R-protein) is Mi-1, in the tomato, which confers resistance to *M. incognita* as well as *M. javanica*, *M. arenaria* and the potato aphid. For *H. schachtii,* the R-gene

$Hs1^{Pro1}$ gene was identified in sugar beet hairy roots as strongly reducing the number of females per plant. In soybeans, a combination of two R-proteins, Rhg1 and Rhg4, radically decreases soybean susceptibility in the field to *H. glycines*. In the potato, Gro1-4 and Gpa2 are two R-proteins that are very effective against potato cyst nematodes *G. rostochiensis* and *G. pallida*.

1.9. Pathogenic compatibility with the host – susceptibility genes

After overcoming all layers of plant defense, pathogens (especially biotrophic ones) need to establish compatible interactions with their host. For the pathogen to survive and complete its life cycle, the host plant needs to have sets of genes that support compatibility. These genes are often called susceptibility (S) genes or susceptibility factors (van Schie and Takken, 2014). Deletion or mutation of this gene can cause pathogen-specific limitations on the spread of disease. The term *S-genes* dates from 2002, when deletion of PMR6 gene caused decreased susceptibility to fungus (Vogel et al., 2002).The phenomenon is described as a novel form of disease resistance based on gene mutation or deletion that is required for compatible interaction (Eckardt, 2002). Unlike R-genes, S-genes are very diverse and do not share common features or domains. In addition, resistance to a pathogen based on R-gene is usually overcome by pathogen, whereas loss-of susceptibility due to deletion of S-gene may provide durable resistance for many decades. A good example for this is S-gene MLO (mildew resistance locus O) in barley, in which a recessive mutant causes loss–of susceptibility to powdery mildew fungus *Blumeria graminis* for more than 70 years. Another famous example is cap binding protein eIF4E which is essential for potyviruses; deletion of this gene removes susceptibility to potyviruses in many plant species.

According to gene function and pathogen phase of development in the life cycle, we can classify S-genes in four major categories:

1. S-genes that facilitate host recognition and penetration
2. S-genes that are targets for pathogen effectors
3. S-genes that are negative regulators of plant immunity
4. S-genes that allow pathogen proliferation and compatibility by facilitating structural and metabolomic requirements for the pathogen

The first step of parasitism for many pathogens is to overcome the plant structural barriers including the wax layer. This process also involves plant S-genes, such as maize gene GROSSY11. The maize mutant of this gene has very long chain aldehyde levels in the leaf cuticles, resulting in very poor germination of powdery mildew spores, making the plant less susceptible to fungal infection (Hansjakob et al., 2011). Some pathogens, such as bacteria, are not able to penetrate the cuticle and thus use wounds and natural openings, such as stomata, to enter the host's apoplast and vasculature. Accordingly, stomatal closure is a plant defence mechanism used against pathogen attack. Several negative regulators of the stomatal closure, such as RIN4 and AHA1, are known; mutants of these genes are less susceptible to bacterial attack (Desclos-Theveniau et al., 2012). As noted before, the first and the best described S-gene is MLO (Mildew resistance Locus O), which is required for powdery mildew penetration of epidermal cells (Büschges et al., 1997). *MLO* mutants perform loss-of susceptibility to powdery mildew with homologs also found in wheat, strawberry, tomato, pea and Arabidopsis that produce the same or similar effect. MLO is characterized as membrane-anchored protein and suppressor of cell death whose resistance is independent of JA, SA and ET.

Many suppressors of PTI are known for many pathogens, including S-genes such as the CERA gene family, whose mutants *cer1*, *cer4*, *cer7* and *cer8* have impared PTI suppress PTI and thus have decreased infection with bacterial and fungal pathogens (Hernandez-Blanco et al., 2007). The Arabidopsis *mpk4* mutant has extremely elevated levels of SA and is less susceptible to a wide range of biotrophic pathogens (Brodersen et al., 2006). In addition, WRKY transcription factors are involved in transcriptional reprogramming upon pathogen infection, and the OsWRKY45-1 is the S-gene for *Xanthomonas oryzae* (Tao et al., 2009).

Pathogens release effectors to disarm plant defence mechanisms and promote parasitism, but some effectors are also used to activate S-genes for that purpose. The TAL (transcription activator–like) family of effectors comprises *Xanthomonas* bacterial effectors that bind to the promoter of the host gene and regulate gene expression (Boch and Bonas, 2010). The promoter of S-gene Upa20, which is a cell size regulator, is a target of TAL-effector AvrBs3. Also, OsSWEET11, OsSWEET14, OsTFIIAgama and OSTFX1 are S-genes that are induced by *Xanthomonas* TAL effectors in rice.

Once the pathogen particularly a biotrophic pathogen establishes an infection site and overcomes the plant defences, it begins proliferation, for which it needs many plant S-genes. SWEET11 and SWEET13 are plasma membrane–located sugar transporters in rice that are required for loading of apoplastic space with the sugars and nutrients that the pathogen needs. Mutants of these genes greatly reduce susceptibility to *X. oryzae*. TOM genes are tonoplast transmembrane proteins required for formation of the RNA replication complex of tomato mosaic virus (TMV). Silencing TOM homologs inhibits TMV multiplication in the plant (Asano et al., 2005).

1.10. Nematode S-genes

Like other pathogens, nematodes have evolved the ability to use plant genes to manoeuvre host functions in their favour. Because research into nematode susceptibility genes is a relatively new scientific field, however, not many nematode S-genes are known. The first characterised S-gene for nematodes was RPE (Favery et al., 1998), whose discovery proved that loss-of-function of a single gene in Arabidopsis can reduce *M. incognita* susceptibility. These S-genes proved their function by infection assays of T-DNA insertion mutants or by knocking out or silencing one or multiple genes. Most of the mutants listed reduce susceptibility rather than offering potential loss of susceptibility, with the strongest reduction observed in the double mutant *Rboh d/f* for *H. schachtii* (Siddique et al., 2014) and the mutants *map65-3* and *pskr1* for *M. incognita* (Quentin et al., 2016).

1.11. Fitness penalties and application of S-genes in modern agriculture

Very commonly, knocking out or silencing a single gene can have a phenotypic effect that can be mild or dramatic. The same is true for mutants of S-genes. In cases such as those of eIF4E and TOM, mutants do not perform any phenotype at all except that they are less susceptible to the viruses. Breeding of crops for loss of susceptibility rather than resistance is a relatively new approach in crop breeding. For an S-gene mutant to be usable in crop breeding and food production, several criteria must be met. First, the mutant should not increase susceptibility to other pathogens. Second, the mutant should not have a strong root or shoot phenotype and should have some undesirable characteristic, such as reduced yield or reduced quality. Third, mutants' interaction with beneficial microorganisms should not be impaired. Of course, this is only a general rule-exact criteria depend on site-specific

conditions. For example, barley *MLO* mutants grown in the field are more susceptible to rice blast *Magnaporthe grisea*. But this problem is avoided by growing barley mutants in Northern Europe, where this fungus is not present. So far, eIF4E and TOM are the only known S-genes that do not have an additional undesired phenotype; this makes identification of an applicable S-gene difficult. Nevertheless, as already noted, after such a gene is found, resistance is much better than that provided by the R-gene effect.

Many pathogen S-genes have been found, many of which have very strong phenotypes and thus are not applicable in agriculture. The Arabidopsis *mpk4* mutant has reduced susceptibility to bacterial and fungal pathogens through over-accumulation of SA acid levels in the plant. As a result, that mutant performs very dwarf phenotype. Alfalfa mutant *gpa7* increases resistance to oomycetes but impairs interactions with mycorrhiza (Wang et al., 2012). *OsSWEET11/14* mutant reduces plant height and suffers from pollen abortion (Antony et al., 2010).

For nematodes, as mentioned in the previous chapter, the number of S-genes is limited. Many genes that greatly reduce susceptibility have severe phenotypes, such as *map65-3* (Callaud et al., 2008) or have increased levels of defence and thus are dwarfed, such as *rbohd/f*. In addition, all these experiments were conducted under sterile conditions – so far, not a single nematode S-gene has been documented or published in a crop. With the emergence of new technologies, such as gene editing through the CRISPR/Cas9 system, genetic engineering of crops will be quicker and more efficient, making this area of research a field ripe with opportunity.

1.12. References

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CHAPTER 2.

Plant-parasitic nematode *Heterodera schachtii* is able to produce vitamin B5

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2.1. Summary

Vitamin B5 (VB5) is an essential nutrient that is synthesized via a three-step process in plants. In Arabidopsis, *AtPANB1* and *AtPANB2* encode the enzyme for the first step and *AtPANC* the enzyme for the last step of the pathway. In comparison to plants, multicellular animals absorb VB5 from their diet. Cyst nematodes are biotrophs, and parasitism is based on the formation of a syncytium in the roots from which nematodes withdraw their nutrients. Here we investigated the role of VB5 during cyst nematode interaction with Arabidopsis. We found that expression of *AtPANB1* and *AtPANB2* is strongly induced upon infection, and this upregulation is essential for nematode development. In comparison to *AtPANB*, *AtPANC* is not upregulated and does not play a role in parasitism. Notably, we identified a nematode PANC gene (HsPANC), and showed that the nematodes are able to perform the last step of VB5 biosynthesis using HsPANC. A comprehensive, biochemical, molecular, and genetic analysis revealed that compartmentalization of VB5 biosynthesis between plants and nematodes is necessary to avoid feedback/feed-forward inhibition and ensures a continuous supply of VB5 to rapidly developing nematodes.

Keywords: Nematode, *Heterodera schachtii*, Arabidopsis, vitamin B5, AtPANB1.

2.2. Introduction

Plant-parasitic nematodes (PPN) are small vermiform animals with a wide range of host plants including many economically important agricultural crops. They are one of the major threats to food production and safety causing an estimated annual loss of $80 billion worldwide (Jones et al., 2013). Although PPN can be categorized into various groups according to their feeding habits and lifestyle, the economically most important is a small group of sedentary endoparasites including cyst nematodes (*Heterodera ssp.* and *Globodera spp.*) and root-knot nematodes (*Meloidogyne spp*.). Among cyst nematodes, *Heterodera glycines* is widespread in soybean, *Globodera pallida* and *G. rostochiensis* in potato, and *H. schachtii* in sugar beet fields (Jones et al., 2013). The life cycle of the cyst nematode begins when J1-stage nematodes hatch and comes out of the cyst as a mobile infective juvenile, J2. The hatching is stimulated by trophic factors such as plant exudates, CO_2, pH etc. Pre-parasitic J2 moves toward the host and once they reach the plant they pierce the root cells with the help of their stylet. Once inside the root, nematode travels through different tissue layers until they reach central cylinder where they select a single cell termed as the initial syncytial cell (ISC). Upon induction of the ISC, nematodes become immobile and release secretions (effectors) inside the ISC to support the formation of a long-term syncytial feeding site (Golinowski et al., 1996; Wyss and Grundler, 1992, Noon et al., 2016, Mitchum et al., 2013). The ISC expands via local dissolution of neighboring cell walls and subsequent fusion of the adjacent protoplasts. Syncytium is hypertrophied containing multiple nuclei, a dense cytoplasm with increased number of mitochondria, ribosomes, ER and numerous vacuoles (Golinowski et al., 1997; Wiezorek et al.,2006). This syncytium is the only food source during the rest of the lifecycle of nematodes and is entirely different from any other plant organ at transcriptome level (Szakasits et al., 2008). As nematode feeds, J2s develop into a swollen J3, which undergo sexual differentiation to develop into males or females (Anjam et al., 2017). However, sexual differentiation is greatly influenced by nutrient availability and resistance level of the plant. In general, under more favorable conditions such as surplus of nutrients or susceptible plant, more females will develop (Trudgill, 1967). In contrary, in the resistant plant with the lack of nutrients, more males will develop. The reason for that is that female needs 29 times more nutrients than males (Müller, 1981). After sex differentiation males become mobile

and fertilize the sedentary female. Following copulation, the female will die and form brown cyst containing 200-300 dormant eggs.

Nematodes, as all other animals, require vitamins in order to complete their life cycle. Since plant parasitic animals are not able to produce vitamins *de novo*, they depend on vitamin sources from their host plants. The big and diverse group of plant vitamins are so-called vitamin B complex. They are class of water-soluble vitamins playing important roles in cell metabolic processes as a precursors or coenzymes (Roje, 2007). In addition, many B vitamins play significant roles as antioxidants in plants (Asensi-Fabado and Munné-Bosch, 2010).

Vitamin B5 (also known as VB5, pantothenic acid or pantothenate) is a universal precursor to the biosynthesis of 4`-phosphopantheteine moiety of acyl carrier protein and coenzyme A (Kleinkauf, 2000). VB5 can only be synthesised by plants and microorganisms. For *E. coli*, this pathway is well understood and consists of three steps. In first step, alfa-ketoisovalerate is converted into ketopantoate by enzyme EcPANB (KPHMT1, ketopantoate hydroxylmethyltransferase). In the second step ketopantoate, is converted into pantoate by EcPANE (KPR, ketopantoate reductase), and in the final step EcPANC (PtS, pantothenate synthetase) converts pantoate into pantothenate or VB5. For *E. coli*, an additional pathway exists in which L-aspartate is converted to beta-alanine by enzyme EcPAND (ADC, L-aspartate-alfa-decarboxylase) (Cronan, 1980; Ottenhof et al., 2004). In plants, VB5 biosynthesis pathway is not fully characterised. Although Arabidopsis possesses the homologues AtPANB1 and AtPANB2 (KPHMT1 and KPHMT2), the EcPANE orthologue from *E. coli* is yet to be identified. Nevertheless, the AtPANC has been identified and characterized. In this study, we characterized the role of VB5 in plants' interaction with cyst nematodes. We found that loss-of-function of AtPANB1 drastically decreases plant s' susceptibility towards beet cyst nematode *H. schachtii*. The mechanism underlying this decreased susceptibility is discussed in this paper.

2.3. Results

2.3.1. AtPANB1 is upregulated in syncytium and supports nematode´s parasitism

A look at the previous transcriptomic and proteomic analysis for pathway involved in VB5 in Arabidopsis showed that transcript as well as protein abundance for AtPANB1 and AtPANB2 is increased strongly in syncytium as compared to control roots (**Figure 1A**). In comparison to AtPANB1 and AtPANB2, expression of AtPANC remained unchanged (**Figure 6A**). To investigate whether VB5 plays a role in cyst nematode parasitism, we ordered two loss-of-function T-DNA insertion lines for *AtPANB1* (*atpanb1-1*, *atpanb1-2*). The homozygous lines were developed and lack of expression for *AtPANB1* was confirmed using RT-PCR (**Figure 1B**). A detailed phenotypic analysis was performed to investigate whether these mutants show a growth-related phenotype. Although mutant plant looks normal, flowering was slightly delayed in *atpanb1-1*. Nevertheless all other tested parameters such as root and shoot fresh weight, siliques sizes, the number of seeds per silique and seed yield, were not altered in *atpanb1-1* compared to Col-0 (**Figure 1C-I**).

Next, we investigated the role of AtPANB1 in cyst nematode parasitism via a detailed infection assays. Plants were grown *in vitro* for 12 days and inoculated with nematodes. The number of males and females were counted at 14 days post inoculation. Our data showed that average number of females was decreased significantly in *atpanb1-1* and *atpanb1-2* as compared to Col-0. In addition, we also measured average number of males per plant, average female sizes, average syncytium sizes, average cyst sizes, pi/pf value and average number of eggs per cyst (**Figure 2**). All these parameters showed a strong and significant decrease in *atpanb1-1* as compared to Col-0 confirming our conclusion that AtPANB1 is an important susceptibility factor for cyst nematode parasitism. To investigate whether AtPANB1 plays a similar role in other plant-pathogen interactions, we analyzed changes in susceptibility of *atpanb1-1* towards necrotrophic fungus *Botrytis cinerea*, and beneficial endophyte *Piriformospora indica.* Notably, *atpanb1-1* did not show a significant change in response to *B. cinerea*. Similarly, plant growth promoting effect of *P. indica* was also not impaired (**Figure 3.**).

Loss-of-function mutants for *atpanb1-1* showed a decreased susceptibility to the nematode. To further validate these results, we produced transgenic lines overexpressing AtPANB1 under the control of a 35S promoter in Col-0 (*35S:AtPANB1*) or in *atpanb1-1* background (*35S:AtPANB1/atpanb1-1*). The increase of transcript abundance was confirmed by qRT-PCR and homozygous lines were analyzed via infection assays. Indeed, we observed that susceptibility of plants overexpressing *AtPANB1* towards cyst nematode was increased significantly as compared to Col-0 (**Figure 4.**).

To investigate whether decrease susceptibility of *atpanb1* to cyst nematodes is due to changes in immune responses, we tested ROS production, a hallmark of basal defence in plants, upon treatment of an immunogenic peptide, flg22. We did not observe any changes in ROS burst, both over time or cumulative (**Figure 5.**). Taken together, these results suggest that loss-of-function of AtPANB1 decreases plant susceptibility towards nematodes, without disturbing plant basal defense and without altered susceptibility to other pathogens.

2.3.2. Cyst nematodes encode a PANC gene

AtPANB1 together with AtPANB2 encode for enzymes catalyzing the first step of VB5 biosynthesis. It converts alfa-KIVA into a ketopantoate, which is further converted into pantoate. At the final step, AtPANC uses pantoate together with beta-alanine, and converts it into VB5 (Ottenhof et al., 2004). According to Microarray results, both AtPANB1 and AtPANB2 are strongly upregulated in syncytia; however, there was no change of expression of AtPANC1 (**Figure 6A**). Considering our data from AtPANB1, VB5 appears to play a key role in cyst nematode interaction with plants. Therefore, no change in expression of AtPANC1 was a bit surprising. To resolve this, we produced loss-of-function homozygous mutants for AtPANC1 (*atpanc1*) and analyzed them via infection assays. However, we did not observe strong changes in susceptibility between Col-0 and *atpanc1* (**Figure 7A-C**).

A previous study suggested that cyst nematode *H. glycines* may possess a gene encoding HgPANC (Craig et al., 2009). This finding combined with our results, prompted us to hypothesize that *H. schachtii* may also encode a PANC (HsPANC), which may contribute towards biosynthesis of VB5. Using HgPANC as the template, we performed similarity searches such as BLAST and found that transcriptome of all

published cyst nematode as well as *H. schachtii* encode single genes that show strong similarity to *HgPANC*. In addition to cyst nematodes, a limited number of other animals also possess genes encoding PanC (**Figure 6B-6E**).

To investigate the expression pattern of *HsPanC* during various life stages of cyst nematodes, we performed qRT-PCR with cDNA extracted from nematode eggs, J2s, J3s and J4 females. We found that *HsPANC* is strongly up-regulated in females suggesting that our hypothesis is correct. A similar trend was observed with RNAseq data from *G. rostochiensis* (Eves-van den Akker et al, 2016) life stages (**Figure 6C and 6D**).

Lastly, we used RNA silencing approach in which we silenced nematode´s HsPANC with siRNA. Arabidopsis plants were infected by nematodes with more than 80% silenced transcript of the gene (**Figure 7D**) and the results showed a reduction in susceptibility similar to that of *atpanb1-1* mutant (**Figure 7E-G**).These results strongly suggest that *H. schachtii* possess homologous of Arabidopsis AtPANC and it is able to intake pantoate and convert it to VB5 by HsPANC (**Figure 8**).

2.4. Discussion and conclusion

Plants synthesise vitamins for a broad range of purposes. Many vitamins, such as C, E, A, B1 and B6, play a central role as antioxidants by scavenging reactive oxygen species, while some serve as coenzymes in many biological processes including ATP production (Asensi-Fabado and Munné-Bosch, 2010). Vitamins are plant chemicals that cannot be synthesised by animals; as such, they are required to be taken in their diet. Therefore, plant parasites, such as insects or nematodes, need to parasitize plants to take in essential vitamins. In addition to antioxidant activity, and being an important part of the animal diet, many vitamins also play a role in the activation of plant defence. Thiamine (vitamin B1)-treated Arabidopsis, rice, tobacco and cucumber show a rapid increase in PR1 and PDF1.2 transcripts, which consequently lead to increased plant resistance against rice blast disease, *Pseudomonas syringe* and pepper mind mottle virus (Ahn et al., 2005). In Arabidopsis, the vitamin C-deficient mutants *vtc1* and *vtc2* lead to stronger activation of cell death and the decrease of susceptibility to the hemi-biotrophic bacterium *P.*

syringae (Pavet et al., 2005). The existing knowledge that many other vitamins directly or indirectly influence plant susceptibility against pathogens (Boubakri et al., 2016) prompted us to investigate the role of vitamin B5 in plant-nematode interaction.

VB5 biosynthesis in Arabidopsis starts with enzymes AtPANB1 and AtPANB2, which are localized exclusively in mitochondria and are responsible for converting alfa-ketoisolavorate into ketopantoate. The last step of VB5 production is catalyzed by AtPANC, which is localized in the cytosol. However, Arabidopsis homologue of *E. coli* KPR or ADC like remains unknown (Cronan, 1980; Ottenhof et al., 2004). Our analysis indicated that expression of both *AtPANB1* and *AtPANB2* is differentially upregulated in syncytium as compared to uninfected roots. Moreover, a loss-of-function mutant for AtPANB1 was significantly less susceptible to cyst nematodes as compared to control. Although we could not measure VB5 content in *atpanb1* mutants, we hypothesize that knocking out AtPANB1 would lead to reduced VB5 levels in the plants, which could be the reason for decreased susceptibility against *H. schachtii* as well as delayed flowering. Nevertheless, the nematode was still able to complete its life cycle probably due to functional redundancy with AtPANB2, and by using pantoate and converting it to VB5 via HsPANC. We did not conduct any experiments with AtPANB2 because the mutant was not available at the time of this project. Interestingly, in addition to cyst nematodes, a limited number of other animals also encode orthologous of PANC including mites and flies. We hypothesise that possessing PANC is part of the evolutionary battle between plant and pathogen. On the one hand, a plant will reduce the expression of PANC and therefore limit VB5 for the pathogen, while on the other hand, a pathogen develops or acquires PANC to convert pantoate into VB5, thereby reducing the effect of decreased VB5 availability. Our results, including the siRNA of HsPANC, support this model.

This discovery is the first evidence that animals are able to synthesize vitamin B5. It is not the first time that we make unorthodox discovery characteristic of the nematodes. Plant-parasitic nematodes are also able to produce cytokinins and release them into the plant´s syncytium in order to promote their parasitism (Siddique et al., 2015). This additional discovery takes us one step closer to solving the puzzle of the complex and fascinating world of plant-nematode interaction.

2.5. Experimental procedures

2.5.1. Arabidopsis growing conditions and nematode infection assays

Seeds of *Arabidopsis thaliana* were sterilized for 3 min in 1.2% NaCl followed by 3 consecutive washings with ddH_2O. Infection assays with *H. schachtii* were conducted in 2% KNOP's nutrients medium as described previously (Siddique et al., 2015). *H. schachtii* J2s were collected from cysts parasitizing on mustard plants (*Sinapsis alba*) that were grown under aseptic conditions in the dark and merged into a modified Baermann funnel containing 3mM $ZnCl_2$ to stimulate hatching. Arabidopsis plants were grown in 9mm Petri-dishes for 12 days in a climate chamber with long-day conditions at temperatures of 22°C during the day and 18°C at night, after which they were infected with 70-80 J2 nematodes sterilized for 5 min with 0.05% $HgCl_2$ and washed with ddH_2O. After 14 days, nematode susceptibility parameters were observed. The number of females and males per plant was counted using binocular S4E (Leica, Germany), while female and syncytia sizes were measured under M 165 C (Leica, Germany) using outline tool (Leica, LAS V4.3). After an additional 2 months, cyst sizes were measured by the same procedure as described for syncytium and females. The number of eggs per cyst was counted by crushing dead cysts into the 1 ml of 6%NaClO to avoid the clustering of eggs. The pi/pf index was calculated by recording the total number of cysts per plant, measuring the average number of eggs per cyst and dividing that figure by the initial inoculum of 70 nematodes.

2.5.2. Genotyping and expression check of the Arabidopsis mutants

All Arabidopsis T-DNA insertion mutants were ordered from the NASC stock centre and were tested for homozygosity by extracting genomic DNA using the CTAB method. DNA concentration was tested on Nanodrop (Thermo Fiser Scientific, Wiltham, MA, USA), while, PCR reactions were conducted on a C100TM Thermal Cycle (Bio-Rad Laboratories, USA). Next, 1.2% agarose gel and a Gel Doc™ (Bio-Rad Laboratories, USA) machine were used to observe homozygosity and expression check via Image Lab 3.0 software.

2.5.3. RNA, cDNA and qRT-PCR preparation

For all plant tissue, RNA was extracted using a Nucleospin RNA XS (Machinery-Nagel, Germany) according to the manufacturer`s protocol. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, UK). Gene expression was measured using the StepOne Plus Real-Time PCR System (Applied Biosynthesis, USA). 18S and UBQ5 was used as a housekeeping gene and data was analysed according to Pfaffl, 2001.

2.5.4 siRNA synthesis and nematode gene silencing protocol

siRNA primers were designed for the *HsPanC* gene with 2 different targets using the Silencer^{TM} siRNA Construction Kit (Thermo Fisher Scientific, Lithuania) and following the manufacturer's protocol. Freshly hatched *H. schachtii* J2s were soaked into the final concentration of 200ng ul^{-1} of siRNA for 1h with constant shaking, before nematodes were surface-sterilized with 0.05% $HgCl_2$ with consecutive washing. siRNA from GFP was used as a negative control. Some of the nematodes were saved for confirmation of down-regulation by qRT-PCR, while an infection assay was conducted on the remainder.

2.5.5. Oxidative burst assay

The measurement of ROS production on a 96-well luminometer (Mithras LB 940; Berthold Technologies) was conducted as described previously (Mendy et al., 2017). 12-day-old Arabidopsis leaf segments of 0.5cm diameter were cut and placed in the ddH_2O for 12h in the dark. After incubation, each leaf segment was placed into a well of a 96-well plate containing 15ul of 20ug ml^{-1} horseradish peroxidase and 35ul of 10mM 8-amino-5chloro-2,3-dihydro-7phenyl(3,4-d) pyridazine sodium salt (L-012, Wako Chemicals). The next step was to use 50µl of flg22 for ROS burst induction, while ddH_2O was used as a negative control. The light emission was measured for 60 min and data was collected using instrument software and Microsoft Excel Office.

2.5.6 *Botrytis cinerea* and *Piriformosphora indica* infection assays

The plant susceptibility for *B. cinerea* was evaluated via a modified protocol according to Lozano-Torres et al., (2014). Bacterial cultures were grown on potato dextrose agar for 30 days until conidia were formed at 20°C in the dark. Then 5µl drops of *B. cinerea* spores of conidia suspension, measuring $5x10^5$ were placed on

the leaves of 4-week-old Arabidopsis plants, grown on the soil in the greenhouse. After inoculation, plants were placed in the dark at 20°C and 100% RH for 3 days. Next, leaves with necrotic lesions were cut and the necrotic area was measured on binoculars using the same method for as the female sizes, as described in the previous chapter. To observe the beneficial effect of *P. indica* fungus on Arabidopsis growth, a modified protocol according to Daneshkhah et al., 2013 was used. Next, 5mm *P. indica* mycelium plugs, grown at 28°C, on CM medium, were inoculated on 7-days-old Arabidopsis seedlings grown on MS medium (Duchefa Biochemie, The Netherlands). After an additional 7 days, fresh roots and shoots weights were measured.

2.5.7. Plant phenotyping

Phenotypic parameters of Col-0 and the *atpanb1-1 m*utant were measured in the greenhouse on plants grown in the soil or in the climate chamber on plants grown on the KNOP medium. Plants' dry weight and yield was measured on naturally matured plants. Every experiment contained at least five different plants and three independent repetitions. Greenhouse parameters were measured by a simple ruler while for KNOP experiments S4E (Leica, Germany) binocular was used. Greenhouse plants were watered every third day with long day conditions and without addition of nutrients. Plant dry weight and seed weight was measured using standard laboratory balance. Flowering time was observed and documented every single day of plant growth.

2.5.8. Gateway cloning and plant transformation

The Gateway cloning approach was used to clone the AtPANB1 gene into the pDONR207 vector by designing Gateway primers and using the Gateway® BP Clonase™ II Enzyme mix (Invitrogen, USA), according to the manufacturer's instructions and using dH5alfa *E. coli* competent cells. Next, pEntry207 was cloned into pMDC32, using Gateway® LR Clonase™ II Enzyme mix (Invitrogen, USA). Agrobacterium transformation into GV3101 was conducted using heat shock. For Agrobacterium mediated plant transformation, protocol according to Clough and Bent, 1998 was used. LB medium was used for growth of all bacteria. KNOP with 25 mg ul^{-1} hygromycin was used for the selection of T0-T3 transformed Arabidopsis plants. qRT-PCR was used to confirm 35S:promoter mediated gene overexpression.

2.5.9. Primer design

Primers for expression used in PCR and qRT-PCR were designed using primer3 online software (http://bioinfo.ut.ee/primer3-0.4.0/). SALK primers used for checking homozygosity of SALK lines were provided from SALK website (http://signal.salk.edu/tdnaprimers.2.html). Gateway and siRNA primers were designed according to manufacturer's instructions by adding Gateway or siRNA extensions to the primers which amplify all gene (Table 1.). For protein alignment ClustalW2 online software was used (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.5.10. Statistical data analysis

All results are presented as a mean ±SE of one individual repetition. Each experiment is repeated at least three times. Statistical significant differences were defined using Microsoft Office Excels and extension XL Statistics with student´s t-test ($p<0.05$).

Competing interests

The authors declare they have no competing interests

Author‘s contributions

ZSR conducted the majority of the experiments and contributed to the writing of the manuscript, MSA conducted Gateway cloning and plant transformation, FMWG and SS designed the experiments and wrote the manuscript.

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2.7. Figures and tables

A

15dpi syncytia	AtPANB1 (AT2G46110)
Microarray data qRT-PCR (18S refrence gene) qRT_PCR (Actin reference gene)	32.0 14.5 5.5
Protein chip	2.9

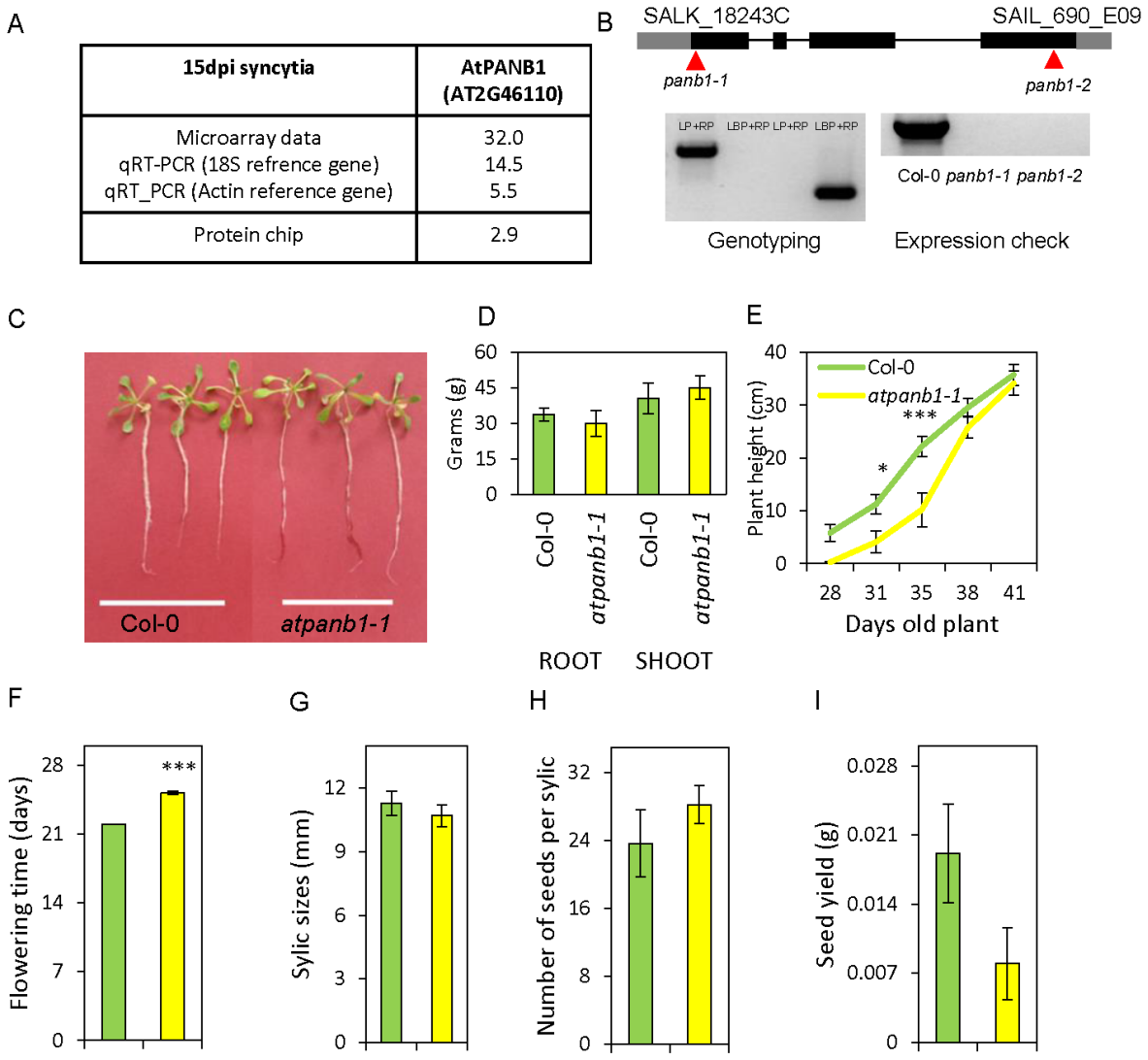

Figure 1: Evaluation of AtPANB1 as an Arabidopsis S-gene for *H. schachtii*. A) Up-regulation of AtPANB1 in gene and protein level. B) Genotyping and expression check. C-I) Phenotypic analysis of the Col-0 and *atpanb1-1* mutant. The mutant did not show strong phenotype except the slight delay in shoot growth which consequently caused delayed in flowering. Asterix indicate statistically significant differences (p<0.05*, p0.01-0.001*, p<0.001***).

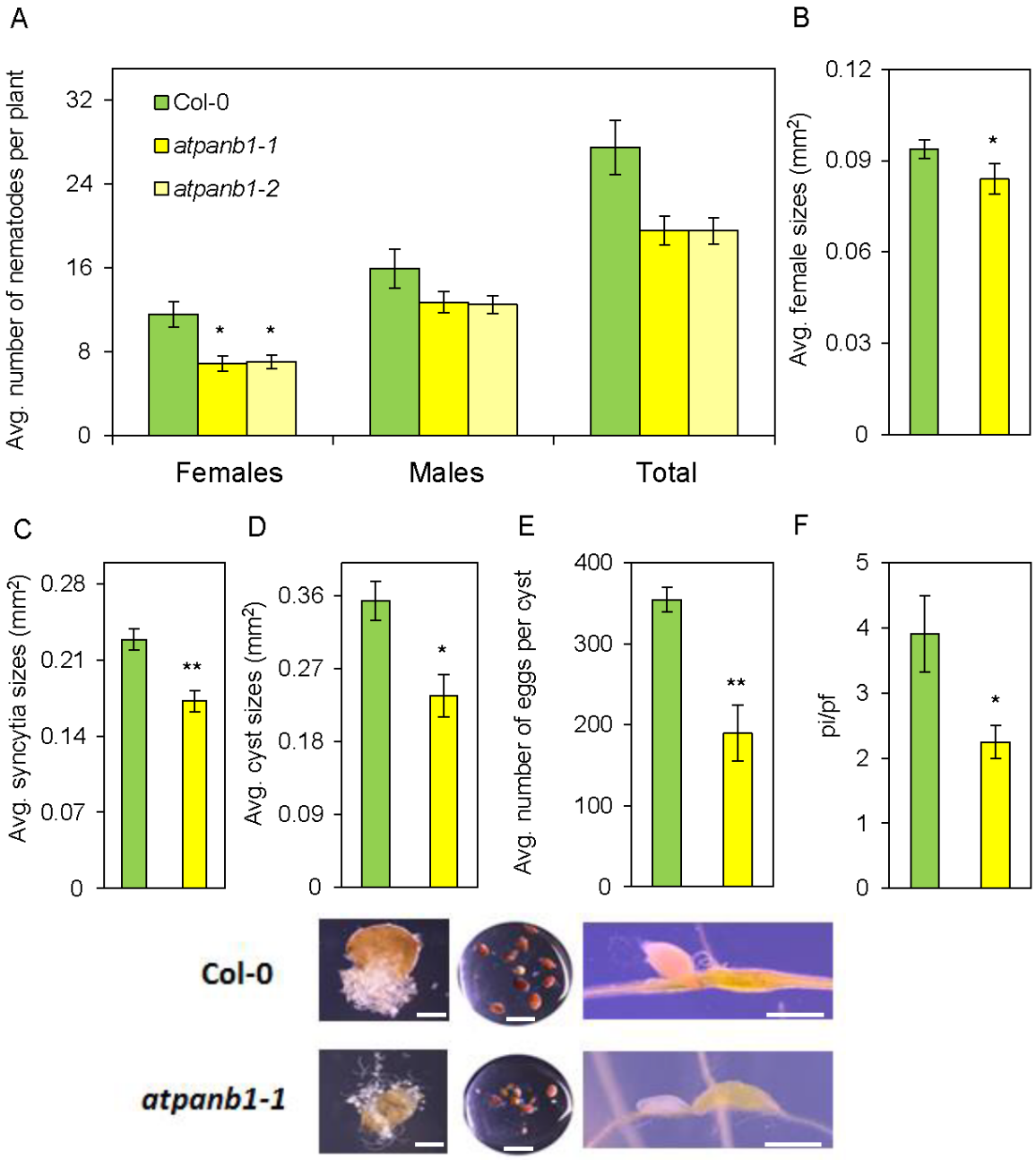

Figure 2: Nematode infection assays in *atpanb1-1* mutants. A-F) Vast majority of nematode susceptibility parameters; the number of females plant, female, syncytia and cyst sizes, pi/pf value and number of eggs per cyst were reduced compared to control. G) Representative samples of a number of eggs per cyst, cyst, female and syncytia sizes. Asterix indicate statistically significant differences ($p<0.05$*, p0.01-0.001*, $p<0.001$***). Scale bars indicate 500um um and 1mm um respectively.

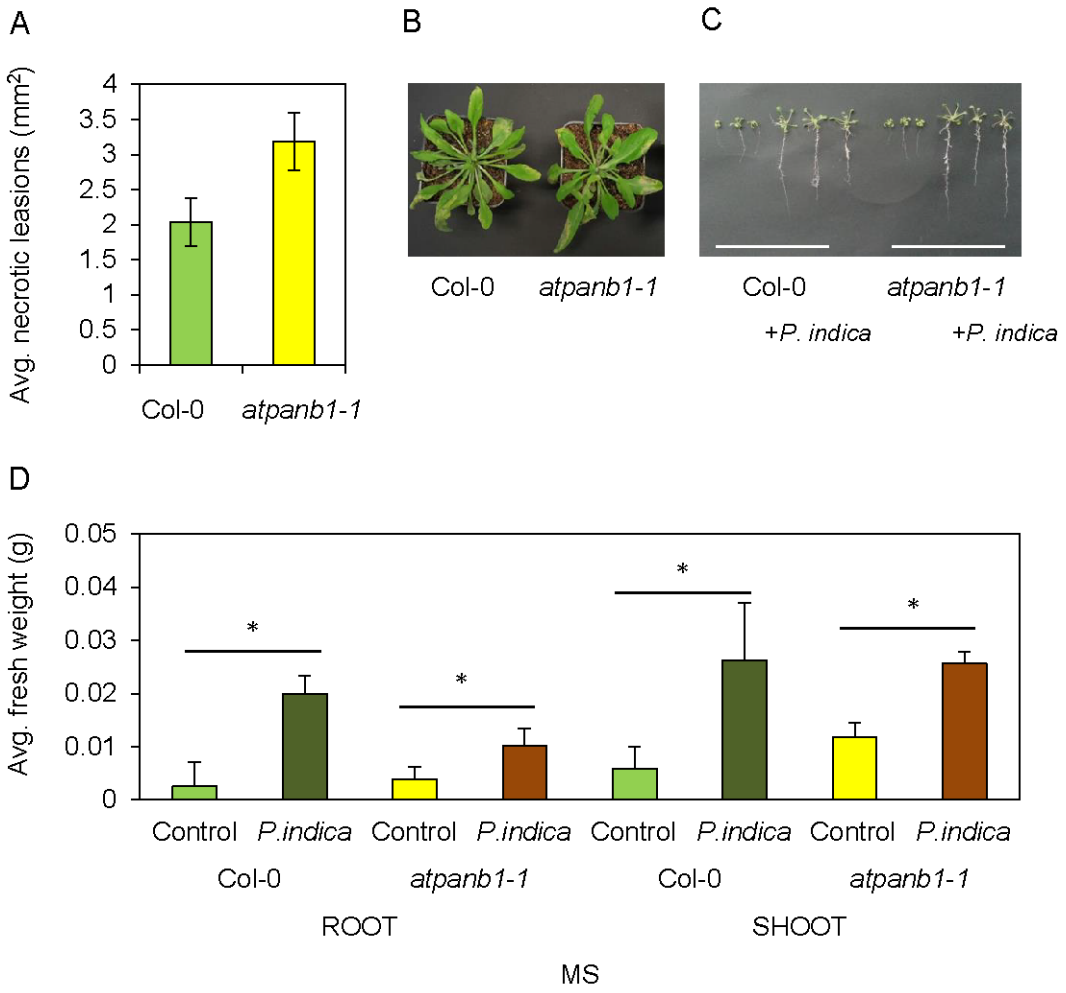

Figure 3: Infection assays with other pathogens. A-B) Infection assay with *B. cinerea* in septic, greenhouse conditions. C-D) Infection assay with *P. indica* for evaluating plant growth promoting effect of the fungus. E) Infection assay with *M. incognita*. Asterisks indicate statistically significant differences ($p<0.05$*):

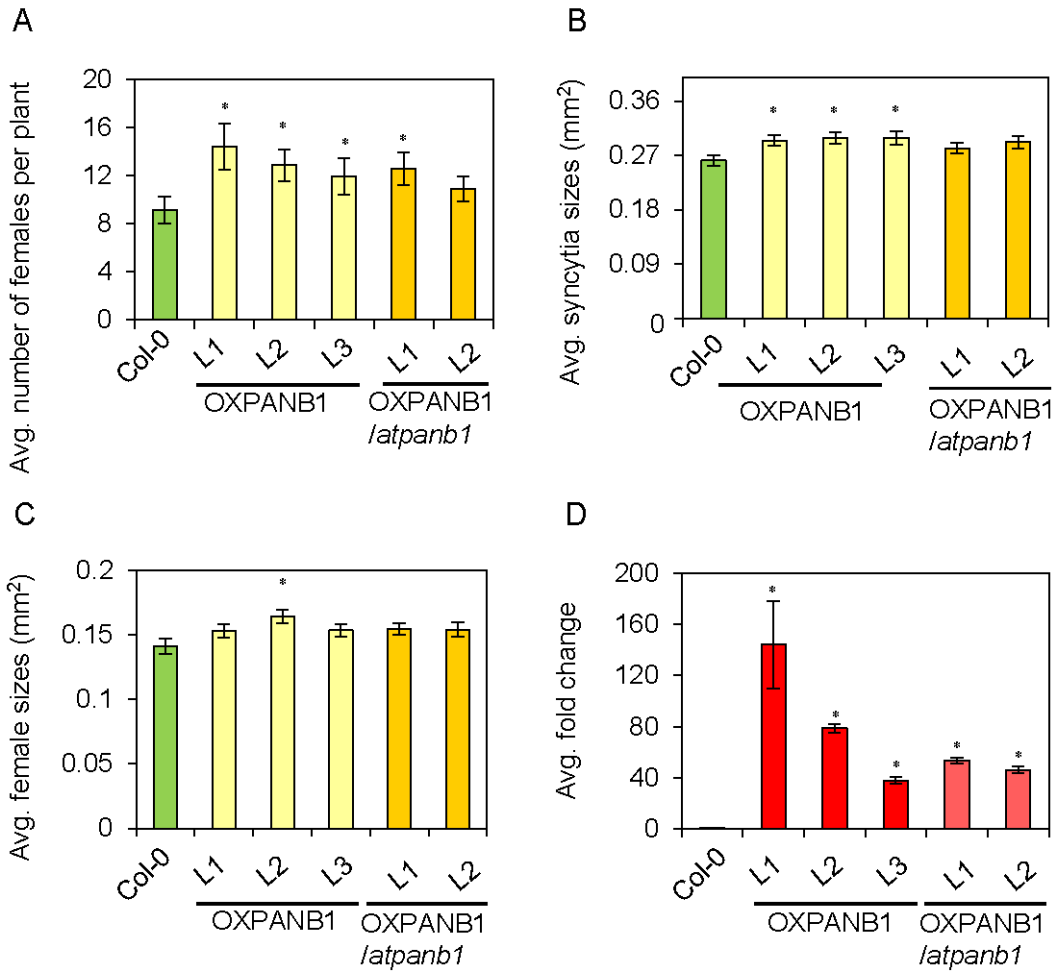

Figure 4: *H. schachtii* infection assays in 35S:AtPANB1 overexpression lines. A-C) Infection assays with three individual overexpression lines (Col-0 background) and two individual recovery overexpression lines (*atpanb1-1* background). D) qRT-PCR confirmation of AtPANB1 up-regulation of the transcript. Asterisks indicate statistically significant differences ($p<0.05$):

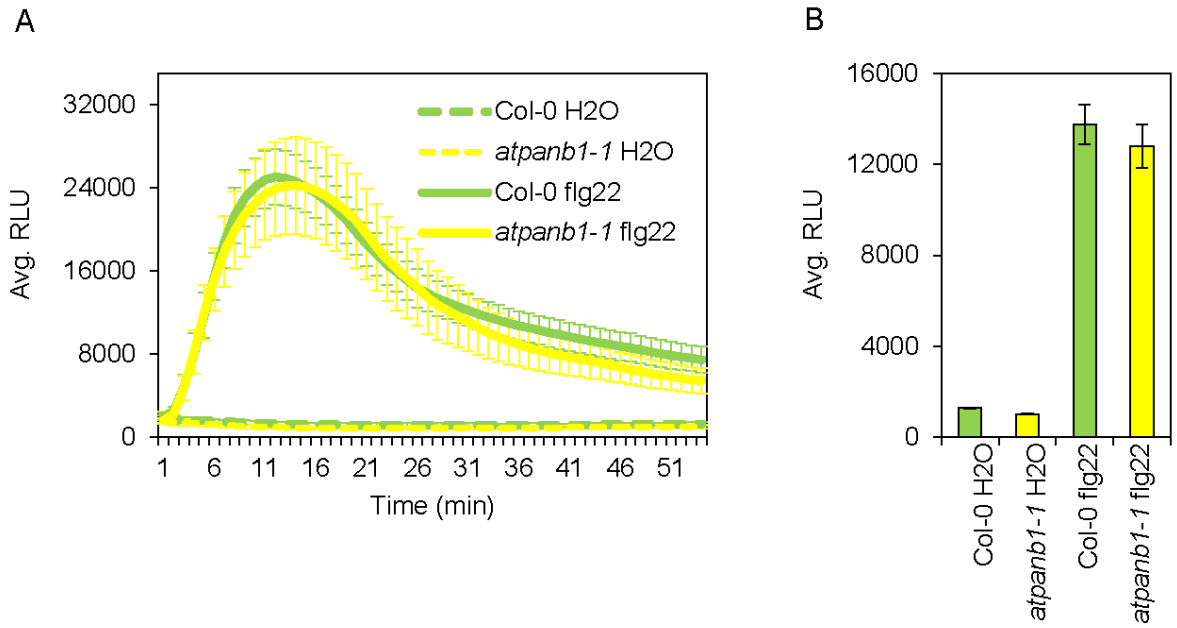

Figure 5: ROS burst induced by flg22 in Col-0 and *atpanb1-1* leaves. A) ROS production curve over 60 minutes. B) Cumulative ROS production in the time period of 60 minutes.

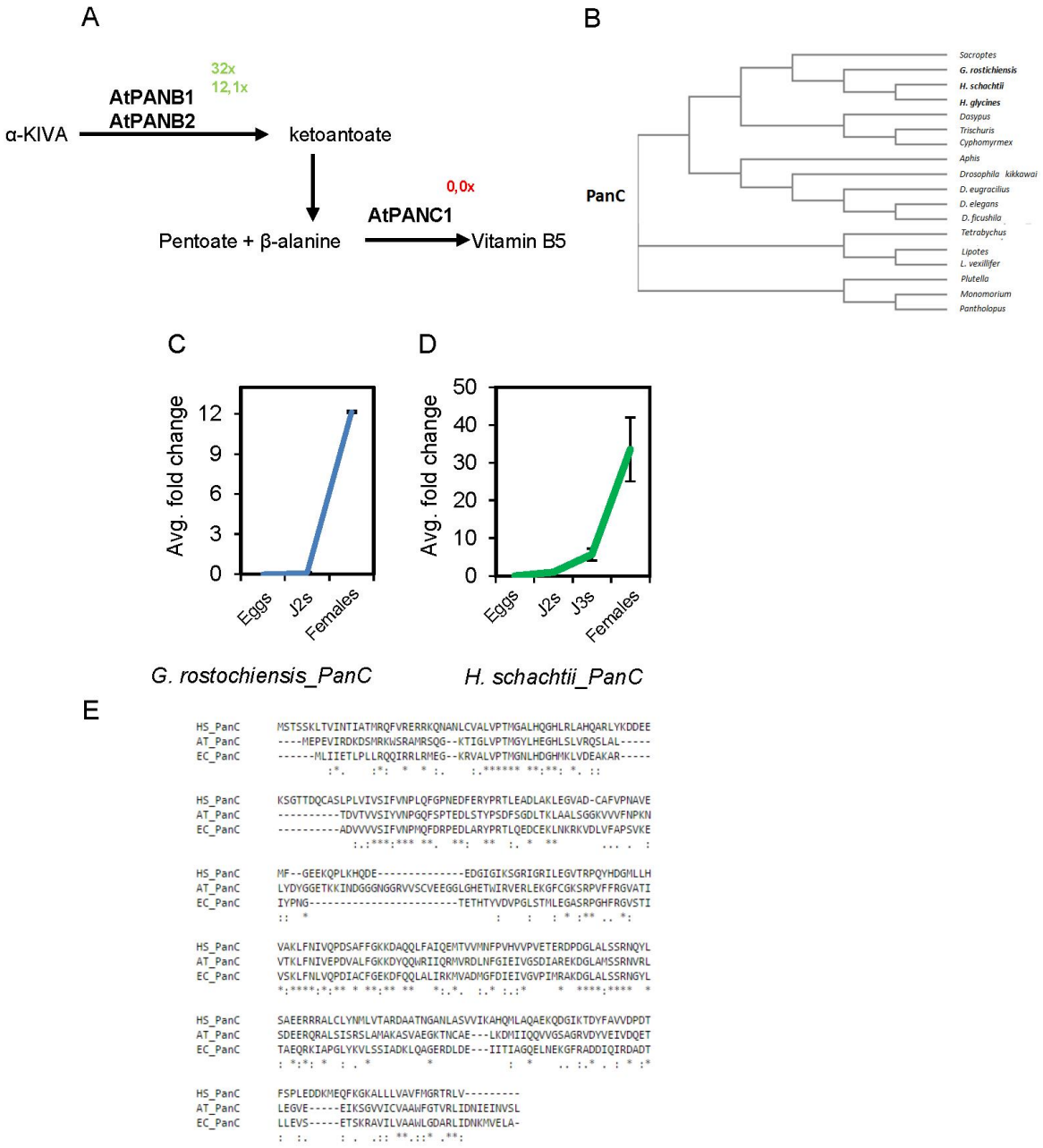

Figure 6: PanC gene characterization. A) VB5 biosynthesis pathway with microarray data of 5+15dpi syncytia. B) Phylogenetic tree of PanC. C) qRT_PCR gene expression of PanC in the *G. rostochiensis* and H. *schachtii* (D). E) Protein alignment of *H. schachtii, H. glycines G. rostochiensis, A. thaliana* and EcPANC.

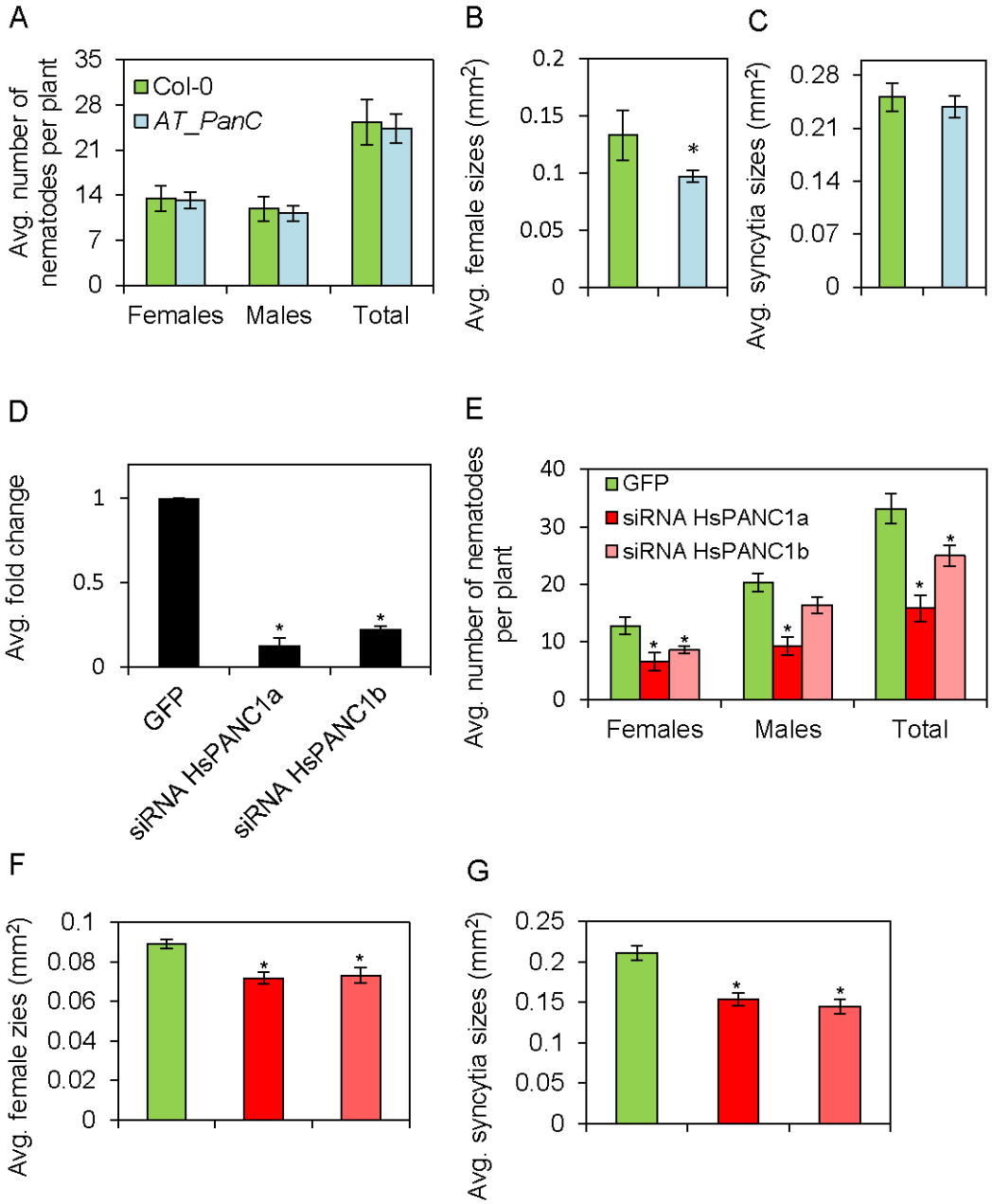

Figure 7: Infection assay in *atpanc1* mutant and siRNA assays. A-C) *atpanc1* infection assays measuring a number of nematodes per plant, female and syncytia sizes. D) qRT-PCR confirmation of down regulation of HsPANC due to siRNA treatment. E-F) siRNA infection assays measuring Col-0 susceptibility with nematodes treated with siRNA. Asterisks indicate statistically significant differences ($p<0.05$*).

Table 1. List of all primer sequences used in this project.

Primer name		Sequence
LBb1	F:	GCGTGGACCGCTTGCTGCAACT
LBp1.3	F:	ATTTTGCCGATTTCGGAAC
AtPANB1	F:	CGGTTAGGGTTTTGAAGGAAG
	R:	AACAACTTCTGAGACCCAGCC
AtPANC	F:	TGCATGGAACTTGTGTTCTTG
	R:	ATTGCATTTGCCTACGCATAC
18S	F:	GGTGGTAACGGGTGACGGAGAAT
	R:	CGCCGACCGAAGGGACAAGCCGA
AtPANB1	F:	ATGCGATTAAGCTCGAAGGA
	R:	AGCAGCTACAGGAGGTGGAA
At_ AtPANC	F:	TCTATGAGCCTGACCGTTCC
	R:	CTACGTCAATCCAGGCCAAT
HsPANC	F:	ACGGTTTGGCATTGTCAAGC
	R:	ACCACCGCAAAATAGTCCGT
Actin	F:	CGTGACCTCACTGACTACCT
	R:	CGTAGCACAACTTCTCCTTG
OE_AtPANB1	F:	GGGGACAAGTTTGTACAAAAAAGCAGGCTG CATGGCGTCCTCACTCACTAG
	R:	GGGGACCACTTTGTACAAGAAAGCTGGGTC TCACTTTGAAGGCTCCATGTTC
HsPANC_siRNA1	F:	AACCGCUGAAGCACCAGGACGCCTGTCTC
	R:	AACGTCCTGGTGCTTCGCGGCCTGTCTC
HsPANC_siRNA2	F:	AACATTGTGCAACCAGATTCGCCTGTCTC
	R:	AACGAATCTGGTTGCACAATGCCTGTCTC

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CHAPTER 3.

3.1. Arabidopsis *HIPP27* is a host susceptibility gene for the beet cyst nematode *Heterodera schachtii*

Authors

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Key words: susceptibility gene, cyst nematode, HIPP27, plant-parasitic nematodes, *Heterodera schachtii*, syncytium, starch

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Arabidopsis *HIPP27* is a host susceptibility gene for the beet cyst nematode *Heterodera schachtii*

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SUMMARY

Sedentary plant-parasitic cyst nematodes are obligate biotrophs that infect the roots of their host plant. Their parasitism is based on the modification of root cells to form a hypermetabolic syncytium from which the nematodes draw their nutrients. The aim of this study was to identify nematode susceptibility genes in *Arabidopsis thaliana* and to characterize their roles in supporting the parasitism of *Heterodera schachtii*. By selecting genes that were most strongly upregulated in response to cyst nematode infection, we identified HIPP27 (*H*EAVY METAL-ASSOCIATED *I*SOPRENYLATED *P*LANT *P*ROTEIN *27*) as a host susceptibility factor required for beet cyst nematode infection and development. Detailed expression analysis revealed that HIPP27 is a cytoplasmic protein and that *HIPP27* is strongly expressed in leaves, young roots and nematode-induced syncytia. Loss-of-function Arabidopsis *hipp27* mutants exhibited severely reduced susceptibility to *H. schachtii* and abnormal starch accumulation in syncytial and peridermal plastids. Our results suggest that *HIPP27* is a susceptibility gene in Arabidopsis whose loss of function reduces plant susceptibility to cyst nematode infection without increasing the susceptibility to other pathogens or negatively affecting the plant phenotype.

Keywords: cyst nematode, *Heterodera schachtii*, HIPP27, plant-parasitic nematodes, starch, susceptibility gene, syncytium.

INTRODUCTION

Cyst nematodes are obligate biotrophs that cause extensive yield losses in almost all economically important crops (Jones *et al.*, 2013). The lifecycle of the cyst nematode begins when an infective-stage juvenile invades the root, preferentially in the elongation zone. Once inside the root, the juvenile beet cyst nematode travels through various tissue layers until it reaches the vascular cylinder, where it selects a single cell that will become the initial syncytial cell (ISC; Wyss and Zunke, 1986). On selection of the ISC, the nematode becomes immobile and releases proteinaceous and non-proteinaceous secretions inside the ISC to promote the formation and function of the syncytium (Gardner *et al.*, 2015; Habash *et al.*, 2017; Hewezi *et al.*, 2015; Siddique *et al.*, 2015). The syncytium is a metabolic sink and serves as the nematode's only source of nutrients for the remainder of its lifecycle. A plethora of metabolic, proteomic and transcriptomic changes accompany the development of the syncytium (Elashry *et al.*, 2013; Hofmann *et al.*, 2010; Hütten *et al.*, 2015; Siddique *et al.*, 2014a; Szakasits *et al.*, 2009). As the syncytium expands, the nematode differentiates into either a male or female (Trudgill, 1967). Although the mechanism of sex determination is not yet fully understood, host factors, such as defence activation and nutrient availability, influence the sexual outcomes of cyst nematodes (Mendy *et al.*, 2017; Shah *et al.*, 2017; Siddique *et al.*, 2014b, 2015). When there is a surplus of nutrients, more females develop. However, most nematodes develop into males under stress conditions, such as those in resistant plants.

A previous transcriptome analysis has shown that expression of a HEAVY METAL-ASSOCIATED ISOPRENYLATED PLANT PROTEIN (HIPP) family gene, *HIPP27*, is strongly upregulated in syncytia induced by the beet cyst nematode *Heterodera schachtii* in Arabidopsis roots (Szakasits *et al.*, 2009). Both prokaryotes and eukaryotes have evolved mechanisms to maintain efficient metal homeostasis inside the cell, including the presence of numerous metal transport proteins, known as metallochaperones. Most metallochaperones contain a heavy metal-binding domain (HMA; pfam00403.6) with a highly conserved CysXXCys motif and a $\beta\alpha\beta\beta\alpha\beta$-fold structure for binding Cu^{+}, Cd^{2+} or Zn^{2+} (Tehseen *et al.*, 2010). Many different families of metallochaperones function in plant defences against the toxicity of metals, such as cadmium, chromium and aluminium. In addition, several plant immune receptors carry HMA domains, indicating that HMA probably plays a role in plant defence against pathogens (Sarris *et al.*, 2016).

In addition to the HMA domain, members of the large HIPP family contain a C-terminal isoprenylation motif. HIPPs are present

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only in vascular plants and are involved in a variety of biological processes, including heavy metal homeostasis and detoxification (Tehseen *et al.*, 2010), transcriptional responses to abiotic stresses, such as drought and cold (Barth *et al.*, 2009), and plant–pathogen interactions (de Abreu-Neto *et al.*, 2013; Zschiesche *et al.*, 2015). However, the mechanistic details underlying the roles of HIPPs in these biological processes remain mostly unknown. In *A. thaliana*, HIPPs make up the largest metallochaperone family, comprising 45 members divided into seven distinct classes (Tehseen *et al.*, 2010). The best-characterized member of this family in Arabidopsis is HIPP3, a zinc-binding protein that functions as an upstream regulator of the salicylate-dependent pathway during pathogen infection (Zschiesche *et al.*, 2015). As described above, the expression of *HIPP27* is strongly upregulated in syncytia induced by *H. schachtii* in Arabidopsis roots. Therefore, we hypothesized that HIPP27 might be an important host susceptibility factor in beet cyst nematode parasitism. In the present study, we investigated the role of HIPP27 in the interaction between *H. schachtii* and Arabidopsis. Our data show that *HIPP27* facilitates cyst nematode infection and the efficient development of nematode feeding sites in Arabidopsis roots.

RESULTS AND DISCUSSION

Selection of *HIPP27* as a candidate Arabidopsis host susceptibility gene for *H. schachtii*

The main objective of this study was to identify new target genes that could play a role in the susceptibility of Arabidopsis to *H. schachtii*. We mined previously published transcriptomic data and selected the top 100 genes that were most strongly upregulated in the syncytium compared with uninfected control roots (Szakasits *et al.*, 2009). We then performed a literature search, and all genes that played a role in crucial metabolic processes, such as photosynthesis and sugar metabolism, were excluded. This step was aimed at minimizing the risk of the selection of genes for which the loss-of-function mutant may show severe growth phenotypes. We also eliminated all genes whose loss-of-function mutants have been shown previously to exhibit severe root or shoot phenotypes. Next, BLAST searches were used to identify candidate genes for which only a single-copy orthologue was present in the genome of the natural host of *H. schachtii*, i.e. *Beta vulgaris* (Dohm *et al.*, 2014). Using this process, we ultimately selected 10 candidate host susceptibility genes (Table 1). The expression of these genes was validated in Arabidopsis plants that were grown *in vitro* and infected with *H. schachtii*. RNA was extracted from hand-dissected root segments containing syncytia that were sampled at 15 days post-infection (dpi), and was used to analyse the expression of the candidate genes via quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The results confirmed that, of the 10 candidate genes, the expression of nine genes was upregulated (Table 1). However, the intensity of fold change was substantially lower than in the microarray data. This discrepancy between the qPCR and microarray data can probably be attributed to the different syncytial material used in the two cases: whereas microaspirated syncytial cell contents were used for microarray analysis, our qRT-PCR analysis was performed using cut syncytia containing the surrounding non-syncytial root cells, which may have diluted syncytium-specific mRNA expression.

Table 1 Validation of the expression of 10 selected candidate genes in syncytia induced in Arabidopsis roots by beet cyst nematode.

		Fold change compared with uninfected control	
Gene	Locus	Microarrays (5 + 15 dpi)	qRT-PCR (15 dpi)
ENH1	AT5G17170	27.9	3.7 ± 0.26
UGP2	AT5G17310	29.9	2.6 ± 0.56
–	AT4G24830	29.9	3.3 ± 0.59
IMD2	AT1G80560	32.0	7.3 ± 4.7
NPC6	AT3G48610	39.3	30.0 ± 11.6
AD11A3	AT2G24270	48.5	44.5 ± 16.9
TRX1	AT3G51030	48.5	11.2 ± 3.28
CCR2	AT1G80820	52.0	15.47 ± 3.48
FAD6	AT4G30950	52.0	5.04 ± 1.71
HIPP27	AT5G66110	68.6	24.81 ± 2.88

For microarrays, data from microaspirated syncytia at 5 and 15 days post-infection (dpi) were pooled and compared with control roots (Szakasits et al., 2009). For quantitative reverse transcription-polymerase chain reaction (qRT-PCR), values represent the relative fold change in infected root segment containing syncytia relative to control uninfected root. 18S and UBP22 were used as housekeeping genes to normalize the data. All values are the means of three biological replicates ± standard error (SE)

We obtained loss-of-function homozygous mutants for the 10 candidate genes and analysed them for beet cyst nematode susceptibility via preliminary pathogenicity assays (Fig. S1, see Supporting Information). Amongst these, a mutant for the candidate gene *HIPP27* showed a particularly significant decrease in susceptibility to beet cyst nematode and was therefore selected for further molecular characterization.

HIPP27 is strongly upregulated in the syncytium

To investigate the expression patterns of *HIPP27* in various organs of Arabidopsis plants, we cloned the putative promoter region (472 bp) upstream of the translation start codon of *HIPP27*, producing the *pHIPP27:GUS* construct. Arabidopsis plants were transformed with *pHIPP27:GUS* and three independent homozygous lines were obtained. The general expression pattern of *pHIPP27:GUS* was then assessed in various plant organs. For uninfected lines, β-glucuronidase (GUS) staining was detected ubiquitously in all tissues examined, with particularly strong expression in young leaves (Fig. 1A–C). GUS expression was observed occasionally in uninfected roots, particularly in the

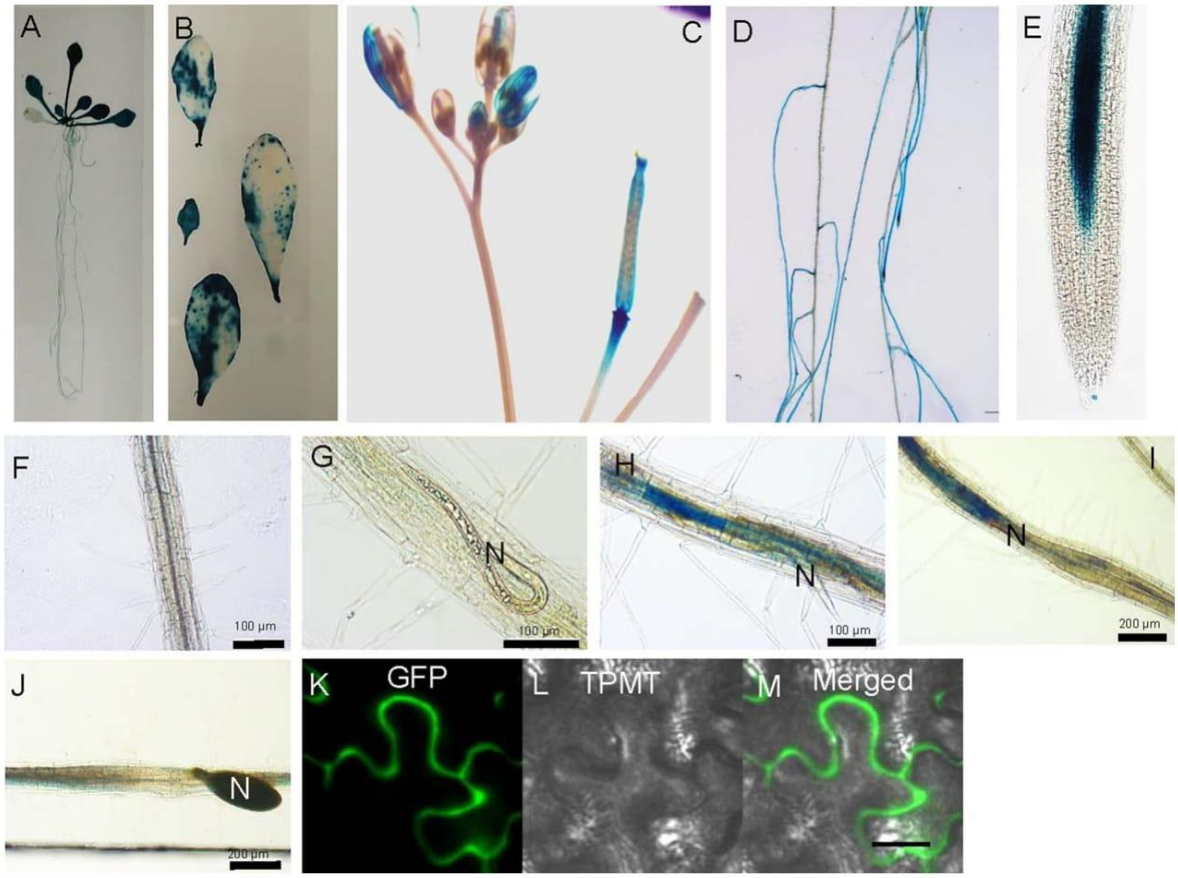

Fig. 1 *HIPP27* is strongly upregulated in the syncytium. Expression of *pHIPP27:GUS* in various organs of Arabidopsis: (A) 14-day-old seedling; (B) leaves from 2–3-week-old plants; (C) inflorescence; (D–F) 12-day-old root (0 days post-infection, dpi); (G–J) nematode-infected root segments at 1 dpi (G), 3 dpi (H), 5 dpi (I) and 10 dpi (J); (K–M) localization of *35S:HIPP27-GFP* in the epidermis of *Nicotiana benthamiana* leaves at 3 days after infiltration. N, nematode. GFP, green fluorescent protein; TPMT, Transmitted light detector.

younger parts (Fig. 1D,E); however, no staining was detected in the primary root, elongation zone and in the root tips (Fig. 1E,F). Plants harbouring *pHIPP27:GUS* were then infected with nematodes and stained for GUS activity at different time points after infection, i.e. 1, 3, 5 and 10 dpi, to cover the different stages of nematode development (Fig. 1G–J). We detected strong GUS expression at 3 dpi (Fig. 1H), which became intense at 5 dpi (Fig. 1I). However, GUS staining was either absent or only faintly detected at 10 dpi (Fig. 1J). GUS expression was generally localized inside the syncytium and extended beyond the syncytium in only a few cases, indicating that the upregulation of *HIPP27* is specific to nematode infection.

To determine the subcellular localization of HIPP27, HIPP27-GFP was transiently expressed in *Nicotiana benthamiana* leaf epidermis under the control of a constitutive promoter (CaMV 2x35S), and its localization was assessed using confocal microscopy. HIPP27-GFP was localized to the cytosolic region of the cell (Fig. 1J–L). Taken together, these results indicate that HIPP27 is a cytoplasm-localized protein and that *HIPP27* expression is strongly upregulated during the early stages of syncytium development. These findings point to the likelihood of importance of this gene in early syncytium development.

Loss of function of *HIPP27* decreases Arabidopsis susceptibility to *H. schachtii*

To further substantiate the role of HIPP27 in the plant's interaction with cyst nematodes, we obtained two loss-of-function T-DNA insertion mutants of *HIPP27* (*hipp27a* and *hipp27b*; Fig. S2, see Supporting Information). Homozygous lines were selected after genotyping and the lack of *HIPP27* expression in the homozygous mutants was confirmed by RT-PCR (Fig. S3, see Supporting Information). To determine whether *hipp27* mutants showed any impairment in growth and development, we analysed phenotypic parameters in plants grown under sterile and glasshouse conditions for *hipp27a*. However, we did not observe any significant

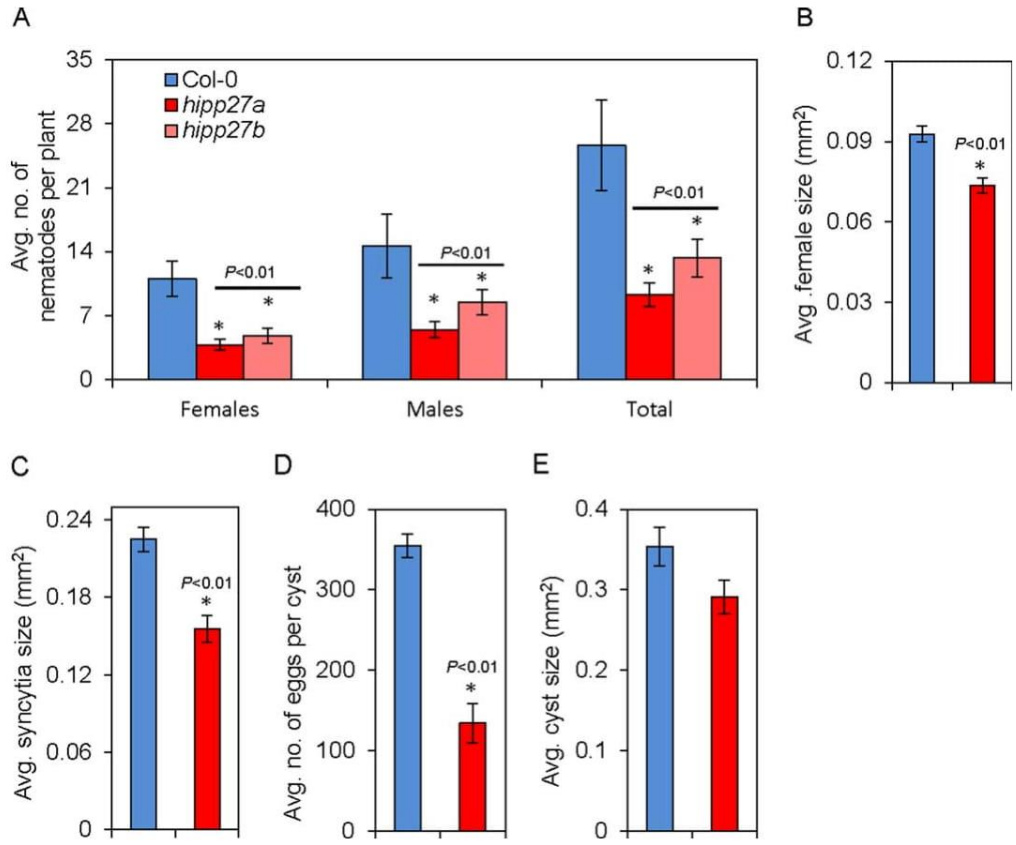

Fig. 2 Loss of function of *HIPP27* decreases Arabidopsis susceptibility to *Heterodera schachtii*. (A) Average number of nematodes per plant present in Col-0 ($n = 26$), *hipp27a* ($n = 45$) and *hipp27b* ($n = 36$) mutant lines at 14 days post-infection (dpi). (B) Average sizes of female nematodes in Col-0 ($n = 181$) and *hipp27a* ($n = 174$) at 14 dpi. (C) Average sizes of plant syncytia in Col-0 ($n = 181$) and *hipp27a* ($n = 174$) at 14 dpi. (D) Average number of eggs per cyst in Col-0 ($n = 66$) and *hipp27a* ($n = 56$) at 42 dpi. (E) Average cyst sizes in Col-0 ($n = 128$) and *hipp27a* ($n = 133$) at 42 dpi. (A–E) Bars represent mean ± standard error (SE). Asterisks represent statistically significant difference from corresponding Col-0 value (*t*-test, $^*P < 0.05$).

difference in average dry weight, average flowering time, average root length or average plant height in the mutant compared with the control (Fig. S4A–D, see Supporting Information).

To analyse the changes in *hipp27* with regard to nematode susceptibility, we performed infection assays in which several susceptibility parameters were measured. The average number of females, a commonly accepted parameter indicating nematode susceptibility under *in vitro* conditions, was strongly reduced in both *hipp27a* (60%, $P < 0.01$) and *hipp27b* (40%, $P < 0.01$) compared with the control (Fig. 2A). In addition, the average numbers of males (*hipp27a*, 58%, $P < 0.01$; *hipp27b*, 48%, $P < 0.01$) and average total number of nematodes (*hipp27a*, 50%, $P < 0.01$; *hipp27b*, 55%, $P < 0.01$) were also significantly reduced in *hipp27* compared with Col-0 (Fig. 2A). Further susceptibility parameters, such as the average sizes of the female and the syncytium, were measured only for *hipp27a* and were also significantly reduced in *hipp27a* ($P < 0.01$) compared with Col-0 (Fig. 2B,C). For field-grown crops, the most common susceptibility parameters were the average number of eggs per cyst and average size of cysts. We found that the average number of eggs per cyst was reduced significantly in *hipp27a* compared with Col-0 (Fig. 2D). Together, these results suggest that HIPP27 plays a vital role in cyst nematode parasitism.

Because loss of function of *HIPP27* resulted in a significant decrease in susceptibility to beet cyst nematodes, we hypothesized that overexpression of this gene might increase susceptibility to nematodes. To test this, we produced transgenic lines expressing *HIPP27* under the control of a constitutive promoter in either the Col-0 (*OXHIPP27*-L1 and *OXHIPP27*-L2) or *hipp27*a (*OXHIPP27*/*hipp27a*-L1 and *OXHIPP27*/*hipp27a*-L2) background. We confirmed the increase in transcript abundance in the homozygous lines by qRT-PCR (Fig. 3A) and performed an infection assay. We found that there was no significant change in average female size and syncytium size in these lines compared with the control ($P > 0.05$) (Fig. 3B,C). However, the average number of female nematodes per plant was significantly higher in the *OXHIPP27* lines (L1 = 13.68 ± 2.09, $P < 0.01$; L2 = 10.85 ± 1.91, $P = 0.02$) as well as one of the *OXHIPP27*/*hipp27a* lines

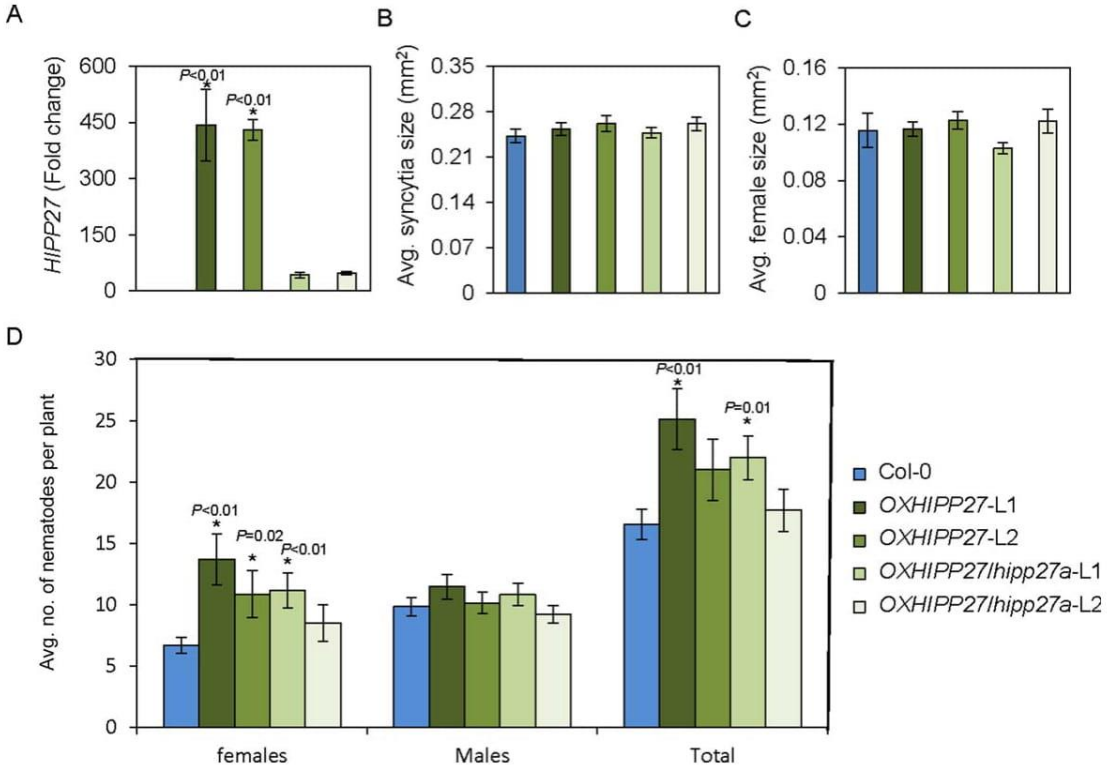

Fig. 3 Overexpression of *HIPP27* leads to increased susceptibility to *Heterodera schachtii*. (A) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) confirmation of the increase in *HIPP27* transcript in overexpression lines of the Col-0 (*OXHIPP27*) or *hipp27a* (*OXHIPP27/hipp27a*) background ($n = 3$). (B, C) Average sizes of syncytia (B) and female nematodes (C) in Col-0 ($n = 77$) and *HIPP27* (*OXHIPP27/L1*, $n = 75$; *OXHIPP27/L2*, $n = 68$; *OXHIPP27hipp27a/L1*, $n = 90$; *OXHIPP27hipp27a/L2*, $n = 75$) overexpression lines at 14 days post-infection (dpi). (D) Average number of nematodes per plant present in Col-0 ($n = 40$) and *HIPP27* overexpression lines (*OXHIPP27/L1*, $n = 24$; *OXHIPP27/L2*, $n = 28$; *OXHIPP27hipp27a/L1*, $n = 26$; *OXHIPP27hipp27a/L2*, $n = 24$) at 14 dpi. (A–D) Bars represent mean ± standard error (SE). Asterisks denote significant difference from corresponding Col-0 value (*t*-test, $*P < 0.05$).

(L1 = 11.15 ± 1.43, $P < 0.01$) compared with Col-0 (6.75 ± 0.65) (Fig. 3D). Moreover, we also found a significant increase in the total number of nematodes in one *OXHIPP27* line (L1 = 25.13 ± 2.46, $P < 0.01$) and one *OXHIPP27/hipp27a* line (L1 = 21.96 ± 1.43, $P = 0.01$) compared with the control (16.52 ± 1.21) (Fig. 3D). Together, these data suggest that the expression of *HIPP27* is activated on cyst nematode infection and is required for proper syncytium and nematode development.

Loss of function of *HIPP27* does not lead to increased susceptibility to other pathogens

We wanted to determine whether the *hipp27* mutant exhibits increased susceptibility to other pathogens. Therefore, we performed infection assays using the gall-forming nematode *Meloidogyne javanica* and the necrotrophic fungus *Botrytis cinerea*. We did not observe differences between the wild-type and mutant in the average size of necrotic lesions caused by *B. cinerea* (Fig. 4A). In addition, we found that the number of galls formed by *M. javanica* in *hipp27a* did not differ significantly relative to that in Col-0 controls ($P > 0.05$) (Fig. 4B). To further rule out a putative role for HIPP27 during gall formation, we compared the diameters of the galls in the wild-type and *hipp27a* mutant lines, finding no significant differences between them (Fig. 4C). Moreover, the reproduction of the root-knot nematodes, measured as the number of egg masses per gall, was statistically unaffected in *hipp27a* ($P > 0.05$) (Fig. 4D). Together, these data point to a cyst nematode-specific role of *HIPP27* in supporting parasitism.

Loss of function of *HIPP27* does not impair plant basal defence

To investigate whether the reduced susceptibility of *hipp27a* plants to *H. schachtii* is a result of altered plant defence responses, we measured the reactive oxygen species (ROS) levels induced over a 60-min period on treatment of leaves with a well-known immunopeptide, flg22. We did not observe significant changes in ROS levels (Fig. 5A,B), indicating that the *hipp27a* mutant does not have impaired plant basal defence responses. To further confirm this finding, we performed qRT-PCR analysis to

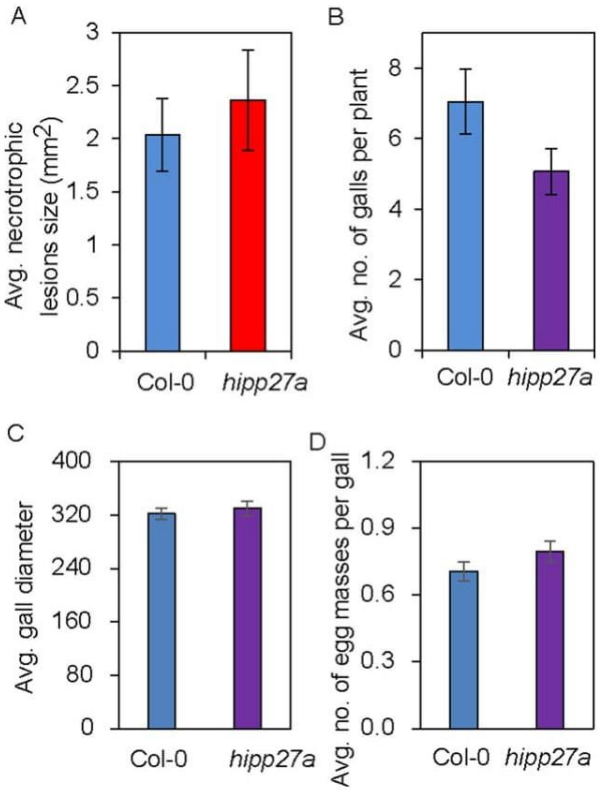

Fig. 4 Loss of function of *HIPP27* does not alter the susceptibility to other pathogens. (A) Average size of lesions induced by *Botrytis cinerea* in Col-0 ($n = 21$) and *hipp27a* ($n = 14$) lines. (B) Average number of galls per plant in Col-0 ($n = 55$) and *hipp27* ($n = 60$) mutant at 21 days post-infection (dpi) with *Meloidogyne javanica*. (C) Gall size calculated as the diameter of galls from Col-0 ($n = 36$) and *hipp27* ($n = 37$) at 14 dpi with *M. javanica*. (D) Number of egg masses formed per gall by *M. javanica* in Col-0 ($n = 363$) and *hipp27a* ($n = 304$) 2 months after infection. Statistical analysis was performed using Student's *t*-test (no significant differences from Col-0 were observed). Bars represent mean ± standard error (SE).

examine the expression of a few plant basal defence marker genes in uninfected shoots: *PR1* and *PR2* are induced by salicylic acid, *PDF1.2* and *HEL1* are marker genes for jasmonic acid signalling, and *ERF6* and *ACS2* are involved in ethylene signalling. The expression levels of all marker genes in the *hipp27* mutant were similar to those of Col-0 (Fig. 5C). These results reinforce the conclusion that *HIPP27* has a specific role in the interaction with cyst nematodes, as an increase in plant basal defence would lead to resistance to other pathogens (Fig. 4A–D).

Loss of function of *HIPP27* causes physiological or metabolic abnormalities

Finally, we examined the anatomical and ultrastructural organization of syncytia induced by the beet cyst nematode in roots of wild-type Col-0 and *hipp27a* mutant plants (Fig. 6). Comparison of light microscopy images of sections taken through similar regions of nematode-induced syncytia showed no apparent differences between the syncytia induced in the two genotypes (Fig. 6A–D versus 6E–H). Next to the nematode head, the syncytia were relatively small on cross-sections and cells not incorporated into them divided at different planes, forming groups of neoplastic cells (Fig. 6A,C,E,G), similar to those described by Sobczak *et al.* (1997). The extent of destruction around the nematode head was also similar in both plant genotypes (Fig. 6A,C,E,G). Sections from the middle and the widest regions of syncytia (distant from the nematode head) showed that, if the syncytium had been induced in young roots without fully differentiated primary xylem, it occupied the central position inside the vascular cylinder and became surrounded by regularly dividing pericyclic cells forming the periderm, a secondary cover tissue (Fig. 6B,F). However, if the syncytium had been induced in the root region in which the primary xylem was fully differentiated, it developed the primary xylem on only one side, whereas secondary xylem elements differentiated on the opposite side (Fig. 6D,H). In both cases, the pericyclic cells divided and formed periderm around the entire vascular cylinder (Fig. 6B,D,F,H). No visible differences in the extent of syncytial element hypertrophy or the number and size of cell wall openings were evident, indicating that the anatomical structure of syncytia induced in the *hipp27* mutant was undisturbed (Fig. 6A–H), although their dimensions were smaller compared with Col-0 (Fig. 2).

At the ultrastructural level, syncytia induced in Col-0 and *hipp27*a plants were similar at 5 dpi (Fig. 6I,K). Their cytoplasm proliferated and became electron dense, with numbers of plastids, mitochondria and endoplasmic reticulum structures increasing, whereas central vacuoles disappeared. Only a few syncytial plastids contained very small starch grains. However, serial sectioning of syncytia showed that syncytia induced in *hipp27a* roots often differed in their ultrastructure along their axis. In the region close to the nematode head and at the leading edge (the most remote part of the syncytium), syncytia had typical ultrastructural organization (Fig. 6K). By contrast, in the middle region of the syncytium, the cytoplasm was relatively electron translucent, endoplasmic reticulum structures were weakly developed and large starch grains were formed in numerous syncytial plastids and plastids of peridermal cells next to the syncytium (Fig. 6L). In samples collected at 10 dpi, syncytial protoplasts had typical ultrastructure in syncytia induced in wild-type Col-0 plants (Fig. 6J). Here again, small starch grains were present in only a few syncytial plastids and were absent in cells surrounding the syncytium. By contrast, starch grains were frequently present in peridermal plastids next to syncytia induced in *hipp27a* roots (Fig. 6M,N). Inside syncytia, plastids present in regions with well-developed endoplasmic reticulum usually did not contain starch grains (Fig. 6M), whereas plastids present in regions with electron

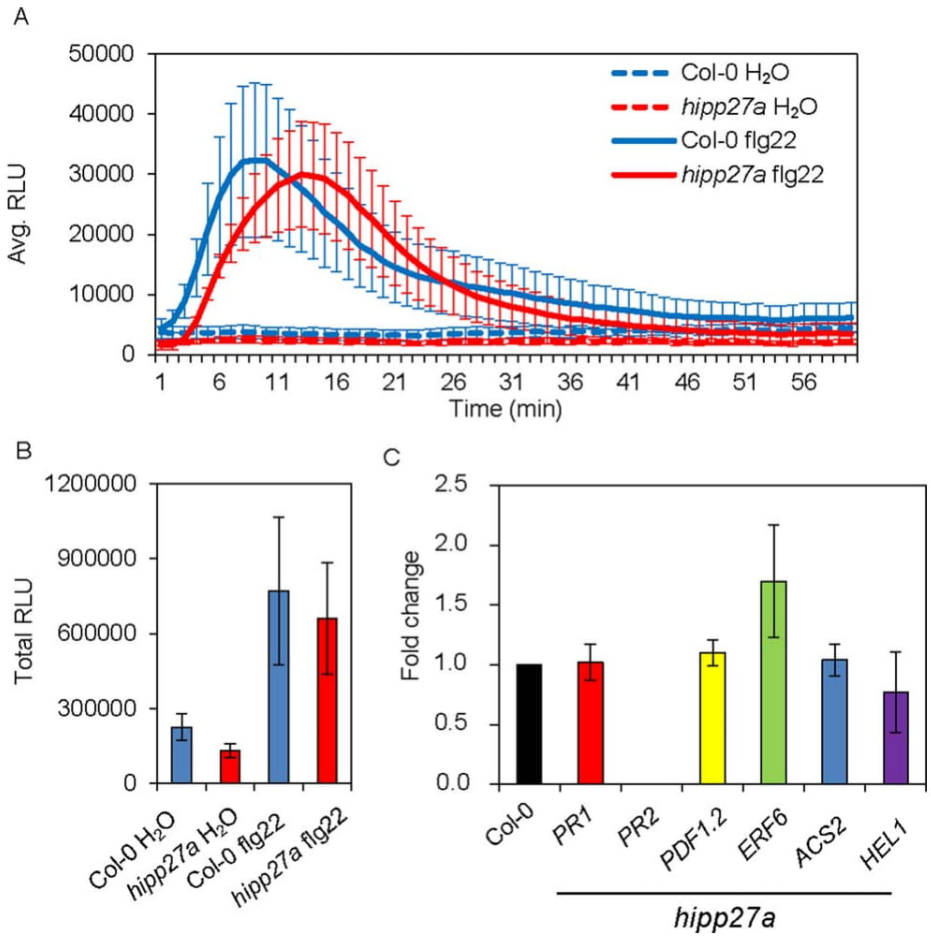

Fig. 5 Loss of function of *HIPP27* does not impair plant basal defence. (A) Reactive oxygen species (ROS) burst induced by flg22 during 60 min of measurement in Col-0 and *hipp27a* mutant expressed in relative light units (RLU) ($n = 3$). (B) Total RLU of ROS during 60 min of exposure to flg22 in Col-0 and *hipp27a* mutant ($n = 3$). (C) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) gene expression analysis of defence-related genes in uninfected shoots of Col-0 and *hipp27a* mutant ($n = 3$). Statistical analysis was performed using Student's *t*-test (no significant differences from Col-0 were observed). Bars represent mean ± standard error (SE).

translucent cytoplasm and poorly developed endoplasmic reticulum usually contained large starch grains (Fig. 6N,O). Some of these plastids acquired enormously large sizes (Fig. 6O) and could be clearly recognized even on anatomical sections (Fig. 6H). Taken together, our ultrastructural analysis shows that loss of function of HIPP27 leads to physiological and metabolic abnormalities, including the accumulation of large starch grains.

CONCLUSION

This study represents the first investigation of the role of an HIPP family protein in plant–nematode interactions. We showed that the expression of *HIPP27* is specifically and highly upregulated in syncytia induced by *H. schachtii* in Arabidopsis roots. The strong GUS expression driven by the *HIPP27* promoter inside the syncytium points to the involvement of *HIPP27* in the establishment and development of syncytia. Indeed, the analysis of loss-of-function mutants and overexpression lines showed that *HIPP27* expression supports infection of the host roots by the beet cyst nematode. In contrast with its expression in cyst nematode-induced syncytia, *HIPP27* expression was not differentially regulated in feeding sites induced by root-knot nematodes (Cabrera *et al.*, 2014). These observations indicate that the role of *HIPP27* in facilitating parasitism is restricted to that of cyst nematodes. This hypothesis is further supported by results from pathogenicity assays with *M. javanica* and *B. cinerea*, in which we did not observe any significant difference in susceptibility between Col-0 and the *hipp27* mutant.

HIPP27 belongs to Cluster IV of the HIPP family in Arabidopsis, which is notable for the presence of a conserved aspartic acid (Asp) residue preceding the metal binding motif (CysXXCys). Notably, the expression of *HIPP27* in yeast confers a slight increase in cadmium (Cd) resistance to a Cd-sensitive yeast strain, probably as a result of binding of Cd by HIPP27 in the cytosol (Tehseen *et al.*, 2010). Several HIPP family members, including HIPP26 and HIPP27, have been shown to interact via the HMA domain with the drought stress-related zinc finger transcription factor 29 (ATHB29) and UBIQUITIN-SPECIFIC PROTEASE 16 (UBP16) (Barth *et al.*, 2009; Zhao *et al.*, 2013). In addition, *hipp26* mutants display altered expression of ATHB29-regulated dehydration response genes compared with the wild-type (Barth *et al.*, 2009). Whether HIPP27 plays a similar role in ATHB29-mediated

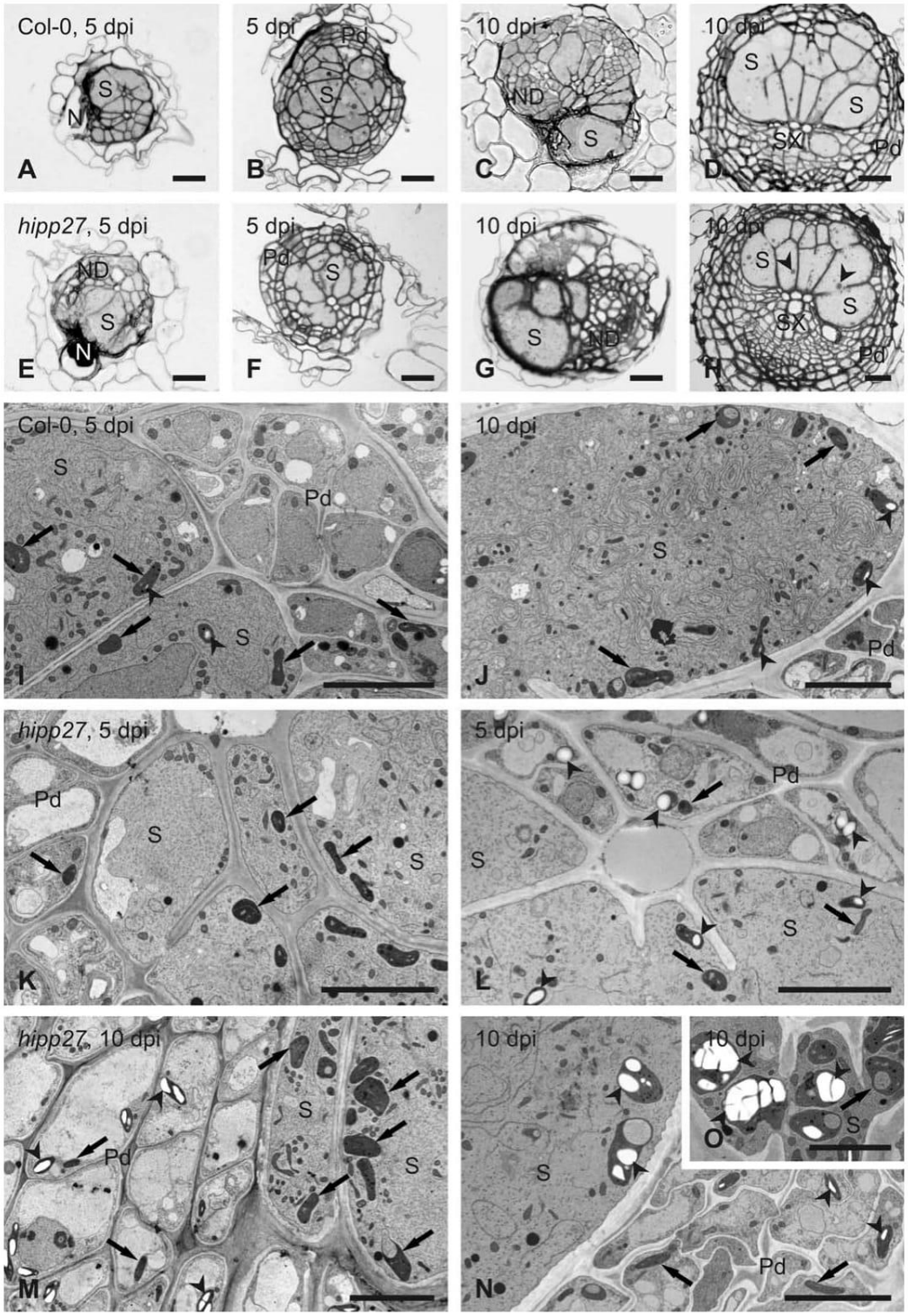

Fig. 6 Loss of function of *HIPP27* causes physiological or metabolic abnormalities. Light (A–H) and transmission electron (I–O) microscopy images of cross-sections of syncytia at 5 days post-infection (dpi) (A, B, E, F, I, K and L) and 10 dpi (C, D, G, H, J and M–O) induced in roots of Col-0 wild-type plants (A–D, I and J) and *hipp27a* mutant (E–H and K–O). Light microscopy images were taken close to the nematode head (A, C, E and G) or through the widest regions of syncytia (B, D, F and H). Arrowheads indicate selected starch grains in plastids; arrows point to plastids. N, nematode; ND, neoplastic divisions; Pd, periderm; S, syncytium; SX, secondary xylem. Scale bars: 20 μm (A–H), 5 μm (I–O).

gene regulation remains to be explored. Previous microarray analysis using microaspirated *H. schachtii* syncytium protoplasts has shown that the expression of both *ATHB29* and *UBP16* is downregulated in syncytia relative to uninfected control roots. By contrast, *HIPP27* is highly upregulated in syncytia (Szakasits *et al.*, 2009). These observations make it likely that HIPP27 acts independently of ATHB29 to regulate infection by *H. schachtii*.

Our data showed no difference in the activation of the ROS burst or in the expression of defence genes between Col-0 and *hipp27a* mutant lines. Based on these results, we propose that the expression of *HIPP27* is required for the maintenance of the optimal development or functioning of the syncytium. This hypothesis is supported by the observation that the syncytium size was significantly reduced in *hipp27a* compared with Col-0. The syncytium is a strong metabolic sink that serves as the only source of nutrients for developing nematodes, and the maintenance of metal homeostasis in the syncytium must be tightly regulated. Considering the role of HIPP family members as metallochaperones, it is plausible that HIPP27 functions in metal transport and homeostasis in the syncytium. Indeed, microscopic observations confirmed that the lack of HIPP27 protein does not influence the general developmental pattern of syncytia, but causes physiological or metabolic disorders, leading to the accumulation of phloem-provided saccharides as starch grains in peridermal and syncytial plastids. These sugars may become unavailable to parasitic juveniles, leading to their disturbed development. Hofmann *et al.* (2008) have shown that the number of starch grains increases in syncytia during nematode moult, when no food is taken up by the associated juvenile, as well as in degraded syncytia of adult males and prematurely degraded syncytia associated with females. As no clearly degraded syncytia were found in our experiments, it can be speculated that starch accumulation and its potential unavailability for the nematodes are very early features of syncytium degradation, and that these processes impair the development of beet cyst nematodes on *hipp27* roots. However, further work is required to elucidate the precise role of HIPP27 in cyst nematode parasitism.

In conclusion, we have identified *HIPP27* as a host susceptibility gene whose deletion reduces plant susceptibility to cyst nematodes without increasing susceptibility to other pathogens. The lack of developmental phenotypes in *hipp27* plants highlights the potential of using *HIPP27* in the breeding of nematode-resistant crop plants.

EXPERIMENTAL PROCEDURES

Plant growth and beet cyst nematode infection

Arabidopsis thaliana seeds were sterilized for 5 min with 1.2% NaClO, followed by three consecutive washes with autoclaved double-distilled H_2O. Sterilized seeds were sown in KNOP medium, and 12-day-old plants were infected with 70–80 surface-sterilized second-stage juveniles (J2s). For surface sterilization, hatched J2s were washed with 0.05% $HgCl_2$, followed by three consecutive washes with autoclaved double-distilled H_2O (Sijmons *et al.*, 1991). Two weeks after inoculation, nematode susceptibility parameters, such as the average number of females, average number of males and average number of total nematodes per plant, were quantified under a Leica S4E stereomicroscope (Leica Microsystems, Wetzlar, Germany), and average female and syncytium sizes were measured under a Leica M 165C stereomicroscope (Leica Microsystems) equipped with Leica LAS v4.3 image analysis software (Leica Microsystems). Cyst size and the average number of eggs per cyst were measured and counted at 42 days after inoculation. All infection assays were repeated at least three times and 10–20 plants were used in each repetition. The data from all individual plants from three experiments were combined to a single mean.

Infection assays with *M. javanica* and *B. cinerea*

Infection assays using *M. javanica* were performed as described previously (Cabrera *et al.*, 2014). In brief, Arabidopsis plants were grown aseptically on 0.3% Gamborg's medium (Gamborg *et al.*, 1968) supplemented with 1.5% (w/v) sucrose for 14 days. Fourteen-day-old plants were inoculated with 70–100 *M. javanica* J2s per plant. The infected plants were incubated at 23 °C and 16 h : 8 h light : dark photoperiod. The number of galls was counted at 21 dpi. Gall diameters were measured at 14 dpi using ImageJ. The infection assays with *B. cinerea* were performed as described previously (Lozano-Torres *et al.*, 2014). In brief, 5-μL drops of fungal spores at a concentration of 5×10^5 were inoculated onto 4-week-old Arabidopsis plants grown in soil under glasshouse conditions. The infected plants were incubated for 3 days in the dark at 20 °C and 100% relative humidity. Plant susceptibility was estimated by measuring the sizes of necrotic areas at 3 dpi in the same manner as used to measure the sizes of syncytia or females (Siddique *et al.*, 2014b).

Genotyping and expression analysis of the mutants

Arabidopsis SAIL lines (*hipp27a*, SAIL_167_B06; *hipp27b*, SAIL_675_E09) were ordered from the Nottingham Arabidopsis Stock Centre (NASC), Nottingham, UK. Homozygosity of the lines was tested by extracting plant DNA using the cetyltrimethylammonium bromide (CTAB) method, after which PCR with SAIL primers was conducted to confirm the T-DNA insertions in these lines. PCR was performed on a C100™ Thermal Cycler (Bio-Rad Laboratories, USA). Visual observation of homozygosity and expression analysis were performed using Gel Doc™ (Bio-Rad Laboratories, Hercules, California, USA), together with Lab 3.0 software. The primer sequences are listed in Table S1 (see Supporting Information).

qRT-PCR gene expression analysis

To measure transcript abundance, plants were grown in Knop medium, and RNA from Col-0 and mutant or overexpression leaves was extracted using Nucleospin RNA XS (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. RNA was extracted from a pool of three to four plants. This RNA was then used to synthesize first-strand cDNA—a procedure repeated three times in three independent experiments. The RNA concentration was measured with a NanoDrop (Thermo Fisher

Scientific, Waltham, MA, USA), and cDNA was prepared using a High Capacity cDNA Reverse Transcript kit (Life Technologies, Waltham, MA, USA). Transcript abundance was measured on a StepOnePlus Real-Time PCR System (Applied Biosynthesis, Waltham, MA, USA) as described by Pfaffl (2001); *18S* and *UBP22* were used as internal controls as recommended previously (Hofmann and Grundler, 2007). Expression in each experiment was measured in three technical replicates.

Plant phenotyping

Phenotypic parameters of Arabidopsis Col-0 and *hipp27a* lines were measured using soil-grown plants in a glasshouse and plants grown in Petri dishes on KNOP medium under sterile conditions. Measurements were conducted under a Leica M 165C stereomicroscope (Leica Microsystems) equipped with Leica LAS v4.3 image analysis software (Leica Microsystems), as described previously, or manually using a ruler and balance following standard phenotyping protocols (Bolle, 2009). The experiments were repeated at least three times and data from all individual plants from all three experiments were combined to a single mean.

Gateway cloning and plant transformation

Gateway cloning was used to clone the *HIPP27* promoter (472 bp upstream of *HIPP27*) and to clone the *HIPP27* CDS into the pDONR207 vector. Briefly, primers with attB extensions were designed and the PCR product was inserted into the pDONR207 vector using Gateway® BP Clonase™ II Enzyme mix (Invitrogen, Waltham, MA, USA). The pEntry207 vector was transformed into *Escherichia. coli* (DH5α) competent cells using the heat shock method. The pEntry207 vector was then extracted using a Nucleospin® plasmid extraction kit (Macherey-Nagel), and homologous recombination of the gene or promoter into the destination vector was performed using Gateway® LR Clonase™ II Enzyme mix (Invitrogen). For *HIPP27:GUS*, the destination vector was pMDC162; for *HIPP27:GFP*, the destination vector was pMDC107; for *35S:HIPP27*, the destination vector was pMDC32 (Curtis and Grossniklaus, 2003). Arabidopsis plants were transformed via the floral dip method (Clough and Bent, 1998).

GUS staining

The *HIPP27:GUS* reporter construct was introduced into the Col-0 background, and homozygous lines for the reporter gene were selected in subsequent generations using selection marker genes and genotyping. Histochemical GUS analysis was performed according to Siddique *et al.* (2009). Briefly, the *HIPP27:GUS* lines were grown in Knop medium as described above. The roots were submerged in X-Gluc (5-Bromo-4-chloro-1*H*-indol-3-yl β-D-glucopyranosiduronic acid) for 6 h at 37 °C at 1, 3, 5 and 10 dpi. The number of stained syncytia was counted, and photographs were taken under a DMI 4000B microscope (Leica Microsystems).

Nicotiana benthamiana infiltration assay

The coding region of *HIPP27* without the stop codon was amplified using Gateway forward and reverse primers as given in Table S1. The amplified product was cloned into pDONR207 using Gateway® BP Clonase™ II Enzyme mix (Invitrogen). The gene was shuttled into the destination vector, i.e. pMDC83, using Gateway® LR Clonase™ II Enzyme mix (Invitrogen). The *HIPP27:GFP* construct was transiently expressed in the epidermis of 6-week-old *N. benthamiana* leaves under the control of the 35S promoter. The GFP fluorescence was observed under an LSM 710 confocal microscope at 3 days after infiltration (Carl Zeiss, Oberkochen, Germany). The wavelength was 488 nm for excitation and 514–550 nm for emission.

Oxidative burst assay

ROS production was measured on a 96-well illuminometer (Mithras LB 940; Berthold Technologies, Bad Wildbad, Germany) according to Mendy *et al.* (2017). Briefly, leaf discs, 0.5 cm in diameter, were cut from 12-day-old Arabidopsis plants and incubated in double-distilled H_2O for 12 h in the dark. Each leaf disc was placed into the well of a 96-well plate containing 15 μL of 20 μg/mL horseradish peroxidase and 35 μL of 10 mM 8-amino-5-chloro-2,3-dihydro-7-phenyl(3,4-d) pyridazine sodium salt (L-012, Wako Chemicals, Neuss, Germany). ROS bursts were induced with 50 μL of flg22 (100 μM), with double-distilled H_2O used as a negative control. Light emission was measured for 60 min, and results were obtained using the software supplied with the instrument.

Anatomical and ultrastructural analyses

Root segments containing syncytia were dissected from *in vitro*-grown and *H. schachtii*-inoculated Arabidopsis Col-0 and *hipp27a* plants at 5 and 10 dpi. They were chemically fixed in a mixture of aldehydes and processed for microscopic examinations as described by Golinowski *et al.* (1996) and Sobczak *et al.* (1997). After embedding in epoxy resin, the samples were serially sectioned for light microscopy examinations; at selected places, ultrathin sections were taken for transmission electron microscopy (Siddique *et al.*, 2014b). The images obtained were cropped, resized and adjusted for similar contrast and brightness using Adobe Photoshop software. Three syncytia induced in *hipp27a* roots at 5 dpi were sectioned and examined. In two of them, starch grains and regions with reduced numbers of endoplasmic reticulum structures were found, whereas, in the third, no such features were observed. In the case of 10-dpi syncytia, five samples were examined and all displayed starch grains and regions with reduced numbers of endoplasmic reticulum structures.

Bioinformatics analysis

To select the 100 most highly upregulated genes in the 5 + 15-dpi syncytium, the NEMATIC program was used (Cabrera *et al.*, 2014). Orthologues of Arabidopsis genes were identified using The *Beta vulgaris* Resource (http://bvseq.mol gen.mpg.de/index.shtml); Arabidopsis genes sharing at least 60% amino acid sequence identity with sugar beet and without additional homologues were selected. Primers were designed using primer3 online software (http://bioinfo.ut.ee/primer3-0.4.0/). SAIL primers were obtained from the SALK website (http://signal.salk.edu/tdnaprimers.2.html). A list of all primers is presented in Table S1.

Statistical data analysis

Results are presented as the mean ± standard error (SE) from at least three independent experiments. Statistically significant differences were defined using Microsoft Office Excel and extension XL Statistics with Student's *t*-test ($P < 0.05$).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Cyst nematode infection assay in Col-0 and mutants of 10 selected candidate genes.

Fig. S2 Genotyping of *hipp27* mutant lines.

Fig. S3 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *HIPP27* expression in Col-0 and *hipp27* mutant lines.

Fig. S4 Phenotyping of Col-0 and *hipp27a* mutant line grown under different growth conditions.

Table S1 List of all primer sequences used in this study.

3.2. Supporting information

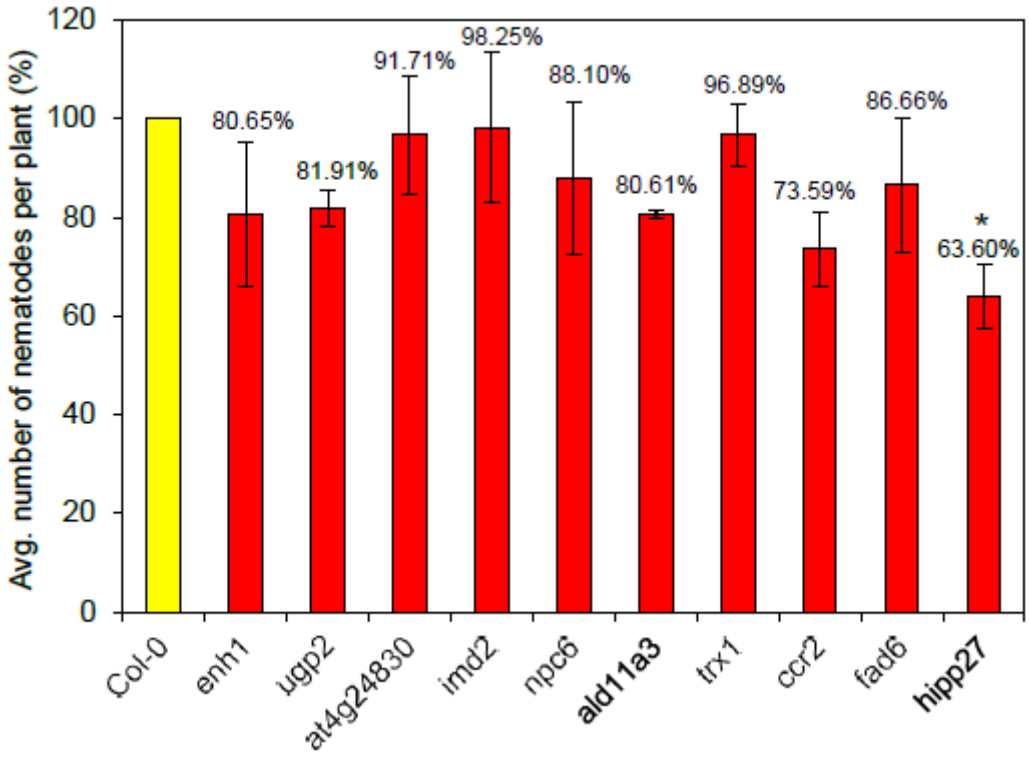

Figure S1: Cyst nematode infection assay in Col-0 and mutants of 10 selected candidate genes. Average number of nematodes per plant present in Col-0 and mutant lines at 14 dpi. Data is normalized to Col-0 which was set to 100%. Bars represent mean + SE of three independent experiments. Asterisks denote significant difference from correspondig Col-0 value (*t-test*, *P < 0.05).

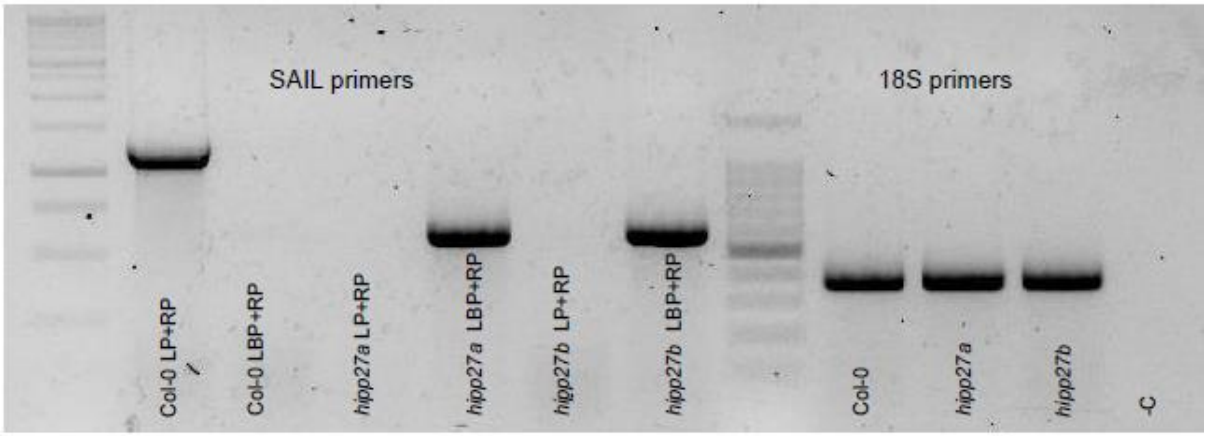

Figure S2: Genotyping of *hipp27* mutant lines. Genomic DNA of Col-0 or knockout lines (*hipp27a*, SAIL_167_B06; *hipp27b*, SAIL_675_E09) was PCR amplified using Left primer (LP), Right Primer (RP), and border primer (BP). The presence or absence of intact wild-type allele is shown. Primer sequences are given in Table S1.

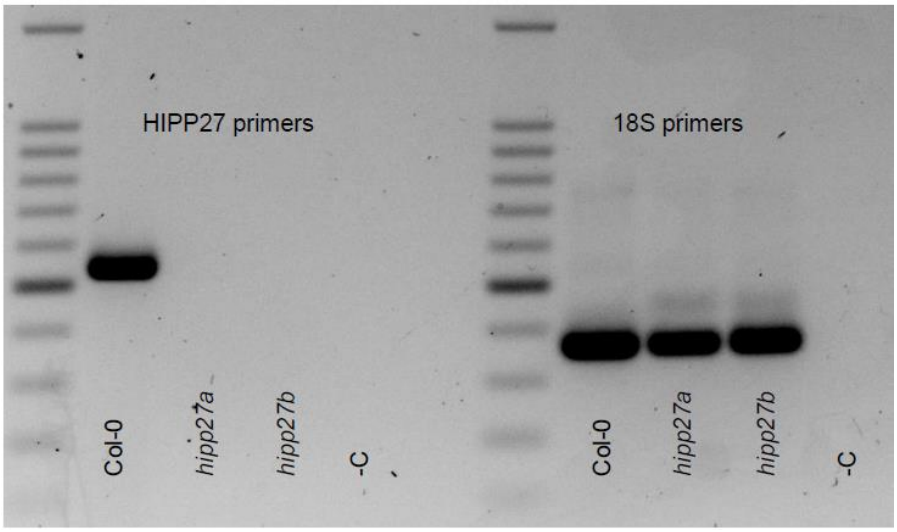

Figure S3: Reverse Transcription-polymerase chain reaction (RT-PCR) analysis of *HIPP27* expression in Col-0 and *hipp27* mutant lines. RNA from Col-0 or knockout lines *(hipp27a, SAIL_167_B06; hipp27b, SAIL_675_E09)* was extracted to synthesize single stranded cDNA. The presence or absence of expression is shown using primers given in Table S1. 18S was used as a positive control.

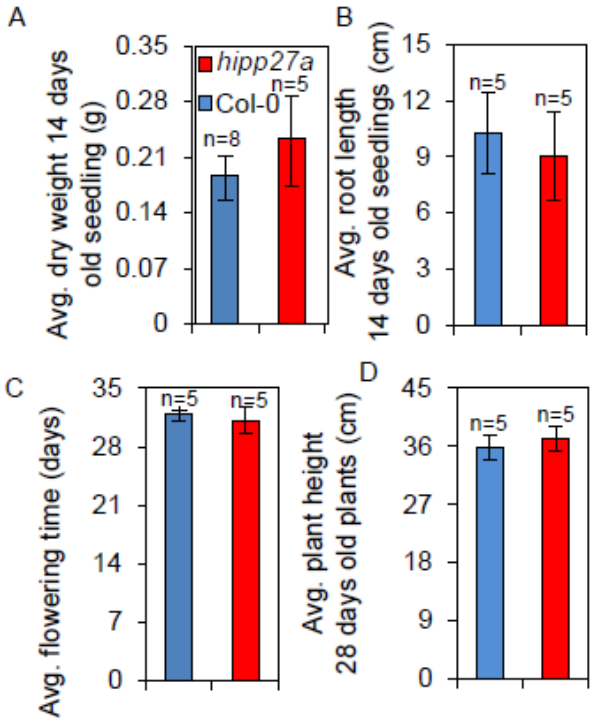

Figure S4: Phenotyping of Col-0 and *hipp27a* mutant line grown under different growth conditions. Bars represent mean +/ SE. Data were analyzed using *t-test* (P < 0.05).

Table S1. List of all primer sequences used in this manuscript.

Gateway primers	
Prom_HIPP27_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTG CGAGAGATGCTACTAAGAGAGAG
Prom_HIPP27_R	GGGGACCACTTTGTACAAGAAGCTGGGTCC CTTCTCTGGTAACTTCAAATTG
OE_HIPP27_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTG CATGGGCTTCCGAGACATTTGT
OE_HIPP27_R-SC	GGGGACCACTTTGTACAAGAAAGCTGGGTC TCACATGATGGTACAAGCGTT
GFP_HIPP27_R_+ Sc	GGGGACCACTTTGTACAAGAAAGCTGGG TCCATGATGGTACAAGCGTTGGG
qRT-PCR primers	
HIPP27_F	CCGGAAACATCACAAAAAGC
HIPP27_R	CATGATGGTACAAGCGTTGG
HIPP27_F2	GCAAAAACAGAGGGGAAAAAG
HIPP27_R2	GCTGGCTATCTCAGCAGTGAC
18S_F	GGTGGTAACGGGTGACGGAGAAT
18S_R	CGCCGACCGAAGGGACAAGCCGA
SAIL-Primers	
SAIL_167_B06	CAGAAGTCGTGGAAATTCAGG
SAIL_167_B06	CTTTGCTGACTCCTTTCATGC
SAIL_675_E09	GCAAAAACAGAGGGGAAAAAG
SAIL_675_E09	GCTGGCTATCTCAGCAGTGAC
LBb1	GCGTGGACCGCTTGCTGCAACT

CHAPTER 4

4.1. General discussion and conclusion

Nematodes are among the most abundant and most adoptive animals on our planet exploiting numerous niches of our ecosystem, from deserts to deep oceans to permafrost regions. As long as a thin film of water is present, the environment is inhabitable for some nematode species. Therefore, they are an important component of every ecosystem. Accordingly, nematodes can be used as indicators of biodiversity and water conditions. In laboratories, the free-living nematode *C. elegans* is used for studies on basic molecular biology of multicellular animals. Animal parasitic nematodes parasitize animals, including humans causing severe diseases such as trichinosis and elephantiasis. On the other hand some nematodes parasitize plants, causing tremendous crop losses in the agriculture worldwide. The most damaging plant parasitic nematodes are root-knot nematodes and cyst nematodes. Both of them are extremely difficult to control; RKNs have a wide host range and crop rotation usually does not have a strong effect on nematode reduction. Cyst nematodes can survive in the cyst for many years and hatch when the suitable host is present. Therefore crop rotation is also not very effective against cyst nematodes. An alternative to crop rotation is soil fumigation and use of nematicides. Since both of them are forbidden in many countries due to their harmful effect on the environment, only option to cope with these nematodes is breeding for resistant crops. Traditional breeding of crops is mostly based on searching for resistance genes (R-gene) against nematodes. But even with the use of R-gene, it is almost impossible to eradicate nematodes from the field. The reason for that is that nematodes have a wide range of adaptations and survival strategies and, in many cases, farmers are forced to use narrow crop rotation which favours nematode populations. In addition, resistance breaking nematode populations or invasive species are emerging more frequently. An example for that is *M. enterolobii* for which resistance is not yet bred and which is spreading world-wide especially in developing countries (Chitambo et al., 2016). Therefore, new breeding strategies need to be developed to ensure stable food production worldwide.

In this project, we used an approach in which we searched for host genes which support nematode parasitism, the so-called susceptibility genes. We hypothesized

that host genes that are strongly expressed in nematodes feeding sites, may play an important role in nematode development. Therefore loss-of-function mutants for these genes may cause the reduction or even elimination of plant susceptibility to the nematode. The background for this approach was the discovery of MLO gene in barley, whose deletion led to a total loss of susceptibility to powdery mildew (Piffanelli et al., 2002). Other examples are TOM and eIF4: their deletion caused loss of susceptibility to potyviruses in a wide range of vegetables. All three genes caused loss-of-susceptibility for several decades and even today the "resistance" is not broken (van Schie and Takken, 2014). To identify beet cyst nematode susceptibility genes in sugar beet, we started with the model plant Arabidopsis, which is also a very good host for this nematode (Sijmons et al., 1991).

By narrowing down Arabidopsis genes highly expressed in *H. schachtii* induced syncytium, we identified and characterized two susceptibility genes; AtPANB1 and HIPP27. We found that loss-of-function mutant of AtPANB1 or HIPP27 does not cause loss-of-susceptibility but instead a radical reduction in all measured nematode susceptibility parameters, suggesting that these genes are important susceptibility factors for completion of *H. schachtii* life cycle.

HIPP27 is a gene which belongs to metallochaperone gene family and is associated with plant tolerance to cadmium (Tehseen et al., 2010). There are also other members of HIPP family induced by abiotic stress (Barth et al., 2009; de Abreu-Neto et al., 2013). Using *pHIPP27::GUS* plants, we found that *HIPP27* is strongly expressed in young leaves, central cylinder of roots as well as in different reproductive organs of plants. On the other hand, expression of *HIPP27* was absent from the plant stem and lateral root. Interestingly, *HIPP27* was strongly induced by wounding in stem and leaves but not in roots, which suggests that increased expression of HIPP27 is independent of nematode-caused wounding. Our data also showed that *hipp27* display a strong decrease in susceptibility to nematodes. The question still arises, why this gene so important for cyst nematode development. We speculate that HMA domain of HIPP27 is a target site for one of the nematodes effectors. There are other HMA domain carrying genes with effector target and whose deletion causes reduction of susceptibility such as Pi21 in rice (Maqbool et al., 2015;, Nakao et al.). HIPP27 might be one more apoplastic gene used by nematodes to hijack plant immunity and in this way enhance nematodes life cycle.

Another hypothesis is that HIPP27 as a metallochaperone plays an important role in syncytium development by transferring heavy metals for activations of other proteins in syncytium.

AtPANB1 is another gene whose deletion caused a strong reduction in susceptibility to nematodes. AtPANB1 is located in the mitochondrion and is an enzyme which converts the first step of VB5 biosynthesis in plants and bacteria (Kleinkauf, 2000; Ottenhof et al., 2004). Deletion of this gene does not cause absence of VB5 biosynthesis but rather only decrease of VB5 levels in the plant, since homologous AtPANB2 can supplement AtPANB1 activity. Nematodes, like other animals, cannot synthesize VB5 *de novo* but are dependent on their host production. Therefore decreased levels of VB5 caused impairments in nematodes life cycle, which consequently led to the reduction of plant susceptibility towards cyst nematode. Creating double-mutant *atpanb1/2* will most probably be embryo lethal due to the VB5 absence or in the best case, will perform strong retardation of plant phenotype. Deletion of just AtPANB1 only caused a slight delay in flowering time without any other obvious phenotypic anomalies, indicating AtPANB2 complementation of AtPANB1 activity. By investigating other genes in the VB5 pathway we discovered that nematodes possess an ortholog of PANC1, which we named HsPANC1 (Craig et al., 2009). Silencing HsPANC by a siRNA approach caused reduction of nematode performance. Therefore, we speculate that cyst nematode takes predominantly not VB5 but its precursor, called pantoate,

Both HIPP27 and AtPANB1 proved to be important susceptibility genes in Arabidopsis. Although, we did not achieve loss-of-susceptibility effect probably due to reason that plant-nematode interaction is a very delicate process guided by the array of genes with different levels of importance. An additional reason could be the fact that we used Arabidopsis as a model organism, which is very suitable for understanding the basic molecular biology of plan-nematode interaction, which does not have resistance against nematodes. The alternative strategy could be to start from the host crop (in our case sugar beet) and once important genes are identified, conduct molecular characterisation and manipulation of genes in Arabidopsis.

By TILLING or ecoTILLING crop varieties can be identified which have malfunction of candidate genes through nucleotide insertion or deletion. Also with the usage of

“new green breeding” such as CRISPR/Cas9, mutants completely missing gene of interest can be identified. Combination of breeding and molecular biology is the key for finding new solutions against nematode threats in modern agriculture.

4.2. References

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