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Identification and characterization of a cystatin-like effector protein from beet cyst nematode *Heterodera schachtii* and its role in plant-nematode interaction

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I. Abbreviations

A. thaliana – *Arabidopsis thaliana*

A. tumefaciens – *Agrobacterium tumefaciens*

ABPP – Activity-based protein profiling

ABPs – Activity-based probes

AD – Activation domain

APNs – Animal parasitic Nematodes

BD – Binding domain

BiFC – Bimolecular Fluorescence Complementation

BSA – Bovine serum albumin

CaMV – Cauliflower Mosaic Virus

CathB – Cathepsin B

CBB – Coomassie Brilliant Blue

cDNA – Complementary DNA

Cf– *Cladosporium fulvum*

CN – cyst nematode

CP – core protease

CRISP – cysteine-rich secretory protein superfamily

DIG – Digoxygenin

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

dNTPs – Deoxynucleotide triphosphates

dpi – Days post infection

DTT – Dithiothreitol

EPIC – Extracellular protease inhibitor with cystatin-like domain

ER – Endoplasmatic reticulum

ETI – Effector-triggered immunity

FLNs – Free living nematodes

G - Glycine

G. spp. – *Globodera spp.*

GFP – green fluorescent protein

GOI – gene of interest

H. spp. – *Heterodera spp.*

H^+-ATPases – hydrogen proton conjugating adenosine triphosphatases

HR – Hypersensitive response

HsCysL1 – *Heterodera schachtii* Cystatin-like protein 1

ISC – Initial syncytial cell

J2,3,4 – juvenile stages

kDa – protein molecular weight [kilodalton]

LAX3 – auxin influx transporter

LRR – Leucine-rich repeats

M. spp. – *Meloidogyne spp.*

MAPK – mitogen-activated protein kinase

MES – Methylesterase

mRNA – messenger RNA

MS – Mass Spectrometry

N. benthamiana – *Nicotiana benthamiana*

NLS – nuclear localization sequence

NPC – Non-probe control

ON – Over night

P. infestans – *Phytophthora infestans*

P. syringae – *Pseudomonas syringae*

PAMP – Pathogen-associated molecular pattern

PBA1, PBB1, PBE1 – Catalytic proteasomal subunits

PCD – Programmed cell death

PCR – Polymerase Chain Reaction

PLCPs – Papain-like cysteine proteases

PPN – Plant-parasitic nematodes

PR – Pathogenesis-related protein

PTI – PAMP-triggered immunity

PTPLA – Protein-tyrosine phosphatase-like A

PVDF – Polyvinylidenfluoride

Q - Glutamine

qRT-PCR – Quantitative real-time PCR

qvpe – Quadruple VPE knockout line of *A. thaliana*

RhFP – Rhodamine-tagged fluorophosphonate probe

RNA – Ribonucleic acid

RNAi – RNA interference

RP – regulatory particle

rRNA – ribosomal RNA

S. cerevisiae – *Saccharomyces cerevisiae*

SDS – sodium dodecyl sulfate

SFGH – S-formyl-glutathione hydrolase

SHs – serine hydrolases

SP – signal peptide

TBS – TRIS-buffered saline

T-DNA – Transfer-DNA

TPP2 – Tripeptidyl peptidase-2

TRIS – 2-amino-2-(hydroxymethyl)-1,3-propanediol

UBC19 – Ubiquitin-conjugating enzyme 19

V - Valine

VAPs – Venome-allergen like proteins

VPEs – Vacuolar processing enzymes

VS – Vinyl sulfone

W – Tryptophane

WT – Wildtype

Y2H – Yeast-2-Hybrid

YFP – Yellow Fluorescence Protein

YVAD-cmk – TYR-VAL-ALA-ASP-chloromethylketone

$\mu Em^{-2}s^{-1}$ –microeinstein per square meter per second

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1. Chapter 1
General introduction

1.1 Nematoda

Nematodes, unsegmented threadlike animals (Greek: *nema* = thread) belonging to the phylum *Nematoda* or *Nemata*, constitute one of the most diverse and abundant animal groups, inhabiting almost every ecological and parasitic niche. Some studies suggest that every four out of five animals on earth are nematodes [1]. Although the existence of animal and human pathogenic nematodes has been known for many centuries, plant-parasitic nematodes in particular long escaped discovery owing to their miniscule size 0.25 to 2 mm, which is impossible to detect without microscopic equipment. Indeed, when Turbevil Needham reported the first plant-parasitic nematode to the British Royal Society in 1743[2], he failed to gain the attention.

In 1918, Nathan Augustus Cobb, a pioneer in the field coined the word “nematology” for this discipline [3], naming a new era for understanding the effects of nematodes on agricultural production. Yet nematodes were overlooked and underestimated for their impact for quite some time. In 1938, Hardrada Harold Hume, the dean of the School of Agriculture at the University of Florida, decried the limited awareness of nematodes and their influence on agriculture as follows:

> “If there would be no nematodes in the South [of United States] and they should suddenly appear in their present numbers, they would be seen as the pestilence they are. […] But the nematode problem arouses no particular interest. Nematodes are always working havoc, taking their toll on crops, sometimes causing complete destruction. We blind ourselves by accepting them as a matter of course. […] There must be a general awakening all along the line to the magnitude of this situation” [4].

Although around 25.000 species of nematodes have been described, the existence of as many as 1 million species has been speculated [5]. All nematode species exhibit a comparatively conserved morphology. They are structurally simple organisms possessing a filamentous, long and cylindrical body, which is round in cross section. The nematode’s muscles are attached longitudinally to its hypodermis,

allowing undulating movement on a dorsoventral plane [6]. Its internal digestive system is separated from the outer body wall by a pseudocoelomic cavity, filled with pressurized fluid that contains several tubular organs. This cavity maintains the body shape and allows movements. Habitus, size and mouthpart, as well as vulva and anus, display main diagnostic characteristics that aid classification of the major groups of nematodes into one of 12 clades within the phylum *Nematoda* [6].

Although nematodes occur in almost every habitat, they are essentially aquatic animals. Humidity in the environment, whatever its form, is essential for locomotion and active life, which indeed appears in diverse forms. Nematodes are either free-living or parasitic on plants, insects or animals [7-9]. Depending on type of organism that a nematode infects, nematodes can be considered as either devastating or beneficial, sitting at the crux of divergent human health or economic interests. Already in the 1930s the potential of many entomopathogenic nematodes as alternatives to chemical insecticides had been recognised [10-12], and they remain in use today as biological control agents for insect pests worldwide [13, 14]. Invasive species of mole crickets, for example, can be successfully controlled using *Steinernema scapterisci*[15] and insecticidal toxins isolated from symbiotic bacteria of entomopathogenic nematodes (*Photorhabdus* and *Xenorhabdus*) have been shown to enhance plant´s resistance against insects [14] and nematodes [16]. However, other members of the phylum *Nematoda* should not be neglected. Animal- and plant-parasitic nematodes cause substantial losses in food production that should spur some research into the interactions between the host immune system and nematode epidemic mechanism.

1.1.1 Plant-parasitic nematodes

Plant-parasitic nematodes (PPN) constitute a comparably small group of the phylum *Nematoda*. To date, around 4.000 species of PPNs have been described, representing 15% of the total number of nematode species known [6]. Nevertheless, their impact on agricultural food production is substantial; causing worldwide yield losses of an estimated $78 billion annually [1]. Parasitism of higher plants by nematodes is mainly confined to two classes of the phylum *Nematoda*: *Adenophorea* and *Secernentea* [17, 18]. All PPNs possess a characteristic stylet, a hollow axial spear that support them colonizing host plants. The stylet is used to puncture plant

cell walls, allowing the nematodes to withdraw nutrients. Moreover, stylet is also involved in secretion of proteins, hormones and metabolites that aid nematodes in parasitism. The presence of stylet distinguishes plant-parasitic from free-living nematodes such as *Ceanorhabditis elegans*[19, 20].
Parasitic members of the class *Adenophorea* are restricted to the two migratory ectoparasitic families *Longidoridae* and *Trichodoridae* within the order *Dorylaimida*. Migratory ectoparasites remain vermiform throughout their life cycle staying outside the root using their stylet to pierce and feed for short periods along the root system, hence their name (Greek: *ecto* = outer,*para* = with at), *siteo* (= feeding) [18, 21]. Those that feed on epidermal cells, such as *Tylenchorhynchus dubius*, possess comparably short stylets that cause only moderate damage to the infected cells. In contrast, those that feed on subsurface tissues use a very long needle-like stylet to reach nutrient-rich cells far below the epidermis. *Longidoridae* and *Trichodoridae* are the only families of plant-parasitic nematodes that are known to be a vector for virus. The economic damage caused by migratory ectoparasites thus comes indirectly for the most part, through vectoring viruses rather than direct feeding on the host [18, 21].

However, the class *Secernentea* not only hosts just animal parasites (subclasses *Rhabditia* and *Spiruria*) but also include the vastly more populous plant-parasitic nematodes (subclass *Diplogasteria*) which are thought to be evolutionary derivatives of *Adenophorea* species [17]. Members of this class are grouped in the suborder *Tylenchina* within the order *Tylenchida* and emanate from ancestral fungus-feeding taxa [17]. Two superfamilies of *Tylenchida*, *Criconematoidea* and *Tylenchoidea*, represent two separate approaches to sedentary obligate root parasitism, the former focusing on ectoparasitic development, and the latter on endoparasitism (Greek: *endon* = inner, *para* = with, *siteo* = feeding).

Sedentary ectoparasites, not surprisingly, garner less attention owing to their minor economic importance. The best-described species of this group is *Criconemella xenoplax* [22]. While migrating along the root surface this species pierces different cortical and epidermal cells with its stylet until it finds a suitable cell that is able to supply it with sufficient nutrients. Leaving its stylet inserted, the nematode becomes sedentary without invading the root, feeding for several days from a single cell [22,

23]. Because they do not enter the root, these nematodes cause only limited damage, restricted to necrosis of those cells actually penetrated by the stylet.

Similar symptoms can be observed for different Tylenchid taxa that behave as semi-endoparasites or facultative ecto-endoparasites, frequently members of family *Hoplolaimidae*. These nematodes remain vermiform throughout their life cycle and feed ectoparasitically on roots. However, they also partially invade the roots to feed on cortical or outer stellar cells. Some individuals may even reproduce inside the root [18, 21].

More devastating than the ectoparasites already mentioned are endoparasitic nematodes that are subdivided, according to their parasitic behavior into migratory and sedentary endoparasites. The nematode families *Pratylenchidae*, *Anguinidae* and *Aphelenchoididae* are adapted to a migratory endoparasitism. Among them, only members of *Pratylenchidae* infect belowground plant parts. Out of *Pratylenchidae*, *Radopholus spp.*, *Hischmanniella spp.* and *Pratylenchus spp.* (family Pratylenchidae) are of highest economic interest [18, 24]. *Pratylenchus* spp., or lesion nematode, has one of the broadest host ranges among plant-parasitic nematodes, being distributed worldwide[25]. Though most species are of only minor economic importance, others are responsible for substantial yield losses in agricultural and horticultural plants. Next to cyst and root-knot nematodes, *Pratylenchus* spp. can safely be called the most damaging genus within the phylum Nematoda [25]. Independent of their life stages, lesion nematodes can invade and leave the root at any time, parasitizing mainly cortical cells [24]. Nematodes in this group feed and reproduce while migrating in between or through plant cells inserting their stylet into a suitable cell sucking out its cytoplasm.

In contrast to their sedentary superfamily, members of migratory endoparasites do not establish a permanent feeding cell. As soon as the infected plant cell ceases supplying nutrients, nematodes leave the lesion and move on to another plant cell. This tendency to move continuously in and out of roots generates new entry points for secondary invaders. Symptoms caused by migratory endoparasites are therefore diverse, ranging from enzymatic degradation of host tissue, via galling, irregular root swelling ("witch´s broom") and many other tissue distortions. These symptoms are mainly caused by hormonal imbalance associated to nematode-induced wounding [24].

The nematodes most devastating to agricultural food production have a sedentary endoparasitic lifestyle. The larvae of these nematodes invade the root, migrating through different tissue layers to reach the vascular cylinder. At the vascular cylinder, they begin piercing individual cells with their stylet to find a suitable cell that supplies nutrients sufficient for the entire life cycle [26, 27]. Members of this group establish a highly complex relationship with their host since they are obligate biotrophic organisms and rely on living host tissue. Infection by sedentary endoparasitic nematodes can thus result in total yield losses. The most economically important nematodes in this group are the cyst (*Heterodera spp.* and *Globodera spp.*) and the root-knot nematodes (*Meloidogyne spp.*).

1.1.2 Cyst nematodes

Cyst nematodes constitute a major group of plant-parasitic nematodes that are of great economic interest throughout the world. Genera of this group can cause immense yield losses in important crop plants, including cereals, rice, potatoes, and soybeans. Eight genera, *Heterodera* (82 species), *Globodera* (12 species), *Cactodera* (13 species), *Dolichodera* (1 species), *Paradolichodera* (1 species), *Betulodera* (1 species), *Punctodera* (4 species) and *Vittatidera* (1 species), and 114 species are currently form this group [28].

Being sedentary endoparasites, all cyst nematodes feed inside the root system of their hosts. Members of this group are cryptobiotic, having the ability to enter a stage of suspended metabolic activity as a survival mechanism during unfavourable environmental conditions. While root-knot nematodes have a very broad host range that allows them to survive on alternate hosts during suboptimal conditions, cyst nematodes persist by producing a tanned brown cyst (hence the name), formed by the dead body of the female after fertilization [26].

The cyst comprises of three different layers: the outer lipoprotein layer, derived from the vitelline layers of the fertilised oocyte, the middle layer consisting mostly of different amino acids, mainly proline, glycine, and alanine [29-31]. These amino acids are substrates for collagen, a structural protein found for example in bones, teeth, cartilages and skin [32]. After polymerisation those cuticular collagens provide the eggshell with its stable protective exoskeleton [33]. Polyphenolic compounds that are present in minor quantities in the cuticle give cyst their characteristic brown colour

[29]. The innermost layer, being the main permeability barrier, protects the eggs from toxic and harmful environmental components while allowing specific root exudates from a suitable host to pass. In this way, the couple of hundred embryonated eggs enclosed by the cyst can persist for many years in the soil in this dormant stage, magnifying the economic importance of this group of nematode in agriculture [26, 31].

The life cycle of the cyst nematodes starts when juveniles in their second stage (J2s) hatch from the cyst leaving either via natural openings or through the neck where the female´s head has broken away [34]. As a survival strategy, not all juveniles hatch and leave the cyst at the same time. A proportion of J2s remain inside the cyst or in external egg masses [34] and those juveniles that were released into the soil begin migrating to the host, primarily following a chemical gradient produced by the host´s root system [31].

Among several cyst nematode species, hatching occurs in response to chemical stimuli such as exudates released by the host roots. In this way, nematodes are able to synchronise their life cycle with growth of the host plant. Several compounds that stimulate hatching are known today. Glycinoeclepin A, isolated from roots of kidney beans, was the first cyst nematode hatching factor to be characterized. This terpene induces hatching of *H. glycines* at very low concentrations [35]. Later, two other structurally related nortriterpenes were isolated and exhibited the same effect as glycinoeclepin A towards *H. glycines* [36]. Similarly, solanoeclepin A, released from tomato and potato roots, and structurally similar to the glycinoeclepins, was identified as a hatching factor *for Globodera* sp. [37]. Beyond chemical compounds, factors such as temperature, soil texture and humidity can influence the hatching process considerably in different cyst nematodes, contributing to the host range and distribution of cyst nematodes worldwide. The genus *Heterodera* provides an example for such diversity: Although *H. cruciferae* invades host plants during winter or early spring, *H. zeae*requires a higher optimal temperature of 30°C and is therefore mainly distributed in tropical regions [38]. In comparison, the sugar beet cyst nematode *H. schachtii* is the most prevalent and economically most important nematode in temperate regions [39, 40].

1.1.2.1 *Heterodera schachtii*

The scientific research on *H. schachtii* began in Germany, in the mid-19th century. In the beginning of 19th century as farmers experienced the economic potential of the *Beta* beet for its use in sugar production, the crop spread quickly across central Germany. Sugar beet has since been cultivated intensively, frequently in monocultures. Nevertheless, only a few years later, the yields were significantly declined in what was called, "beet weariness". In certain patches of the sugar beet field, the fruiting body grew significantly smaller than those of healthy plants, their leaves wilted and their lateral roots increased in numbers leading to the appearance of so called "root beard" [41, 42]. Searching for the factor causing a steady decline in yields, Prof. Hermann Schacht (1814-1864) in 1859 found “little white dots” on the root surface of symptomatic sugar beets of the tail-off in yields. After careful consideration, Schacht identified those dots as gravid females of a species within the phylum *Nematoda* [41].

Although Schacht intensively investigated the morphology and biology of this parasite, he did not himself name the newly discovered nematode. Only 11 years later, Adolf Schmidt (1871) corrected this oversight and named the new species *Heterodera schachtii* Schmidt in honor of its discoverer. The name was also given taken into the account the fact that the genus Heterodera describes the dimorphic cuticle texture of female and cyst (greek: *hetero* = different; *dera* =skin) [43, 44]. Because the original sugar beet *Beta vulgaris* L. spp. *maritima* is native to the Mediterranean area, *H. schachtii* is also suspected to originate from same area [44]. However, *H. schachtii* has been recently shown to cause substantial yield losses in sugar beet cultivation around the world [39, 40]. In addition to sugar beet, *H. schachtii* can parasitize several other plant species including the member of the families *Amaranthaceae* and *Brassicaceae*[45].

1.1.2.2 Life cycle

After hatching from the cysts infective J2s migrate toward the host root, following a gradient of stimuli present around the host roots. Some of those stimuli, including gradients for CO_2, amino acids, pH and sugars, act as nonspecific attractants prompting long-distance migration of nematodes [46]. However, detailed knowledge about the localized attraction of J2s to sites of root invasion is still missing. Once they

reached the host, J2s invade the roots, predominantly in the elongation zone and migrate intracellularly across the cortical tissue toward the pericycle. Using their protrusible stylet, they begin piercing cells until they select a suitable initial syncytial cell (ISC) [19, 31]. Because the ISC ensures a continuous supply of nutrients to the nematode, the parasitism must be performed nondisruptively. Accordingly, the juveniles carefully insert the stylet into the host cell, taking care not to destruct the plasmalemma, and then cease movement for 6-8 hours [19]. Afterwards, J2s begin establishing a feeding tube made of saliva produced by pharyngeal glands [31]. This feeding tube serves as a filter membrane controlling the uptake and release of specific molecules from and into the plant cell, regulated by the pharyngeal pump. After successful establishment of the feeding tube, the infected root cell is massively reprogrammed. Several neighbouring cells are incorporated through local cell wall dissolutions leading to the formation of a large, multinucleated and hypertrophied syncytium. During the formation of syncytium, a large central vacuole is replaced by several smaller ones, the nuclei are hypertrophied and the number of organelles including smooth ER increases significantly resulting in a metabolic highly active nutrient source[47].

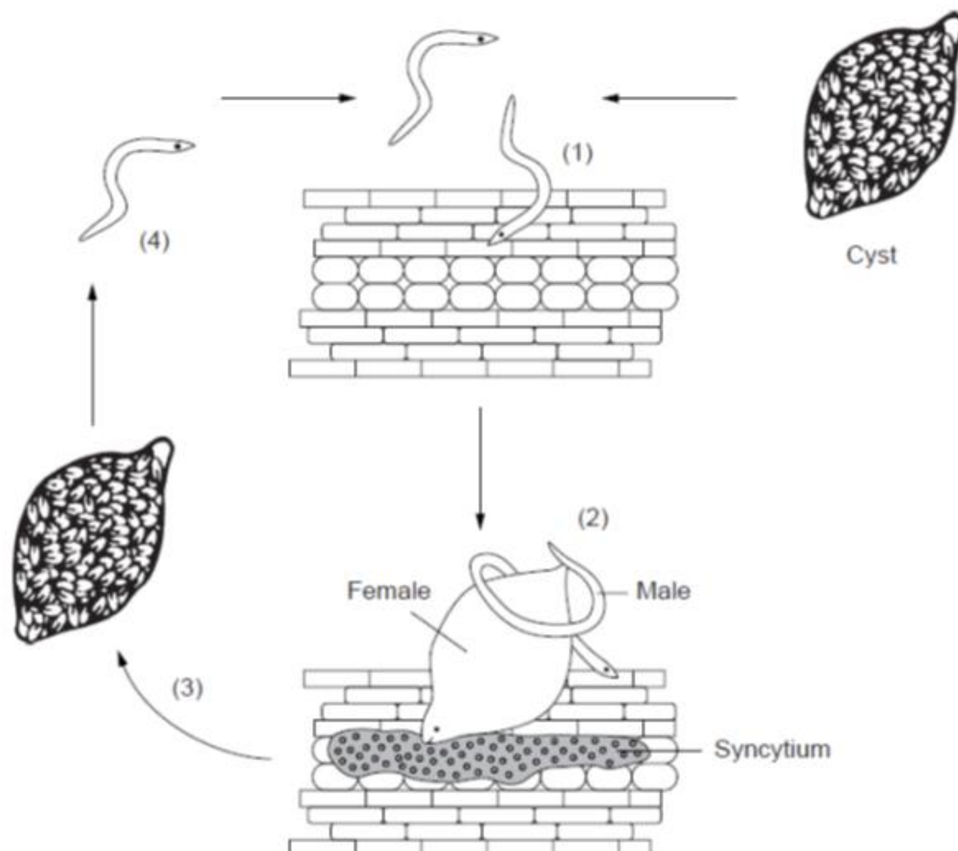

Figure1: Life cycle of *H. schachtii.* Infectious juveniles invade the roots (1). After induction of syncytia and sex differentiation, males leave the root to fertilize the females (2), which develop into cysts filled with eggs (3). After hatching, infectious juveniles can start a new life cycle (4); modified after Jung *et al.* [48].

In contrast to *H. schachtii*, the root-knot nematode *Meloidogyne incognita* induces giant cells that are formed through repeated rounds of nuclear division and cell growth in absence of cytokinesis[27]. Despite their different ontogeny, these two types of feeding cells have similar physiological functions. During next two weeks, the asexual juveniles of *H. schachtii* develop either into males or females. As they approach the adult stage, juvenile developing to females moult three times, compared to two moultings undergone by males [19, 49]. While moulting into their juvenile´s stage, females develop their genital primordial and rectum. Male nematodes become vermiform again during the moult to J4 and leave the root to copulate with females after their last moult.

The females, by contrast, remain sedentary, feeding continuously from the syncytium. The globular shape of females facilitates the rapid growth of their developing ovaries, allowing them to rupture the roots after their fourth moult, exposing the reproductive system to males–who are attracted to the females via sexual pheromones. After copulation, the embryonated eggs inside the female develop until the formation of next generation of J2s, at this point female dies and forms a robust protective cyst, as discussed earlier[31].

The factors that are involved in determining the sexual outcome of nematodes into either males or females are not yet fully known. However, Molz reported in 1920 that the epigenetic sexual development of *H. schachtii* is strongly influenced by the physiological condition of the host plant [50]. Later, Von Sengbusch figured out that females have a 3-times greater nutritional requirement than males, indicating that the amount of nutrients supplied by the syncytium influences to the sexual development of *H. schachtii* [51-53]. Under optimal conditions more than 90% of infective juveniles of *H. schachtii* may develop as females [54]. Accordingly, intraspecific competition due to high infection density results in adverse conditions in food supply and therefore less female formation [52].

1.1.2.3 Management

Because of cyst nematode´s ubiquitous appearance, soil-base life cycle and well-protected eggs, their control presents a particular problem and many or all eggs remain dormant within the protective cyst wall for many years. The first management strategies for *H. schachtii* were established in the year of its discovery. In 1871, Kühn suggested a break in cultivation to reduce the population density of *H. schachtii*, though the duration of this cultivation break threatened to lower sugar beet production below an economically reasonable threshold[55]. In the following years opinions were divided over the ideal length of such cultivation break [56-58]. However, this strategy to reduce the yield loss caused by nematode infection was a landmark for nematode pest management in modern agriculture.

As the word management implies, a combination of several measures is needed to reduce nematode densities to a non-injurious or sub-economic threshold, since an elimination of nematode pests is not possible [59]. Today, management of plant-parasitic cyst nematodes combines cultural and biological strategies, to achieve the greatest possible reduction of pest populations [60].

Compared to root-knot nematodes, cyst nematodes have a narrow host range, making crop rotation one of the main cultural control agents used in modern agriculture [31]. Crop rotation extends the interval between planting of hosts susceptible to the same nematodes [61, 62]. In the interim, trap cropping or cultivation of antagonistic catch crops can reduce the population density of nematodes to a non-damaging level [60].

Trap cropping is a very effective, though equally costly measure for controlling sedentary endoparasitic nematodes [63]. Either highly susceptible or resistant cultivars are grown that allow nematode hatching and invasion but not completion of their life cycle. In the former case, growing of the plants induces fast and efficient hatching, but requires proper planting and precise timing, since susceptible plants need to be physically destroyed before nematodes reproduce [60]. Tolerant plants instead are not recommended for use in a trap cropping system [64], though they are defined as plants that are able to endure the same amount of pathogens as compared to susceptible plants without reducing yield and quality [65, 66]. However,

tolerant plants do not diminish reproduction of the pathogen and growing those plants therefore might also increase the nematode population within a few years [67, 68].

In sugar beet cultivation resistant trap crops like mustard or oil radishare commonly used in combination with resistant and tolerant sugar beet varieties in a wide crop rotation to control the population density of *H. schachtii* [60, 68].Although the risk of using tolerant plants is well known, the abandonment of those varieties is not yet conceivable, since resistant sugar beet cultivars gain less yield and lower inner quality at low infestation level compared to susceptible or tolerant varieties [64].

Antagonistic plants, on the other hand, release specific root exudates that are toxic for nematodes; marigold (*Tagetes spp.*) is one of the most thoroughly studied plants in this category [69]. This plant produces allelopathic compounds such as alpha-terthienyl that are toxic to or that inhibit the development of nematodes [69, 70]. However, *Tagetes* spp. has only been shown to affect *Meloidogyne* spp. and *Pratylenchus* spp. [71].

Root exudates of different cultivars can improve soil quality and plant health creating an environment that may favours nematode-antagonistic flora and fauna [70]. In addition to their nutritional benefits, the advantageous effects of the incorporation of organic amendments into the soil on nematode-antagonistic microorganisms were known early on [72, 73]. The regulation of pH, temperature, moisture, nutrients, and heavy metals was found to influence the distribution and occurrence of nematode-antagonistic species [74-76]. Although many microorganisms have been shown to feed on nematodes such as predatory nematodes, mites, insects and other vertebrates, fungi and bacteria are the most studied natural enemies of nematodes [77]. However, little is known about their development in soil, apart from carbohydrate sources found, for example, in nematodes´ cuticle that they need to proliferate [60].

Similar to some toxin-producing fungi, bacteria such as *Burkholderia spp.*, *Pseudomonas spp.*, *Bacillus spp.* and *Agrobacterium radiobacter* interfere with nematodes indirectly by producing antibiotics, enzymes or toxins [78-82]. Many compounds antagonistic to nematodes are formed by those bacteria during decomposition of organic material, indicating the effectiveness of organic amendments in nematode control [72, 73]. Yet, little is known about the production and functionality of such compounds in the rhizosphere. So far, the most

economically promising bacteria known to parasitize nematodes are the endospore forming bacteria *Pasteuria spp.*, whose endospores adhere to the nematode cuticle and germinate inside the host. In *Meloidogyne spp.*, infected females have been seen to further develop but cannot reproduce, their reproductive system having been destroyed by as many as 2 million endospores [83]. After decomposition of both roots and nematodes, those spores are released into the soil environment, ready to infect new nematodes. Nevertheless, a narrow host range for a number of the bacterium isolates makes it difficult to produce sufficient number of endospores for large-scale trials, putting their application as biocontrol agents in agriculture out of reach.

Due to their potential health and environmental risks, public sentiment and government policies have pressed for a reduced use of nematicides. Yet, alternatives are rare. A few organisms have been identified as potential practical control agents, but limitations on their mass production so far have prohibited their widespread use. Furthermore, research suggests that biological control agents alone are not sufficient to reduce nematode infection but must be combined with other control measures for successful use in sustainable agriculture [84, 85]. In addition to the described cultural control practices, genetic resistance is a primary control measure that can be combined with biological agents for sustainable control of nematodes.

Resistance is defined as the ability of a plant to reduce pathogenic population density by blocking the completion of their life cycle after invasion [86]. Numerous individual plant resistance genes have been identified and are used in research programs to create effective and economically as well as environmentally reasonable alternative to chemical control agents. However, researchers face challenges in breeding new resistant crop plants, since nematodes are evolutionarily able to overcome the resistance after some time. Furthermore, resistant varieties often differ in yield or taste, making cultivation along these lines impractical. The key to breeding resistant plants that also meet the consumer´s requirements lies in knowing the details of the interaction between plant and pathogens. Accordingly, progress is still needed particularly in fundamental areas of research to find a sustainable and reliable strategy for nematode management.

1.2 Plant-nematode interaction

Plants have evolved different strategies for water and nutrients uptake to aquire sufficient macro-and micronutrients from the soil. These strategies involve passive and active transporter systems as well as the release of specific chemical signals interacting with symbiotic organisms. All these mechanisms influence the biochemical composition of the rhizosphere. Iron, for example, is an essential macronutrient that - though highly abundant in soils - is only of limited availability for plants, since it tends to form insoluble complexes (Fe^{3+}) under aerobic and low pH conditions. Accordingly, plants use H^{+}-ATPases and ferric chelate reductases to acidify the rhizosphere and reduce Fe^{3+} to the more soluble form Fe^{2+}[87]. Further, for better nitrogen uptake, some legumes release flavonoids to attract nitrogen-fixing bacteria (rhizobia) that convert atmospheric nitrogen into ammonium, thus, avoiding nitrogen deficiency even when other nitrogen sources are not available in the soil [88]. Using these mechanisms, however, plants become conspicuous within their environment, unavoidably presenting themselves to antagonistic organisms as potential hosts.

Plant-parasitic nematodes sense changes in their environment through internal and cuticular sense organs or sensilla. Compared to internal sensilla, which are mechanoreceptors or (less frequently) photoreceptors, cuticular sensilla detected a wider range of stimuli, including chemical, mechanical, temperature and osmotic pressure [89]. The highest concentration of cuticular sensilla is found on the head of the nematode, where different types of semiochemicals can be sensed [90]. Semiochemicals are defined as chemicals that induce an interaction between two organisms by transmitting chemical messages; they include allelochemicals and pheromones [91]. The former mediate interspecific responses such as the responses of nematodes to diffusates from host roots, whereas the latter mediate intraspecific responses regulating, for example, the attraction of males to female nematodes during fertilisation [92]. Detection of semiochemicals regulates nematode behaviour. Some plant attractants stimulate the hatching of juveniles, whereas others enable the nematode to migrate to the root area and invade the roots. Once, the nematode enters the host root system, the interaction between host and parasite moves to a direct level. Independent of different life cycles each plant-parasitic species must get in direct contact with the host plant and thus provokes plant cell responses during

infection. Sedentary endoparasitic nematodes are the most invasive nematode species and accordingly need to interact with the host plant in complex ways [27].

1.2.1 Morphological changes and molecular background of host cells during syncytium development

Being endoparasitic organisms, cyst nematodes establish a highly complex long-term relationship with their hosts that require massive cytological modifications of the feeding cell. The juveniles enter the epidermis of the roots and migrate intracellularly through the cortex, piercing and rupturing surrounding cell walls with their stylet during this phase [47]. After the nematode reaches the vascular cylinder, it selects an initial syncytial cell (ISC) near the primary xylem elements and punctures carefully with the stylet without destroying the plasmalemma [93]. By forming a feeding plug, the nematode anchors the stylet at the point of insertion.

The selected cell immediately undergoes striking morphological changes: After insertion of the stylet, the nematode remains motionless on the infection sites and electron-translucent cell wall material becomes visible [94]. Plasmodesmata between the pericycle gradually widen, forming the first connection from the ISC to neighbouring cells. The protoplasts of these adjacent cells amalgamate, and the granular cytoplasm becomes denser through hypertrophy, representing the syncytial structure; later, cell walls are dissolved through enzymatic digestion, and neighbouring cells are incorporated, expanding the syncytial structure along the vascular cylinder [47, 94]. In *Arabidopsis* roots, a single syncytium consists of up to 200 individual cells [95]. Furthermore, the central vacuole is fragmented into several smaller ones [47], and the syncytium becomes metabolically highly active, as indicated by the presence of a large number of mitochondria, and free ribosomes and the proliferated structure of the endoplasmatic reticulum (ER) [47, 96-98]. During nematode development from J2 to J4, ER structure changes from rough in the early stages to smooth with dilated cisternae in later developmental stages, apparently forming the small vacuoles previously described [47, 98]. Whereas the rough ER is studded with ribosomes responsible for protein biosynthesis, the smooth ER without any ribosomes serves mainly as a source of Ca^{2+}-signalling molecules (reviewed in[99]) and is responsible for the production of lipids [100]. Consequently, the number of lipid bodies also increases in later syncytial elements. To cope with such high level

of metabolic activity, enlarged nuclei and nucleoli can be found in syncytial structures due to endoreduplication of DNA without mitosis [101, 102].
All morphological changes in the plant cell during a compatible interaction require a very ingenious parasitism strategy on the nematode´s part. Cyst nematodes are well equipped for plant parasitism: Using their strong hollow stylet, they mechanically disrupt the tough epidermal cell layer in the zone of elongation via repeated forceful and highly coordinated thrusts to enter and migrate intracellularly towards the vascular cylinder. They also use the stylet to introduce secretions into the plant tissue and to suck nutrients and other plant cell contents during feeding [27].

These released secretions comprise a mixture of different enzymes with different functions. In 1998, Smant et al. found the first plant cell wall-degrading enzymes in nematodes [103], a finding confirmed by de Meutter et al. [104]. Endo-ß1-4-glucanase has been shown to break the ß1-4 links within the cellulose polysaccharide chain, the most abundant polymer in the plant cell wall [103]. Subsequently, pectate lyases, xylanases, expansins, polygalacturonases, arabinases and arabinogalactan galactosidases have been identified in the secretions of different phylogenetic groups [105-110], all enzymes capable of breaking down specific plant cell wall components and thus facilitate nematode migration inside the roots. Complementary studies have since confirmed that endoglucanase isolated from cyst nematodes is expressed specifically during the migratory phase, including during the redeployment of expression within males, which retain mobility after their third juvenile stage [111, 112].

The discovery of the nematode endoglucanases by Smant et al. [103] was a landmark in understanding the process of host plant invasion by nematodes. Previously, plant cell wall-degrading enzymes have only been found in plants themselves or in plant-pathogenic bacteria or fungi [113-116], prompting the initial assumption that nematode cellulase originates from endosymbiotic bacteria. However, several convincing pieces of evidence have confirmed its endogenous origin. The leucine-rich hydrophobic core of the identified endoglucanase confirms its eukaryotic origin; furthermore, the expression of the gene could only be localized in the subventral glands, which do not contain symbiotic bacteria [103].

Still, nematode cellulases show a close similarity to those of bacteria, provoking the question of whether nematodes have acquired their cellulases by horizontal gene

transfer from a prokaryote [103, 117, 118]. Horizontal gene transfer is defined as the asexual exchange of genetic material between different species [119]. After incorporation into the genome, the foreign DNA is expressed as functional proteins. Considering the differences in gene structure and organisation between eukaryotes and prokaryotes, the limited reports of horizontal gene transfer between bacteria and animals are understandable. However, Hotopp et al. [120] later supported the current proof of lateral gene transfer from prokaryotes to eukaryotes given by Smant et al. [103], by confirming bacterial *Wolbachia pipientis* genes inside the genomes of different insect and nematode species. Within the past decade, several reports of horizontal gene transfer between prokaryotes and eukaryotes, respectively, have emerged leading to the suggestion that it probably allows specialisation to evolve [119, 121-125]. Plant parasitism in nematodes is one case of specialisation that has been shown to have arisen at least three times independently during evolution [126, 127].

1.2.2 The role of effector proteins in plant-nematode interaction

Mechanical and enzymatic dissolution of cell walls during invasion results in recognition by plants and activation of plant-defence mechanisms. Secretions introduced by the nematode are thus of special relevance. In addition to plant cell-wall-modifying proteins, enzymes manipulating the plant defence, growth and metabolism pathways have been found in nematodes [123, 124, 128-130]. Lambert et al. [131] and Jones et al. [132], for example, characterized a chorismate mutase in *M. javanica* and *G. pallida*, a regulatory enzyme in the shikimate pathway in plants and bacteria, which has not been described before in any animal. Chorismate mutase has been shown to catalyse the conversion of chorismate to prephenate, which is a precursor for a variety of compounds including salicylic acid, a key defence signalling molecule. Chorismate itself is a precursor of auxin, an important plant hormone essential for cell differentiation [133, 134]. So far, chorismate mutase has been found only in plant-parasitic nematodes, suggesting that nematodes do not need this enzyme for their own metabolic purposes but use it to function outside the nematode body in the host-parasite interaction. These virulence factors are called effector proteins [135].

Most effector proteins are secreted cysteine-rich proteins that suppress defence responses to enable parasitism. In bacteria, fungi and oomyces, the existence of

these avirulence genes has been well accepted for decades [136-139] and molecular understanding of these host-pathogen interactions has progressed far more than that of plant-nematode interactions. Although the infection process of plant-parasitic nematodes is only partially understood, the introduction of molecular methods to the field of nematology during the past decade has led to many successes in identifying and functionally characterizing several nematode effectors [140].

The nematode organs most important for producing secretions are three esophageal glands, the hypodermis and two amphids [140-142]. Although amphids help the nematode to localize the host through chemoreception [143], one dorsal and two subventral oesophageal glands are the main sources of most of the secreted effector proteins. Interestingly, dorsal and subventral glands show varied activities during different stages of the parasitic cycle. While the subventral glands are strongly active during nematode penetration and migration in roots, the activity gets higher in the dorsal gland during feeding cell formation and maintenance throughout the sedentary life stages of the nematode [118]. Another origin of effectors is the hypodermis, which synthesizes proteins that are present on the cuticle surface. Those proteins, as well as the secretions released by the amphids during recognition processes, are directly exposed to the environment and can thus be detected by the host. However, the ability to invade the host and induce a feeding structure without being detected or impeded by the host is a key moment in the life cycle of the nematode. Invader and host thus are continuously under strong selection pressure to maintain their respective benefits.

According to the “Zig-Zag-Model” proposed by Jones and Dangl[137], plant-pathogen interaction could be described as a constantly recurring overcoming of immune responses through the release and reception of effector proteins on behalf of both interacting partners. Molecules on the exterior of the invading pathogen betray their presence to plants and initiate a basal or PAMP (Pathogen-associated molecular pattern)-triggered immunity (PTI) [137, 144]. PAMPs are mostly associated with molecules that are essential for the infectivity, reproduction or survival of different pathogens and thus are not easily changed or lost by mutations, being generally conserved across taxa [145]. PTI is thereby relatively durable and is often effective against a range of distantly related pathogens [146]. However, in case of successful infection, effectors produced by biotic pathogens suppress host´s PTI. Host

resistance proteins in turn detect these effectors and initiate an effector triggered immunity (ETI), often invoking a strong, localised cell death known as the hypersensitive response (HR) [147].

Within the past two decades several genes coding for putative nematode effector proteins have been identified, some of them are common in different nematode species, but others differing based on their specialized parasitism [148-150]. For example, cyst nematode´s 19C07 effector has no similarity to other nematode sequences. 19C07 interacts with an auxin influx transporter (LAX3) to promote syncytium development [151].

Recently, the first effector targeting the plant peroxisome from *G. pallida* was reported. Peroxisome is a major contributor to metabolic processes producing auxin, jasmonic acid or hydrogen peroxide, indicating an involvement of effectors in the suppression of plant defences [152]. However, only a limited number of putative effectors have so far been functionally characterized, presenting a great challenge for future research.

1.3 Cysteine proteases

Proteases (also called peptidases or proteinases) are enzymes that can cleave proteins via the hydrolysis of peptide bonds [153]. So far, only a few of several hundred proteases encoded in plants have been characterized. The biological role of proteases is mostly unknown, but along with their ubiquitous appearance in plant cells these enzymes have been found to be involved in striking variety of biological processes, including development and local and systemic defence responses [153, 154]. Based on their catalytic activity they are divided into five major classes: cysteine, serine, metallo, threonine, and aspartic proteases [154, 155]. According to the MEROPS protease database, these classes have been subdivided into families and clans based on evolutionary relationships (http://merops.sanger.ac.uk) [156]. More than 800 proteases are encoded in Arabidopsis, distributed over almost 60 families and 30 different clans.

Cysteine proteases are usually 21-30 kDa in size and present in all living organisms. The discovery of caspase-1 like proteolytic activity during programmed cell death of tobacco due to the infection induced by tobacco mosaic virus [157] led to intensive studies to find enzymes with those properties in plants. Although several reports

confirmed caspase-like activities in the meantime in plants during plant defence [158], sequencing of the whole genomes of *Arabidopsis thaliana* L. and rice *Oryzasativa* L. did not reveal any caspase orthologs. Accordingly, the existence of plant proteolytic enzymes that possess caspase-like activities without being ortholog of caspase was assumed. A vacuolar processing enzyme (VPE) was the first identified plant protease with caspase-1 like activity involved in programmed cell death [159, 160]. A VPE is a legumain-like cysteine protease categorized by the MEROPS peptidase database into the C13 family of clan CD. Collapse of the vacuole induced by a VPE is considered to be one of the key factors in programmed cell death in plants [159, 161].

In total, four genes encoding for VPEs were found in the genome of *Arabidopsis thaliana: αVPE, βVPE, γVPE* and *δVPE.* Expression of *αVPE* and *γVPE* was found in vegetative organs of the plants, whereas *βVPE* is expressed in embryos and*δVPE* is expressed during seed coat formation [162, 163].Since then, metacaspases belonging to the same CD clan as VPEs have been identified in plants and have been shown to play essential roles during the induction of programmed cell death induced by different biotic and abiotic factors [164-167].

1.3.1 Papain-like cysteine proteases (PLCPs)

Papain-like cysteine proteases (PLCPs), the best-characterized family of cysteine proteases (C1 family of CA clan), show phylogenetic similarities to cathepsins from animals. Protease families belonging to this `CA-clan´ are structurally related to papain, the best characterized family of this class, denoted by a two-domain structure with inlying catalytic domain [168]. However, clan CA proteases show significant diversity at the protein sequence level. Proteases having significant sequence homology to papain have thus been grouped into family C1, which in turn has been subdivided into extracytoplasmic (C1A) and cytoplasmic (C1B) PLCPs. PLCPs contain an autoinhibitoryprodomain that needs to be proteolytically removed to activate the enzyme [169, 170]. Additionally, many PLCPs are secreted or localized in the endomembrane system due to an N-terminal signal peptide. PLCPs are relatively stable proteins that can resist proteolytically harsh cell environment, such as the apoplast, the vacuole and lysosomes [171]. They use a catalytic cysteine residue to cleave peptide bonds in their protein substrates and have been found to play a role for both parties during plant-parasite interaction [172].

In plants, PLCPs have been shown to interact with specific pathogenic effectors and thus play substantial roles in parasitism. Recently it has been discovered, that one of the most conserved effector proteins among all parasitic nematodes, the so-called venom-allergen like proteins (VAPs), interacts with host PLCPs during infection [173, 174]. VAPs belong to the SCP/TAPS protein family within the cysteine-rich secretory protein superfamily (CRISP). A highly abundant example of the SCP/TRAPS family is the pathogenesis-related protein PR-1, which is frequently used as a marker protein for systemic resistance in plants. Although this protein like many other members of the SCP/TRAPS protein family appears to play an important role in immunity, the detailed mode of action remains largely elusive [175, 176]. Recently, Lozano-Torres et al. [174] were able to knock-down VAPs in the potato cyst nematode *G. rostochiensis*, showing significantly reduced infectivity of the nematode on potato plants. Furthermore, heterologous expression of Gr-VAP1 in Arabidopsis undermines the basal immunity as shown by higher susceptibility of *A. thaliana* to *H. schachtii*, supporting the assumption that VAPs are indeed required for parasitism. Similar to the effector protein Avr2 of *Cladosporium fulvum*, also Gr-VAP1 of *G. rostochiensis* inactivates, among others, the extracellular PLCP $Rcr3^{pim}$ of *Solanum pimpinellifolium*, which itself is recognized by the extracellular plant immune receptor protein Cf-2 [173].

1.3.2 Cystatins

The catalytic activity of proteases depends on the highly reactive thiol group of a cysteine residue at the catalytic site [177]. To ensure controlled degradation of peptides and proteins, regulation of the activity of cysteine proteases is essential for each living organism. This can be achieved by the synthesis and degradation of proteases at the transcriptional level and also by inhibitors known as cysteine protease inhibitors or cystatins that bind specifically and reversibly to the catalytic site of target proteases [178-180]. The first identified cystatin is an inhibitor of papain found in chicken egg white [181]. Beside animal cystatins (stefins, cystatins and kininogens), the superfamily of cystatins also includes plant cystatins, known as phytocystatins. The subdivision of the cystatin superfamily is based on its sequence homology, structure and molecular mass [177]. During the past two decades significant progresses in identifying phytocystatins in several monocots and dicots

has shown their potential in defence against pests and pathogens, as well as in response to various abiotic stresses [182, 183].

Phytocystatins include more than 80 members within a family of specific cysteine protease inhibitors found only in plants [180, 184]. The first identified and characterized phytocystatins were oryzacystatin I [185] and oryzacystatin II [186], which have been found to be involved in the regulation of storage proteins during development and germination of rice seeds. However, since then, many other phytocystatins have been isolated from different plants, such as potato [187], corn [188], soybean [189, 190] and wheat [191] and have also been associated with diverse physiological processes, including programmed cell death [192], fruit development [193], seed germination and development [185, 190, 194] and defence against biotic and abiotic stresses [195-199]. Additionally, transgenic plants overexpressing the cystatin-form Oc-IΔD86 (oryzacystatin I with a deletion of an aspartic acid residue at position 86), targeting intestinal proteases in nematodes, have been shown to reduce the growth and fecundity of cyst and root-knot nematodes [200, 201].

Cystatins bind directly to the active-site cleft of the target protease resulting in a tight inhibition by the presence of a three-point interaction between the inhibitor and the protease [202]. Two contact points are achieved by five-stranded antiparallel β-sheets forming hairpin loops. The first hairpin loop contains one out of three motifs found within all cystatins, including (i) the highly conserved QxVxG motif. The second binding loop, less conserved, may contain (ii) a tryptophan near the carboxy-terminal [203]. The third motif and contact point is represented by (iii) a conserved glycine residue, placed at the extremity of the N-terminal region [202, 203].

1.4 Objectives

Infection by *Phytophthora infestans* (which causes late blight) results in a different expression level of cysteine proteases in susceptible and resistant potato varieties, respectively [204], suggesting that the activity of these proteases modulates resistance. This has led to the assumption by Tian et al. [205], that *P. infestans* has evolved counter-defence protease inhibitors to target cysteine proteases. Doing motif searches they were able to isolate a novel family of putative protease inhibitors having cystatin-like domains (EPIC1 to EPIC4; "extracellular protease inhibitor with

cystatin-like domain") and in doing so confirmed the inhibition of a novel papain-like cysteine protease, termed Phytophthora Inhibited Protease 1 (PIP1).

The discovery of this effector protein of *P. infestans* laid one of the foundations for present work, as we found similar cystatin motifs in a genomic sequence of *H. schachtii*. This work aims to show that nematodes modulate plant defence at a posttranslational level using effector proteins, supporting the statement that plants and plant pathogens have coevolved diverse defence strategies for survival. Accordingly, we showed in chapter 3 that the activity of different cysteine proteases Arabidopsis roots is downregulated upon infection by *H. schachtii*. In chapter 4 we characterized a novel effector protein from *H. schachtii* (HsCysL1) having cystatin-like motifs, and further identify corresponding interacting proteins in plant.

1.5 References

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2. Chapter 2

Activity profiling reveals changes in the diversity and activity of proteins in Arabidopsis roots in response to nematode infection

Marion Hütten, Melanie Geukes, Johana C. Misas-Villamil, Renier A.L. van der Hoorn, Florian M.W. Grundler, Shahid Siddique

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ABSTRACT

Cyst nematodes are obligate, sedentary endoparasites with a highly specialised biology and a huge economic impact in agriculture. Successful parasitism involves morphological and physiological modifications of the host cells which lead to the formation of specialised syncytial feeding structures in roots. The development of the syncytium is aided by a cocktail of nematode effectors that manipulate the host plant activities in a complex network of interactions through post-translational modifications. Traditional transcriptomic and proteomic approaches cannot display this functional proteomic information. Activity-based protein profiling (ABPP) is a powerful technology that can be used to investigate the activity of the proteome through activity-based probes. To better understand the functional proteomics of syncytium, ABPP was conducted on syncytia induced by the beet cyst nematode *Heterodera schachtii* in Arabidopsis roots. Our results demonstrated that the activity of several enzymes is differentially regulated in the syncytium compared to the control roots. Among those specifically activated in the syncytium are a putative S-formyl-glutathione hydrolase (SFGH), a putative methylesterase (MES) and two unidentified enzymes. In contrast, the activities of vacuolar processing enzymes (VPEs) are specifically suppressed in the syncytium. Competition labelling, quantitative gene expression and T-DNA knock-out mutants were used to further characterise the roles of the differentially regulated enzymes during plant–nematode interaction. In conclusion, our study will open the door to generate a comprehensive and integrated view of the host-pathogen warfare that results in the formation of long-term feeding sites for pathogens.

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1. Introduction

Biotrophic plant parasites have developed lifestyles that allow them to penetrate and establish specific structures for nutrient uptake within the host while avoiding the activation of defence responses. The sugar beet cyst nematode *Heterodera schachtii* Schmidt is a biotrophic endoparasite with a highly specialised biology. This parasite induces modifications in the root system that impede the nutrient and water supply of the host plant, leading to substantial yield losses (Sasser and Freckman, 1986). Due to their wide range of hosts, these nematodes are able to infect different crops within the families Chenopodiaceae and Brassicaceae, including the plant *Arabidopsis thaliana*, which has been established as a model organism for analysing the molecular aspects of the plant–nematode interaction (Sijmons et al., 1991).

The infective stage juveniles (J2) of *H. schachtii* hatch from eggs that are stored in the cyst, the modified dead body of the females. The J2 worms invade the host roots near the tip and move intracellularly towards the central cylinder. During penetration, the nematodes pierce single cells with their stylets, resulting in the spontaneous collapse of the cytoplasm of these cells; therefore, the paths of the invading J2 are delineated by necrotic cells. Having reached the vascular cylinder, they probe the individual cells by gentle stylet stabbing. In cases of cell collapse, they continue

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moving until they succeed in inducing an initial syncytial cell (ISC) (Sobczak et al., 1997; Wyss and Grundler, 1992). Within 24 h after selection, the ISC fuses with adjacent cells by local dissolution of cell walls, and the formed syncytium hypertrophies. Two days after selection of the ISC, the cells incorporated into the syncytium are enlarged and exhibit features of a typical syncytium. The cytoplasm is condensed and enriched with ribosomes, endoplasmic reticulum, mitochondria and plastids. Additionally, the nuclei are enlarged, cytoskeleton is rearranged, and the central vacuole is replaced by several smaller vacuoles in the syncytium (Golinowski et al., 1996; Kyndt et al., 2013). Solutes are withdrawn by the nematode throughout its parasitic life stages, and the syncytium induces a strong sink for assimilates in the plant. The development of the syncytium is accompanied by massive transcriptomic and metabolic changes in the infected tissue, and these changes have been studied in detail in our previous works (Hofmann et al., 2010; Siddique et al., 2009; Szakasits et al., 2009; Wieczorek et al., 2006). During the following two weeks, the nematodes continue to draw nutrients from the roots and develop into males and females after moulting three times (J3, J4 and adult). A female-associated syncytium is composed of approximately two hundred cells and reaches its maximum size approximately 10 days after infection. Syncytia of females remain functional for several weeks until egg production is completed, the females die afterwards and transform into typical brown cysts, which contain several hundred eggs. Syncytia of males are much smaller and short living (Sobczak et al., 1997). After the third developmental stage male juveniles stop feeding, their syncytia degenerate, and the animals become vermiform. Adult males hatch from the juvenile cuticle and migrate in search of adult females for copulation.

The whole process of penetration, migration and feeding site establishment is aided by secretions, which act on the host plant as effectors (reviewed by Mitchum et al., 2013). The identification of these effectors has been significantly facilitated by the development of new sequencing technologies in recent years. However, it remains largely unknown how these nematode effectors induce and orchestrate the massive physiological and structural changes in the plants. Proteomic studies concerning the host side of the plant–nematode interaction are rare. In 1995, the protein composition of the feeding sites of *H. schachtii* in *A. thaliana* was studied (Schmidt, 1995). There was a significant increase in the abundance of the protein encoded by the myrosinase gene *PYK10* around the syncytia compared to the non-infected roots. Similarly, a root proteomic study was performed by analysing nematode resistant and susceptible cotton (*Gossypium hirsutum* L.) cultivars infected with the root-knot nematode *Meloidogyne incognita* (Callahan et al., 1997). Several polypeptides were found to be regulated differentially as a result of the infection; for example, a novel 14 kDa polypeptide was expressed at higher levels in young galls of the resistant isoline at 8 dpi. In a similar study, the roots of nematode-resistant genotypes of cotton (*Gossypium hirsutum* L.) and coffee (*Coffea canephora*) infected with *M. incognita* and *Meloidogyne paranaensis* were compared to their corresponding non-infected roots using two-dimensional gel electrophoresis, and this analysis led to the identification of several differentially regulated proteins (Franco et al., 2010).

Conventional transcriptomic and proteomic analyses do not cover the complete cellular regulatory mechanism, which also includes posttranslational modifications. The activities and functions of proteins are not only determined by phosphorylation, but also by other post-translational modification, such as glycosylation, acetylation, carbonylation, and certainly in the case of disease these modifications are known to play an important role (Huber and Hardin, 2004; Pastore and Piemonte, 2013). Therefore, the amount of cellular mRNA does not necessarily result in a higher level of corresponding functional protein. A recently developed method, which has turned out to be highly useful for the identification and annotation of enzyme activities, is "Activity-Based Protein Profiling" (ABPP). Pioneered by Cravatt, Bogyo and co-workers (Cravatt et al., 2008; Kato et al., 2005; Verhelst and Bogyo, 2005), it has evolved into an effective tool for the identification and functional characterisation of proteins in extracts and living cells (Edgington et al., 2009; Gu et al., 2010; Hang et al., 2006; Nodwell and Sieber, 2012; Uttamchandani et al., 2008; van der Hoorn et al., 2004; van der Hoorn and Kaiser, 2012; Weerapana et al., 2010, 2011). ABPP is based on the design of biotinylated or fluorescent active-site-directed small molecules (probes) that irreversibly bind to the active side residues of enzymes in complex proteomes; thus, this method gathers information on the functional state of the enzymes rather than on their abundance. Most activity-based probes (ABPs) target a large, but manageable, fraction of the proteome with shared catalytic features by achieving a desired level of intraclass coverage and minimal extra-class cross-reactivity (Cravatt et al., 2008). The labelling is covalent and irreversible, facilitating the imaging of the labelled enzymes on protein gels by fluorescent scanning and the identification of labelled proteins by affinity capture and mass spectrometry (MS) (Gu et al., 2010; Nodwell and Sieber, 2012; van der Hoorn et al., 2004; Kolodziejek and van der Hoorn, 2010). Van der Hoorn et al. (van der Hoorn et al., 2004) introduced DCG-04 to plant science, which is an activity-based probe for papain-like cysteine proteases, and this probe illustrated the potential of ABPP as it has been used to reveal senescence-induced protease activities (Martinez et al., 2007), defence-related protease activation (Gilroy et al., 2007) and various pathogen-derived inhibitors that target tomato proteases (Rooney et al., 2005; Song et al., 2009; Tian et al., 2007; van Esse et al., 2008).

In this study, we used two different probes to determine the differential activities of vacuolar processing enzymes (VPEs) and serine hydrolases (SHs) in root tissues upon infection with *H. schachtii*. These enzyme families (VPEs and SHs) were chosen for analyses because of their previously described role in plant–pathogen interactions and availability of reliable probes to perform ABPP (Rojo et al., 2004; Misas-Villamil, 2010; Liu et al., 1999). Furthermore, gene expression analysis was performed for the differentially activated enzymes to generate an integrated view of pre- and posttranslational regulation events in the syncytia. T-DNA loss-of-function mutants were used to study the importance of these differentially regulated enzymes during nematode and syncytium development. In this way, we were able to show that the activity of the various enzymes was differentially regulated in female-associated syncytia compared to the control roots. Furthermore, our results revealed that the functional activity of these enzymes did not necessarily correlate with their gene expression.

2. Material and methods

2.1. Plant and nematode culture

Seeds of *A. thaliana* ecotype Columbia were surface-sterilised for 5 min in 0.6% sodium hypochlorite, then incubated for 3 min in 70% ethanol and subsequently rinsed four times with sterile water. Knop medium was prepared as previously described (Siddique et al., 2009). Five seeds for each treatment were transferred onto an agar layer in 9 cm Petri dishes and grown at 25 °C with a photoperiod of 16 h at 700 μE m^2 s^{-1} for 12 days. The quadruple mutant genotype *qvpe* (Gruis et al., 2002) that lacks all known VPEs in *A. thaliana* (At2g25940, At1g62710, At4g32940 and At3g20210) has been previously described.

H. schachtii was cultivated in vitro on mustard (*Sinapsis alba* cv.

Albatros) roots growing in Knop medium supplemented with 2% sucrose (Sijmons et al., 1991). Second-stage juveniles (J2) of *H. schachtii* were hatched in a funnel containing 3 mM zinc chloride. After surface sterilisation with 0.05% $HgCl_2$ and four washes with sterile water, they were transferred in a water suspension to the roots of the grown Arabidopsis plants. For optimal development of the nematodes and a sufficient infection rate, each plant was inoculated with 60–70 nematodes and was stored under the same light conditions for more than 10 days. Sex and stage of the developing nematodes was determined under a dissecting microscope on a ground glass screen. Females usually were in the third developmental stage when syncytia were sampled at 10 dpi.

Afterwards, the female-associated syncytia were cut under a microscope considering that the females were carefully removed from syncytia and were immediately frozen in liquid nitrogen. Corresponding root sections of the non-infected Arabidopsis plants served as a reference (Fig. 1). All experiments were replicated three times.

2.2. Activity-based protein profiling

Proteins from the root samples were extracted by grinding the roots in an Eppendorf tube and were quantitatively analysed by photometric measurement using the RC DC™ Protein Assay (Pharmacia LKB Ultraspec III Spetrophotometer) at 750 nm to ensure equal amounts of proteins in each sample during subsequent steps.

The probes used for ABPP were provided by the van der Hoorn lab at the Max-Planck-Institute for Plant Breeding Research (Cologne). Labelling was usually performed by incubating the extracted proteins in 50 µl containing 125 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) buffer (pH 7.5), 2 mM dithiothreitol (DTT) and 2 µM of a rhodamine-tagged fluorophosphonate probe (RhFP) for 1–2 h at room temperature in the dark. The samples labelled with 2 µM AMS101 were incubated in 125 mM sodium acetate (NaAc, pH 5.5) buffer containing 2 mM DTT and were labelled for 3 h under the same conditions as the RhFP probes. In the case of competition labelling, the samples were preincubated with the corresponding inhibitors at 50 µM for 30 min prior to labelling with the probe. Phenylmethanesulfonylfluoride (PMSF) prevents the subsequent labelling of RhFP, and TYR-VAL-ALA-ASP-chloromethylketone (YVAD-cmk) competes for the same targets as AMS101. The same volume of dimethyl sulfoxide (DMSO) was used as a non-probe control.

After incubation, the labelled proteins were separated on 12% sodium dodecyl sulfate (SDS) gels and visualised by in-gel fluorescence scanning using a Typhoon FLA 9000 scanner. Fluorescence intensity was measured using the ImageQuant TL software (GE Healthcare Life Sciences, http://www.gelifesciences.com).

2.3. Quantitative real-time PCR

RNA was extracted from the syncytial and control root material using a Nucleospin RNA Xs (Macherey–Nagel, Germany) kit according to the manufacturer's instructions and was transcribed into cDNA using random primers and a High Capacity cDNA Reverse Transcription Kit (Life-technologies catalogue number, 4368814). 18S rRNA (3 biological replicates) and Actin (1 biological replicate) were used as an internal reference, as previously described (Hofmann et al., 2010). The samples were analysed using quantitative real-time PCR in 20 µl reactions containing 10 µl of Fast SYBR Green Master Mix (Applied BioSystems), 2 mM $MgCl_2$, 0.5 µl each of forward and reverse primers (10 µM), 2 µl of complementary DNA (cDNA), and water in 20 µl total reaction volume. For the internal reference, the cDNA was diluted 1:100. qRT-PCR was carried out at 95 °C for 20 s, followed by 40 cycles each with 95 °C for 3 s and 60 °C for 30 s. The melting curve analysis was conducted at 95 °C for 15 s, 60 °C for 1 min with increments of 0.3 °C every 15 s up to 95 °C. The expression of 18S and Actin was used to analyse the changes in transcript levels using the formula $(1 + E)^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

2.4. Nematode infection assay

Plants were grown and inoculated as described above. The number of nematodes was counted at 14 dpi. The sizes of the females and their associated syncytium were measured using the Leica Application Suite (4.3.0) software (LAS: Leica Microsystems, http://www.leica-microsystems.com). All experiments were repeated three times.

3. Results

We used ABPP to analyse the changes in the active proteome of

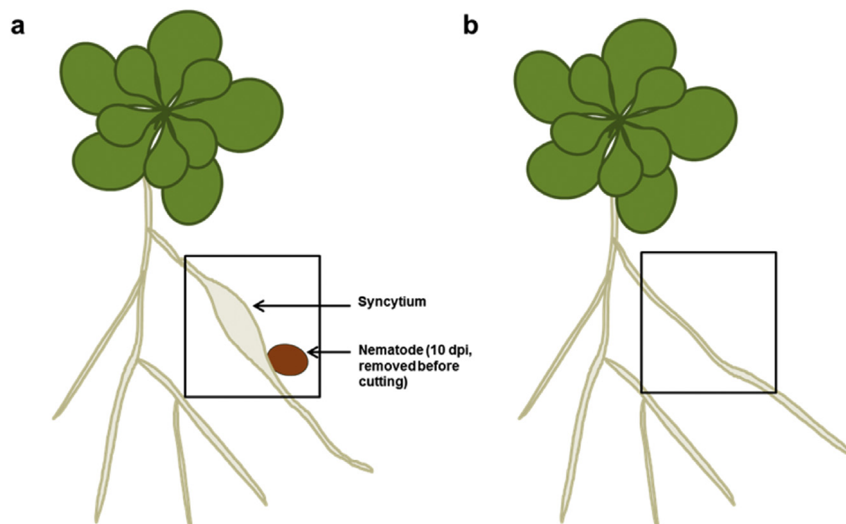

Fig. 1. Scheme of root sampling for ABPP analysis. (a) Syncytium samples were cut from the infected roots after carefully removing the nematodes. (b) Root sections from the uninfected roots were used as control.

roots after infection with *H. schachtii*. Root sections containing female- associated syncytium were collected at 10 days post infection (dpi), as described in the methods section. Corresponding root sections from uninfected plants were used as controls (Fig. 1). For conduction of ABPP labelling a total of 100 μg of protein was required.

3.1. Vacuolar processing enzymes (VPEs)

Vacuolar processing enzymes (VPEs) are cysteine proteases that are classified in the legumain family C13 (clan CD). In Arabidopsis, a total of four VPE-encoding genes (αVPE, βVPE, γVPE and δVPE) are known and are subdivided into seed-type and vegetative-type VPEs (Nakaune et al., 2005; Yamada et al., 2005). Seed-type βVPE is responsible for the maturation of the seed storage proteins and the activation of antimicrobial peptides, whereas the vegetative-type αVPE and γVPE play pivotal roles during stress and senescence conditions (Hara-Nishimura et al., 1998, 2005). δVPE is specifically expressed in the seed coat and regulates cell death (Nakaune et al., 2005).

The fluorescent activity-based probe AMS101 is potent and highly specific for all four VPEs (Misas-Villamil et al., 2013). This probe contains an aza-epoxide reactive group and a Bodipy fluorescent tag. Labelling with AMS101 resulted in a strong signal at 43 kDa and two weak signals at 40 kDa and 38 kDa in control roots (Fig. 2a). Compared to the control, the intensity of the signal at 43 kDa was weaker in the female-associated syncytia, while the other two signals remained unchanged. Surprisingly, an additional weak fluorescent band at 37 kDa was present only in the syncytium samples (Fig. 2a). We further validated these observations by quantifying the fluorescence intensity from the protein gels (Fig. 2b).

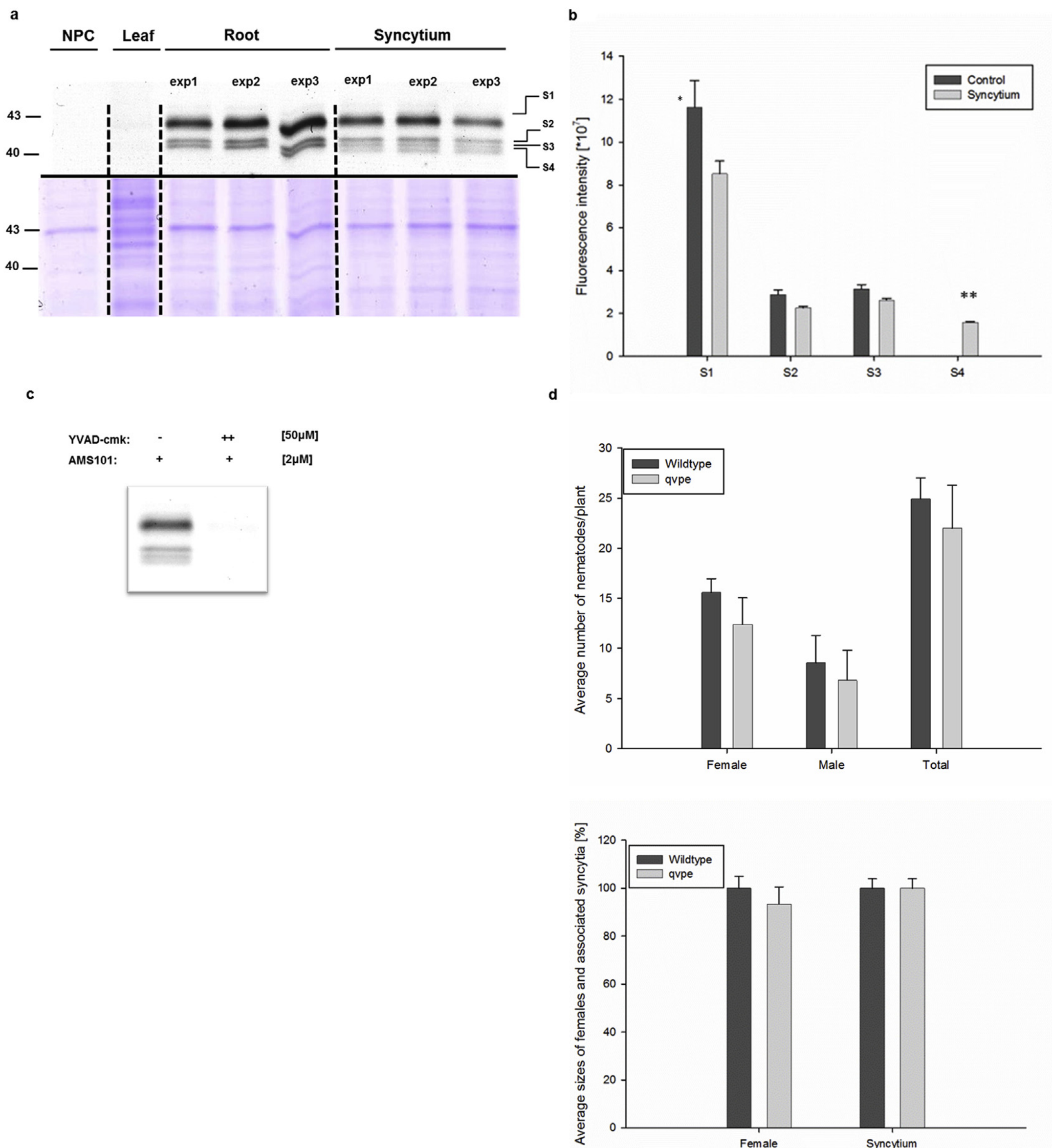

Fig. 2. AMS101 labelling of the syncytium, root and leaf. (a) Comparative labelling of VPEs in leaf, syncytium and non-infected root material with AMS101. Experiment was repeated in three biological replicates (exp1, exp2 and exp3) and blue colour gel shows staining of total protein with coomassie blue. NPC, non-probe control. (b) Fluorescence intensity of the gel signals from Fig. 2a. Asteriks indicate significant difference to control (t-test; $p < 0.05$). (c) Competition labelling of VPEs with inhibitor YVAD-cmk. (d) Nematode infection assay using the quadruple knockout mutant (qvpe), which shows the number and sizes of the nematodes and associated syncytia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Caspase-1 or VPE-specific inhibitors bind to both intermediate and mature forms of VPE (Hara-Nishimura et al., 2005; Hatsugai et al., 2004). Therefore, we performed a competition labelling with the covalent, irreversible chlorometylketone-based caspase-1, 4 and 5 inhibitor YVAD-cmk (Fig. 2c). The assay confirmed the specificity and performance of the AMS101 labelling as pre-incubation with the inhibitor prevented labelling with the probe.

Next, we investigated the changes at the transcript level for the genes encoding all four VPEs in Arabidopsis after nematode infection. A recent transcriptome analysis by Szakasits et al. (Szakasits et al., 2009) showed that αVPE and γVPE transcripts were significantly reduced in the syncytia compared to the non-infected roots, whereas βVPE transcripts were significantly upregulated. To confirm the reliability of the microarray analysis, we performed quantitative real-time-PCR (qPCR) with root sections that were cut from infected roots at 10 dpi. The results obtained from the qPCR analysis are in line with the gene chip analysis (Table 1).

We further characterised the role of VPEs in nematode and syncytium development by performing infection assays with *H. schachtii* using a quadruple knock-out mutant of VPE (qvpe) (Gruis et al., 2002). The numbers of females and males were counted at 14 dpi. Furthermore, the average sizes of the females and corresponding syncytia were measured. We expected that the roots of the *A. thaliana* knock-out mutants would exhibit a higher infection rate compared to the wild-type plants. However, knocking out the VPE-encoding genes did not result in significant changes in the infection rate or nematode development (Fig. 2d).

3.2. Serine hydrolases (SHs)

Serine hydrolases comprise a large collection of enzymes from different structural classes and are known to be involved in numerous physiological and pathological processes (Nodwell and Sieber, 2012; van der Hoorn and Kaiser, 2012; Liu et al., 1999). To study the role of serine hydrolases in the plant–nematode interaction, we labelled total protein extracted from infected and uninfected roots at 10 dpi using a fluorophosphonate (FP)-based probe with a rhodamine (Rh) reporter tag (RhFP, (Liu et al., 1999)). This probe was previously used to identify the activities of over 50 serine hydrolases in Arabidopsis leaf extracts (Kaschani et al., 2009). After labelling and separating on a protein gel, nine fluorescent signals were detected by scanning (Fig. 3a). Four fluorescent signals (s_4, s_6, s_8 and s_9) exhibited an increased protein activity in the syncytia compared to the non-infected roots. Of these signals, s_4 and s_8 had not been previously described. However, s_6 is a putative S-formylglutathione hydrolase (SFGH), and s_9 is a putative methylesterase (Kaschani et al., 2009). Similarly, there were five signals that remained unaffected (s_1, s_2, s_3, s_5 and s_7) in the syncytium. Of these five signals, s_1, s_2, s_3 and s_5 were previously identified as tripeptidyl peptidase-II (TPP2), prolyl oligopeptidases-like proteins (POPL), serine carboxypeptidase-like proteins (SCPL) and carboxylesterase-like proteins (CXE), respectively. The identity of s_7 is currently unknown. The measurements of the fluorescence intensity confirmed our observations. In particular, the methylesterase (s_9) showed a significant increase in activity (Fig. 3b). Preincubation with Ser protease inhibitor PMSF suppresses RhFP labelling of some of these proteins (Fig. 3c). This selective suppression is consistent with the selectivity of PMSF and consistent with previous findings (Kaschani et al., 2012).

To generate an integrated view of the SH activities in the syncytium, we looked at the transcriptome data (Szakasits et al., 2009) for the genes encoding the SHs detected in this experiment. Of the signals that showed increased activity, SFGH (s_6) is encoded by a single gene in Arabidopsis (At2g41530), and this enzyme catalyses the last step in the detoxification of formaldehyde by hydrolysing S-formylglutathione to formic acid and glutathione (Kordic et al., 2002). Transcriptome data revealed that there was also an upregulation in the expression of SFGH mRNA in the syncytium compared to the control roots (Supplementary Table ST1). Similarly, Kaschani et al. (Kaschani et al., 2009) identified methylesterase (s_9) as a product of the MES2 (At2g23600) and MES3 (At2g23610) genes. MES hydrolyses methylated phytohormones, such as indoleacetic acid, salicylic acid and jasmonic acid. Transcriptome analysis data revealed that, while MES3 is upregulated in the syncytium compared to the control roots, probe sets for MES2 were not specific; therefore, these data were not included in the analysis (Szakasits et al., 2009). In contrast, no significant differences were observed in transcript activity between the syncytia and control roots for TPP2 (At4g20850). For POPL (s_2), SCPL (s_3) and CXE (s_5), different isoforms have been simultaneously detected in previous studies (Kaschani et al., 2009), which makes it difficult to identify the gene/s encoding the detected SHs. Nonetheless, a look at the expression of all the genes encoding the previously detected SCPL, CXE, and POPL (Kaschani et al., 2009) did not reveal significant changes at the transcript level (Supplementary Table ST1).

Further characterisation of the detected SHs using knockout mutants was not performed in this study due to a lack of prior identification, the unavailability of homozygous T-DNA mutants, and the functional redundancy among the multigene SH families.

4. Discussion

The cyst nematode *H. schachtii* induces metabolically active syncytial feeding sites in roots. These syncytia are the sole source of nutrients for the nematodes throughout their lives. In this paper, we studied the functional proteomics of the syncytium induced by *H. schachtii* in Arabidopsis roots using Activity-based Protein Profiling (ABPP).

4.1. Activities of vacuolar processing enzymes are reduced in the syncytium

Vacuolar processing enzymes (VPEs) are cysteine proteases that were originally found to be the processing enzymes responsible for the maturation of seed storage proteins (Hara-Nishimura et al., 1991). In Arabidopsis, four VPEs have been identified, and these VPEs have been subdivided into seed-type (βVPE, At1g62710) and vegetative-type VPEs (αVPE, At2g25940 and γVPE, At4g32940). δVPE (At3g20210) was found in Arabidopsis (36) and belongs to neither of these two groups. It is considered an uncharacterised-

Table 1
Gene expression of the VPEs in 5- and 15-dpi syncytia analysed by Szakasits et al. (2009) and confirmed by qRT-PCR. Statistically significant fold changes in syncytia compared to non-infected control roots are indicated by stars (Fisher's t-test and Bonferroni correction, q < 5%). ∞ indicate that signal was below level of detection.

Affimetrix chip (Szakasits et al., 2009)					qRT-PCR	
Name	Locus	Gene symbol	M value (log2)	Fold change	ddCt value	Fold change
α	At2g25940	VPE	−2.4	−5.28*	−1.95	−3.86
β	At1g62710	VPE	2	4.00*	1.0	2.0
γ	At4g32940	VPE	−1.3	−2.46*	∞	∞
δ	At3g20210	VPE	0.1	0.93	−0.77	−1.7

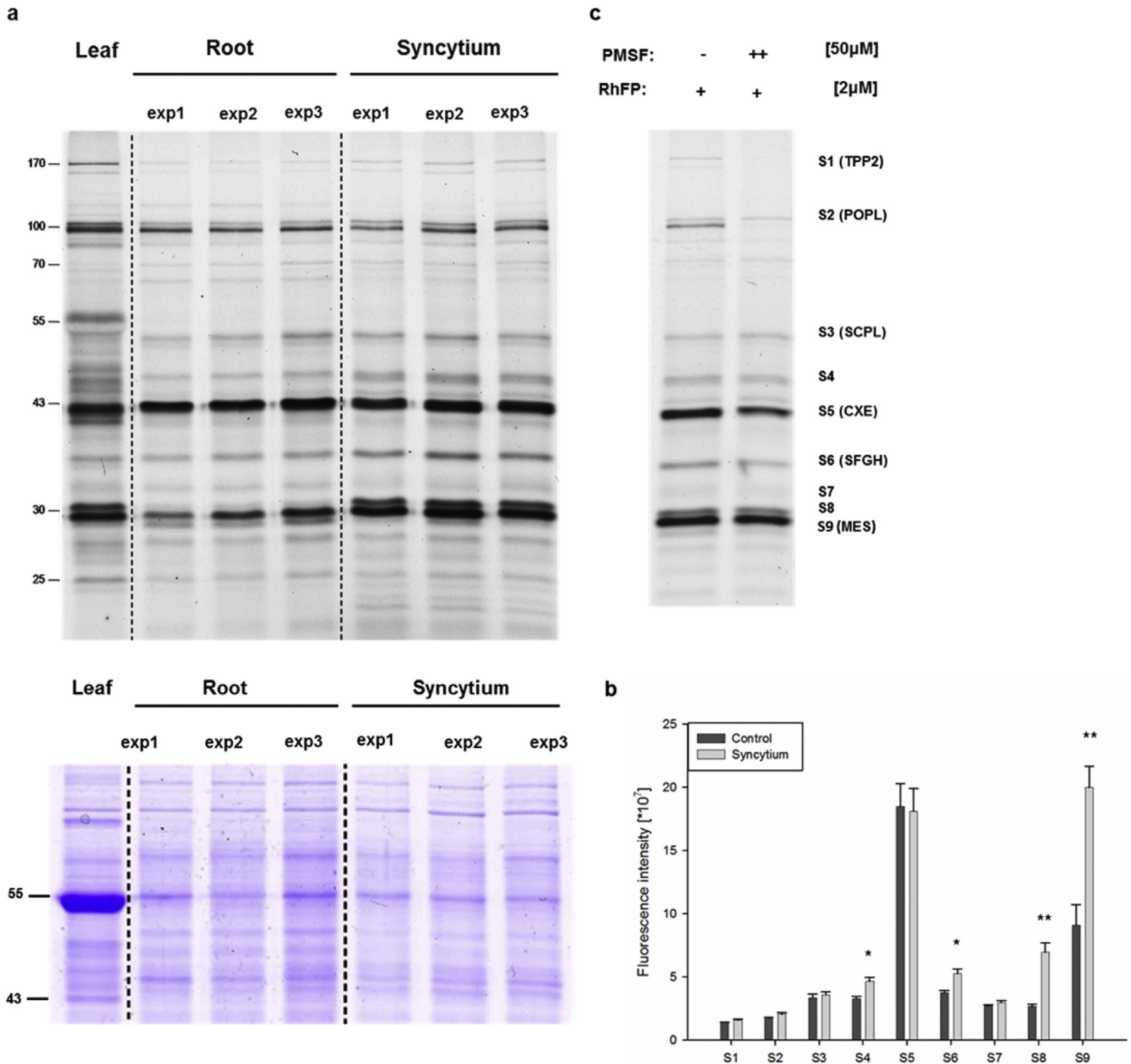

Fig. 3. RhFP labelling of the syncytium, root and leaf. (a) Comparative labelling of the SHs in leaf, syncytium and non-infected root material with RhFP. Experiment was repeated in three biological replicates (exp1, exp2 and exp3) and blue colour gel shows staining of total protein with coomassie blue. (b) Fluorescence intensity of the gel signals from 3a. Asteriks indicate significant difference to control (t-test; $p < 0.05$). (c) Competition labelling with the inhibitor PMSF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

type VPE. Vegetative-type VPEs have been shown to be upregulated in vegetative organs under stress conditions and during senescence (Hara-Nishimura et al., 1998). The plant Hypersensitive response is an efficient defence tool that leads to well-organised programmed cell death (PCD). In animals, PCD is mediated by caspases, which are cysteine proteases. VPE was the first described proteinase in plants to exhibit caspase-like activity and has been shown to be involved in vacuole-mediated hypersensitive cell death in TMV-infected tobacco leaves (*Nicotiana benthamiana*) (Rojo et al., 2004; Hatsugai et al., 2004). Similarly, it was recently shown that colonisation-associated cell death in Arabidopsis roots caused by the mutualistic fungus *Piriformospora indica* is mediated by VPEs (Qiang et al., 2012).

Because plant-parasitic nematodes rely on living plant tissues for parasitism, they need to avoid the activation of the plant cell death machinery. Indeed, after labelling with AMS101, we observed a significant decrease in the activity of the VPEs in the syncytia compared to the control roots (Fig. 2a and b). These results suggested that nematode might be able to overcome the VPE-mediated defence responses by injecting inhibitor proteins into the host cells. Interestingly, knocking out the VPEs did not affect the susceptibility of the plants to nematodes (Fig. 2d). At least two hypotheses could account for this result. First was already made in previous study (Gruis et al., 2002). Gruis et al. (Gruis et al., 2002) did not observe any phenotype or accumulation of seed proteins after knocking out β- and δ-VPE probably due to functionally redundant proteolytic enzymes other than VPE homologs. Although disputed in the literature, support for these proteolytic enzymes has been shown in

soybean. Scott et al. isolated a protein from seeds capable of processing legumin, whose molecular mass was distinctly different than VPE (Scott et al., 1992). Second hypothesis might suggest that the special and temporal suppression of VPEs by the nematodes is essential and therefore close to complete, so that any further decrease in activity does not result in any change in plant susceptibility. For future work, however, it would be interesting to study the effect of overexpression lines on the plant–nematode interaction.

4.2. Selective activation of serine hydrolases in the syncytium

The serine hydrolase (SH) family is one of the largest and most diverse classes of enzymes found in nature, and these proteins are involved in a wide range of physiological processes, including metabolism, development, and immunity (Nodwell and Sieber, 2012; Liu et al., 1999; Kaschani et al., 2012). All SHs feature an active site that contains an activated serine residue, which performs nucleophilic attack on the substrate, resulting in a covalent intermediate. The Arabidopsis genome encodes hundreds of SHs that belong to a dozen of large multigene families, such as proteases, lipases, transferases and esterases (Kaul et al., 2000). We applied the fluorophosphonate probe (RhFP) to the syncytium protein extracts to profile the activities of the SHs. After labelling, we observed significant changes in the activities of four different SHs: SFGH (s_6), MES (s_9), and two unidentified proteins (s_4 and s_8) (Fig. 3a and c). Kaschani et al. (Kaschani et al., 2009) observed the increased activity of SFGH in Arabidopsis leaves after infection with the fungal pathogen *Botrytis cinerea* in *pad3* mutants compared to Col-0. *pad3* plants are deficient in camalexin production and exhibit enhanced susceptibility to *B. cinerea*. The increased activity of SFGH in the syncytium, as well as in the leaves of infected *pad3* mutants, suggests that SFGH might play an important but as yet unknown role in plant–pathogen interactions. Similarly, the activity of a methylesterase (s_9) was increased in the female-associated syncytium compared to the control roots. MES hydrolyses methylated phytohormones, such as indoleacetic acid, salicylic acid and jasmonic acid; therefore, it is possible that increased activity is important for the maintenance of syncytial functions. Unfortunately, loss of function homozygous mutants for SFGH and MES3 were not available and, therefore, could not be used in this study.

ABPP with FP-probes identified the differential activities of SHs in the root and syncytium proteomes. These enzymes represent diverse families of enzymes, as previously shown (Kaschani et al., 2009). However, not all SHs were detected in our analysis. This could be due to several reasons. First, many Arabidopsis genes are not expressed in the tissues and conditions tested. Second, the abundance of some enzymes may be under the detection limit and third, some enzymes might not be active under the tested conditions. For example, it has been shown that RhFP labelling is strongly influenced by pH (Kaschani et al., 2009). Finally, RhFP may not react with every serine hydrolase. Nevertheless, the differential activities of the detected enzymes suggest changes in a variety of biochemical pathways in the syncytium. Unfortunately, the biological functions and significance of a majority of the enzymes are unknown. Accordingly, annotation of their biological and biochemical functions would require functional characterisation using reverse genetic approaches. This may not be an easy task, considering that the majority of SHs belong to large gene families that may have redundant members.

5. Conclusions

Sedentary parasitic nematodes manipulate plant functions to induce and maintain a highly active nurse cell system in the roots. This manipulation leads to changes in the abundance as well as activity of several proteins, such as serine hydrolases and vacuolar processing enzymes in the nematode feeding site that may not be detected by traditional transcriptomic or proteomic approaches. In this study, we have shown the proof-of-concept for the utility of ABPP-method to display the differential activities of various enzymes upon nematode infection. Our results hinted towards the existence of nematode effectors that may inhibit or activate enzyme function at post-translational level. Future work will aim to characterize the identity and functions of the differentially activated host as well as nematode proteins, which may provide new exciting insights into the plant–nematode interactions. Considering that there is not much known about functions of nematode effector proteins in host, application of ABPP in future will provide a powerful tool to characterize functions of such effectors within the host. This in turn will help generating an integrate picture of changes during plant–nematode interaction at pre- and post-translational level. To start with, more probes should be used to study interactions involving different hosts and nematodes species at various time-points of infection process. For example, it will be interesting to investigate and compare functional proteomics using ABPP during a compatible and incompatible plant-interaction.

Contribution

Marion Hütten and Melanie Geukes performed the experiments. Johana C. Misas-Villamil supervised ABPP experiments. Renier van der Hoorn and Florian M. W. Grundler critically reviewed the manuscript. Shahid Siddique and Marion Hütten wrote the manuscript. Shahid Siddique designed the research.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2015.09.008.

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3. Chapter 3

Papain-like cysteine proteases and proteasomal activities are suppressed in syncytium induced by cyst nematodes

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3.1. Abstract

Cyst nematodes are obligate biotrophs that cause substantial yield losses in agriculture. Having a complex biology, they spend the major time of their life cycle inside the host root where they establish a hypertrophic and hypermetabolic syncytial nurse cell system. To establish syncytium inside the root, they need to circumvent plant´s defence mechanisms. Papain-like cysteine proteases (PLCPs) and the proteasome are known to play important roles in plant defence and would need to be suppressed in case of successful parasitism. Using Activity-based protein profiling (ABPP), we were able to show in this study that the beet cyst nematode *Heterodera schachtii* is able to suppress the activity of PLCPs and proteasomal subunits at posttranslational level thus facilitating infection. We further show a differential regulation of the proteasomal activity between female- or male nematode-associated syncytium, whereas the activity of PLCPs is reduced in both samples.

3.2. Introduction

Plant-parasitic nematodes raise high economic importance in global agriculture and therefore gained increasing attention over the past decades. Among plant-parasitic nematodes, root-knot (*Meloidogyne* spp.) and cyst nematodes (*Heterodera* spp. and *Globodera* spp.) are economically most devastating species. The beet cyst nematode (BCN) *Heterodera schachtii* is a sedentary, biotrophic endoparasite with a wide host range within the *Amaranthaceae* and *Brassicaceae*, including *Arabidopsis thaliana* [1].

As an obligate parasite, *H. schachtii* possesses a complex biology allowing the nematode to remain the majority of its life cycle inside the root. The life cycle starts, when upon arrival of favourable conditions, the pre-infective juveniles of nematodes (J2s) hatch from the cyst, and invade the host root. They migrate intracellularly towards the vascular cylinder piercing host cells with their characteristic needle-like stylet until they find a suitable host cell inside the vascular cylinder to establish an initial syncytial cell (ISC) [2]. This ISC constitutes the origin of the nurse cell system of nematodes, the syncytium [3]. During nematode development, syncytium undergoes diverse ultrastructural, transcriptomic and metabolic changes, which have previously

documented [4-7]. The syncytium expands primarily through local dissolution of adjacent cell walls [8].

There are only few studies done to investigate the changes in protein level in host plants after nematode infection [9, 10]. Infection of potato roots by cyst nematode *Globodera rostochiensis* carrying H1 resistance genes did not cause any change in protein level during nematode development as analysed by two-dimensional gel analysis [11]. Root proteomic studies were carried out in nematode-resistant coffee and cotton cultivars after infection with root-knot nematode, *Meloidygyne incognita* and *M. paranaensis*, which led to identification of some pathogen responsive proteins [12]. An analysis of syncytium developing on the oil radish roots revealed a change in pattern of free amino acid during the development of *H. schachtii* [13]. A biochemical analysis of syncytia was performed by Grundler et al. (1991) and Betka et al. (1991) revealing a strong increase in the protein content and profound changes in composition of free amino acids [4, 14]. A comparative protein analysis of Arabidopsis roots and syncytia led to the identification of the myrosinase gene pyk10, which was shown to be increasingly produced around syncytia [15]. Later the root specific promoter of this gene was cloned [9]. Strategies aiming at measuring plant responses to nematode infection at the proteomic level are still at their infancy. However, technical advancement in high-throughput protein separation and analytical mass spectrometry has facilitated the performance of proteomic analyses and should therefore be promoted also in the context of plant-nematode interactions.

It is generally believed that nematodes secrete a cocktail of proteinaceous and non-proteinaceous effectors to manipulate the host plant activities through various post-translational modifications e.g. inhibition and activation. The current knowledge on plant-nematode interaction in general and development of syncytium in particular is mainly based on transcriptomic and genomic analysis of host plants. There are also few proteomic studies done as mentioned above. These approaches detect the changes in abundance of transcripts or proteins but not the protein activity. However, the function of a protein is dependent on its activity, which is regulated by pH, co-factors, and temperature etc. Therefore, an approach that takes into account the post-translational modifications at proteomic level is much desired.

Activity-based protein profiling (ABPP) is a method to investigate the enzyme activity in an extract or in living tissues [16]. ABPP is based on small molecular probes (biotinylated or fluorescent) that react with the active site of subsets of enzymes in complex proteomes in an activity-dependent manner [16]. The size of the enzyme class can range from a few to several hundred individual proteins. The labelling of probes is irreversible and covalent, which facilitates the detection of labelled proteins in a protein gel or by mass spectrometry. Since these probes react in a strictly activity-depended manner, all those proteins, which are inhibited, lack cofactors or are inactive for other reasons, are not labelled during ABPP. Therefore, labelling reflects the information on activity of proteins rather than their abundance.

Application of ABPP is relatively new in plant science. Several proof-of-concept studies have been carried out with a number of probes, which illustrate the utility of ABPP to study plant enzymes [17-20]. ABPP in context of plant-pathogen interaction can reveal functional information at proteomic level, which is of vital importance to understand a particular interaction. For instance, ABPP of botrytis-infected Arabidopsis leaf extracts with fluorophosphonate (FP) identified several serine hydrolases, which contribute to pathogenicity [21]. Similarly, we recently showed a reduced activity of serine hydrolases and vacuolar processing enzymes (VPEs) in syncytia showing a suppression of plant defences during nematode infection [22]. ABPP with the probe DCG-04 led to the identification of several papain-like cysteine proteases (PLCPs) that have been shown to play crucial roles in plant-pathogen interaction in Arabidopsis, tomato, tobacco and wheat [23-26].

PLCPs are produced as pro-proteases containing an auto activation domain, which is removed to release a 20-30 kDa mature protease peptide. PLCPs act on non-self substrates and both host and their pathogens employ PLCPs on the molecular battlefield [27]. As described above, the role of PLCPs in plant defence against pathogen attack has been studied in detail in Arabidopsis, tomato, tobacco and wheat [23-26]. On the other hand, it has been shown that bacterial PLCPs manipulate host defence responses in the plant cytoplasm to cause infection [28-30]. Arabidopsis contains 30 genes encoding PLCPs and role of only few have been studied in detail. In context of plant nematode interaction, it has been shown that PLCPs are present in digestive system of

plant parasitic nematode [31-33]. Feeding of nematodes on roots of transgenic Arabidopsis plants overexpressing the PLCPs inhibitor "oryzacystatin'' reduced the number and size of nematodes [31, 32]. However, role of plant PLCPs in plant-nematode interaction remains obscure till to date.

Being the main protein degradation machinery in plants, the 26S proteasome plays a very important role in plant-pathogen interactions. The 26S proteasome is a protease complex consisting of multiple components localized in the nucleus and cytosol. The structure of proteasome resembles to that of a cylindrical complex consisting of a 20S core protease (CP) and two 19S regulatory particles (RP) covering the CP. The RP unfold the substrates that are already ubiquitinated and feeds them into CP [34]. The 20S CP consists of four rings that are stacked together [35]. Whereas the two outer rings of CP consist of seven different α subunits being mainly responsible for recognition of substrates, two inner rings consist of seven different β subunits. The proteolytic activity of the proteasome resides in three β subunits: β1, β2 and β5. These three subunits cleave the protein substrate into smaller peptides ranging from 3-20 amino acids, which are then released into nucleus and cytosol. Plant pathogens have been shown to manipulate the host proteasome machinery to degrade immunity-associated proteins and facilitate infection of plants [36, 37]. An increasing number of studies show that the proteasome mediated degradation pathway is essential for successful mount of plant defence [38, 39]. However, successful pathogens have been shown to possess virulence factors that cause direct or indirect inhibition of the proteasome [20, 35]. Activity-based probes (ABPs) based on vinyl sulfone (VS) reactive groups have been shown to label catalytic subunits of the Arabidopsis proteasome and several PLCPs [40, 41]. In this paper, we used the VS-reactive ABP called ´Bodipy TMR-Ahx_3L_3VS´ (MV151) to analyze the activity of PLCPs and proteasome in syncytia. This probe contains a Bodipy fluorescent group to enable fluorescent imaging.

3.3. Material and Methods

Collection of syncytial and root material and for proteome analysis was performed as previously described [22]. Protein was extracted and ABPP was performed using 2 µM MV151 for 3-4 hours (MV151) as described previously [22]. In case of competition

labelling, samples were incubated with probe E64 (50 µM) for 30 min and followed by labelling with MV151. DMSO was used as a non-probe control. After labelling, the proteins were separated on SDS gels (12%) and fluorescence was visualised in-gel by using a fluorescent scanner (Typhoon FLA 9000). The intensity of fluorescent was measured using the ImageQuant TL software (GE Healthcare Life Sciences, www.gelifesciences.com).

To confirm that the observed diverse signal intensities are due to the activity of the proteins rather than their abundance, both, Coomassi Brilliant Blue staining and Western Blot analysis was conducted. For Western Blot a PVDF membrane was incubated in 100% methanol for 30 seconds and washed with water for 2 minutes before it was assembled with the SDS gel and transferred for 1 hour at 200mA. Transfer buffer consisted of 25mM Tris (pH 8.0), 190 mM glycine and 20% methanol. Blocking of the membrane was achieved by 20 minutes incubation in 10ml 1X TBS + 3% BSA. Afterwards, 200µl Tween20 and 2µl αPBA-1 (1:5000) antibody was added and incubated ON at 4°C.

After washing five times in 1X TBS + 3% BSA, the second antibody Anti-Mouse IgG Peroxidase (1:10.000) was added and incubated for 1 hour at 4°C. Finally, after washing with 1X TBS and 0.1% Tween20, the membrane was developed with SuperSignal® West Pico/Femto Chemilumincent Substrate (ThermoFisher, Prod# 34080) according to the instruction manual.

3.4. Results

3.4.1. Activity of papain-like cysteine proteases (PLCPs) is suppressed in female and male associated syncytium

PLCPs have been shown to protect plants against pests and pathogen attacks [42-44]. Arabidopsis contains 30 genes encoding PLCPs and role of only few have been studied in detail. We used an activity-based probe based on vinyl-sulfone (VS) reactive groups called ´Bodipy TMR-Ahx$_3$L$_3$VS´ (MV151) to analyze the activity of PLCPs in syncytia. The profiling was performed with syncytia associated with females. As shown in **Figure 1a** and **1d** labelling of female-associated syncytial samples resulted in comparatively weaker signals at 40 kDa and 30 kDa (s_1 and s_2), which reflect reduced PLCP activity in

syncytia compared to control roots. Competition labelling with E64 confirmed that PLCPs are causing the signals at 30 (s_2) and 40 (s_1) kDa in the MV151 labeling profile, as pre-incubation with this inhibitor prevented subsequent labelling with MV151 (**Figure 1c**). Previous studies with MVA178, an acid-labelled version of MV151, suggested that the signal around 40 kDa signal represents an intermediate isoform of desiccation-induced RD21 (iRD21). RD21 is a cysteine protease that is abundantly present in Arabidopsis leaf extracts [17], whereas the 30 kDa signal represents a mixture of PLCPs, including the mature isoform of RD21 (mRD21), xylem specific XCP2 and cathepsin B-like proteases [17, 45]. Next, we analysed the PLCP activity in syncytia-associated with males. The data analysis indicated that similar to that of female-associated activity of PLCPs is suppressed strongly as compared with control root (**Figure 2**).

Microarray performed by Szakasits *et al.* [6] and our qPCR analyses did not show dramatic changes in transcription profiles of PLCPs except for three encoding cathepsin B (CathB1, CathB2, CathB3), which showed a slight downregulation (**Supplementary Table ST1 and Table 1**). To further confirm change in activity of CathB, we performed another labelling with FH11, which was recently designed by Lu *et al.* [46] to display the activity of bacterial type III effector protease AvrPphB. It contains an acyloxymethylketone (AOMK)-reactive group and a rhodamine reporter tag for fluorescent detection. The profiling of syncytia associated with females showed less activity of cathepsin (CathB) compared to non-infected roots (**Figure 3a and 3c**). Pre-incubation of the samples with E64 inhibited the binding of FH11 to the active sides of enzymes, showing the specificity of this probe (**Figure 3b**).

3.4.2. Activity of proteasomal subunits is suppressed in female associated syncytium but not in male associated syncytium

As one of the main protein degradation machinery in cells, the proteasome plays an essential role in plant defence and development [41]. In addition to PLCPs, ABPs based on VS-reactive groups were also shown to label the catalytic subunits of the mammalian proteasome [40, 47]. Therefore, we used the probe MV151 to analyse the activity of the three catalytic subunits β_1 (PBA1, At4g31300), β_2 (PBB1, At3g27430; PBB2, At5g40580) and β_5 (PBE1, At1g13060) of the proteasome. After labelling of infected and

non-infected Arabidopsis root material several differences could be observed. As shown in **Figure 1a**, labelling of samples from female associated syncytia resulted in three fluorescent signals around 25 kDa (s_{3-5}), which are weaker in syncytia compared to uninfected roots (s_3 6.6 to 19.2, s_4 12.8 to 34.5 and s_5 6.3 to 13.4 fluorescence intensity) (**Figure 1d**). According to mass spectrometry (MS) studies of Arabidopsis leaf extracts s_3 represents the β_2 (PBB1) catalytic subunit of the proteasome, s_4 subunit β_5 (PBE1) and s_5 subunit β_1 (PBA1) [41]. These changes were not observed in male-associated syncytium (**Figure 2**). In contrast to our data, transcriptome analysis showed a significant increase in amount of transcripts of not only 5 genes encoding these three subunits but also other proteasome genes in infected root material (**Supplementary Table ST2**; [6]). Western Blot analyses using α-PBA1 antibody confirmed that there was no significant change in total protein content (**Figure 1b**).

3.5. Discussion

3.5.1. Papain-like cysteine proteases are inactivated in syncytium

PLCPs use a catalytic cysteine residue to cleave peptide bonds in protein substrates [27] and are therefore thought to play a role in plant pathogen interaction. Interestingly, pathogens as well as their hosts use proteases as well as protease inhibitors to overcome each other [48]. Plants express PLCPs to defend themselves not only against pathogens attack but also during water stress and senescence like RD21 (At1g47128). On the other hand, pathogens need to circumvent this cellular defence to establish infection within their hosts. It has recently been demonstrated for the biotrophic fungus *Cladosporium fulvum* (syn. *Passalora fulva*) that it uses secretory proteins (effectors) to inhibit the extracellular PLCP, Rcr3 (required for *Cladosporium* resistance 3; [49]), which is essential for the function of the resistance gene *Cf*-2 in tomato. Similar effector proteins with protease inhibitory activity have also been identified from *Phytophthora infestans* in tomato plants [50-52], for example the Cys protease inhibitor EPIC2, which targets the Rcr3-like Cys protease Pip1 (*Phytophthora*-inhibited protease 1; [52]). Based on these findings we assumed that also nematodes could use protease inhibitors, such as *C. fulvum* Avr2 and *P. infestans* EPIC2, to inactivate the basal plant defence.

In fact, the activity of PLCPs was reduced in syncytia produced by females as well as in those produced by males. The profile of MV151 showed lower activity of proteins at 30 kDa and 40 kDa. In reference to Gu *et al.* [41, 53] and after implementation of a competition labelling with the well-known protease inhibitor E64, we identified these signals as RD21 (40 kDa) and a mixture of other PLCPs (30 kDa). This mixture contains among others the mature isoform of RD21 (mRD21), xylem specific (XCP2) and Cathepsin B-like (CathB) proteases. In animals, i.e. Cathepsin B is known to be involved in many different processes, including programmed cell deaths (PCD) [54], which led Gilroy *et al.* [23] to study the function of Cathepsin B in plant disease resistance. They were able to show that this enzyme plays a role in both host and non-host resistance of plants, as transcription and enzymatic activity is induced during the HR [23]. Based on this information and to itemize the PLCP mixture at 30 kDa another labelling with the activity-based probe FH11 was conducted. Thereby, the assumption was confirmed that CathB was less active in syncytia compared to non-infected roots. Obviously, CathB and other PLCPs constitute a plant defence mechanism that needs to be down-regulated by nematode to establish functional feeding cells. This hypothesis is supported by a recent study showing that loss-of-function mutants for PLCPs are hypersusceptible to cyst nematode infection [55].

3.5.2. Inhibition of proteasome in syncytium

The plant 26S proteasome is a large, multicomponent protease complex residing in nucleus and cytosol. The inner cavity of this complex contains three catalytic subunits (β1, β2 and β5) that are responsible for the proteolytic activity of proteasome [41]. The role of proteasomes in many cellular processes including activation of defence against pathogens has been well characterized [41, 44, 56, 57]. Therefore, pathogens such as *P. syringae* inhibit host proteasome activity by releasing effectors into host cells to facilitate infection [35-37]. More recently, it has been shown that *Xanthomonas campestris* Type III effector XopJ targets the host cell proteasome to suppress salicylic acid mediated defence responses [58]. In context of plant-nematode interaction, it was recently shown that an ubiquitin carboxy extension protein secreted by potato cyst nematode *Globodera rostochiensis* promotes formation of syncytium by interfering in host proteasome function [59]. Our results after ABPP with the vinyl sulfone probe

MV151 are mainly compatible with these findings. This probe was used to label the three catalytic subunits (β1, β2 and β5) of plant proteasome in syncytia associated with females and males. Interestingly, we observed different activity regulation in both types of syncytia. Female associated syncytia showed a strong reduction in the activity of these three subunits as compared to control root. In comparison to activity of proteins, expression of 5 genes encoding these and other components of proteasome is upregulated as compared to control roots [6]. In contrast to females, male associated syncytium did not show any change in activity of proteasome subunits. In *H. schachtii*, sex is thought to be determined epigenetically: poor environmental conditions e.g. resistant plants or active plant defence mechanisms lead to development of majority males. Our results suggest that suppression of proteasome activity by nematodes might be a pre-requisite for development. However, more work will be needed to understand the role of proteasome in development of females.

3.6. Conclusion

Summarizing this work we used an activity-based profiling analysis to visualize the altered activity of specific proteins in plants after nematode infection. According to our results the nematodes are able to modify essential plant defence-related enzymes, such as PLCPs and proteasomal subunits, at the post-transcriptional level to enable successful parasitism. We also showed for the first time that the activity of the proteasomal subunits is differentially regulated in the male and female associated syncytium. Considering the prevalence of male nematodes in resistant plants this observation is of major interest. Although evidences are still missing the existence of nematode effector proteins inhibiting those plant-defence related proteins to establish and maintain nurse-cell systems is obvious and provides exciting paths to study the complex interaction between nematode and host plant in more detail. Further analysis should be conducted using protein-protein interaction tools to allow a deeper understanding of the circumvention of plant defence mechanisms and the successful parasitism of nematodes to enable thereby the development of new control measures.

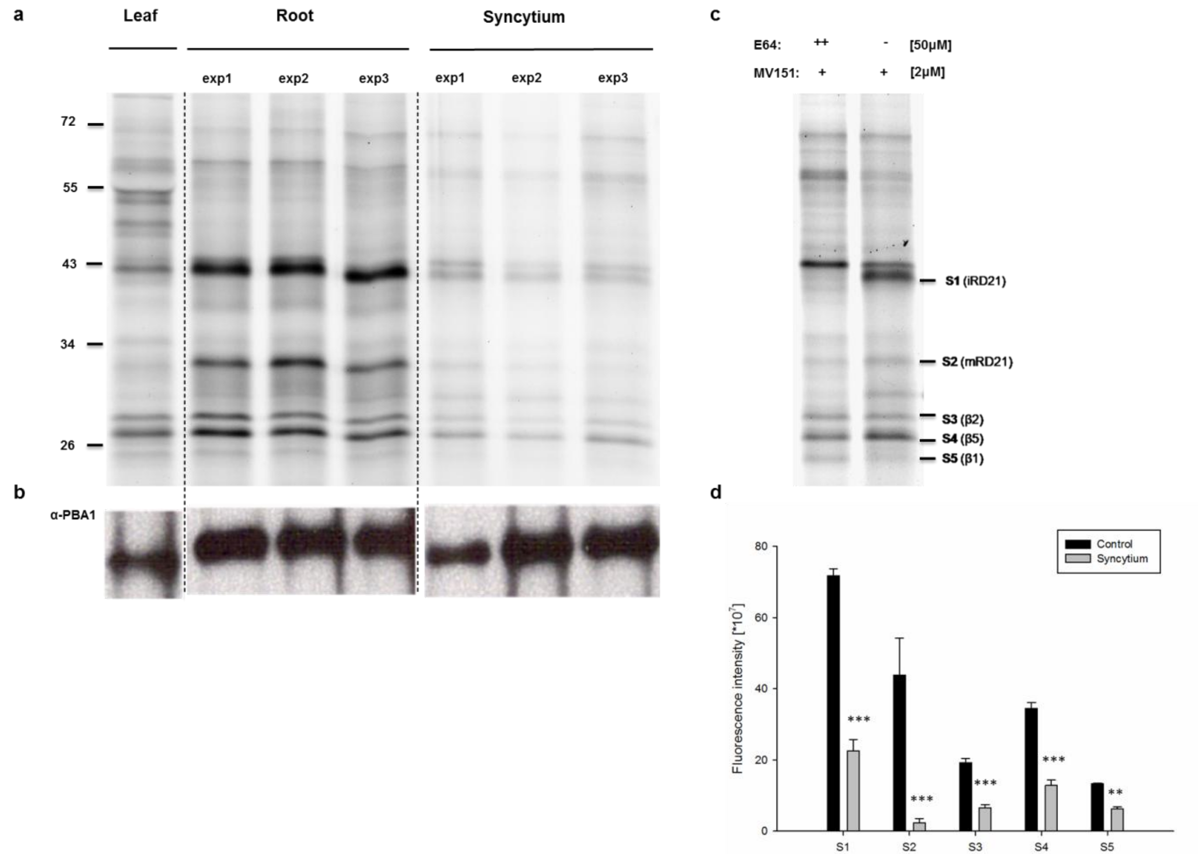

Figure 1: Comparative **(a)** and competitive **(c)** labelling of proteasome and PLCPs in female associated syncytia and non-infected root material with MV151 and inhibitor E64. **(b)** Western Blot with α-PBA1 antibody of syncytia and non-infected root material. **(d)** Fluorescence intensity of gel signals.

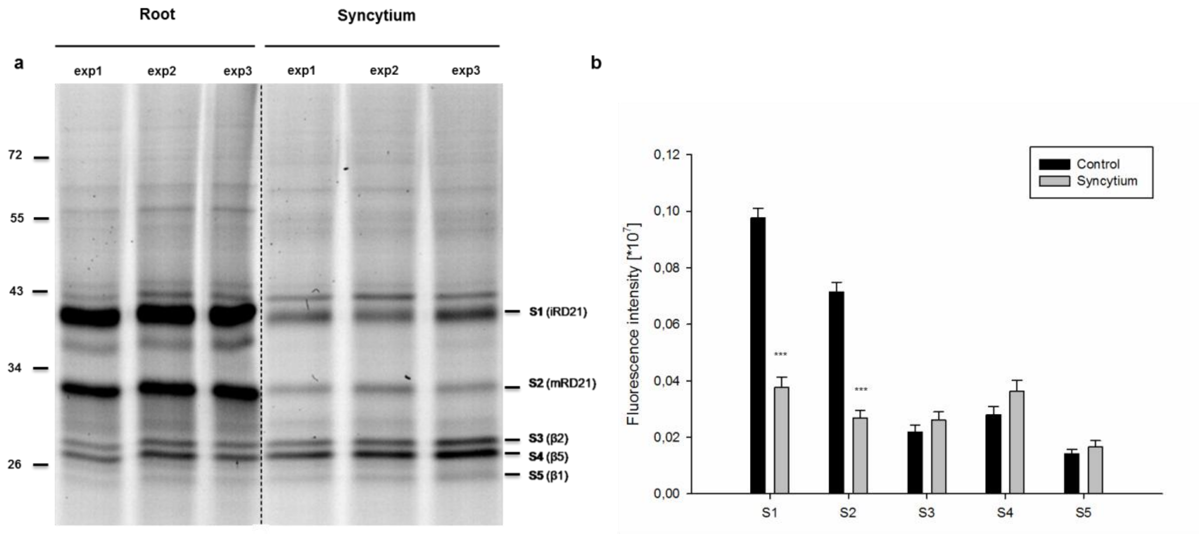

Figure 2: (a) Comparative labelling of male associated syncytium and non-infected root material with MV151. **(b)** Fluorescence intensity of gel signals.

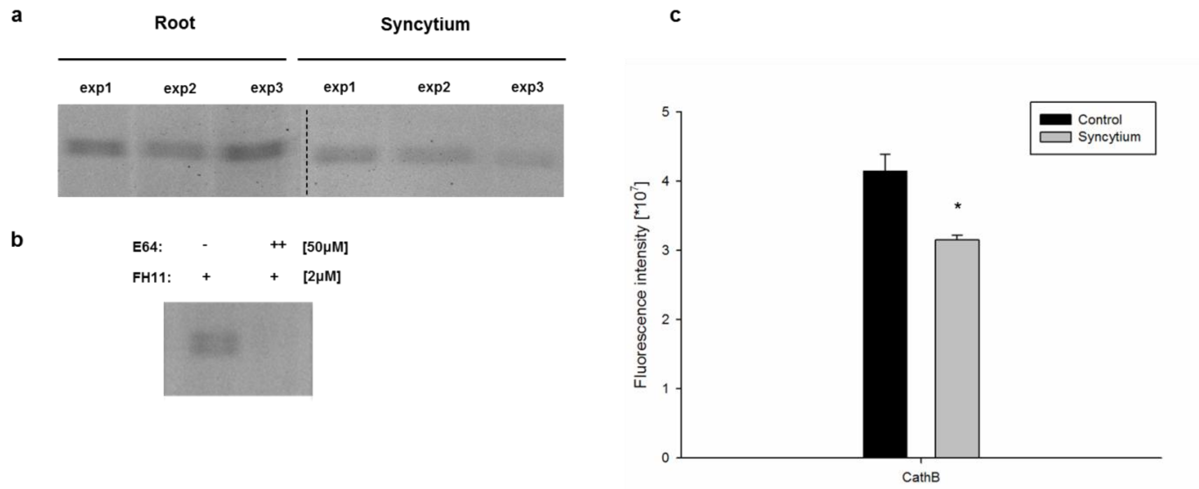

Figure 3: Comparative **(a)** and competitive **(b)** labelling of female associated syncytium and non-infected root material with FH11. **(c)** Fluorescence intensity of gel signals.

Table 1: Quantitative RT-PCR of CathB transcription in 5dpi and 10dpi syncytia.

Name	Locus	Gene symbol	ddCT value	Fold change
Cathepsin B	At1g02300	CathB1	-1.01	-2.01
	At1g02305	CathB2	-0.54	-1.45
	At4g01610	CathB3	-1.23	-2.35

Supplementary Table ST1: Affimetrix chip analysis data: PLCP transcription in syncytia (5 and 15 dpi) [6]

Name	Locus	Gene symbol	M value (log_2)	Fold change
Cathepsin B	At1g02300	CathB1	-0.6	-1.52
	At1g02305	CathB2	-0.6	-1.52
	At4g01610	CathB3	-0.5	-1.41
XCP	At4g35350	XCP1	-4.9	-29.86*
	At1g20850	XCP2	-5.3	-39.40*
RD21	At1g47128	RD21A	-2.6	-6.06*
	At5g43060	RD21B	0.6	1.52
	At1g09850	RD21D	0,6	1.52

Supplementary Table ST2: Affimetrix chip analysis data: Proteasome transcription in syncytia (5 and 15 dpi) [6]

Name	Locus	Gene symbol	M value (log_2)	Foldchange
α1	At5g35590	PAA1	0.9	1.87*
	At2g05840	PAA2	0.9	1.87*
α2	At1g16470	PAB1	1.6	3.03
	At1g79210	PAB2	1.6	3.03*

α3	At3g22110	PAC1	1.9	3.73*
	At4g15160	PAC2	0.6	1.52
α4	At3g51260	PAD1	3	8.00*
	At5g66140	PAD2	2.1	4.29*
α5	At1g53850	PAE1	1.3	2.46*
α6	At3g14290	PAE2	1.4	2.64*
	At5g42790	PAF1	2.1	4.29*
α7	At1g47250	PAF2	3.3	9.85*
	At2g27020	PAG1	2.9	7.46*
β1	At4g31300	PBA1	1.8	3.48*
β2	At3g27430	PBB1	2.8	6.96*
	At5g40580	PBB2	2.1	4.29
β3	At1g21720	PBC1	3.9	14.93*
	At1g77440	PBC2	2.3	4.92
β4	At3g22630	PBD1	3.1	8.57*
	At4g14800	PBD2	2.4	5.28*
β5	At1g13060	PBE1	1.7	3.25*
β6	At3g26340	PBF1	1.7	3.25*
	At3g60820	PBF1	2.1	4.29*
β7	At1g56450	PBG1	2.8	6.96*

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4. Chapter 4

A cystatin-like effector protein of beet cyst nematode *Heterodera schachtii* performs diverse roles during plant-nematode interaction

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4.1 Abstract

Plant-parasitic nematodes constitute an important parasitic group causing substantial yield losses in global agriculture. Most devastating species within this class are cyst and root-knot nematodes. As sedentary endoparasites, these nematodes induce nurse cell systems in host´s roots from which they feed for their entire life cycle. To establish and maintain these metabolically highly active feeding cells, they secrete effector proteins inside the host´s root to suppress plant defence mechanisms and to modify cellular processes for their own benefit. In this study, we identified and functional characterized the secreted effector protein HsCysL1 from the beet cyst nematode *Heterodera schachtii* that contains cystatin-like domains. Contrary to our assumption, HsCysL1 may not only inhibit papain-like cysteine proteases (PLCPs), but more likely has a dual function due to diverse cellular localizations.

4.2 Introduction

Although plant-parasitic nematodes make up a comparably small part of the described nematode species (around 15%), they infect a broad range of crops and cause substantial yield losses in global agriculture. Most devastating is a small group of sedentary cyst (*Globodera* spp. and *Heterodera* spp.) and root-knot (*Meloidogyne* spp.) nematodes. The sugar beet cyst nematode *Heterodera schachtii* is a sedentary biotrophic endoparasite that spends the majority of its life within the root. With a wide range of hosts this nematode can infect crops within the Amaranthaceae and Brassicaceae and cause severe agricultural problems in temperate regions [1-3]. *H. schachtii* can also infect the model plant *Arabidopsis thaliana*, and this pathosystem has been used intensively to study the molecular aspects of plant-nematode interactions [4, 5].

As with all cyst nematodes, *H. schachtii* possesses a highly specialized biology. Upon the arrival of suitable environmental conditions, second stage juveniles of these nematodes hatch from the cyst and invade the roots guided by root exudates. After entering the roots, nematodes migrate intracellularly through different tissue layers to reach the vascular cylinder, where they probe individual cells with the help of their characteristic stylet until they find a suitable initial syncytial cell (ISC) for feeding [5, 6]. The factors involved in selecting a particular cell to be an ISC are still unknown. The ISC expands through local dissolution of cell walls with neighbouring cells, thus

leading to the formation of a multinucleate and hypertrophied syncytium. Upon ISC selection, *H. schachtii* becomes sedentary and syncytium serves as the only nutrient source through the entire life cycle of the nematode [1, 7-9]. The development of syncytium is accompanied by massive cytological and histological changes, which have been studied in detail [10-13]. The central vacuole is replaced by several smaller ones, and a highly pronounced smooth endoplasmatic reticulum is present inside syncytium. Moreover, nuclei are hypertrophied, and the number of organelles increases significantly [6], resulting in a metabolic highly active nutrient source. Once the sexually dimorphic nematode becomes sedentary, the nematode undergoes three developmental stages (J2, J3, J4), interrupted by three moults, before females take on a lemon-shaped body [6]. Meanwhile, males become vermiform and leave the roots to copulate with the females. After fertilization, the females die and form a robust cyst that protects the up to 300 eggs within from environmental factors for many years [14, 15].

As a sedentary biotrophic endoparasite, *H. schachtii* depends on living host cells and establishes a highly complex relationship with its host. During nematode invasion and selection of the ISC, host tissue gets damaged, which, in turn, may activate basal defence mechanisms of the plant through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) located on the cell surface. Although the existence of PAMPs on behalf of the nematode has not been proven so far, the similarity of highly-specific resistance genes found in nematode resistant plants to those induced by other pathogens, suggests a similar mechanism [16, 17].

One of the main defence strategies by plants is the release of apoplastic proteases upon infection. These proteases can detect secreted proteins from pathogens (effectors), leading to the activation of a strong defence response, that may culminate in a form of programmed cell death (PCD) known as hypersensitive response (HR) [18-21]. One well-known example of such apoplastic proteases is *RCR3* (Required for Cladosporium Resistance 3), a small secreted extracellular cysteine protease from tomato (*Lycopersicon esculentum*). *RCR3* is transcriptionally upregulated upon infection by leaf mold causing fungus *Cladosporium fulvum* (Cf) and has been previously shown to play a role in host defence against infection. Interestingly, inhibition of *RCR3* by *Avr2*, a cysteine-rich protein secreted by *C. fulvum* results in

activation of HR that is mediated by plasma membrane-localized R-gene *Cf-2* [22]. *Cf-2* encodes a transmembrane protein with extracellular leucine-rich repeats (LRRs) and short (23 to 36 amino acids) cytoplasmic domains. However, the exact mechanism of by which Cf-2 detects AVR2-RCR3 interaction is not fully known.

Papain-like cysteine proteases (PLCPs) are proteolytic enzymes involved in development, immunity and senescence [23]. They use a catalytic cysteine residue to cleave peptide bonds in protein substrates and have been found to be used by both parties in plant-parasite interactions [24]. In Arabidopsis, around 30 genes encode for PLCPs, but only a few have been studied in detail so far [23-26]. To successfully invade a host, biotrophic pathogens need a strategy to inhibit PLCPs. Indeed, it has been shown that PLCPs are targeted by effectors of several pathogens [27-31]. For instance, RD21 (Responsive to Desiccation 21; At1g47128) is involved in defence mechanism of *A. thaliana* against the necrotrophic fungus *Botrytis cinerea* [32]. Furthermore, effector proteins secreted by *H. schachtii* and *Meloidogyne chitwoodi* have been identified that target RD21 [26], supporting the role of PLCPs in plant-nematode interaction. The yellow potato cyst nematode *Globodera rostochiensis* also secretes an effector (Gr-VAP1) that targets the PLCP Rcr3^{pim} in *Solanum pimpinellifolium*, triggering a Cf-2 mediated resistance response [33].

All these effector proteins act as cysteine-protease inhibitors and show common phytocystatin-like characteristics. Phytocystatins are reversible inhibitors of papain-like cysteine proteases possessing consensus amino acid residues that are indispensable for their inhibitory activity: (1) QxVxG located at the active site; (2) a G near the N-terminus; (3) a conserved W in the second half of the protein [34, 35].

Two apoplastic, cystatin-like effector proteins, EPIC1 and EPIC2B, have been identified in *P. infestans* targeting the PLCPs PIP1 (Phytophthora Inhibited Protease 1), RCR3 and C14 in tomato [22, 36-38]. Supported by our recent findings indicating a reduced activity and thus reduced functionality of PLCPs in Arabidopsis roots after infection with *H. schachtii* [c.f. chapter 3], we assumed that the nematode secretes effector proteins that function similarly to EPIC1 and EPIC2B of *P. infestans*. Using bioinformatic approaches we found a sequence within the genome of *H. schachtii* termed HsCysL1 (*Heterodera schachtii* Cystatin-Like-1) that obtains cystatin-like motifs, indicating an inhibitory function. To investigate the role of HsCysL1 during

plant-nematode interaction, we performed a detailed characterization of this putative effector in this manuscript.

4.3 Material and Methods

All primers used in these experiments are given numbered in supplementary table **ST1**.

4.3.1 Bioinformatic approaches

Transcriptome assembly using RNA of *H. schachtii* juveniles (J2) enabled the identification of an ORF (396bp) containing a signal peptide on its N-terminal determined by SignalP server [39] and cystatin domains [40] predicted by Pfam. This transcript was sequenced by next generation sequencing (illumina) and encodes for a 132aa protein, designated as HsCysL1 that was later analysed by TMHMM server to predict transmembrane domains of this protein [41].

Available sequences of other nematode species were tested by tBLASTn to identify HsCysL1 homologues. Included species covered plant-parasitic, free-living and animal-parasitic nematodes. Considered characteristics during this test included the presence of a putative signal peptide, pfam domains as well as predicted transmembrane domains. Only homologues pointing up these features were taken into account of further analyses. Isoforms within the same species were minimized to a single representing HsCysL1-like sequence per species. The final homologues were aligned to each other (CLC genomics workbench Version 8.5). All aligned sequences were used to build up a phylogenetic tree using the maximum likelihood phylogeny 1.2 algorithm (construction method = Neighbour Joining, Nucleotide substitution modes = Jukes Cantor, Protein substitution model = WAG, and a 100 bootstrap) (**Figure 1**).

4.3.2 Expression of HsCysL1 in *H. schachtii*

To analyze the expression of HsCysL1 at different juvenile stages, RNA was extracted from juveniles (J2) and nematodes at different days after infection (5dpi and 10dpi). Quality of RNA was checked by Bioanalyzer and cDNA was produced using reverse transcriptase and random primers (Applied Biosystems, prod.nr. 4368814). qRT-PCR was conducted using the primers given in **ST1** (number 1) and protocol previously described [42].

4.3.3 Localization of HsCysL1

To localize the HsCysL1 transcripts in nematode organs *in situ* hybridization was conducted on pre-parasitic J2s. Digoxigenin (DIG)-labelled probes were amplified from the cloned HsCysL1 in the pGEM-T vector (Promega, Madison, WI, USA). The probes were generated by PCR using specific primers (**ST1**, number 2) in the presence of DIG-labelled deoxynucleotide triphosphates (dNTPs) (Roche). The RW-primer probe was used for the localization while the FW-primer probe served as a negative control. Thehybridization was performed according to the protocol of de Boer et al. [43] using a hybridization temperature of 47 °C. Afterwards, the hybridized nematodes were examined using a Leica DMI2000 compound microscope.

Localization of HsCysL1 in plants was investigated in *Nicotiana benthamiana* and leek cells independently. The signal peptide (SP) attached to HsCysL1 has a nematode origin and is usually removed as soon as the protein leaves the oesophageal gland cells. However, to investigate whether the signal peptide is processed during secretion and also functional in the plant´s translocation pathway, we conducted all localisation experiments with both constructs (HsCysL1 with and without SP). Full-length genes were amplified from a *H. schachtii* DNA library and modified by PCR according to the Gateway cloning system (Invitrogen) (**ST1**, number 3 (+SP) and 4 (-SP)). The fragments were cloned into the donor vector pDONR207 through a BP-reaction and then transferred into 35S::GFP::pmdc83 through a LR-reaction, resulting in binary vectors GFP-HsCysL1^{+SP} and GFP-HsCysL1^{-SP}, respectively. The sequences were confirmed by sequencing. Both binary vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 pMP90 [44]. For this purpose competent cells of *A. tumefaciens* were thawed on ice, mixed with 100ng DNA and incubated on ice for 30 minutes. After deep freezing in liquid nitrogen for 10 seconds the cells were heat shocked at 37°C for 5 minutes and cooled again on ice for 3 minutes. Finally, 950 µl liquid LB-medium was added and the cells were incubated for 2-3 hours at 26°C, continuously shaking at 200rpm. Selection for the construct was achieved using LB-agar plates containing kanamycin [50µg/ml], gentamycin [10µg/ml] and rifampicin [50µg/ml]. Plates were incubated at 26°C for 40 hours.

Transient overexpression of GFP-tagged HsCysL1^{+SP} and HsCysL1^{-SP} was attained by co-infiltrating cultures of Agrobacterium strains carrying the constructs with

cultures that contained the silencing inhibitor p19 from the tomato bushy stunt virus [45] in fully expanded leaves of six-weeks-old tobacco plants (*N. benthamiana*) according to the previously described protocol [46]. Leaves were harvested 3 days after infection and analysed using confocal microscopy.

Expression of $HsCysL1^{+SP}$ and $HsCysL1^{-SP}$ in leek cells was achieved by particle bombardment [47]. Gold particles were washed in 100% ethanol and vortexed for 2 minutes. Afterwards, suspension was sonicated on ice for 1 minute and centrifuged at 10.000 rpm for 1 minute. Supernatant was discarded and pellet got resuspended in 50% glycerol. 1µg DNA was mixed with 12.5 µl gold, 5 µl spermidin [1M] and 12.5 µl $CaCl_2$ [2.5M] and vortexed for 3 minutes. After pelleting the DNA-binded gold particles by centrifuging for 30 seconds, particles got washed with 100µl absolute ethanol and vortexed for 3 minutes. Then particles were pelleted again and resuspended in 37.5 µl absolute ethanol. Macro carriers were washed in 100% ethanol and dried on sterile tissue paper before transferring the DNA-carrying gold particles onto the macro carrier. Fresh leek cells were bombarded at 1350 psi and stored in the dark for 12 hours before being exposed to a 488 nm wavelength on confocal microscopy.

4.3.4 Y2H

To functionally characterise HsCysL1, a Yeast-2-Hybrid (Y2H) screening was performed as described in the BD Matchmaker Library Construction and Screening Kits (Clontech, Matchmaker® Gold, Cat. Nos. 630466, 630498 & 630499). The complete coding region of $HsCysL1^{-SP}$ was fused to the GAL4 DNA binding domain (BD) of pGBKT7 to generate pGBKT7-$HsCysL1^{-SP}$ and then transformed into *Saccharomyces cerevisiae* strain Y187 to form the bait strain. An Arabidopsis DNA library from the roots of ecotype Col-0 before and 10 days after infection with *H. schachtii* was generated in *S. cerevisiae* strain Y2H-Gold (AH109) and fused to the GAL4 activation domain (AD) of pGADT7-Rec2 vector. Screening for interacting proteins and subsequent analyses were performed according to the Clontech protocols.

4.3.5 BiFC

In order to confirm the interaction of $HsCysL1^{-SP}$ with the candidate proteins yielded from Y2H screening, DNA sequences of $HsCysL1^{-SP}$, $HsCysL1^{+SP}$, UBC19 and

PTPLA were amplified with modified primers to conduct cloning into split-YFP vectors pSAT4-nEYFP-C1 and pSAT4-cEYFP-C1(B), respectively. Including HsCysL1^{+SP} should confirm the specificity of the protein interaction between HsCysL1^{-SP} and the target genes. *Hind*III restriction site including additionally random basepairs was attached to the 5´end of both HsCysL1 constructs, and *BamH*I to the 3´end (**ST1**, number 5 (+SP) and 6 (-SP)). Appropriate enzymes were used to digest the gel-purified and modified DNA fragments as well as pSAT4-nEYFP-C1 and ligated via T4 ligase. The same procedure was carried out with DNA of UBC19 and PTPLA, as well as pSAT4-cEYFP-C1(B), using *Sal*I and *Xma*I restriction sites (**ST1**, number 7 and 8). Afterwards, plasmid constructs were co-bombarded at leek cells according to the aforementioned protocol. Particle bombardment of each individual plasmid separately served as the negative control. Meanwhile, plasmids were transformed into *A. tumefaciens* strain GV3101 and infiltrated into *N. benthamiana* using the same method previously mentioned. In case of positive protein interaction, signals were observed using confocal microscopy 5 days after infection.

4.3.6 Functional characterization of target genes

To estimate the importance of the identified interacting protein PTPLA during nematode infection, an Arabidopsis knock-out mutant of this gene was infected with *H. schachtii* J2s and the number of females and the sizes of the associated syncytium were both determined. The procedure of this infection assay was conducted using the same method as described in Hütten et al. [39]. Genotyping and expression analysis was conducted by PCR using genomic and cDNA, respectively (**ST1**, number 9 and 10) to confirm that the genome of the described knock-out mutant of PTPLA contains a T-DNA insertion and therefore no expression of the gene on the proteomic level. However, for the second identified interacting protein UBC19, however, no mutant was available.

4.4 Results

4.4.1 Sugar beet cyst nematodes encode a cystatin-like protein

We have recently performed Activity Based Protein Profiling (ABPP) and found that activity of several PLCPs is reduced in syncytium as compared to non-infected roots [c.f. chapter 3]. Based on these data and previous literature [22, 36-38], we assumed a putative cystatin-like effector in *H. schachtii* with supposed functions similar to

those described of *P. infestans* (EPIC1 and EPIC2B). Bioinformatic approaches identified a cystatin-like transcript (396bp) in the transcriptome of *H. schachtii* that encodes for a protein of 132 amino acids, hereafter referred to as HsCysL1 (*Heterodera schachtii* Cystatin-Like-1). Next, we performed sequence alignment through a phylogenetic tree comparing HsCysL1 from plant-parasitic, free-living and animal-parasitic nematodes for their homology. This analysis indicates that HsCysL1-like protein forms a separate cluster with other plant-parasitic nematodes (PPN-specific cluster) (**Figure 1A**). Interestingly, within the PPN-specific cluster, HsCysL1-like proteins from cyst nematodes (*H. schachtii*, *H. glycines*, *G. pallidae* and *G. rostochiensis*) were the closest to each other, forming a cyst nematode specific subcluster (CN-specific cluster), whereas *Heterodera spp*. and *Globodera spp*. subclustered independently. The nearest likelihood to the CN-specific cluster was found in *Pratylenchus coffeae* followed by *Nacobbus aberrans*. Although a homologue of HsCysL1 was also found in *Meloidogyne hapla* (MhA1_Contig41.frz3.gene10), we did not include this gene in our data analysis due to the lack of a signal peptide. Transcriptomes of *M. incognita* and *H. avenae* did not reveal any HsCysL1-like homologues. Animal Parasitic Nematodes (APNs) and Free Living Nematodes (FLNs) were clustered separately from PPNs and showed relatively low similarity to HsCysL1.

HsCysL1 contains a signal peptide at the N-terminus, but no transmembrane domain, which supports the hypothesis that it is secreted into the host to facilitate parasitism. Similar to the homologues EPIC1 and EPIC2B from *P. infestans*, conserved amino acid residues being characteristic for the inhibitory activity of phytocystatins can also be found within the CN-specific cluster including *H. schachtii* and *H. glycines*: glycine (G) is located near the N-terminus (114 bp) and an active site QxVxG motif is present within the sequence (**Figure 1B**). Interestingly, only HsCysL1 lacks a conserved tryptophan (W) in the second half of the protein. However, we assumed this protein as a promising candidate for the inhibition of PLCPs.

4.4.2 HsCysL1 is secreted into the host tissues

One of the hallmark characteristics of parasitism genes is their spatial and temporal expression in the secretory oesophageal gland cells of the nematode. We used in-situ hybridization to localize the spatial expression of HsCysL1 transcript in pre-infective J2 nematodes. The antisense riboprobe of HsCysL1 labelled with

digoxigenin hybridized particularly strongly within the oesophageal gland cells (**Figure 2A**) supporting the idea of HsCysL1 being a secreted protein. However, a signal specifying the expression either in the dorsal or the subventral gland cells could not be observed. Based on the presence of cystatin-like motifs, we expected HsCysL1 to be involved in inhibition of PLCPs, and therefore to be specifically expressed during migratory stages of the nematode to supress early plant defence responses. Although quantitative RT-PCR revealed an expression of HsCysL1 during all developmental stages analysed in this study (J2, 5dpi and 10dpi), a particularly high expression was observed at 5dpi (**Figure 2B**). At 5dpi, syncytium is already established and nematode has started feeding. Therefore, a particular high expression at this time point hints to an additional role for HsCysL1 in parasitism other than PLCPs suppression. The effectors that are directly or indirectly involved in maintaining nurse cell systems are thought to be injected into the cytoplasm of syncytium via stylet [48, 49]. PLCPs instead are known to function within the apoplast [24, 31, 50]. Accordingly, we performed *in planta* localization to get insights into the destination and putative function of HsCysL1.

4.4.3 HsCysL1 shows diverse localization in plant

We generated constructs fusing HsCysL1 gene with or without signal peptide to GFP (green fluorescent protein) under the control of the CaMV 35S promoter (35S::HsCysL1^{+SP}_GFP; 35S::HsCysL1^{-SP}_GFP). Although it is expected that the signal peptide of HsCysL1 gets cleaved off in the endoplasmatic reticulum of the dorsal gland cell prior to secretion from the nematode stylet, inclusion of the signal peptide expressing a pre-protein was expected to be helpful to analyse the functionality of the signal peptide and the translocation through the plant´s secretory pathway. These constructs were then infiltrated into epidermal cells of *Nicotiana benthamiana* to detect their sub-cellular localization. Due to the signal peptide we expected an apoplastic localization of 35S::HsCysL1^{+SP}_GFP. Indeed, co-transformation of 35S::HsCysL1^{+SP}_GFP with mCherry-apoplastic marker showed a clear co-localisation of the signal in the outer periphery of the cells (**Figure 3A**), confirming that the nematode´s origin signal peptide can be processed through plant´s secretory pathway. In contrast, GFP-HsCysL1 without signal peptide (35S::HsCysL1^{-SP}_GFP) was localized in cytoplasm as well as in nucleus (**Figure 3B**). The presence of a strong signal inside the nucleus for 35S::HsCysL1^{-SP}_GFP

raises the question whether this protein in localized into nucleus due to passive diffusion of by using plant trafficking machinery or due to the presence of a nuclear-localization-sequence (NLS) found at C-terminal region of HsCysL1 (**Figure 3C**). To confirm the utility of this NLS, we generated a fusion protein lacking NLS (35S::HsCysL1^{-SP}-NLS_GFP). Nevertheless, our microscopic analyses found that nuclear signal could still be detected for HsCysL1 (**Figure 3D**). This result indicates that the nuclear signal observed in 35S::HsCysL1^{-SP}_GFP may arise from passive diffusion of the tagged GFP, as the predicted protein size (38 kDa) does not cross the nuclear exclusion size of 60 kDa [51]. These observations were further confirmed by bombarding GFP-tagged 35S::HsCysL1^{+SP} and 35S::HsCysL1^{-SP} on leek cells (**Figure 3E** and **3F**). Based on our data indicating dual localization of HsCysL1 during different staged of parasitism, we speculated that HsCysL1 might have obtained diverse roles during plant-nematode interaction.

4.4.4 HsCysL1 interacts with PTPLA and UBC19

To investigate the potential interacting partners of HsCysL1^{-SP} inside the host cell, we performed a Yeast-2-Hybrid (Y2H) analysis. Around 14 million colonies of an *Arabidopsis* cDNA library which was generated from non-infected roots and syncytia (10 days after *H. schachtii* infection) were screened using HsCysL1^{-SP} as bait.

After selection on high-stringency medium and exclusion of false positive proteins interacting with the empty prey vector we identified five target proteins (**Table 1**) interacting with HsCysL1^{-SP} in yeast. Considering that interaction between proteins can just take place once they are in the same cell compartment, we excluded ER-ANT1 and PGL5, which are localized in the ER and chloroplast, respectively. These interactions of the remaining three target genes were further confirmed by yeast co-transformation analysis **(Figure 4A-C**), showing a strong interaction of OTU2 with the empty prey vector (**Figure 4C**). This way, we ended up with two target genes interacting with HsCysL1^{-SP}, PTPLA and UBC19.

To further confirm the identified target genes as true positives interacting with HsCysL1^{-SP}, Bimolecular Fluorescence Complementation (BiFC) was performed. BiFC is based on the reconstitution of a split yellow fluorescence protein (split-YFP) independently attached to two proteins. In case these proteins interact with each other the YFP gets recombined and a fluorescent signal can be detected (**Figure**

5A). HsCysL1^{+SP} was included in this experiment to underscore the specificity of protein interactions. Both constructs, HsCysL1^{+SP} and HsCysL1^{-SP}, were independently fused to the C-terminal half of yellow fluorescent protein (YFP), whereas PTPLA and UBC19 were fused to the N-terminal half, respectively. Via co-bombardment of one C-terminal construct and one N-terminal construct, the activity of YFP was reconstituted in leek cells and confirmed the interaction between HsCysL^{-SP} and PTPLA as well as UBC19 through cytoplasmic expression (**Figure 5B**). However, no interaction was observed between HsCysL1^{+SP} and both target genes (**Figure 5C**). Furthermore, no YFP signal was obtained when the YFP fragment constructs harbouring HsCysL1$^{+/-SP}$ or PTPLA/UBC19 alone (**Figure 5D and 5E**).

Additionally, all BiFC vector constructs were transformed into *A. tumefaciens* and co-expressed in *N. benthamiana*. YFP signals were observed in cytoplasm when HsCysL1^{-SP} was co-infiltrated with PTPLA and UBC19 (**Figure 5F**). In case of HsCysL1^{+SP} no interaction between our gene of interest (GOI) and target genes could be obtained (**Figure 5G**), not either in YFP fragment constructs harbouring one of the genes alone (**Figure 5H** and **5I**).

4.4.5 Knock-out of PTPLA does not affect nematode development

To functionally characterize the identified target genes with regard to their importance in plant-nematode interaction, we conducted a nematode infection assay using Arabidopsis single-knockout mutants and infective J2s of *H. schachtii*. However, a knockout line was only available for PTPLA. The T-DNA insertion is located in the intron region (**Figure 6A**) and homozygosity was confirmed by PCR (**Figure 6B**). Additionally, no expression could be detected (**Figure 6C**). The number of females and males as well as sizes of females and associated syncytium should give information about changed susceptibility of the transgenic lines compared to wildtype plants. As indicated in **Figure 6D and 6E**, no significant change could be observed in the infection rate or nematode development.

4.5 Discussion

Papain-like cysteine proteases (PLCPs) have been shown to play diverse roles in plants. Beside protein remobilization during seed germination and organ senescence, they are also essentially involved in plant-pathogen interactions [18, 24, 25]. In

contrast to other proteolytic enzymes, PLCPs possess the peculiar characteristic of being reversibly inhibited by natural peptides like cystatins [52]. Previously, Tian et al. [37] described the protein EPIC2B of *P. infestans*, which possesses all signature sequences of cystatin-like protease inhibitors, as an inhibitor of PIP1 and other apoplastic cysteine proteases in tomato. In this study we biochemically and functionally characterized a putative effector protein of *H. schachtii*, termed HsCysL1, which was identified based on bioinformatic approaches. HsCysL1 does contain a signal peptide but no predicted transmembrane domain, allowing the protein to be translocated through the nematode´s secretory pathway into host cells. Furthermore, the cystatin motifs present in the sequence of HsCysL1 suggested that HsCysL1 inhibits cysteine proteases and therefore plays a role in inhibiting plant defences. The phylogenetic tree (**Figure 1A**) reveals that HsCysL1-like homologues are uniquely found in plant-parasitic nematodes, whereas the closed likelihood is shown within cyst nematodes. Interestingly, the homologues of *Heterodera spp*. and *Globodera spp*. subclustered separately, hinting at an essential functional specialization for parasitism strategy of cyst nematodes.

The presence of a signal peptide and lack of transmembrane domain characterizes HsCysL1 as a putative effector protein. *In-situ* hybridization further validated the secretion of HsCysL1 as the hybridization signal was localized in the secretory oesophageal gland cells of pre-infective J2s. Because PLCPs have been shown to constitute one of the main plant defence proteins in the apoplast, we assumed HsCysL1 to be pre-dominantly expressed during the migratory stage of infection process. Contrary to our expectations, HsCysL1 is expressed during all developmental stages that were tested in this study (J2, 5 dpi, 10 dpi), showing highest transcriptional expression at 5dpi. This time point represents the syncytium expansion stage [53], which is characterized by massive restructuring of the host cells. These results suggest that although HsCysL1 has cystatin motifs, it may perform an additional function other than inhibiting PLCPs.

We propose that HyCysL1 may perform diverse roles during the different phases of infections. At the migratory stage of infection, nematode invasion and movement inside the root causes damage and activates PLCPs. HsCysL1 is secreted into the apoplast and inhibits the activity of PLCPs, thereby suppressing the activation of plant defence responses. To confirm this role of HsCysL1, we attempted to express

and purify HsCysL1 heterologously in bacteria as well as in planta. The purified HsCysL1 could then have been tested to confirm the protease inhibitory activity *in vitro*. However, we were unable to purify sufficient amount of HyCysL1 for such assays. Alternatively, ABPP, using the proteome of Arabidopsis plants overexpressing HsCysL1, could also provide information about a putative inhibitory effect of HsCysL1. These experiments are currently underway.

In the second phase of nematode infection during syncytium establishment and expansion, HsCysL1 is assumed to be secreted into the cytoplasm [48, 49], and play a role in syncytium formation and maintenance. To validate this hypothesis, first of all we conducted localization of HsCysL1 with or without signal peptide *in planta*. Interestingly, including the signal peptide resulted in a translocation of HsCysL1^{+SP} into the apoplast, whereas the construct without signal peptide led to localization of HsCysL1 in the cytoplasm and nucleus. The nuclear expression was expected to result from a nuclear-localisation-sequence (NLS) uniquely identified in HsCysL1. This NLS could not be found in any homologues of other nematode species, hinting at a high specificity of this effector. However, removal of the NLS from HsCysL1^{-SP} did not result in elimination of nuclear expression. These data suggested that the nuclear signal might have arisen from passive diffusion of the fused GFP-protein, as the predicted size of the fusion protein (38 kDa) was smaller than the nuclear exclusion size (60 kDa) [51]. Another possibility is that HsCysL1^{-SP} forms a protein complex with an interacting partner that leads to its transport into the nucleus.

Functional data presented here show that HsCysL1^{-SP} interacts with two proteins in cytoplasm, PTPLA (Protein tyrosine phosphatase-like A) and UBC19 (Ubiquitin-conjugating enzyme 19). Both proteins are known to play significant roles in various signalling and regulatory processes through dephosphorylation and ubiquitination of proteins, respectively [54]. Phosphorylation and ubiquitination have been identified as fundamental posttranslational modification processes controlling immune signalling pathways [55-58], which underscores the importance of these targets to be modulated by pathogens´ effectors for successful parasitism.

In line with localization studies of HsCysL1^{-SP}, the interaction between HsCysL1^{-SP} and PTPLA could only be confirmed in cytoplasm, although PTPLA is assumed to be located in both, cytoplasm and nucleus, based on a SV40-like NLS [59, 60]. It is possible that HsCysL1 interacts with PTPLAs to promote their translocation into the

host´s nucleus: recently, it has been reported that dual specificity phosphatases [54, 61], as well as Ser/Thr phosphatases, can dephosphorylate and inactivate stress-activated mitogen-activated protein kinases (MAPKs) in plants [62, 63]. Even though, to date, only a single tyrosine-specific protein phosphatase (AtPTP1) dephosphorylating AtMPK4 has been characterized in plants [64, 65], the role of this phosphatase family should not be underestimated, especially since the role of dual-specificity PTPs and PTPs in the inactivation of MAPKs has been widely reported in mammals [66, 67]. In Arabidopsis, MPK3 and MPK6 are positive regulators of plant defence responses and essential for resistance against several pathogens like *Botrytis cinerea* [68, 69] or *H. schachtii* [70] by inducing cell signalling cascades. Interestingly, during nematode migration inside the host plant and selection of the ISC, the host MAPK phosphatase AP2C1 is induced and plants lacking the gene of AP2C1 significantly reduced the susceptibility of *A. thaliana* towards *H. schachtii* [70], indicating that nematodes may enhance host phosphatase expression during the infection process to minimize MAPK activities, and therefore, signalling cascades within the plant. Interaction of MAPKs and AP2C3 has been shown to take place in the nucleus of host cells [62]. It could therefore be guessed, that HsCysL1 promotes the translocation of PTPLA into the host´s nucleus to affect MAPK-like proteins and thereby inactivate host´s signalling cascades. Indeed, detailed information about the molecular function of PTPLA in plants so far remains unknown. Regardless of biological function of HsCysL1-PTPLA interaction, depletion of PTPLA in Arabidopsis did not affect the susceptibility of the plant against *H. schachtii*, which is likely due to functional redundancy in PTPLA gene family in Arabidopsis. This would imply that there are other PTPLs present in Arabidopsis, which are not analysed so far, which are also able to interact with HsCysL1 and are therefore able to compensate for the function of PTPLA. Regarding this complex interaction, it would be worthwhile to analyse the susceptibility of the plant against *H. schachtii* after silencing HsCysL1. Based on the assumption that HsCysL1 could be involved in suppression of signalling cascades, lack of this putative effector would be expected to result in lower nematode infection. However, soaking nematodes in dsRNA of HsCysL1 did not result in reduced gene expression. Other specific silencing methods like small interfering RNA (siRNA) should still be used in future, to enable a better estimation of the function of HsCysL1.

UBC19 is known to act as an ubiquitin conjugating enzyme (E2-C), which seems to be specifically involved in cyclin B degradation, a regulatory protein playing an essential role during the mitotic cell cycle [71-73]. Cyclin B forms complexes with specific cyclin-dependent kinases (CDKs) and is known to be expressed during late G2-to-M transition, initiating the mitosis process. At the same time, destruction of cyclin B is essential to the progress of the cell to leave mitosis by affecting chromosome decondensation, nuclear envelope reformation and cytokinesis [74, 75]. Previously, studies showed that sister chromatid separation, rather than the inactivation of cyclinB/CDK complex, induces the transition between metaphase and anaphase [76, 77]. However, it was deduced that the same machinery being responsible for cyclin B destruction also destroys the linkage of sister chromatids [76], suggesting that both processes, cyclin B degradation and sister chromatid separation, are required for the exit from mitosis into G1 of the next cell cycle [78]. Inhibition of the ubiquitination machinery responsible for the degradation of cyclin B would arrest the cell in mitosis, resulting in DNA duplication without cytokinesis (endoreduplication). A human homolog of E2-C, termed UbcH10, has been shown to block cyclin ubiquitination and cause the destruction of one or more proteins responsible for sister chromatin separation, which causes endoreduplication in cells [71]. UBC19 is highly expressed in dividing cells and substitutes the function of UbcP4 in yeast [73], which is known to regulate both a G2/M and a metaphase/anaphase cell cycle progress [79, 80], accentuating the importance of UBC19 during the cell cycle and explaining the unavailability of knockout-lines in Arabidopsis.

Endoreduplication is a well-accepted phenomenon in giant cells and syncytia, generating numbers of enlarged nuclei to sustain the enhanced metabolic activity in feeding cells [81, 82]. Increased expression of CDK-inhibitory Kip-related proteins (KRPs) [84] or the upregulation of anaphase-promoting complex (APC) components like CCS52A [84], which degrades cyclin B before entry into the M-phase, have been identified as key regulators switching the mitotic to the endoreduplication cycle. Based on the present data, we hypothesise that HsCysL1 may inhibit UBC19 in syncytium, resulting in an accumulation of cyclin B and arresting of the cells in mitosis until late G2 without onset of anaphase. This hypothesis is supported by previous studies showing an accumulation of cyclin B, as well as an enhanced DNA synthesis and enlarged nuclei in syncytium [85, 86]. Furthermore, an increased cell

division activity could also be observed in neighbouring cells of syncytia [87, 88], whereas no mitosis was observed in syncytia themselves [89]. Interestingly, an enhanced expression of cyclin B shifts from the initial syncytial cell during early stages to the cells surrounding the syncytium at later stages of syncytium development [90]. Maybe in addition to our previous hypothesis we therefore assume that an inhibition of UBC19 by HsCysL1 could also induce a higher mitotic activity in surrounding cells that are supposed to be incorporated during syncytium development.

4.6 Conclusion

Concluding our data we were able to identify a putative effector protein from *H. schachtii*, termed HsCysL1 that is uniquely found in plant-parasitic nematodes and has closest homology within cyst nematodes, highlighting the putative specificity of this effector in nematode parasitism. HsCysL1 is expressed in the secretory oesophageal gland cells and shows inhibitory involvement in plant defence and signalling. Expressed at different time points during syncytium establishment, HsCysL1 might influence signalling pathways and induces metabolic activity in syncytia and surrounding cells by interacting with PTPLA and UBC19, respectively. The presence of NLS and the depletion of the cystatin-motif tryptophan in the second half of the protein uniquely found in *H. schachtii* might significantly influence the multifunctionality of this effector protein and enable host-specific infection. However, evidences are missing so far and further analyses need to be conducted regarding the activation or inactivation of the target proteins in host cells and the detailed molecular function of these modifications by characterizing Arabidopsis plants overexpressing HsCysL1. Furthermore, putative inhibition of PLCPs in apoplast during the migratory stage should be examined through ABPP of Arabidopsis plants overexpressing HsCysL1^{+SP}, which would hint to a dual function of HsCysL1.

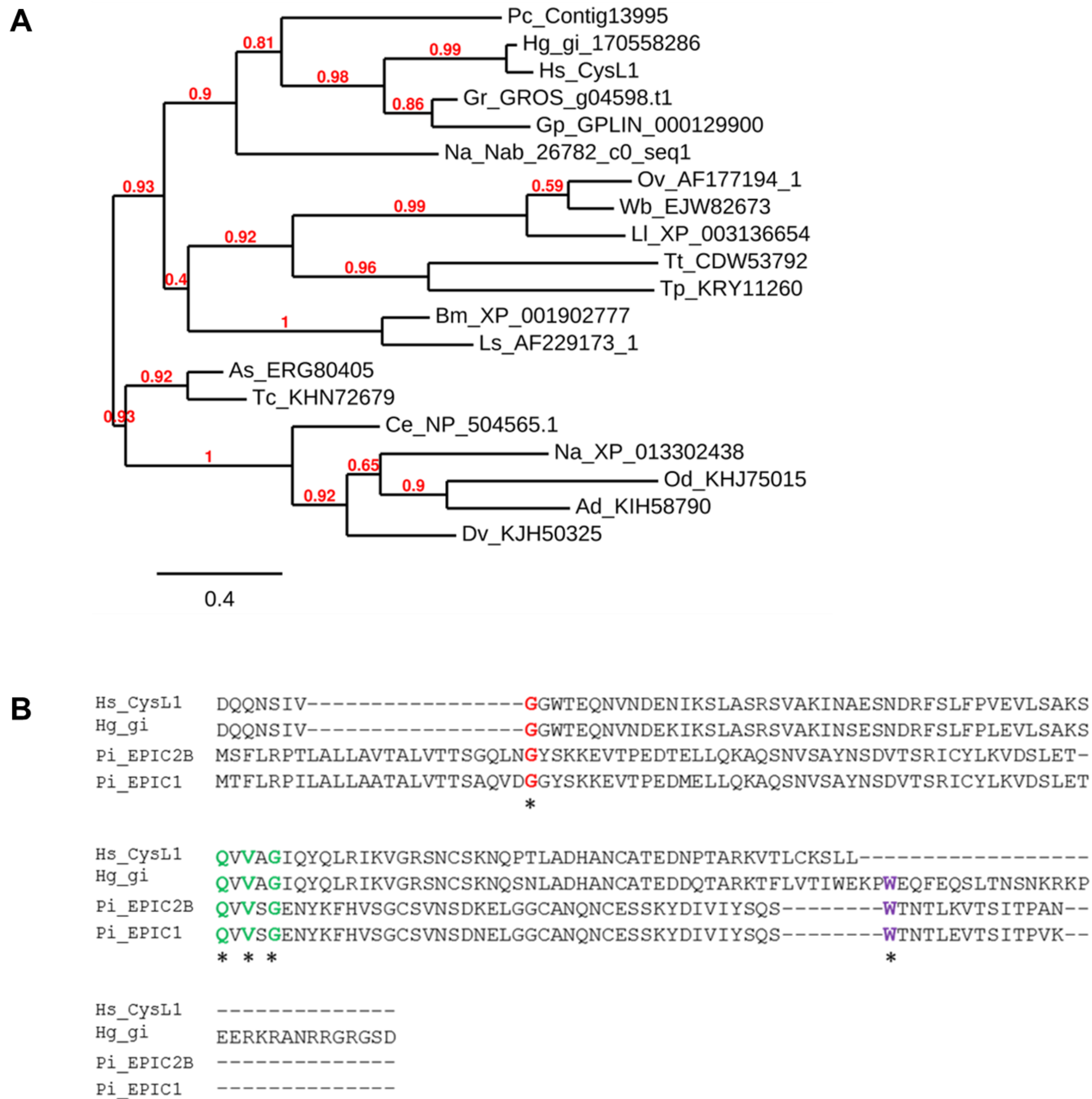

Figure 1: (A) Phylogenetic tree showing HsCysL1-like homologues in different nematode species. The tree was generated as described in "Material and Methods". The scale bar indicates 40% weighted sequence divergence. Pc (*Pratylenchus coffeae*); Hg (*Heterodera glycines*); Hs (*Heterodera schachtii*); Gr (*Globodera rostochiensis*); Gp (*Globodera pallida*); Na_Nab (*Nacobbus aberrans*); Ov (*Onchocerca volvulus*); Wb (*Wuchereria bancrofti*); Ll (*Loa loa*); Tt (*Trichuris trichiura*); Tp (*Trichinella patagoniensis*); Bm (*Burgia malayi*); Ls (*Litomosoides sigmodontis*); As (*Ascaris suum*); Tc (*Toxocara canis*); Ce (*Caenorhabditis elegans*); Na_XP (*Necator americanus*); Od (*Oesophagostomum dentatum*); Ad (*Ancylostoma duodenale*); Dv (*Dictyocaulus viviparus*); **(B)** Sequence alignment of HsCysL1 with homologue sequence of *H. glycines* as well as *P. infestans* EPIC2B and EPIC1. Amino acid residues that are characteristic for cystatins are marked with asterisks.

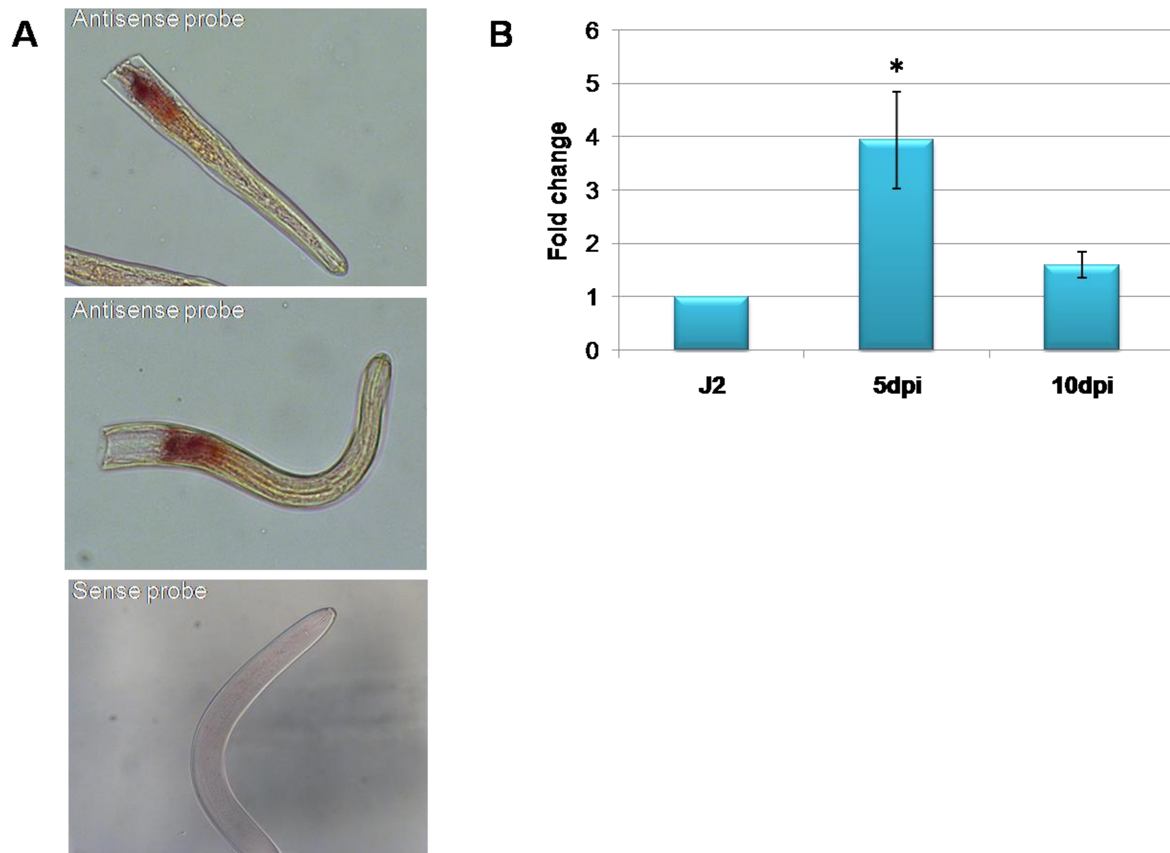

Figure 2: (A) *In situ* hybridization showing localization of HsCysL1 in J2 of *H. schachtii*. **(B)** Quantitative RT-PCR revealing the expression of HsCysL1 during different developmental stages of *H. schachtii*. Asterisk indicates statistically significance when using P-value<0.05 as threshold (Student´s t-test). Bars represent relative fold change with standard error of mean.

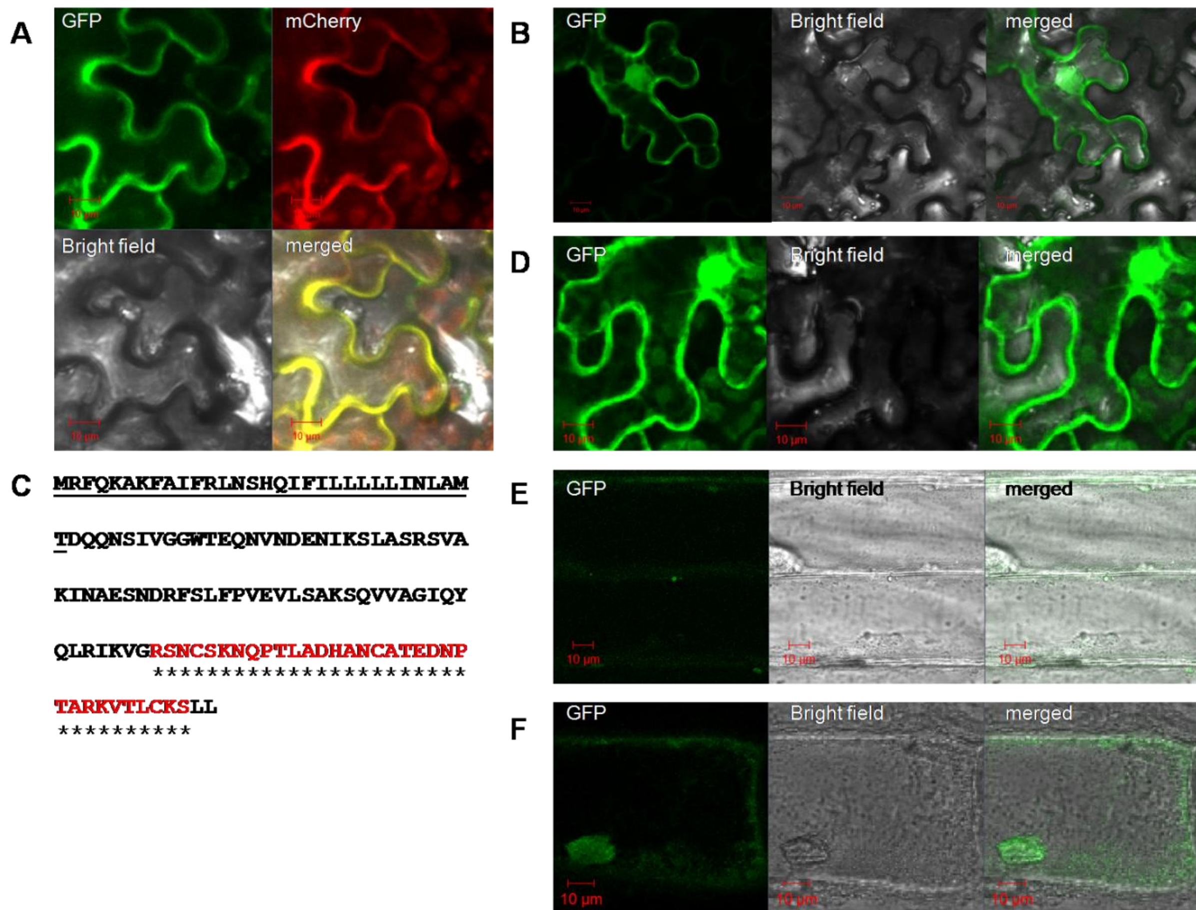

Figure 3: Confocal pictures showing **(A)** the *A. tumefaciens* mediated transient expression of 35S::HsCysL1^{+SP}_GFP that revealed apoplastic expression overlapping with mCherry-apoplastic marker in *N. benthamiana* leaves and **(B)** the *A. tumefaciens* mediated transient expression of 35S::HsCysL1^{-SP}_GFP that reveals cytoplasmic and nuclear expression in *N. benthamiana* leaves. **(C)** Amino acid sequence of HsCysL1^{+SP} indicating the presence of a predicted NLS; signal peptide is underlined, the predicted NLS is highlighted in red and by asterisks. **(D)** Removal of NLS from HsCysL1^{-SP} still resulted in cytoplasmic and nuclear expression of 35S::HsCysL1^{-SP}_NLS_GFP in tobacco leaves. **(E-F)** Confocal pictures confirming the apoplastic localization of 35S::HsCysL1^{+SP}_GFP **(E)** and 35S::HsCysL1^{-SP}_GFP **(F)** in leek. Bars=10µm.

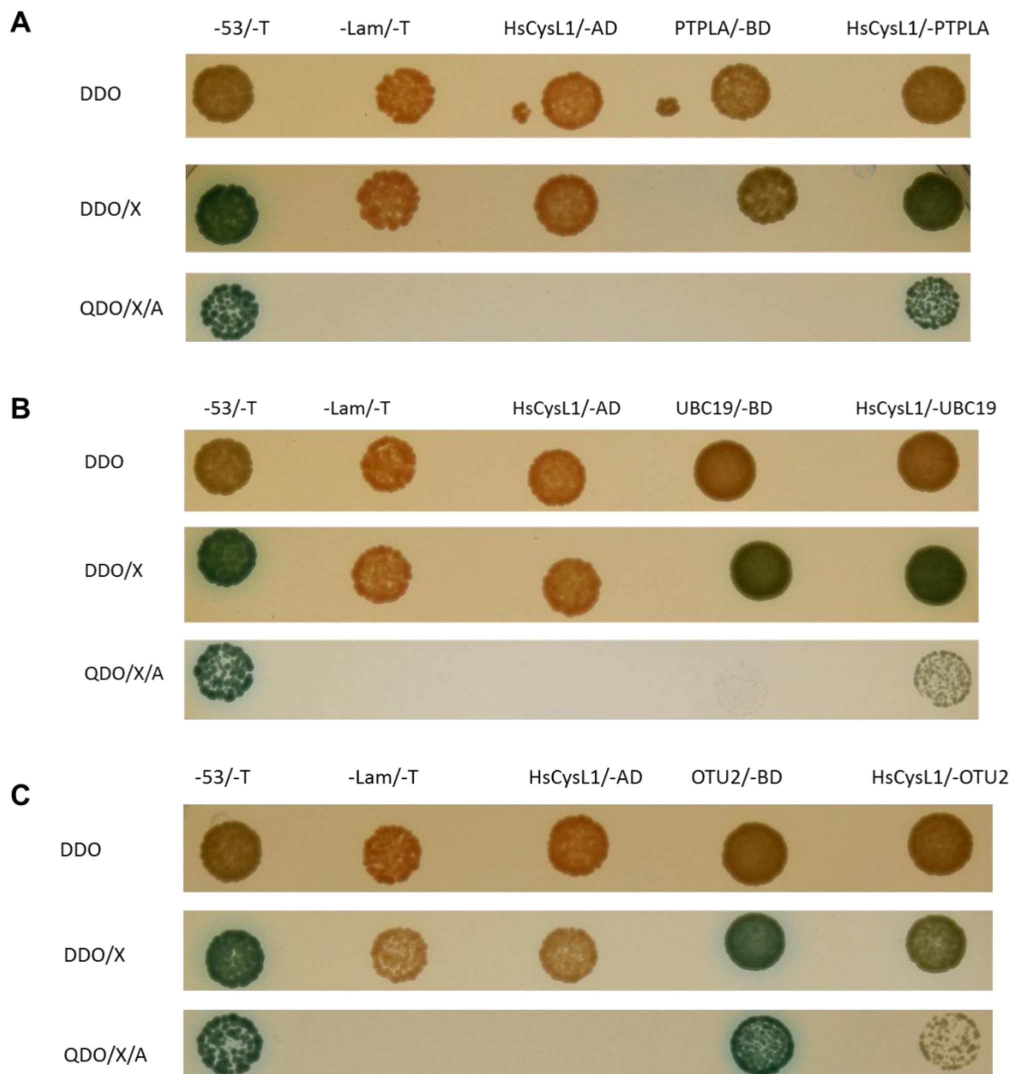

Figure 4: Interaction of HsCysL1 with three target genes of *Arabidopsis thaliana* (HsCysL1/PTPLA **(A)**, HsCysL1/UBC19 **(B)** and HsCysL1/OTU2 **(C)**) in yeast identified by Yeast-2-Hybrid screening. Murine p53 fused to binding domain (BD) interacts with T-antigen fused to activation domain (AD) and served as positive control (-53/-T). Lamin and T-antigen were used for negative control (-Lam/-T). Both, negative and positive control, are shown in each set **(A-C).** HsCysL1 as well as all three target genes were co-transformed with the empty vector of the corresponding interacting partner (HsCysL1/-AD **(A-C)**; PTPLA/-BD **(A)**; UBC19/-BD **(B)**; OTU2/-BD **(C)**). Growth on low stringency medium (DDO) selects for expression of AD and BD constructs. In case of positive protein interaction α-galactosidase is encoded resulting in blue colonies in presence of X-α-Gal (DDO/X). Growth of all strains on high stringency medium including X-α-Gal and Aureobasidin (QDO/X/A) indicates interaction of the co-expressed proteins through blue coloured colonies that are able to grow because of induced encoding of aureobasidin resistance.

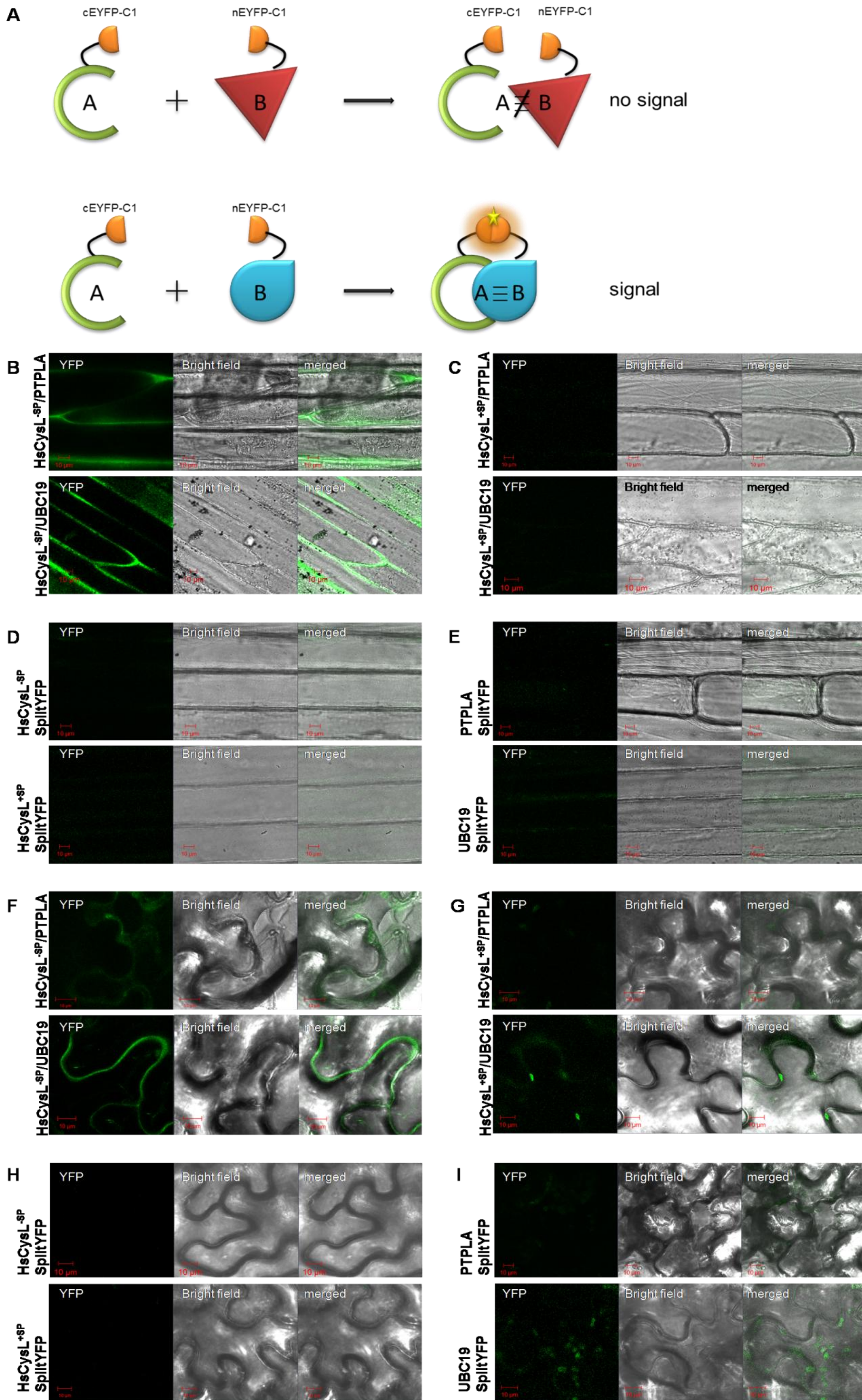
A
cEYFP-C1
nEYFP-C1
A
B
no signal
signal
B
HsCysL-SP/PTPLA
HsCysL-SP/UBC19
YFP
Bright field
merged
C
HsCysL+SP/PTPLA
HsCysL+SP/UBC19
D
HsCysL-SP SplitYFP
HsCysL+SP SplitYFP
E
PTPLA SplitYFP
UBC19 SplitYFP
F
G
H
I
10 μm

Figure 5: (A) Schematic illustration of the BiFC principle. **(B-E)** Confocal pictures showing BiFC results of HsCysL1^{-SP}**(B)** and HsCysL1^{+SP}**(C)** with both interacting partners, PTPLA (upper panel) and UBC19 (lower panel) in leek cells. SplitYFP-constructs of HsCysL1^{-SP} (**D**, upper panel), HsCysL1^{+SP} (**D**, lower panel), PTPLA (**E**, upper panel) and UBC19 (**E**, lower panel) were used to exclude autofluorescence. Same constructs were used to conduct BiFC in *N. benthamiana* leaves **(F-I)**. Bars = 10µm.

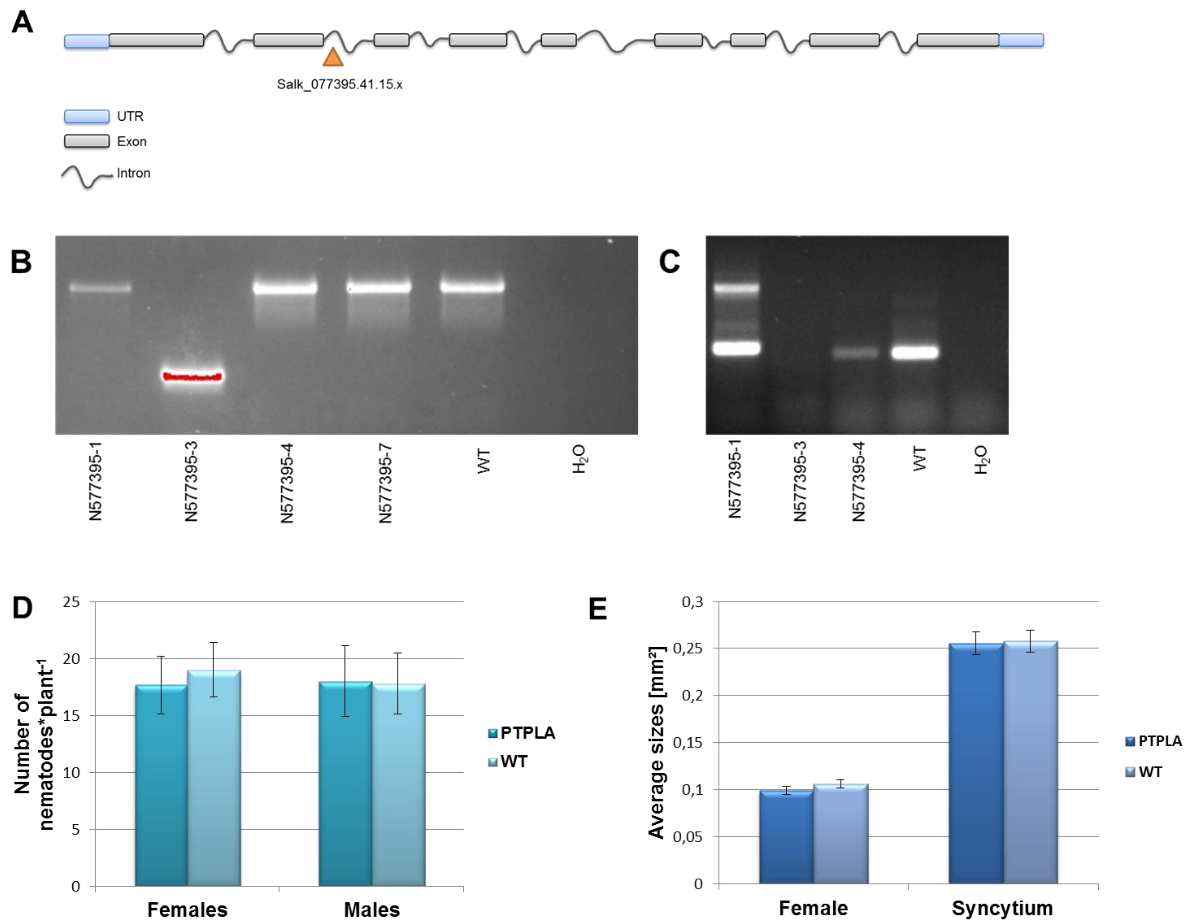

Figure 6: (A) Schematic illustration of PTPLA single-knockout line of *A. thaliana* (Salk_077395.41.15.x). Orange triangle indicates the location of T-DNA insertion. **(B-C)** Genotypic characterization of PTPLA single-knockout line N577395 **(B)** and expression analysis **(C)** of transgenic (PTPLA) compared to wildtype (WT) plants of *A. thaliana* Col-0. **(D-E)** Infection assay of PTPLA single-knockout line compared to wildtype (WT) indicating the average number of females and males per plant **(D)** as well as the average sizes of females and associated syncytia **(E)**. Bars are shown with standard error of mean.

Table 1: Putative interacting targets of HsCysL1 identified through Yeast-2-Hybrid screening.

Name	Gene	Localization in plant
ER-ANT1	At5g17400	Endoplasmatic reticulum
OTU2	At1g50670	Nucleus, Cytoplasm
PGL5	At5g24420	Chloropast
PTPLA	At5g59770	Cytoplasm
UBC19	At3g20060	Cytoplasm

ST1: Primer sequences used during experiments conducted in presented work.

Number	Forward primer sequence	Reverse primer sequence	Used for…
1	GAGCAAAATGTGAACGACGA	CCAAGGTTGGCTGATTCTTC	Expression in J2
2	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCATGCGT TTTCAAAAAGCCAA	GGGGACCACTTTGTACAAGAAA GCTGGGTCCAGTAATGATTTGC ACAGTG	HyCysL1^{+SP} in pDONR207 for pMDC83
3	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGATCAG CAGAATTCAATTGT	GGGGACCACTTTGTACAAGAAA GCTGGGTCCAGTAATGATTTGC ACAGTG	HsCysL1^{-SP} in pDOR207 for pMDC83
4	CTAGCTTCAAGCTTATGCGTTTTCAAAAAG	ACGCCTGGGATCCCTACAGTAA TGATTTGCA	BiFC-HsCysL1^{+SP}
5	CATGCTTCAAGCTTATGGATCAGCAGAATTC	ACGCCTGGGATCCCTACAGTAA TGATTTGCA	BiFC-HsCysL1^{-SP}
6	ACTAGCTTCGTCGACATGGCCACACAAAG	ACTGTATCGCCCGGGTCACATT CTCTTTCTCT	BiFC-PTPLA
7	ACTAGCTTCGTCGACATGGCGACGGTTAATGGG	ACTGTATCGCCCGGGTCATGC GTTTAAAGGCT	BiFC-UBC19
8	AGGTACACGGGATTCATCGT	TTCTTTAACATATGGAAGTGCTT	Genotyping PTPLA
9	ATTTTGCCGATTTCGGAAC	-	Genotyping PTPLA
10	TGCTGGATTCCTGATTAGTCTGT	TCTCTTGCCGTGAAGCTTCT	Expression check PTPLA
11	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCATGCGT TTTCAAAAAGCCAA	GGGGACCACTTTGTACAAGAAA GCTGGGTCCTACAGTAATGATT TGCACA	HsCysL1^{+SP} in pDONR207 for pMDC32
12	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCATGGAT CAGCAGAATTCAAT	GGGGACCACTTTGTACAAGAAA GCTGGGTCCTACAGTAATGATT TGCACA	HyCysL1^{-SP} in pDONR207 for pMDC32
13	GAGCAAAATGTGAACGACGA	CCAAGGTTGGCTGATTCTTC	HsCysL1$^{+/-SP}$ qRT-PCR for overexpression check

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5. Chapter 5

General discussion

As sedentary endoparasites, cyst nematodes remain inside the host root and feed from specifically induced feeding sites. Initiating this feeding site, while avoiding activation of host defence reactions, requires a dynamic process involving an active communication between the nematodes and their host plants. This communication between nematodes and their hosts is facilitated through the secretion of effector proteins synthesised in nematodes´ oesophageal glands. During the last decades, much effort has been investigated into studying the mechanism involved in induction of feeding cells by cyst nematodes. A number of effector proteins have been shown to be involved in reprogramming of the host cell on ultrastructural [1],transciptomic [2-4], metabolomic [5] and proteomic [6, 7] levels. Nevertheless, due to lack of technology, posttranslational modification, a process that substantially regulates the activity and therefore functionality of all cellular changes, could not be considered previously. Posttranslational modifications occur during or after the protein biosynthesis and include processes such as changes in pH or dephosphorylation, glycolisation and lipidation.

Activity-based protein profiling (ABPP) analysis is a technology that identifies changes in activity of enzyme classes within a complex proteome, thus revealing functional information, which is difficult to find from traditional transcriptomic or proteomic data. ABPP uses small reactive probes, that are biotinylated or fluorescent tagged and react irreversibly with active site residues of proteins in an activity-dependent manner. This way, only active proteins can be visualized as independent from their transcriptomic abundance [8]. Invented by Cravatt and Bogyo and co-workers [9, 10] and comprehensively used in medical science [9-15], ABPP was introduced into plant science few years ago [16] and has already provided a wealth of information regarding plant-pathogen interaction. For example, this technology made it possible to demonstrate that the fungal AVR2 effector promotes susceptibility, not only for the biotrophic leaf mold fungus *Cladosporium fulvum*, but also for other pathogens like *Botrytis cinerea* and *Verticillium dahliae*, through the inhibition of several cysteine proteases required for plant basal defence [17]. Beside cysteine proteases, also other enzymes like serine hydrolases, vacuolar processing enzymes or proteasome subunits, being essential for the plant immunity system have been

shown to be affected by different virulent and avirulent factors [18-23]. However, changes of the active proteome during plant-nematode interaction remain yet unexplored. In this work, the impact of nematode infection on the activity of several host defence related proteins is displayed for the first time using ABPP. Furthermore, we described a putative effector protein found in *H. schachtii* that may not only alter protein activity observed during ABPP, but seem to play a surprisingly dual function in regulation of cell metabolism and suppressing signalling cascades.

5.1. Activity of vacuolar processing enzymes (VPEs) is reduced upon nematode infection

One of the main defence strategies employed of host plants is the induction of programmed cell death (PCD). Caspase activities are major mechanisms regulating the PCD. For example, in tomato, chemical-induced apoptosis induces caspase activity [24]; same effect was observed during bacterial infection in tobacco [25]. A vacuolar processing enzyme (VPE) was the first cysteine protease described in plants that exhibit caspase-1 activity and has been shown to be transiently activated in resistant tobacco leaves during tobacco mosaic virus (TMV) infection to induce cell death and confine the virus to a limited area [26]. Interestingly, silencing of VPE in these plants resulted in the suppression of the hypersensitive cell death leading to successful virus-infection. As obligate biotrophic organisms, plant-parasitic nematodes relay on living plant tissue and need to avoid the activation of the plant´s cell death machinery. The significant decrease of VPE activity in syncytia compared to non-infected roots, which we observed during ABPP, is therefore in line with previous findings [26-28], underscoring the importance of VPEs in plant basal immunity.

5.2. Serine hydrolases (SHs) are involved in metabolic processes during nematode infection

Changes of protein activity due to nematode infection were also observed within the serine hydrolase (SH) family in syncytium compared to non-infected roots. Serine hydrolases constitute one of the largest and most diverse enzyme families found in nature and are involved in many different physiological processes, including metabolism, development, and immunity [29-31]. Arabidopsis encodes hundreds of serine hydrolases that belong to dozens of large multigene families [32]. Although not

all SHs could be detected in our studies for technical reasons, we were able to highlight an increased activity in some interesting enzymes that may play essential roles during parasitism. S-formylglutathione hydrolase (SFGH) is an enzyme with a putative role in formaldehyde detoxification, a by-product of cellular metabolism of one-carbon compounds´ metabolism [33, 34]. Detoxification is therefore an essential mechanism to prevent cytotoxicity and maintain the vital function of host cells. During syncytium induction, metabolic activity in invaded cells is significantly altered as shown in different studies [1, 5, 35]. Through secretion of chorismate mutase for example, the invading nematode is able to regulate the synthesis of cellular aromatic amino acids and several secondary metabolites by influencing the shikimate pathway [35, 36]. Although the detailed function of SFGH in plants is not completely known, results given from bacteria and yeast [37, 38] indicate a similar detoxifying function of this protein in plants. Accordingly, an increased activity of SFGH in syncytium might play an essential role in the regulation of host´s metabolism for successful parasitism. Increased SFGH activity was also observed by Kaschani et al. [39] after infection of *pad3* mutant of Arabidopsis with *Botrytis cinerea*. *pad3* plants are deficient in camalexin production, a cytotoxin that usually protects the plants against the fungus. Even though *B. cinerea* is a necrotrophic fungus and does therefore not rely on living host tissue, infection by this fungus induces several metabolic changes in host tissues [40-42], which indeed supports the assumption of SFGH being involved in metabolic processes.

In addition to SFGH also methylesterases (MES) showed an increased activity in syncytia compared to non-infected roots after ABP profiling. MES are thought to play a regulatory role in plant signalling cascades since they hydrolase methylated phytohormones like indoleactic acid (IAA), salicylic acid (SA), jasmonic acid (JA) or ethylene (ET) [43, 44]. Interestingly, during interaction with other pathogens the activity of MES in susceptible plants was downregulated, indicating that phytohormone signalling within the host should be avoided during infection [39, 45]. Recently, Kammerhofer et al. [46] observed an increased biosynthesis of JA-related genes during nematode migration within the roots but no altered SA-expression during early nematode infection. Indeed, at later time points the expression of SA marker genes PR-1, PR-2, and PR-5 was up-regulated in infected roots, suggesting a role for SA during nutrition acquisition stages of infection [46, 47]. These observations are also in line with previous findings indicating that SA is a key

component in the defence against biotrophic pathogens [48], whereas the JA/ET pathway is mainly activated during necrotrophic parasitism or cell destruction [48, 49]. In addition to JA and SA, several studies have also shown a positive involvement of phytohormones, in particular IAA and ET, in attracting nematodes and establishing feeding sites [50-55]. Similar positive role for other growth promoting phytohormones like gibberellin (GA) or cytokinin (CK) in syncytium formation is also being suggested [56-58]. Taken together, phytohormones play diverse roles during plant-nematode interaction that have not yet been clarified to full extent. Based on our data and previous literature, we propose that increased MES activity is required to meet the increased demand of phytohormones, particularly those involved in growth promotion such as IAA during syncytium formation. However, a detailed functional analysis using loss-of-function and overexpression lines is required to shed light on the utility of MES´ exceptional increased activity in nematode-induced feeding cells.

5.3. The plant proteasome constitutes a defence mechanism that is circumvented by *H. schachtii*

As one of the main proteolytic degradation machineries of the plant, the 26S proteasome is involved in almost every cellular process, including the activation of defence response against pathogens [59]. The proteasome consists of a 20S core protease (CP) and a 19S regulatory particle (RP) and is located in the cytosol and nucleus [60]. Proteins that need to be degraded by the plant become ubiquitinated and accepted by the RP. The RP unfolds the proteins and transfers them into the CP, where three catalytic subunits (β_1, β_2, and β_5) are responsible for the degradation of the polypeptide chains into small peptide substrates of 3-20 amino acids [22, 61]. This ubiquitin/proteasome pathway appears to be involved in different steps of the phytohormone signalling cascades [62-65], but has also been shown to obtain catalytic RNAse activity [66, 67] implicating the proteasome in plant antiviral defence. Several virus movement proteins such as from *Tobacco mosaic virus* (TMV) [68], *Turnip yellow mosaic virus* (TYMV) [69] and *Potato leafroll virus* (PLRV) [70] have been shown to be degraded by the 26S proteasome pathway. Other pathogens would also initiate the proteasome activity due to host´s defence response and need to suppress it to promote infection. Strains of *Pseudomonas syringae* pv. *syringae* secrete syringolin A, which irreversibly inhibits all three catalytic subunits of the host´s proteasome [23, 71]. Contrary to viral and bacterial infection, information about

the proteasome activity during plant-nematode interaction is limited. A ubiquitin carboxyl extension protein from the potato cyst nematode *Globodera rostochiensis* (GrUBCEP12) is processed into free ubiquitin and a CEP12 peptide *in planta* [72]. Changes in the cellular ubiquitin level induce altered proteasome composition [73] and the suppression of RPN2a, a gene encoding a subunit of the 26S proteasome, in GrUBCEP12 overexpression lines also hints to a direct involvement of this effector protein in suppressing plant immunity by manipulating the functionality of the host 26S proteasome [72]. In resistant plants infected by *H. glycines* the expression of RPN2a is upregulated [74], leading to similar conclusions. According to our results from ABPP, also *H. schachtii* is able to suppress the proteasomal activity during syncytium establishment. While genes encoding for proteasomal subunits were shown to be upregulated in syncytium [2], the activity of β_1, β_2, and β_5 was reduced in syncytium shown by the vinyl sulfone (VS)-based probe MV151. Similar observations were made during exogenous application of a proteasome inhibitor in Arabidopsis leaves, which led to the accumulation of the proteasome subunit genes [75]. However, detailed functional knowledge about the involvement of proteasome in plant-nematode interaction as including involvement of effector proteins in the inactivation remains missing. Considering the diverse cellular processes in which the plant proteasome is involved, it is difficult to create mutations affecting one of the central players of the proteasome functions.

5.4. *Heterodera schachtii* suppresses several papain-like cysteine proteases (PLCPs) to enable infection

In addition to the proteasome, the probe MV151 is also able to target papain-like cysteine proteases (PLCPs) and therefore to provide information about their activity during different biological events. PLCPs constitute a big class of proteolytic enzymes in plants associated with different cellular processes such as development, senescence and immunity, whereas only a comparably small number of PLCPs has been described in detail [76]. Because of their stable structure, which consists of an alpha-helix and a beta sheet domain, these proteins can resist proteolytically harsh environments and are therefore mainly found in the apoplast, the vacuole and lysosomes [77, 78]. Considering that the plant apoplastis invaded by many pathogens, this compartment of the cell is like a molecular battlefield that contributes to deciding successful parasitism or plant resistance. Therefore, it is not surprising

that PLCPs have garnered more attention in several studies regarding plant-pathogen interaction. PLCPs are shown to use catalytic cysteine residues to cleave peptide bonds in proteins and might play essential roles in defence, but also act during signalling cascades. For example, the plant PLCP cathepsin B is required for the development of the hypersensitive response in *Nicotiana benthamiana*, and the secreted protease CDR1 probably releases systemic signalling molecules that initiate defence responses in *Arabidopsis thaliana*. The suppression of PLCPs is correspondingly imperative for pathogens to induce infection. Our findings regarding the inhibition of several PLCPs, namely, the mature and intermediate form of RD21 (mRD21 and iRD21), XCP2 and a cathepsin B-like protease, in syncytia induced by *H. schachtii* are in line with this assumption. Further support is given by Lozano-Torres et al. [79] who exhibited an increased infection of PLCP knockout lines of Arabiopsis plants through *H. schachtii*.

RD21 (Responsive to Desiccation 21) is a PLCPs that was found to be expressed during senescence [16, 80]. RD21 is located in ER-bodies that fuse with the vacuole upon stress [81]. During hypersensitive response the vacuolar content is released [82] and RD21 is therefore thought to be involved in this process, although the exact function of this PLCP remains unknown. Lack of RD21 in Arabidopsis did not affect the susceptibility of the plant against the biotrophic oomyete *Hyaloperonospora arabidopsidis* or against hemitrophic bacteria *P. syringae*. However, the necrotrophic fungus *Botrytis cinerea* showed increased infectivity in the absence of RD21, indicating that this cysteine protease might play a role in the defending against necrotrophic pathogens [83]. Interestingly, the genome of *B. cinerea* does not seem to encode obvious inhibitors of PLCPs [84, 85], which may be due to the fact that this fungus infects a wide unspecialized host range and rather post-harvest fruits than healthy leaves [86]. This is in contrast to the biotrophic pathogens, which have been shown to be armed with tools to suppress cysteine proteases. The biotrophic fungus *Cladosporium fulvum* secretes an effector protein Avr2 that inhibits, among others, the extracellular PLCP Rcr3^{pim} of *Solanum pimpinellifolium*, which itself is essential for the function of the tomato resistance gene Cf-2 [87, 88]. *Phytophthorainfestans* was also found to secrete PLCP inhibitors during tomato infection to suppress the activity of Rcr3 and PIP1, two closely related PR proteins [89, 90]. These effectors, EPIC1 and EPIC2B, obtain cystatin motifs, which are known to inhibit cysteine proteases.

5.5. Cystatins inhibit PLCPs

The investigation into biological function of cystatins keeps researchers busy for many years. The first identified and characterized phytocystatins were oryzocystatin I [91] and II [92]. Those plant deriving cystatins are involved in the regulation of storage proteins during development and germination and is also involved in other physiological plant processes, including programmed cell death [93], fruit development [94], and defence responses [95-97]. Three motifs found in all cystatins enable a three-point interaction with their target protein and are therefore characteristic for their inhibitory function: (i) the highly conserved QxVxG motif, (ii) a tryptophan near the carboxy-terminal, and (iii) a conserved glycine residue [98, 99]. Interestingly, both host and invader use proteins with a cystatin-like structure to combat each other. Previous reports describe the inhibition of digestive proteins by cystatins during insect infestation [100, 101], whereas pathogens have also been shown to use host cystatins for their own benefit as compatibility of susceptibility factor. The Arabidopsis cystatin, AtCYS1, for example, is induced by wounding or from avirulent pathogen attack and suppresses hypersensitive cell death [93]. Also, the biotrophic maize smut pathogen *Ustilago maydis* benefits from the maize gene Cystatin 9 (Corn Cystatin-9 [CC9]), as this host compatibility factor inhibits apoplastic cysteine proteases and therefore suppresses maize immunity to *U. maydis* [102]. However, pathogens also obtain effector proteins with cystatin-like structure, as indicated by EPIC1 and EPIC2B of *P. infestans*. Accordingly, we assume that *H. schachtii* might also elicit cystatin-like effector proteins as the activity of several PLCPs was reduced in syncytia compared to non-infected roots.

5.6. A putative effector found in *H. schachtii* obtains cystatin characteristics

Using bioinformatics approaches we found a gene transcript in the genome of *H. schachtii* (HsCysL1) that contains cystatin motifs and a secretory signal peptide but no transmembrane domain, indicating that this putative effector protein is translocated through the nematode´s secretory pathway. Sequence alignment studies revealed homologues in other cyst nematodes such as *H. glycines*, *G. pallida*, and *G. rostochiensis*, but interestingly two main features were only present in the genome of *H. schachtii*: First, the characteristic cystatin motif of a tryptophan (Trp) near the carboxy-terminal is missing in HsCysL1 but present in the homologues of all other species. Bacterial studies have shown that the positions of Trp within a

three-dimensional structure of proteins are highly conserved and take active parts in the translocation of proteins through the membrane [103, 104]. Due to a hydrophobic benzene ring and a spatially separated aromatic indole side chain, Trp obtains two contrary characters that can take place independently [105, 106]. By its lipophilic nature, Trp is assumed to facilitate the translocation of the periplasmic portion of a protein and thereby determine the orientation of the protein in the membrane. Afterwards, the aromatic chain likely helps anchor the protein through hydrogen bonding [106]. The addition or depletion of Trp residue could strongly affect the structure and orientation of a protein and therefore also its function. The missing Trp in the genome of *H. schachtii* could substantially contribute to the secretion and localization of HsCysL1 apart from cell membranes. Furthermore conserved Trp residue in Domain IV of a gain-of function mutant of *iaa* (Osiaa23) has recently shown to be responsible for the protein-protein interaction between AUX/IAA and ARF and therefore for the suppression of auxin synthesis [107]. Accordingly, the depletion of Trp in HsCysL1 could also be considered as a strategy to circumvent the formation of protein complexes either to promote protein expression, or to avoid being recognized by the host and induce host´s immune response. In addition to the depleted Trp the sequence of HsCysL1 is the only homologue among all tested species that contains a nuclear localization sequence (NLS). Considering the lack of a lipophilic Trp, both features could go hand in hand to enable HsCysL1´s translocation to the nucleus of the host tissues. The fact that those two sequence characteristics are uniquely found in *H. schachtii* supports its potential to play key role in specialized parasitism.

5.7. HsCysL1 may have a surprisingly dual function during syncytium establishment

The conserved cystatin motifs present in HsCysL1 suggest that HsCysL1 might have a cystatin-like function and is secreted to suppress plant immunity via inhibiting PLCPs. Our results show that HsCysL1 is expressed in the oesophageal gland cells of the nematode supporting the hypothesis that it is a putative effector protein. Oesophageal gland cells have previously shown to be active at different times during infection. Whereas the two subventral gland cells are mainly active in the preparasitic juveniles, which corresponds to the migratory phase and the initiation of the feeding cell, the activity of the dorsal gland cell dramatically increases at the onset of the

parasitic life stages [108, 109]. Although, we were not able to determine the define gland cell in which HsCysL1 is expressed, the qRT-PCR results showed that HsCysL1 is expressed throughout all tested developmental stages (J2, 5dpi, 10dpi) with highest expression at 5dpi. Furthermore, we observed different localization of HsCysL1 in the host cell when the signal peptide was removed. The HsCysL1 construct containing the signal peptide (HsCysL1^{+SP}) got transferred into the apoplast, whereas HsCysL1^{-SP} showed a cytoplasmic and nuclear localization, indicating that the nematode origin signal peptide is also functional in plant secretory pathway. Similar results were also found by Jaouannet et al. [110], who also identified apoplastic and cytoplasmic distribution, respectively, depending on the signal peptide of the secreted calreticulin effector of *M. incognita* (Mi-CRT). Based on these results, HsCysL1 appears to have a dual function. Being expressed during the migratory phase and secreted into the apoplast, we assume that HsCysL1 interacts with PLCPs, which are highly expressed in the apoplast, to suppress host defence responses. However the expression of HsCysL1 reaches its maximum at 5 dpi when syncytium is established and needs to be expanded. This increased expression at 5 dpi suggests that HsCysL1 may play a role in syncytium formation other than inhibition of PLCPs.

5.8. Target genes of HsCysL1 reveal an involvement in signalling and regulatory processes

Indeed, we were able to identify two proteins located in the cytoplasm, PTPLA and UBC19, as interacting targets of HsCysL1. Both proteins are known to be key factors during various signalling and regulatory processes through dephosphorylation and ubiquitination of proteins [111-115]. PTPLA is assumed to be localized in both cell compartments - cytoplasm and nucleus - due to a SV40-like NLS [116, 117]. Combined with previous findings concerning the inhibitory effect of PTPs on stress-activated MAP-kinases, this attribute hints to a second suggestion: being targeted by HsCysL1, PTPLA could be activated and/or guided to the nucleus to fulfil its function of inhibiting MAPK induced signalling cascades. Considering the nuclear expression of the GFP tagged HsCysL1^{-SP} after removing NLS and the exclusively cytoplasmic interaction between PTPLA and HsCysL1, this latter hypothesis seems to be the more likely one. PTPLA is the only protein tyrosine phosphatase in which the active motif HCxxGxxP contains an arginine-to-proline replacement [117], suggesting a high

protein interaction specificity. However, the absence of PTPLA in Arabidopsis did not affect the susceptibility of the plant against *H. schachtii*, which may be explained by redundant function of PTPLA by other PTPLs present in Arabidopsis. Even though evidences for detailed molecular function of PTPLA are missing, the interaction between HsCysL1 and PTPLA seems to be an important mechanism to initiate syncytium formation.

UBC19 seems to be substantially involved in the endoreduplication, a well-accepted phenomenon observed in giant cells and syncytia, which describes the process of cell division, including DNA duplication, but without cytokinesis [118, 119]. Studies regarding the molecular function of UBC19 are rare. However, UBC19 is expected to act as an ubiquitin conjugating enzyme (E2-C) degrading cyclin B, a regulatory protein playing an essential role during the mitotic cell cycle [120-122]. Destruction of cyclin B progresses the cell to leave the mitotic phase and to initiate the entry into G1 of the new cell cycle [123, 124]. The accumulation of cyclin B and an enhanced progress of mitosis until late G2 in nematode feeding sites [125, 126] indicate the inhibition of UBC19 through nematode effector proteins. Consequently, the cell would be arrested in mitosis, resulting in a multinuclear cell with enhanced metabolic activity. Also neighbouring cells seem to be affected by a cyclin B accumulation in syncytium, as the cyclin B expression increases substantially in syncytium surrounding cells [50]. Furthermore, previous findings described an increased cell division activity in neighbouring cells [1, 127] correlated with increased expression of UBC19 in dividing cells [128], which in total promotes high metabolic activity in cells that are going to be incorporated into the syncytial structure. Accordingly, the accumulation of cyclin B and associated with the inhibition of UBC19 seems to be the most likely function of HsCysL1^{-SP} regarding the interaction with UBC19. Nevertheless, a more detailed functional characterization would be needed to know the precise role of UBC19 during plant-nematode interaction. In this context, characterization of a knockout mutant would have been very useful. However, no loss-of-function mutant is available for UBC19. Therefore, it will be important in the future to generate and characterize lines overexpressing UBC19.

Regarding this work it would be worthwhile to invest more effort in analysing the molecular function of HsCysL1. Arabidopsis plants overexpressing this effector, which are currently in progress, constitute an encouraging fundament for future

research. ABPP using the proteome of these plants could provide exciting insights into the posttranslational modification of PLCPs and other proteins and may support our hypothesis of HsCysL1 having a multiple function during infection. Furthermore, infection assays comparing HsCysL1 overexpression lines with Arabidopsis wildtype plants could reveal knowledge about the importance of this effector protein in terms of successful parasitism.

Conclusively, one should consider that successful parasitism is the result of a highly complex interaction between pathogen-derived effectors and host-derived defence proteins. Evolutionary induced both interacting partners have developed an enormous diversity of proteins that combat each other. Displaying the altered protein activity of various enzymes in syncytium as well as the identification and characterization of a single effector protein of *H. schachtii* does not even rudimentarily complete the molecular understanding of nematode infection, but it enables the contribution to an exciting chapter of host-parasite interaction.

5.9. References

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6. Summary

Sedentary cyst nematodes are of high economic interest as they can cause substantial yield losses in important crop plants. Due to their complex soil-based life cycle and severe restrictions on the application of nematicides management strategies are rare. To date, resistant plants are the most effective and economically as well as environmentally reasonable alternative to chemical control agents. However, nematodes are evolutionarily able to overcome the resistance after some time, facing researchers with the challenge to breed new resistant lines. Therefore, knowing the details of the interaction between plant and pathogens is fundamentally.

Cyst nematodes establish a highly complex long-term relationship with their hosts that requires massive cytological modifications of the host cell to form a syncytial feeding structure. Therefore, plant defence mechanisms need to be circumvented by the nematode. Using their stylet, cyst nematodes introduce a mixture of different effector proteins into the host cells that manipulate the activity of host derived proteins. Since enzymes are only functional in their active form, one objective of presented work was to visualize spezific proteins of the active proteome of syncytium induced by *Heterodera schachtii* in Arabidopsis roots. Using Activity-based Protein Profiling (ABPP) it could be shown that the activity of serine hydrolases are differently regulated, whereas the activity of vacuolar processing enzymes (VPEs) is supressed in syncytium. Furthermore Papain-like cysteine proteases (PLCPs) and all catalytic proteasomal subunits, both known to be involved in plant defence, are suppressed in case of successful parasitism.

PLCPs are inhibited by cystatins, which guided to the second main objective of presented work: the identification and functional characterization of a putative cystatin-like effector protein in *H. schachtii* (HsCysL1). HsCysL1 shows involvement in plant defence and signalling by interacting with PTPLA (Protein tyrosine phosphatase-like A) and UBC19 (Ubiquitin-conjugating enzyme 19). Both proteins are known to play significant roles in various signalling and regulatory processes.

Although these findings do not rudimentarily complete the understanding of the complex plant-nematode interaction, they definitely open an exciting chapter for researchers to find new management strategies against cyst nematodes.

7. Zusammenfassung

Sedentäre Zystnematoden sind von großer Bedeutung, da sie erhebliche Ertragsausfälle in landwirtschaftlichen Kulturen verursachen können. Aufgrund ihres komplexen, bodenbürtigen Lebenszyklusses und strengen Auflagen bei der Anwendung von Nematiziden sind Bekämpfungsstrategien rar. Resistente Pflanzen stellen die effizienteste und ökonomisch wie auch ökologisch vertretbarste Alternative zur chemischen Bekämpfung dar, allerdings sind Nematoden nach einiger Zeit in der Lage, Resistenzen zu durchbrechen. Dadurch stehen Forscher kontinuierlich vor der Herausforderung, neue resistente Pflanzen zu züchten. Detailliertes Wissen über die Interaktion zwischen Pflanze und Pathogenen ist daher essentiell.

Zystnematoden entwickeln hochkomplexe Langzeitbeziehungen mit ihren Wirten, was die Bildung eines Nährzellensystems durch massive Veränderungen der Wirtszellen voraussetzt. Dazu muss die pflanzliche Abwehr vom Nematoden umgangen werden. Mittels ihres Mundstachels geben sie verschiedene Effektorproteine in die Wirtszelle ab, die die Aktivität von Proteinen beeinflussen. Da Enzyme nur in aktive Form funktionieren, war ein Ziel der vorgestellten Arbeit, das aktive Proteom eines Syncytiums darzustellen. Durch Activity-based Protein Profiling (ABPP) konnte gezeigt werden, dass die Aktivität von Serinhydrolasen unterschiedlich reguliert wird, während die von vakuolar verarbeitenden Enzymen (VPEs) im Syncytium runterreguliert ist. Des Weiteren sind Papain-ähnliche Cysteinproteasen (PLCPs) sowie alle katalytischen Untereinheiten des Proteasoms runterreguliert; beide Enzymgruppen sind wichtig für die pflanzliche Abwehr.

PLCPs werden durch Cystatine gehemmt, was zum zweiten Hauptpunkt der vorliegenden Arbeit führte: die Identifizierung und funktionelle Charakterisierung eines cystatin-ähnlichen Effektorproteins in *H. schachtii* (HsCysL1). HsCysL1 zeigt eine Beteiligung an der pflanzlichen Abwehr und Signalwegen durch die Interaktion mit PTPLA (Protein-Tyrosinphosphatase-ähnlich A) und UBC19 (ubiquitin-konjugierendes Enzym 19).

Auch wenn diese Erkenntnisse nicht annähernd einen Gesamtüberblich über die komplexe Interaktion zwischen Nematoden und Pflanzen geben können, eröffnen sie dennoch spannende Möglichkeiten für Forscher, neue Bekämpfungsstrategien gegen Zystnematoden zu entwickeln.

8. Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorstehende Arbeit selbstständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus Veröffentlichungen entnommen sind, wurden als solche kenntlich gemacht.

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

Bonn, den ___________________ ___________________________

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