Always remember that you are absolutely unique.

Just like everyone else.'

Margaret Mead (1901-1978)

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How the plant-parasitic nematode *Hirschmanniella oryzae* is able to subdue the defense system of rice; a molecular analysis

Lander Bauters

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) of Applied Biological Sciences

Het onderdrukken van het afweersysteem in rijst door de plantparasitaire nematode *Hirschmanniella oryzae*; een moleculaire analyse

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List of abbreviations

2,3-DHBA	2,3-dihydroxybenzoate
ABA	Abscisic acid
ADH	Arogenate dehydrogenase
ADP	Adenosine diphosphate
ADT	Arogenate dehydratase
AGP	Arabinogalactan protein
APN	Animal-parasitic nematode
Asp-Lys-Cys	Aspartic acid, lysine, cysteine
ATP	Adenosine triphosphate
AUX	Auxin
Blast	Basic local alignment search tool
bp	Base pair
BR	Brassinosteroid
CBD	Cellulose binding domain
CDD	Conserved domain database
cDNA	copy-DNA
CK	Cytokinin
CLV	CLAVATA
CM	Chorismate mutase
CM*	Cytosolic chorismate mutase
CMD	Chorismate mutase domain
CN	Cyst nematode
CWMP	Cell wall modifying proteins
DAMP	Damage associated molecular pattern
DDHB	2,3-dihydro-2,3-dihydroxybenzoate
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
dpi	Days post infection
DP	Diterpenoid phytoalexin
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
ET	Ethylene
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
FC	Fold change
FDR	False discovery rate
FPP	Farnesyl pyrophosphate
g	Gram
GA	Gibberellic acid
GFP	Green fluorescent protein
GGPP	Geranylgeranyl pyrophosphate
GGPPS	Geranylgeranyl pyrophosphate synthase
GHF	Glycosyl hydrolase family
GLM	Generalized linear model

Glutper	Glutathione peroxidase
GO	Gene ontology
GRAVY	Grand average of hydropathy
GST	Glutathione-S-transferase
h	Hours
HGT	Horizontal gene transfer
HMM	Hidden Markov model
HPP-AT	hydroxyphenylpyruvate aminotransferase
HR	Hypersensitive response
IAA	Indole-3-acetic acid
ICM	Isochorismatase
ICS	Isochorismate synthase
id	Indentifier
IRGSP	International rice genome sequencing project
JA	Jasmonic acid
J2	Stage 2 juvenile
kDa	kilo Dalton
L	Litre
LB	Luria-Bertani
LEA	Late embryogenesis abundant
M	Molar concentration
Mb	Megabase
MEP	2-C-methyl-D-erythritol 4-phosphate
MES	2-(N-morpholino)-ethanesulfonic acid
mRFP	Monomeric red fluorescent protein
mRNA	messenger RNA
MS	Murashige and Skoog
MVA	Mevalonate
NCBI	National Center for Biotechnology Information
NERICA	New rice for Africa
NLS	Nuclear localization signal
NPR	Non-expressor of PR genes 1
nt	Nucleotides
OD	Optical density
OGA	Oligogalacturonide
ORF	Open reading frame
PAGE	Parametric analysis of gene set enrichment
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen associated molecular pattern
PAT	Prephenate aminotransferase
PCR	Polymerase chain reaction
PDB	Protein databank
PDH	Prephenate dehydrogenase
PDT	Prephenate dehydratase
PG	Polygalacturonase
PGIP	PG-inhibiting protein

Phe	Phenylalanine
Phyre	Protein homology/anology recognition engine
PL	Pectate lyase
PPN	Plant-parasitic nematode
PPY-AT	Phenylpyruvate aminotransferase
PR	Pathogenesis-related
PTI	PAMP triggered immunity
Q-RT-PCR	Quantitative reverse transcriptase PCR
RKN	Root-knot nematode
RMSD	Root mean square deviation
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RRM	RNA recognition motif
s	Second
S/HRD	Serine/histidine rich domain
SA	Salicylic acid
SAP	Sand and absorbent polymer
SCOP	Structural classification of proteins
SP	Signal peptide
SRA	Sequence read archive
STAR	Spliced transcripts alignment to a refeence
SXP	SXP/RAL-2
t-CA	trans-cinnamic acid
TE	Transposable element
TLP	Thaumatin-like protein
Trp	Tryptophan
Tyr	Tyrosine
VAP	Venom allergen protein
Y2H	Yeast two-hybrid
YEB	Yeast extract broth

Chapter 1. Introduction

1.1. Rice: the host

1.1.1. General facts about rice

Looking at primary crops, cereals comprise over 25% of the total world production, growing on almost 55% of the total crop area. Wheat, maize and rice are the three most important cereal crops; together they accounted for almost 90% of total cereal production in 2013 and they are responsible for 60% of the world's food energy intake. Rice is the primary staple food for more than half the world's population and over 740 million tonnes were produced in 2013, providing 21% of the global human per capita energy. Most of this rice is grown in Asia, with China and India as top producers (http://faostat3.fao.org/). While maize and wheat are partially used as feed, most rice grown around the world is consumed by humans, making it the most important staple food. Two important rice species in human nutrition are *Oryza sativa* and *O. glaberrima*. *O. sativa* is grown worldwide while *O. glaberrima* is only grown in parts of West Africa. The unique traits of *O. glaberrima*, like drought tolerance and weed competiveness, have been combined with *O. sativa* to develop the NEw RIce for AfriCA (NERICA). These hybrids can increase yield by almost 50% (Sarla and Mallikarjuna Swamy, 2005). Rice is a relatively easy crop to grow since there are hundreds of different varieties that can be cultivated all over the world in a wide range of soil types and hydrological conditions (IRRI, 2002).

The most important factor to take into consideration when cultivating rice is water. When the soil water content drops below saturation level, growth and yield levels are impaired (Bouman and Tuong, 2001). Rice is grown in regions where the farmers can cope with this huge water demand. There are four environments/ecosystems in which rice is cultivated. The most important ecosystem is irrigated rice, comprising about half of the area used for rice production. Rainfed lowland and upland are two other ecosystems that heavily rely on rainfall and are easily affected by periods of drought. Another ecosystem is the flood-prone rice ecosystem where plants have to adapt to longer periods of flooding and submergence of a big part of the shoot (up to 100cm) (Bouman et al., 2007).

1.1.2. Pests and diseases

Although rice yield can be high, it depends on several environmental factors specific to the region where it is grown. These factors include water availability, climate and the types of

pathogens present. It has been calculated that the combination of several rice pests and diseases can cause a yield loss of almost 40%. Weeds are the most important cause of yield loss, they are responsible for about 20% of the total loss, competing for light, nutrients and space (Savary et al., 2000). Next to weeds, other major problems are bacterial and fungal pathogens, among which *Xanthomonas oryzae pv oryzae* (bacterial blight), *Magnaporthe oryzae* (rice blast) and *Rhizoctonia solani* (sheath blight) cause the most important rice diseases with a world-wide distribution. A whole array of insect species is able to attack rice plants, but only few of them cause significant yield losses. The most important ones among them are the stem borers, the planthoppers and the rice gall midge (IRRI, 2002).

A type of pathogens that has not been mentioned above is nematodes. Nematodes are not among the most famous pathogens and are largely unknown to the general public. Nevertheless, they have the ability to cause severe yield losses in rice fields all over the world. Rice-parasitic nematodes can be divided into two distinct classes; shoot parasites and root parasites. The first group can cause distinctive symptoms on the above-ground part of the plant, while the symptoms produced by the latter group are rather variable and difficult to diagnose. Some species are restricted to a particular rice-growing environment, while others occur throughout the rice growing regions of the world. Most important shoot nematode parasites are *Ditylenchus angustus* and *Aphelenchoides besseyi* (causing Ufra and white tip disease respectively). Since this study focusses on nematodes attacking the roots of the rice plant, root nematodes are discussed in more detail below.

Most important parasitic genera on rice roots are *Heterodera*, *Hirschmanniella*, *Meloidogyne* and *Pratylenchus* spp. (Bridge et al., 2005; Coyne and Plowright, 2004). Not all species within these genera have the capability to infect rice roots. A long, but non-comprehensive, list of species reported to infect rice roots has been published by Fortuner and Merny (1979). It is difficult to assess yield losses caused by these species. Several studies have tried to address this topic with different results. Earlier investigations have reported severe yield reductions attributed to nematode infections, summarized by Padgham et al. (2004) for *M. graminicola* and by Maung et al. (2010) for *H. oryzae*. Observed yield losses varied between 20-80% due to infestations by either one of these nematodes. Most of these numbers were achieved by comparing natural fields to fields treated with nematicides. These uncontrolled conditions can give a false indication about the severity in yield reductions, since nematicides can have detrimental effects on other organisms in the field as well. This, in turn, can have an effect on yield. However, there are some controlled studies that describe a yield loss of 23-28%

due to *M. graminicola* infection (Tandingan et al., 1996). Although numbers on yield losses caused by nematode infections can vary, there is still a consensus that this type of pest has to be taken into consideration when thinking about improving yield.

1.1.3. Rice: an ideal crop model

Although plant-parasitic nematodes can infect many types of plants, rice is an ideal model to investigate plant-nematode interactions. Rice is a crop under intensive study, generating vast amounts of data which can be used in further studies. This is not only due to the fact that rice is one of the most important agricultural crops, but also because rice was the first crop plant to have its genome sequenced (Goff et al., 2002; Yu et al., 2002). The genome size of rice is about 420 or 466 megabases (Mb) in size (respectively for the japonica and indica variety). The genome is larger than the 125Mb genome of the dicot plant *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000). This observation is also reflected in the number of predicted protein coding genes: about 26,000 and 36,000 for Arabidopsis and rice respectively. While synteny between these two model plants is rather low, 85% of all predicted Arabidopsis proteins have a homologue in the rice dataset (Goff et al., 2002). When comparing the genome of rice to the recently sequenced genomes of other cereal crops, a significant difference in size is observed. Maize has a genome size of about 2,300Mb, barley 5,000Mb and wheat 17,000Mb (Chapman et al., 2015; Mayer et al., 2012; Schnable et al., 2009). This is quite stunning if taken into account that the amount of predicted genes is comparable for maize (32,000 predicted genes) and barley (26,000 genes), meaning that those two crops have a much lower average gene density. Wheat is hexaploid and contains about 32,000 to 38,000 functional protein coding genes on each of the three diploid subgenomes (IWGSC, 2014). In total, wheat is predicted to have 106,000 protein coding genes, which is three times more than rice, but its genome is also more than twenty times larger (Chapman et al., 2015). Thus average gene density is much higher in rice compared to other cereal crops. This is due to the portion of transposable elements (TEs); while only 25% of the rice genome is considered to consist of TEs, more than 80% of the genomes of other cereals consist of mobile elements or repeats (Mayer et al., 2012; Schnable et al., 2009).

In spite of the differences in genome size and gene density, the homology, together with the high degree of synteny among these cereals, indicates that rice could function as an excellent model, representing the monocotyledonous plants in further studies. Nguyen et al.

(2014) already established a working model with rice to study the plant-root-knot nematode interactions in monocotyledons.

1.2. Nematodes: the pathogens

1.2.1. General introduction

The phylum Nematoda, or roundworms, is a very abundant and diverse group of organisms, inhabiting many different ecosystems around the globe. Although not all species have been characterized, the estimated total of different species in this phylum ranges from 100,000 to 1 million (Lambshead, 1993). While sizes can vary, basically all nematode species have a similar anatomy; a long hollow outer tube containing another tube functioning as the alimentary canal and reproductive organs. Although all nematodes look very similar, some species have specialized to exploit a variety of ecological niches. Nematodes can thrive as free-living terrestrial and marine microbivores, meiofaunal predators and animal and plant parasites. The phylum was divided into five different clades based on small subunit ribosomal RNA phylogenetics (Blaxter et al., 1998). Later this phylogeny was updated and nematodes were divided over twelve clades (van Megen et al., 2009). Nematodes with a different lifestyle occur randomly throughout the clades, meaning that parasitism has arisen multiple times during nematode evolution (Figure 1.1) (Parkinson et al., 2004).

Interest in nematology studies increased in 1998, when the genome sequence of *Caenorhabditis elegans*, the free-living soil dwelling nematode, was published (The C.elegans Sequencing Consortium, 1998). This was the first sequenced genome for a multicellular organism and it proved to be a valuable basis for further genome research in nematodes and other species. In the meantime other genomes of nematodes have been sequenced as well. The genome of the animal-parasitic nematodes (APN) *Ascaris suum* and *Brugia malayi*, as well as the plant-parasitic nematodes (PPN) *Meloidogyne incognita*, *M. hapla* and *Globodera pallida* among others have been sequenced and can now serve as template for comparative studies (Abad et al., 2008; Cotton et al., 2014; Ghedin et al., 2007; Jex et al., 2011; Opperman et al., 2008).

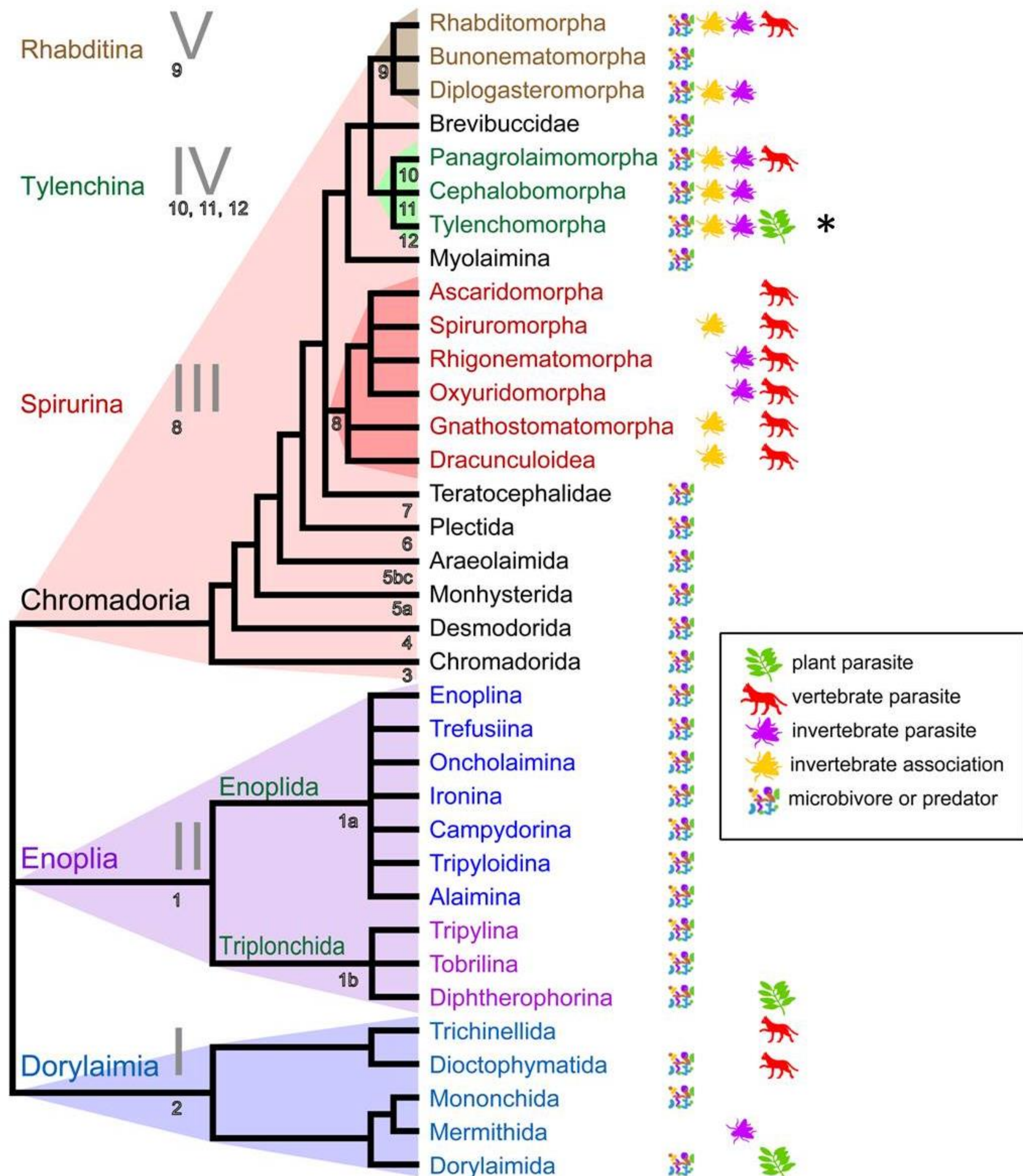

Figure 1.1: Presentation of a phylogenetic tree of the phylum Nematoda based on molecular analyses utilizing the small subunit ribosomal RNA gene (adapted from Blaxter (2011)). Roman numerals indicate the intitial five clades. Arabic numerals show the updated version of the tree with twelve clades. *Hirschmanniella oryzae* and *Meloidogyne graminicola* are both members of clade 12 (Tylenchomorpha, indicated with an asterisk).

1.2.2. Plant-parasitic nematodes

In this study, the focus will be on PPNs, with an emphasis on PPNs that are able to infect rice. Nematodes can attack the root system of the plant (root pathogens), but some species also

colonize the upper part (foliar pathogens). Root pathogens can be divided into two different types according to their lifestyle. Some nematodes induce the formation of a specialized feeding cell in the plant and remain sedentary once this feeding cell has been established. Others migrate through the roots during their whole life cycle, feeding on cells along their path. Sedentary nematodes can be classified as either root-knot nematodes (RKN) or cyst nematodes (CN). So far, molecular studies have mainly focused on sedentary species, and much less on the migratory ones. Up till now no genome sequences are available for any of the migratory PPNs. Fortunately lots of transcriptomic data is accessible to study these migratory species in more detail (Bauters et al., 2013; Haegeman et al., 2011b; Jacob et al., 2008; Nicol et al., 2012). In the following paragraphs each group of nematodes will be discussed briefly, highlighting the importance of each group in rice cultivation.

Root-knot nematodes

Sedentary nematodes penetrate the plant roots to establish a specialized feeding site where they reside for almost their whole life cycle. J2 of RKNs (*Meloidogyne* spp.) penetrate the root and migrate intercellularly until they have found an appropriate root cell to initiate feeding site formation. Feeding cells, or giant cells, are formed by induction of a series of cell divisions uncoupled from cytokinesis. Nematodes feed on these cells throughout their life cycle. Due to the hyperplasy and hypertrophy of cells surrounding the nematode and the giant cells, the roots form the typical gall or root-knot associated with the genus *Meloidogyne* (Bird, 1961). Most root-knot species reproduce by parthenogenesis. After three moults, adult females deposit eggs outside their body. These eggs are protected by a proteinaceous matrix until they are ready to hatch. This life cycle takes approximately 20-30 days (Curtis, 2007; von Mende, 1997). A representation of the life cycle of RKN can be found in Figure 1.2. The two main species of RKNs that have been associated with rice are *M. graminicola* and *M. incognita* (Bridge et al., 2005). Of these two species, *M. graminicola* is the most devastating pest in rice fields. Both species thrive in rice grown under aerobic conditions, but only *M. graminicola* is somehow adapted to flooding conditions, while *M. incognita* is susceptible to anaerobic conditions (Fortuner and Merny, 1979). *M. graminicola* can be prominent in flooded rice ecosystems since it has the ability to infect the roots before flooding occurs. By laying its eggs inside the roots (instead of on the surface as other *Meloidogyne* species do) it can reproduce within the roots during flooded conditions, which increases its survival rate (Bridge and Page, 1982).

Cyst nematodes

Cyst nematodes (*Heterodera* and *Globodera* spp.) have a similar life cycle compared to RKN (Figure 1.2). J2 of cyst nematodes penetrate the plant root and migrate through the cells to the vascular cylinder to initiate the syncytium. In contrast to giant cell ontogeny, a syncytium is formed by the incorporation of neighbouring cells through cell wall digestion. This process is able to assemble as many as 200 cells to form one multinucleate cell (Jones, 1981). After three additional moulting stages, the adult female is fertilized by the male and produces eggs which are contained in the body. The female body that protrudes from the root becomes hard and brown to form the typical cyst to which these nematodes owe their name (Andrews and Curtis, 2005; Jones, 1981). Up till now, no *Globodera* spp. infecting rice roots have been found. Some *Heterodera* spp. have been reported to be able to colonize rice roots; *H. oryzae*, *H. sacchari*, *H. graminophila*, *H. oryzicola* and *H. elachista* (Fortuner and Merny, 1979; Mcgawley and Overstreet, 1998). Although locally in West Africa severe yield losses were reported for CNs (Babatola, 1983; Kumari and Kuriyan, 1981), their global economic importance is low compared to RKNs.

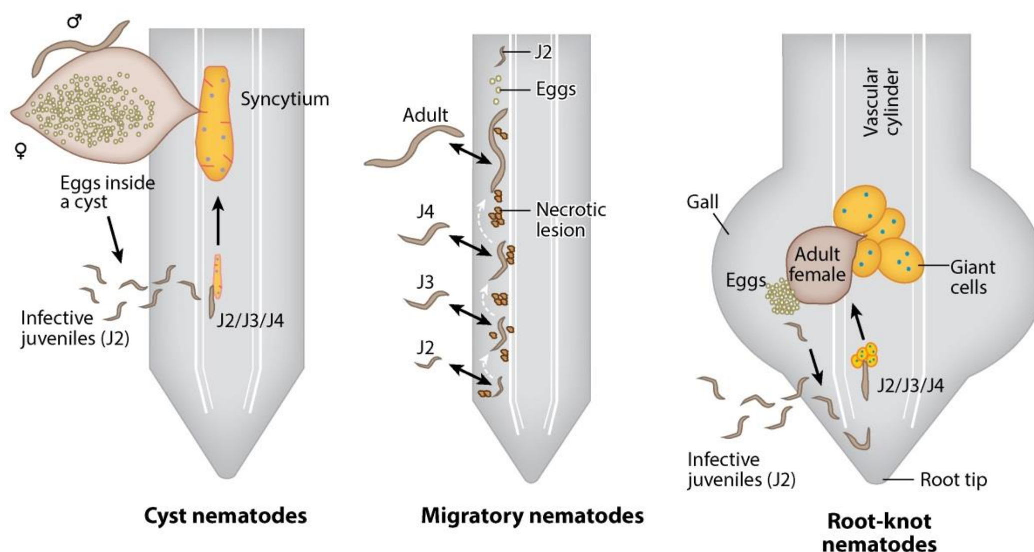

Figure 1.2: Life cycle of different types of nematodes, adapted from Kyndt et al. (2014b)

Migratory nematodes

In addition to the sedentary species, some migratory nematodes are important pests on rice plants as well. Root lesion nematodes (*Pratylenchus* spp.) have been recorded in rice fields in

Africa, South America and Southeast Asia (Bridge et al., 2005). Some species of this genus, for instance *P. zeae* and *P. indicus*, have been responsible for severe yield losses, mainly in upland rice (Aung and Prot, 1990; Prasad and Rao, 1978; Prot and Savary, 1993).

The research described in this thesis will focus on another type of migratory nematodes, the rice root rot nematodes (*Hirschmanniella* spp.). This genus contains some of the largest PPNs and most species are able to successfully infect rice. A total of 15 species have been associated with rice, *H. oryzae* being the most predominant one (Liao et al., 2000), especially in flooded ecosystems (Bridge et al., 2005). Yield reductions up to 87% have been associated with *H. oryzae* (Mathur and Prasad, 1972). When yield losses were estimated in a controlled experiment for this nematode and two related species (*H. spinicaudata* and *H. imamuri*), the estimated loss varied around 40% (Babatola and Bridge, 1979). All life stages of *H. oryzae* can enter the root, often using the same opening. While migrating through the root, they leave a path of lesions along the burrowed channels. If these nematodes infect roots in flooded soils, roots are prone to secondary infection of rot-inducing micro-organisms, hence the name “root rot nematode” (Babatola and Bridge, 1980). Above ground symptoms are rather scarce, but a general chlorosis of the leaves occurs in most cases. The nematode enters the roots some distance from the tip and moves freely in the air channels (Figure 1.3). Sexual reproduction is obligatory for *H. oryzae*. After fertilization, female nematodes lay eggs which will hatch inside the roots within 4-5 days (Figure 1.2). A full life cycle comprises 4 moulting stages and takes about 33 days under ideal conditions (Karakas, 2004).

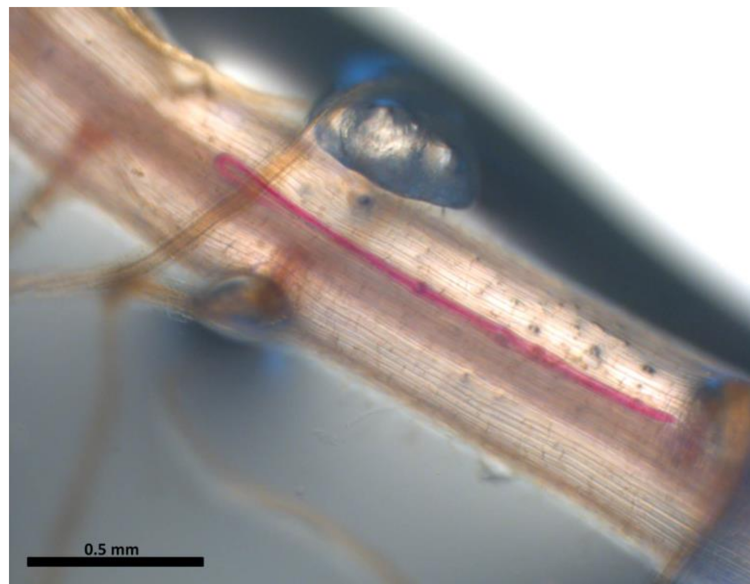

Figure 1.3: *H. oryzae* inside rice roots, stained with acid fuchsin.

One of the reasons that this nematode is so successful in being one of the major pests of rice is that it can easily survive in several weeds that are common in rice fields (Anwar et al., 2011). Another reason is that it is well adapted to conditions of flooding; it is one of the few nematodes able to survive under anaerobic conditions (Babatola, 1981). Different strategies have been tested to try to keep this pest under control. The application of nematicides, like Carbofuran, has been applied with success, but they pollute the water and can pose serious health risks (Kuriyan and Sheela, 1981). Crop rotation with the green manure crops *Sesbania rostrata* and *Aeschynomene afraspera* reduces the nematode population in the field, but this method is not thought to be economically viable. Fertilization of the rice field has unwanted side-effects. Application of nitrogen to the field results in an increased grain weight, but also the number of nematodes inside the roots was elevated, raising nematode population in the field for the next crop (Poussing et al., 2015). To develop effective and economical control measurements against *H. oryzae*, more research is needed regarding the interaction between this nematode and its host.

1.3. The general plant-pathogen interaction model adapted to nematodes

The attack-defense relationship between pathogens and their host can be presented in a zig-zag model (Figure 1.4) (Jones and Dangl, 2006). This model shows the evolution of pathogen attack and plant defense. Initially the host can recognize the pathogen by specific molecules on the exterior of the pathogen, functioning as a sort of molecular signature: Pathogen Associated Molecular Patterns (PAMP). Once these PAMPs are recognized by cell surface receptors, PAMP triggered immunity is activated (PTI). The typical defense response is callose deposition near the infection site to contain the pathogen within the infection site. The best known example of a PAMP is an epitope in flagellin (flg22), a protein in bacterial flagella (Zipfel et al., 2004). Pathogens have evolved to evade PTI by secreting effectors able to suppress PTI. Host plants, in turn, have acquired the ability to specifically recognize these effectors, thereby activating Effector Triggered Immunity (ETI). Receptors detecting pathogen effectors are very specific, even a slight difference of two amino acids in the effector can be enough to evade detection by the receptor, thereby rendering the pathogen virulent again (Bos et al., 2006). Consecutively, pathogens have evolved other effectors over

time to suppress the invoked ETI response. And so the evolutionary battle between plant and pathogen keeps continuing (Jones and Dangl, 2006; Smant and Jones, 2011).

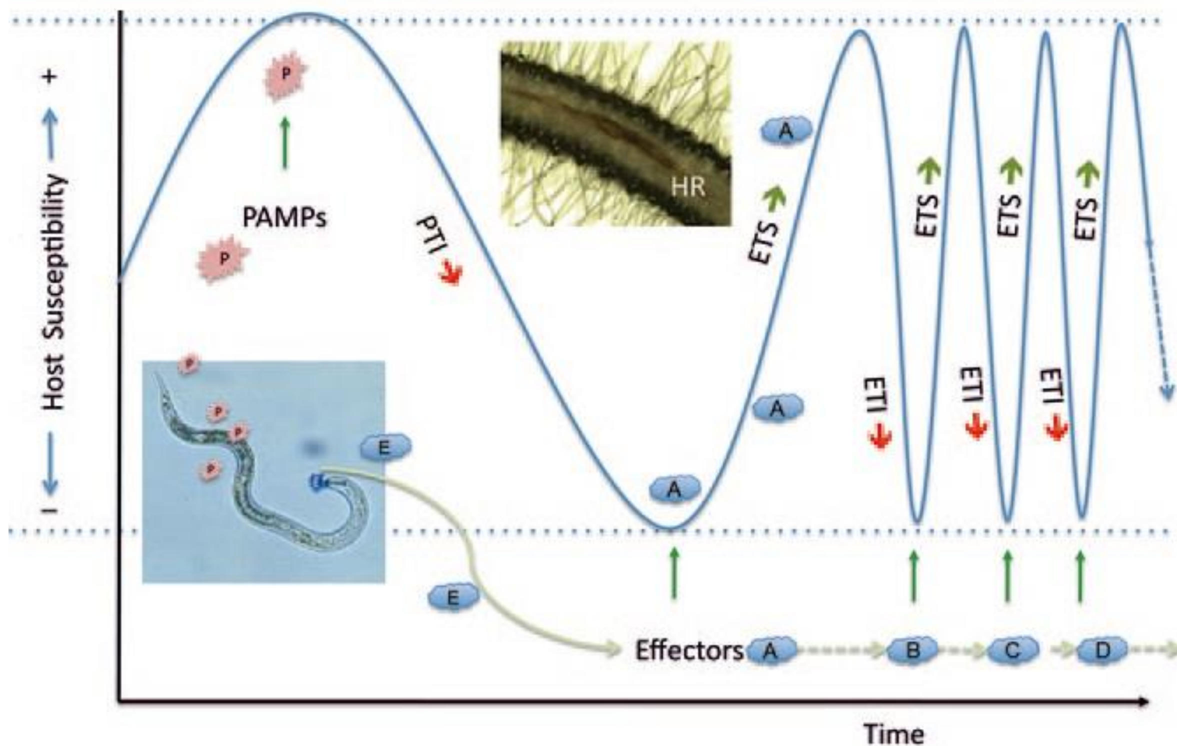

Figure 1.4: Hypothetical zig-zag model of plant-nematode interaction. A pathogen is detected by the plant, which will trigger PAMP triggered immunity (PTI). Pathogens have evolved to secrete effectors (E) that can inhibit PTI and induce effector triggered susceptibility (ETS). If plants are able to detect effectors, effector triggered immunity (ETI) is induced. A secreted effector (A) is able to inhibit PTI responses, thereby activating ETS. Over time, plants have adapted to recognize these effectors, triggering ETI. Pathogens will evolve to secrete additional/mutated effectors (B) to trigger ETS again. Adapted from Smant et al. (2011).

The zig-zag model to describe host-susceptibility was validated in plant-microbial pathogen interactions, but the question remains whether it is also applicable to plant-nematode interactions. The answer to this question remains elusive. To date, no typical PAMPs have been found for PPNs. The suggestion has been made that chitin molecules might serve as PAMPs in nematodes (Libault et al., 2007). Chitin may be present in the stylet of PPNs, which can be the cause of local callose depositions, the typical PTI response, in the region of infection (Golinowski et al., 1997; Hussey et al., 1992). This is an indication that PTI may indeed play a role during nematode infection. In contrast to PTI, clear examples of ETI or effector triggered susceptibility (ETS) have been described for plant-nematode interactions. For instance, the GrCEP12 peptide from *G. rostochiensis* is able to suppress the PTI response triggered by flg22 (Chen et al., 2013), but the best example is probably the Gp-RBP-1 effector

of *G. pallida*. This effector is recognized by Gpa2, a CC-NB-LRR type receptor originating from potato (Sacco et al., 2009). If these two genes are co-expressed in leaf tissue, a hypersensitive response (HR) occurs, which is a typical ETI response. Interestingly, in the same research it was reported that virulent populations of this nematode possess RBP-1 variants that are also capable to elicit an HR. This discovery implicates that the virulent populations must have evolved other effectors capable of suppressing the Gpa2 mediated ETI (Sacco et al., 2009). Taken together, these results imply that the zig-zag model is applicable for plant-nematode interactions. The main hiatus in this model is that, to date, no clear PAMPs have been identified for nematodes (Smant and Jones, 2011).

Both RKNs and CNs need to keep cells alive whilst feeding. To date, no detailed description of feeding habits of *H. oryzae* exists. Since *H. oryzae* induces a lot of necrosis in rice roots during migration, it is possible that this nematode does not need to keep cells alive while feeding, which undermines the relevancy of the zig-zag model for *H. oryzae*. However, research has shown that necrosis upon infection, is due to secondary infection by micro-organisms. Rice roots infected with sterilized *H. oryzae*, did not show necrosis (Babatola and Bridge, 1980). Feeding behavior has been studied in *Pratylenchus* species, which are closely related to *Hirschmanniella spp*.. Although migratory species move through the cells, thereby eventually killing them, they have periods of feeding. During a feeding period, a cell is gently pierced by the stylet, keeping it alive. Cells only die hours after nematodes have moved away (Zunke, 1990). Since also migratory nematodes keep cells alive whilst feeding, they would also benefit from trying to attenuate elicited defense responses. This underlines the relevancy of the zig-zag model applied to migratory nematodes.

1.4. A rigid cell wall and hormonal signaling: adequate defense systems?

Underground, a large-scale molecular warfare is carried out between the nematode and its host roots. Nematodes have developed an arsenal of enzymatically active weaponry to penetrate the plant cell wall, but they have also evolved sophisticated mechanisms to evade the defense system of the plant. In its turn, the host plant does not remain passive upon infection, but it activates a whole series of countermeasures in an attempt to stop the invading pathogen.

1.4.1. The cell wall: protecting the frontiers

The first barrier a PPN has to overcome is the plant cell wall. Cell walls are a complex and dynamic network of extended polysaccharides with high molecular weight. In essence, the cell wall is composed of three main components: cellulose, hemicelluloses and pectic polysaccharides, organized in different layers (O'Neill and York, 2003). Cellulose is a homopolymer consisting of successive glucose residues. In contrast to cellulose, hemicelluloses are branched heteropolysaccharides. Pectins, heteropolysaccharides, have uronic acids as major components, but composition differs among plants (Somerville et al., 2004).

Cell walls (Figure 1.5) are composed of three different types of layers; the middle lamella, the primary cell wall and the secondary cell wall. The latter is only present in some types of plant cells. The middle lamella is the layer between adjacent cells and is mainly composed of pectins. This layer can be seen as a type of glue preventing cells from sliding under tension. Underneath this layer, the primary cell wall provides a rigid structure to contain the cell. This strong layer is formed while cells are growing. Some cells that need additional support (for instance fiber cells in wood) synthesize an additional secondary cell wall, interior to the primary cell wall, after the cell is fully grown. The cell wall is composed of cellulose microfibrils that are tightly linked together with hemicellulose chains. This structure is embedded in a matrix of pectins (O'Neill and York, 2003). Secondary cell walls can be strengthened by lignin, synthesized by polymerization of monolignols, products from the phenylpropanoid pathway. The hemicellulose and pectin composition differs in grasses compared to dicotyledonous plants (Vogel, 2008).

In addition to providing a physical barrier between pathogen and plant cell to prevent penetration, the cell wall might also have another function related to plant defense. There is growing evidence that cell wall polysaccharide composition is an important factor in host-pathogen interaction. The composition of the cell wall can be responsible for the difference between a host and non-host for a certain type of pathogen (Vorwerk et al., 2004). In addition, degradation products formed during cell wall digestion by pathogens can act as signal molecules, thereby eliciting defense responses to prepare the host for an imminent pathogen invasion (1.4.2) (Esquerre-Tugayé et al., 2000). In this light, the cell wall should not only be seen as a physical barrier, but also as a plant component that actively participates in plant defense.

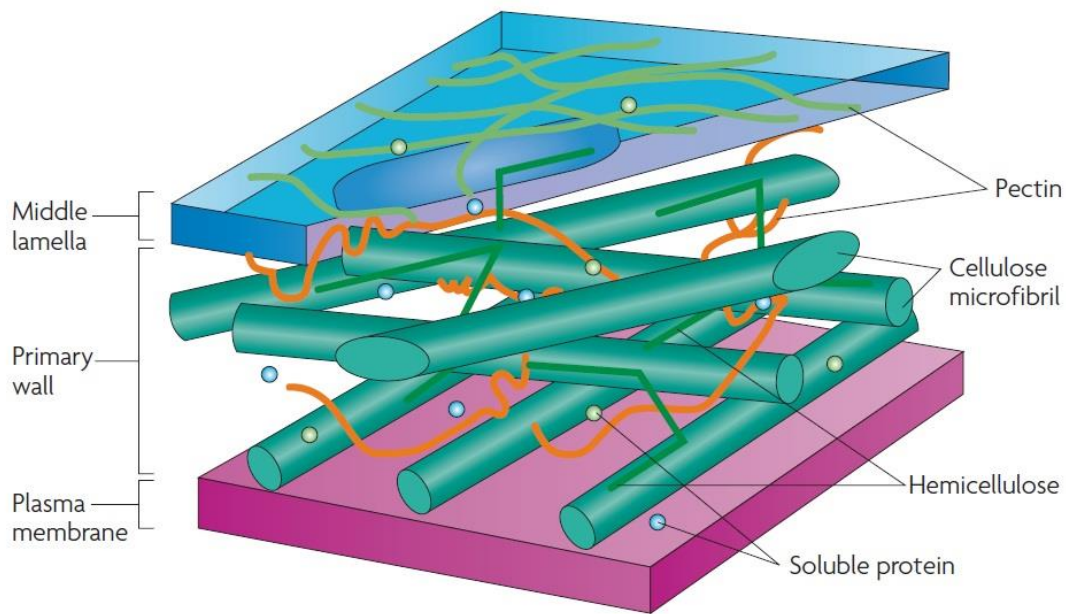

Figure 1.5: Model of the plant cell wall. Cell types possessing a secondary cell wall fit this extra layer in between plasma membrane and primary wall (this layer is not presented in this model). Adapted from Sticklen (2008).

1.4.2. Active defense mechanisms at the vanguard

The vast amount of cell wall modifying proteins (CWMP) that nematodes secrete through their stylet into the host tissue shows that PPNs have evolved to become specialists in degrading plants cell walls (1.5.1). Breaching this first barrier does not mean that the pathogen can freely infect the plant, since it has other defense mechanisms as well.

Plants are equipped with several layers of defense responses. If a pathogen is able to breach the cell wall, it can be recognized by the plant and defense responses are activated. Even during the process of cell wall degradation the pathogen can trigger defense responses. During cell wall hydrolysis, cell wall fragments are generated, which can be sensed by the plant and can trigger defense responses. These fragments are grouped under the term “Damage Associated Molecular Patterns” (DAMPs) (Denoux et al., 2008; Esquerre-Tugayé et al., 2000; Grundler et al., 1997). DAMPs act as signal molecules able to activate a local defense response. This defense mechanism can be extremely important for sedentary species, since they want to prevent an adequate local defense response. The migratory species on the other hand might be able to outrun DAMP-triggered immunity (Smant and Jones, 2011). Plants are also able to directly detect CWMP secreted by the nematodes. The

cellulose binding domains (CBDs) which are present in many cellulases, like β-1,4-endoglucanase, are potent elicitors of plant defenses. So far this phenomenon has been reported in oomycetes, but it remains uncertain whether the same mechanism applies during nematode infection (Dumas et al., 2008). Likewise, bacterial endoxylanases have been reported to trigger the plant defense system (Belien et al., 2006). Though no reports have been made of CWMP of nematode origin triggering defense responses, the possibility cannot be ruled out without further investigation. It has been suggested that the activity of polygalacturonases secreted by the nematode or the activity of plant polygalacturonases induced by the nematode can be attenuated by plant polygalacturonase-inhibiting proteins. These proteins are highly expressed in cells surrounding the feeding site of *H. goettingiana*, implying their important role during defense (Veronico et al., 2011).

1.4.3. Plant hormones: instructors of defense

Plant hormones are key factors in regulating plant defenses. In the following paragraphs the role of some plant hormones during nematode infection will be discussed. As the focus of this thesis is on the relationship of the migratory nematode *H. oryzae* and its host plant rice, the emphasis will lie on hormones that are involved in this interaction. Since two effectors that might have an effect on the SA biosynthesis pathway in rice have been found in the transcriptome of *H. oryzae* (see Chapter 2), the role of salicylic acid (SA) during infection will be especially highlighted.

The typical immunity related hormones are SA, jasmonic acid (JA) and ethylene (ET). Other hormones like abscisic acid (ABA), auxin (AUX), gibberellins (GA), cytokinins (CK) and brassinosteroids (BR) are thought to be more important during abiotic stress responses and general plant development (Kyndt et al., 2014b; Pieterse et al., 2012). Most of the published hormone-related research has been conducted on shoot tissue of dicotyledonous plants and the scope of this thesis is on a root-invading pathogen of rice, a monocotyledonous plant. The model that was put forward in dicotyledonous plants cannot be fully extrapolated to rice, but it forms a good base to start from (De Vleesschauwer et al., 2013; Kyndt et al., 2014b; Yang et al., 2013). Recently more and more data has become available regarding hormone signaling in rice upon pathogen infection. A recent review by De Vleesschauwer et al. (2014) has gathered all available information, thereby constructing a hormone signaling model in rice.

Salicylic acid biosynthesis

SA biosynthesis starts from chorismate, a product from the shikimate pathway. The complete biosynthetic pathway has yet to be revealed, but a lot is known already from investigations in *A. thaliana*. With chorismate as a precursor, SA can be formed through two distinct pathways (Figure 1.6); a first route that uses isochorismate as intermediate (ICS-route) and a second route with the aromatic amino acid phenylalanine as intermediate (PAL-route) (Dempsey et al., 2011). Chorismate is converted into phenylalanine by a multistep process in the chloroplast. Phenylalanine is transported out of the chloroplast and metabolized in the phenylpropanoid pathway. Phenylalanine is processed into trans-cinnamic acid (t-CA) by phenylalanine ammonia-lyase (PAL). From here t-CA can be converted to SA via several sub routes, depending on the plant species (Dempsey et al., 2011). Several secondary metabolites (e.g. flavonoids and monolignols) involved in defense can also be produced through this pathway. Another route uses isochorismate synthase (ICS) to convert chorismate to SA. Synthesis of SA through the ICS-route takes place in the chloroplast, after which it is transported to the cytosol (Serrano et al., 2013). Experimental evidence suggests that the ICS-route is responsible for most of the produced SA, basal SA as well as SA production induced by (a)biotic stress (Vlot et al., 2009). Increased resistance when overexpressing *ICS* in Arabidopsis confirmed the importance of the ICS-route (Matthews et al., 2014). The predominance of this route has not yet been confirmed in rice. In the cytosol SA can function as a signaling molecule in its free form, or it can undergo several modifications like methylation to make it more volatile or it can be glucosylated and stored in the vacuole (Widhalm and Dudareva, 2015).

Recent results have undermined the model whereby the ICS-route is the predominant route to synthesize SA. Although it was shown that the ICS-route accounts for 90% of SA production, quadruple Arabidopsis *pal* mutants produced 50% less SA upon infection with *Pseudomonas syringae* (Huang et al., 2010). If the majority of SA was produced by the ICS-route, silencing of *pal* genes should not have had such a detrimental effect on SA content. These results imply that *pal*-genes are important for SA production, basal as well as pathogen induced. However, it should be mentioned that the SA level was only reduced in the quadruple mutants, it remained at a normal level in all single, double and triple mutants. This means that the reaction catalyzed by PAL in the SA biosynthesis pathway is not a rate-limiting step (Huang et al., 2010).

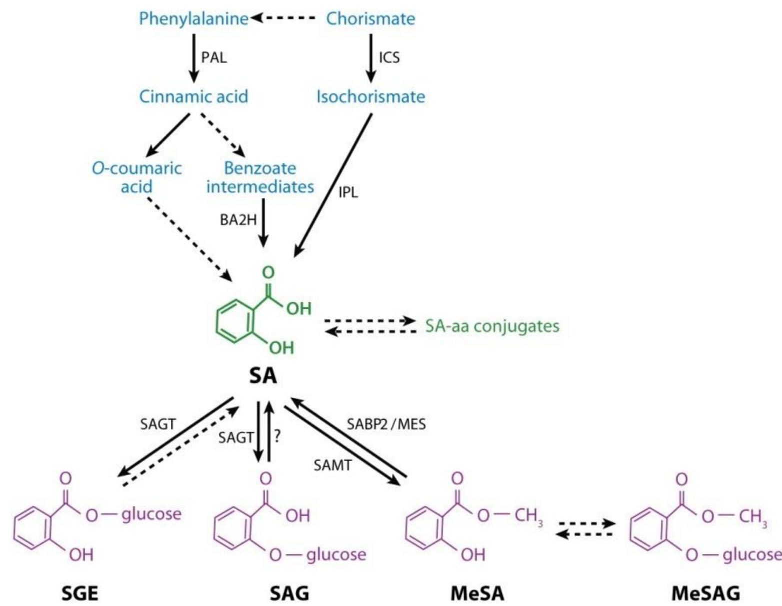

Figure 1.6: Salicylic acid (SA) biosynthesis pathway in plants (Vlot et al., 2009). SA can exist in a free form or it can be stored as methylated (MeSA), glucosylated (SAG) or esterified (SGE) SA. Dashed lines indicate multistep or unknown processes.

Role of salicylic acid during nematode infection of rice

Rice plants usually have very high levels of endogenous SA in leaf tissue, while SA levels in roots are lower. Although endogenous SA is kept at a high level, rice plants still respond to exogenous SA treatment (Silverman et al., 1995). This observation, taken together with the fact that SA-deficient NahG rice plants are more susceptible to nematode infection, indicates that SA plays a major role in stress responses (Yang et al., 2004). In *A. thaliana*, SA can mediate defense responses by activating NPR1, which triggers downstream defense mechanisms, including WRKY transcription factors (Vlot et al., 2009). Rice has NPR1 and WRKY homologs, but they do not act downstream of each other. They appear to be activated independently by SA, splitting the SA-responsive pathway in two branches, an NPR1-dependent pathway and a WRKY45-dependent pathway. It seems that WRKY45 is mainly involved in the up regulation of genes, while NPR1 does the opposite (Figure 1.7) (Takatsuji et al., 2010).

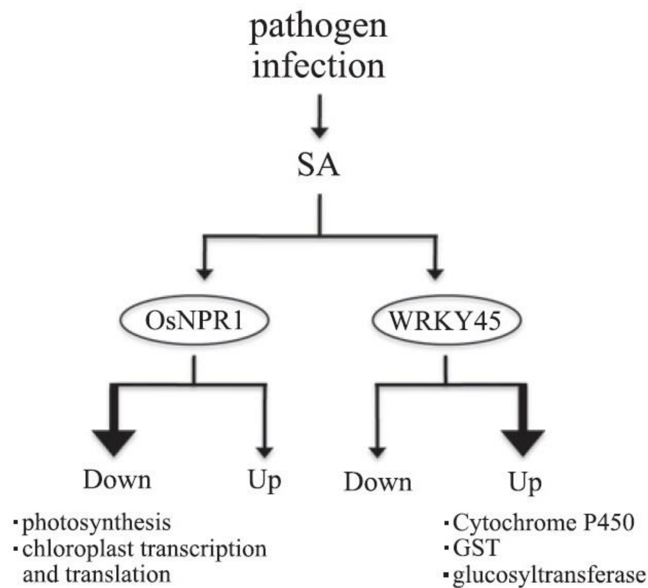

Figure 1.7: Salicylic acid signaling pathway in rice studied in leaves, adapted from Takatsuji et al. (2010). The SA-responsive pathway in rice is split into two branches: an NPR1-dependent and a WRKY45 dependent pathway. The thickness of the arrows gives an indication of the relative amount of up- or downregulated genes.

Experimental evidence has shown that SA is an important factor during nematode infection of rice. Spraying plants with an SA analog has a strong adverse effect on *H. oryzae* infection (Nahar et al., 2012). Although SA seems important during migratory nematode infection, exogenous application of SA analogs to rice plants did not result in a high increase of resistance to *M. graminicola* (Nahar et al., 2011). However, transcriptome data showed that *M. graminicola* is able to suppress the SA pathway in galls, while the SA pathway seems to be upregulated initially upon *H. oryzae* infection (Kyndt et al., 2012a). If samples are taken at later time points it seems that *H. oryzae* is able to subdue the SA biosynthesis pathway as well (Kyndt et al., 2012b). Both *H. oryzae* and *M. graminicola* were able to decrease expression of the isochorismate synthase gene systemically at 3 dpi and 7 dpi (days post infection). Isochorismate synthase is a key factor in the SA biosynthesis pathway (Vlot et al., 2009). In rice infected with *H. oryzae*, the shikimate pathway is repressed in shoots. Since the shikimate pathway produces chorismate, a precursor for SA and other defense related secondary metabolites (e.g. flavonoids and phenolics), it is possible that less of these compounds are synthesized, making the plant more vulnerable (Kyndt et al., 2014a).

Role of other hormones during nematode infection

Most of the research regarding hormonal regulation upon nematode infection has been focusing on RKNs, so information about hormonal regulation during *H. oryzae* infection is scarce. SA is one of the best studied examples of hormonal regulation of defense, but of course other hormones also play a role during infection. Next to SA, JA and ET complete the trio of phytohormones which are considered as the main protagonists in defense to nematode infection. This general idea was confirmed by Nahar et al. (2011). The activation of these three hormonal biosynthesis pathways through external hormone application– and especially the JA pathway – led to a decreased susceptibility of the rice plant to *M. graminicola*. ET seems to activate the JA pathway, thereby promoting resistance against RKNs (Nahar et al., 2011). In addition, the application of ABA to rice plants induces susceptibility *to H. oryzae* infection, probably caused by an antagonistic reaction with the SA, JA and ET pathways (Nahar et al., 2012). For *H. oryzae* and *M. graminicola*, it has been shown that, at early time points after infection, both JA and ET pathways are induced, underlining their importance during defense responses (Kyndt et al., 2012b; Kyndt et al., 2012a).

The biosynthesis and signaling pathways of GA and BR are activated in galls during the early stages of infection by *M. graminicola* (Kyndt et al., 2012a). GA and BR play important roles in developmental processes in the plant, so it is tempting to speculate that RKNs recruit these pathways to aid in the formation of specialized feeding cells (Jammes et al., 2005). BR can also participate in susceptibility of the plant. BR is an antagonist of JA, which is a promoter of resistance against RKN, hence a higher concentration of BR can lead to plants that are more susceptible to RKN infection (Nahar et al., 2013). Some effectors secreted by plant pathogens target the BR-signaling pathway. For instance, AVR2 from *Phytophthora infestans* interacts with BSL1, a protein phosphatase involved in BR signal transduction (Saunders et al., 2012). The BR-signaling pathway inducing growth and the PTI pathway upon flg22 perception are quite similar and share some components. These similarities led to the hypothesis that there is crosstalk between the two pathways, meaning that BR could possibly have a central role in the trade-off between growth and defense (Lozano-Duran and Zipfel, 2015). GA will also play a role during defense. The GA pathway is strongly induced upon the incompatible interaction of soybean with *M. javanica*, suggesting that GA is also involved in plant defense and not only feeding site formation (Beneventi et al., 2013).

The phytohormone AUX is more involved in developmental processes than defense responses. It appears that nematodes have found ways to use this feature to their advantage. AUX levels are increased in feeding cells and the surrounding tissue, thereby supporting feeding site establishment (Absmanner et al., 2013). Experimental evidence suggests that Hs19C07, an effector secreted by *H. schachtii*, is probably involved in this process (Lee et al., 2011). Similar observations were made for CK and root knot nematodes. A CK-inducible gene (*ARR5*) was highly expressed in cells surrounding the giant cells and smaller galls were formed if CK was degraded by overexpressing a CK oxidase (Lohar et al., 2004). These results suggest that nematodes developed mechanisms to manipulate hormonal pathways to use them during feeding site establishment.

Hormonal regulation; a difficult subject

The results described in the previous paragraphs show that hormonal regulation of defense is a very complex subject. It is difficult to specify the role of each hormone separately since one hormone can influence the function of one or more other hormones upon nematode infection, the cross-talk of phytohormones. In addition, hormone responses can differ not only with the type of pathogen, but also with the stage of infection (e.g. RKN in their migratory phase versus RKN during feeding site establishment). Furthermore, nematodes (and plant pathogens in general) have evolved to hijack or manipulate hormonal biosynthetic pathways and signaling in the host. This makes it hard to distinguish the difference between endogenous plant responses and responses that are actively induced by the nematode to their own benefit. It is a difficult subject to study since even the investigation method can influence the outcome. If plants are treated by hormone spraying, the effects on plants can be dependent on the administered dose, whereby higher doses have opposite effects compared to low doses. (Nahar et al., 2013). Hormonal pathways are quite complex, with crosstalks at different steps in the pathway (Li et al., 2015). Therefore depending on the gene/protein that is being analyzed, a different result can be obtained. This problem can be solved by using transcriptomics, whereby expression of all genes is investigated in stead of only a few. In addition, endogenous hormone concentrations can be measured directly by using chromatography and mass spectrometry. Hormonal regulation in rice upon nematode infection remains an elusive topic, so additional research is needed to complete the complicated puzzle.

1.5. Nematode effectors: effective weaponry?

1.5.1. Cell wall modifying proteins: hydrolyzing the first barrier

The cell wall is a formidable barrier, but nematodes have found ways to overcome it. The first and most clear adaptation to this hurdle is clearly visible in the anatomy of the nematode. All PPNs have a distinct aspect in their anatomy that separates them from most other nematodes. PPNs are armed with a stylet, which is a protrusible spear in their oral cavity. Some fungivorous nematodes, like *Aphelenchus* and *Aphelenchoides* species, and even some insect-feeding species (like *Peraphelechus orientalis*) also have this feature. The stylet can be used to mechanically pierce through the cell wall of the host. Once the cell wall is pierced, the nematode is able to withdraw the highly needed nutrients for its survival. The stylet is also used in biochemical warfare. Most PPNs are equipped with three pharyngeal glands, one dorsal and two subventral (Figure 1.8) (Davis et al., 2004; Hussey, 1989), but some species can have up to five pharyngeal glands (Loof and Coomans, 1972; Siddiqi and Brown, 1965). The gland cells produce secretory proteins that are transported through the stylet into the plant tissue. This mechanism is the basis of the ability of the nematode to infect the plant. Secreted proteins are grouped under the name of “effectors” or “parasitism proteins”. Effectors aid in penetration by degradation of the cell wall, are responsible for the establishment of a feeding site in sedentary nematodes and help in evading the defense mechanism of the plant (Davis et al., 2004).

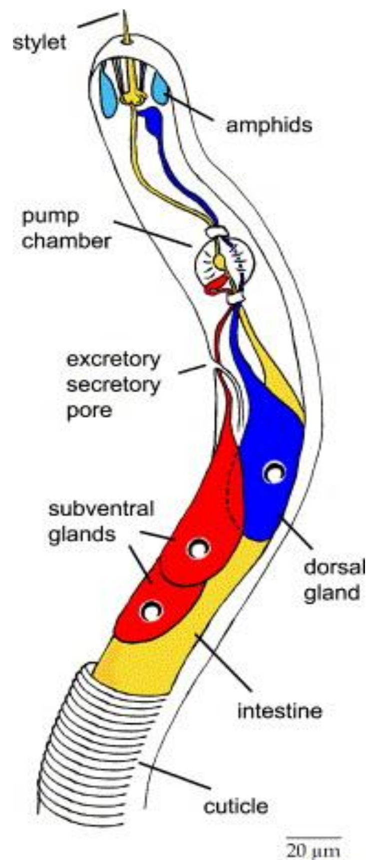

Figure 1.8: Schematic representation of the typical PPN anatomy (Vanholme et al., 2004).

Characteristics of cell wall modifying proteins

The study and discovery of CWMP has been a hot topic in nematodes. A lot of genomic and transcriptomic research has focused on identifying CWMP as well as other effector proteins (Haegeman et al., 2011b; Haegeman et al., 2012; Opperman et al., 2008). It is relatively easy to search for those types of genes through similarity searches with publicly available sequence data. CWMP also share some common features.

- CWMP have a distinct N-terminal signal, targeting the protein for secretion through the stylet.
- Possible secretion can be indicated by *in situ* hybridization, showing a clear signal in the pharyngeal glands (de Boer et al., 1998).
- CWMP tend to be present in PPNs in large gene families (Haegeman et al., 2008; Kikuchi et al., 2004).
- Many of the genes encoding CWMP resemble bacterial sequences; suggesting that they are probably acquired from bacterial plant parasites through horizontal gene

transfer (HGT). Not only HGT through bacteria is possible, but also fungi can be the source of genes for certain CWMP (Kikuchi et al., 2004). This phenomenon has been described and discussed by Haegeman et al. (2011a).

These above-mentioned characteristics have made it fairly easy to discover new potential CWMP in PPNs. To discover new effectors which might have an effect on plant defense, it is not always possible to rely on similarity searches. In this case the focus will lie on the presence of an N-terminal secretion signal and expression in the pharyngeal glands. Another technique which might be used is expression profiling. Gene expression is measured in different nematode stages to define the role/importance of the gene product in each stage (Thorpe et al., 2014). This is a good technique for identifying genes with, for instance, a role in feeding site initiation or maintenance. Since all stages of *H. oryzae* are migratory and feed on roots, expression profiling might not be that relevant for migratory compared to sedentary nematodes.

Cellulose and hemicellulose degrading enzymes

The first cell wall modifying protein reported in nematodes was a β-1,4-endoglucanase, member of the glycosyl hydrolase family 5 (GHF5). This endoglucanase is able to degrade the key structural component of the plant cell wall, cellulose, which is composed of a β-1,4-glucan backbone. This enzyme was the first endoglucanase discovered in animals that did not originate from symbiotic micro-organisms (Smant et al., 1998). Due to the high overall amino acid identity with endoglucanases of bacteria, for the first time, it was put forward that some effectors might be acquired by horizontal gene transfer from a prokaryotic organism. After endoglucanase was found in *G. rostochiensis* and *H. glycines* (Smant et al., 1998), it was subsequently discovered in other PPNs including *M. incognita* (Rosso et al., 1999), *Radopholus similis* (Haegeman et al., 2008) and *P. penetrans* (Uehara et al., 2001). All endoglucanases belonged to GHF5 and were members of a gene family. Endoglucanases reported in *Bursaphelenchus* spp. are members of GHF45, suggesting a different origin, hence an independent horizontal gene transfer (Kikuchi et al., 2004). The importance of these endoglucanases during infection has been proven by Chen and colleagues (Chen et al., 2005). Nematodes treated with double stranded RNA to reduce expression of a gene coding for endoglucanase, were impaired in their ability to invade roots of host plants.

Another type of endoglucanase, a β-1,3-endoglucanase, has been found in *Bursaphelenchus* spp. and in *Aphelenchus avenae* (Karim et al., 2009; Kikuchi et al., 2005). Both nematodes are fungivorous and use this enzyme to degrade β-1,3-glucan, a polymer which is a major component of fungal cell walls. Intriguingly a β-1,3-endoglucanase has been found in the transcriptome of *P. coffeae* although this nematode has not been reported to feed on fungi (Haegeman et al., 2011b). Plants can deposit large callose depositions upon pathogen infection, making it difficult for the invading pathogen to migrate through the plant tissue (Flors et al., 2005; Luna et al., 2011). Callose is composed of β-1,3-glucans, making it tempting to speculate that β-1,3-endoglucanases play a role in the degradation of callose. This type of countermeasure against plant defense mechanisms has already been proposed in the plant pathogenic fungus *Gaeumannomyces graminis* (Yu et al., 2010). More research is needed to understand the role of β-1,3-endoglucanases of non-fungivorous nematodes. Once more genomes and better covered transcriptomes are available for PPNs, it will be easier to analyze the presence of β-1,3-endoglucanase genes in other nematodes.

Next to cellulose, some GHF5 endoglucanases are capable of degrading hemicellulose components that are β-1,4 linked (Gao et al., 2004). A more specialized enzyme to degrade hemicellulose is endoxylanase. The gene coding for endoxylanase has been reported in several PPNs, the protein of which is thought to degrade xylan, the major component of hemicellulose (Haegeman et al., 2009b; Mitreva-Dautova et al., 2006; Opperman et al., 2008).

Pectin degrading enzymes

PPNs have acquired genes that enable them to degrade pectins, present in the primary cell wall and middle lamella. Pectins form the matrix in which cellulose and hemicellulose fibers are embedded (Figure 1.5). If pectin is degraded by a pathogen, the tissue develops a characteristic symptom called soft-rot (Lietzke et al., 1994). The two main enzymes present in PPNs to depolymerize pectins, are pectate lyase and polygalacturonase.

Pectate lyases are present in plant pathogenic fungi and bacteria as well as nematodes (Barras et al., 1994). They help in a general degradation or softening of the cell wall to give the pathogen the opportunity to pierce through it. Pectate lyase was originally discovered in *G. rostochiensis*, as such being the first pectate lyase from animal origin reported to aid in cell wall digestion (Popeijus et al., 2000). The important role of pectate lyases during

nematode infection was made clear by Vanholme et al. (2007). RNAi mediated knock down of a gene coding for pectate lyase of *H. schachtii* resulted in a lower infection rate, proving the importance of this enzyme in the secreted arsenal of PPNs. Another pectin degrading enzyme, present in many *Meloidogyne* spp., is polygalacturonase. This enzyme was discovered in *M. incognita*, which was also the first report of a polygalacturonase of animal origin (Jaubert et al., 2002a). At first it was thought that polygalacturonase might be restricted to *Meloidogyne* spp., but recently the corresponding gene was also discovered in EST data of *H. oryzae* and *P. coffeae* (Bauters et al., 2013; Haegeman et al., 2011b). This implies that polygalacturonase was probably acquired by HGT that occurred after the speciation event that separated Meloidogynidae and Pratylenchidae from Heteroderidae.

Pectin molecules are branched, which makes the pectin backbone less accessible to enzymes. This might pose a problem for pectate lyase, which will actively cleave the α-1,4-linkages in the backbone of pectin. Some nematodes have acquired genes, coding for enzymes able to solve this problem. One of these enzymes is arabinogalactan endo-1,4-β-galactosidase, an enzyme capable of hydrolyzing β-1,4-galactan in the branches of pectin. The gene has been cloned from the cyst nematode *H. schachtii* and was reported in the EST data of *P. coffeae* (Haegeman et al., 2011b; Vanholme et al., 2009a). So far it has not been detected in other PPNs. It is peculiar that this gene was not detected in the genome of *Meloidogyne* spp. since *Pratylenchus* spp. are more closely related to *Meloidogyne* spp. than to *Hetorodera* spp.. This could be an indication that the gene was acquired by two independent HGTs in both species, or by one HGT in a common ancestor, followed by gene loss after speciation in Meloidogynidae. RKNs made up for the absence of arabinogalactan galactosidase by expressing arabinases, which have been discovered in the genome of *M. incognita*. Arabinase can hydrolyze the α-1,5-linkages of arabinan polysaccharides, present in the branches of pectin (Abad et al., 2008; Davis et al., 2011).

Non-enzymatic cell wall modifications

In the previous paragraphs cell wall degrading enzymes produced by nematodes have been discussed. All these secreted proteins have an enzymatic activity using a plant cell wall component as substrate. Most PPNs also secrete another protein through their stylet that helps in the degradation process of the cell wall, but it does so without enzymatic modifications. Expansin was discovered in the cyst nematode *G. rostochiensis* (Qin et al., 2004). Previously, expansins were reported to be present throughout the plant kingdom, but

they had not been detected in animals before. In plants they are most likely used to disrupt non-covalent bounds between cell wall fibrils and the hemicellulose matrix in which these fibrils are embedded. In this way expansins are able to loosen up the cell wall, enabling the cell to extend (Cosgrove, 2000; Queen-Mason and Cosgrove, 1995). Experimental evidence suggests that nematode expansins have the same effect on the cell wall as the endogenous plant expansins (Qin et al., 2004). This type of cell wall modifying protein is indispensable during nematode infection. It loosens the cell wall, generating space for other cell wall degrading enzymes to access their substrate. The importance is also reflected in the presence of genes encoding expansin in several PPNs, migratory as well as sedentary endoparasites (Davis et al., 2011). In addition to expansin, some PPNs have been found to secrete effector proteins consisting of just a predicted N-terminal secretion signal and a CBD. A secreted CBD from *H. schachtii* was shown to interact with Arabidopsis pectin methylesterase, thereby increasing its activity (Hewezi et al., 2008). Overexpression of *CBD* in *A. thaliana* induced susceptibility. The mechanism by which the interaction of CBD with pectin methylesterase promotes susceptibility remains uncertain, but it probably reduces methylesterification of pectin in the cell wall (Hewezi et al., 2008) .

1.5.2. Other effectors: a more sophisticated attack

Once the cell wall has been breached, the biological warfare between plant and pathogen continues. In addition to CWMP, nematodes also secrete many other effectors as well. Effectors secreted by sedentary nematodes are crucial for their survival. They are needed to induce the formation of a specialized feeding cell from a normal plant cell and they have to make sure the plant remains unaware of this process in order to prevent defense responses. Migratory nematodes do not have to establish a specialized feeding site, hence most secreted effectors have a role in penetration and evading/attenuating defense responses. Since the scope of this research is mainly about the migratory nematode *H. oryzae*, the emphasis will be on effectors that have a role in attenuating defense responses of the plant. In the following paragraph a brief overview will be given of some of the most discussed effectors.

Secreted antioxidants

Plants have developed multiple sophisticated ways to defend themselves, making it almost impossible for the nematode to infect the host without triggering any response. Some

nematodes have adapted to these circumstances by secreting effectors to protect themselves. A glutathione-S-transferase (GST) expressed in the pharyngeal glands, has been found in *M. incognita*. It is thought to be secreted by the nematode into the host tissue to aid in the detoxification of a wide range of nematotoxic compounds produced by the plant upon infection (e.g. phytoalexins, isoflavonoids and terpenoids) (Campbell et al., 2001; Dubreuil et al., 2007). Upon nematode infection, the plant can produce reactive oxygen species (ROS), this phenomenon is known as the oxidative burst (Waetzig et al., 1999). ROS are able to attack the nematode directly but they can also activate signaling pathways which lead to strengthening of the cell walls. As means of protection, nematodes can secrete antioxidant proteins. *M. incognita* secretes superoxide dismutase and peroxiredoxin. These proteins are strong antioxidants that can metabolize superoxide and hydrogen peroxide respectively (Bellafiore et al., 2008; Dubreuil et al., 2011). A secreted glutathione peroxidase, a peroxiredoxin and a superoxide dismutase have been found in the secretome of *G. rostochiensis* as well (Jones et al., 2004; Robertson et al., 1999; Robertson et al., 2000). This group of secreted enzymes makes it possible for the nematode to survive under constant pressure from the defense system of the plant.

Venom allergen-like proteins

It was discussed that DAMPs are potent elicitors of local defense responses. DAMPS can be produced in large quantities when a nematode is penetrating the plant and when it is migrating trough the tissue. Many PPNs secrete venom allergen-like proteins (VAPs). Experimental evidence suggests that these secreted VAPs can modulate the defense response activated by this tissue damage, hence this is another important protective factor during the migratory phase of nematodes (Lozano-Torres et al., 2014).

Suppressing PTI and ETI

The zig-zag model has been clearly demonstrated in plant pathogenic bacteria. The validation of this model in nematodes is still ongoing. There are some effectors which have been linked to ETI by nematodes, like the *Map-1* and the *Mi-Cg1* gene of *M. incognita*. Both genes are expressed in avirulent populations while the expression is absent in the virulent populations of *Meloidogyne* spp.. These observations suggest that both proteins are involved in the early steps of recognition by the plant and this recognition was linked to the Mi-1 receptor in plants (Gleason et al., 2008; Semblat et al., 2001). The best known example regarding ETI in nematodes is the recognition of the RBP-1 protein from *G. pallida* by Gpa2,

an NB-LRR receptor from potato. RBP-1 is a sprysec which can provoke a HR upon reaction with Gpa2. This interaction is also mediated by RanGAP2, which is needed to elicit the HR. Recognition is dependent on a single amino acid polymorphism on position 187 in the spry domain. Rbp-1 is also expressed in virulent populations, implying that *G. pallida* also secretes other effectors to inhibit the RBP-1/Gpa2 interaction, thereby provoking ETS (Sacco et al., 2009).

There are also some examples of secreted effectors that are responsible for the attenuation of defense, but the mechanisms by which this is accomplished remain unclear. For instance, a member of the SPRYSEC family of *Globodera* spp. (SPRYSEC19) interacts with a tomato resistance gene homologue (SW5F). Since this interaction does not cause any HR, it is hypothesized that SPRYSEC19 can suppress ETI (Jones et al., 2009; Postma et al., 2012; Rehman et al., 2009). Calreticulin, secreted by the RKN *M. incognita*, is able to suppress the PTI triggered by the PAMP elf18 in *A. thaliana*. When *A. thaliana* lines overexpressing calreticulin were treated with elf18, expression of defense marker genes was repressed and callose deposition was inhibited compared to wild type plants (Jaouannet et al., 2013). An example regarding cyst nematodes is the secreted GrCEP12 peptide of *G. rostochiensis*. Experimental evidence showed that this peptide is able to suppress flg22-induced PTI responses in leaves of *Nicotiana benthamiana*, hereby reducing ROS production and inhibiting expression of PTI marker genes (Chen et al., 2013). These results show nematodes secrete effectors that play important roles in overcoming basal plant defense systems, but the mechanisms remain unclear.

Effectors acting on hormonal pathways

Sedentary nematodes as well as migratory nematodes secrete chorismate mutase (CM) (Haegeman et al., 2011c). CM is involved in the SA biosynthesis pathway. SA is an important signaling hormone regulating defense responses. Chorismate can be converted into SA in a multistep process. It is hypothesized that CM depletes the chorismate pool, thereby reducing the available chorismate for conversion to SA (Haegeman et al., 2011c). This hypothesis was proven for *Ustilago maydis*, a fungal plant pathogen. Experimental evidence showed that the secreted CM of *U. maydis* was able to reduce total SA content in plants (Djamei et al., 2011). Since SA is an important player in plant defense, the SA biosynthesis pathway is a popular target for effectors. The nematode effector 10A06 of *H. schachtii* was able to elevate spermidine levels and increased polyamine oxidase activity through its

interaction with SPDS2 from *A. thaliana*. These compounds might aid in protecting the nematode from ROS. Although the SA pathway is not directly targeted, it was shown that overexpression of *10A06* in plants repressed SA signaling, making the plant more vulnerable to nematode infection (Hewezi et al., 2010).

CM can also have an effect on the auxin signaling pathway. Experimental evidence provided by Doyle and Lambert (2003) suggested that CM alters auxin homeostasis. The distribution of this hormone can also be regulated by secreted nematode effectors. The effector Hs19C07 from *H. schachtii* is able to bind LAX3, an auxin influx transporter. Overexpression of the nematode effector increased the rate of lateral root formation, which is a sign of a higher auxin influx, indicating that Hs19C07 increases the activity of LAX3 (Lee et al., 2011).

Effectors as mimics

PPNs have evolved to deceive their hosts by mimicry. During convergent evolution, pathogens have evolved to produce proteins that resemble proteins of their host. During pathogenesis, these mimics can have an effect on certain pathways in the host. Some nematode effectors can mimic plant peptide hormones. One of the best studied examples is a group of effectors that mimic the CLAVATA (CLV) signaling components. These CLV mimics have been reported in sedentary nematodes. Experimental evidence showed that these proteins are processed in the same way as the endogenous CLV peptides. Silencing of these peptides in nematodes led to a decrease in infectivity (Huang et al., 2006). The precise role and mechanism by which these peptides operate have yet to be elucidated, but their role during infection is indisputable (Haegeman et al., 2011c; Mitchum et al., 2008). Besides peptide hormone mimicking, some nematodes produce hormones, secreting them into the plant tissue. For instance, cytokinins and conjugated auxins have been discovered in the secretions of *M. incognita* and *H. schachtii*. To date, researchers are still unsure about the synthesis and function of these latter two hormone mimics. Hormones have also been found in non-PPNs. Further studies are needed to investigate the role of nematode-secreted hormones during infection (De Meutter et al., 2003; De Meutter et al., 2005).

1.6. Scope and outline of the thesis

Sedentary nematodes have always been the center of attention in the world of nematologists studying PPNs. They have been the subject of ample field experiments, molecular studies, transcriptome studies, and lately also genomic studies. Research on migratory PPNs has lagged behind, but recently more transcriptome studies have been conducted, providing the research community with additional data about these migratory species.

In this thesis, the transcriptome of the migratory nematode *H. oryzae* is described. In chapter 2 an attempt was made to uncover the arsenal of CWMP used by this nematode to successfully infect rice roots. This approach revealed a new cell wall degrading enzyme that was not reported in nematodes before and that is probably involved in hemicellulose degradation; β-mannanase (GHF5). The transcriptome data was also used to give more insight into potential survival strategies in the field. Interestingly two effector genes were discovered that could have an effect on the SA and phenylpropanoid pathway in plants, CM and ICM.

In chapter 3 it is shown that both CM and ICM have *in vitro* enzymatic activity. In addition, a 3D model of the protein structure was predicted. The potential effect on plant defense was tested by infiltration studies, showing that both genes can have an effect on susceptibility of the plant. In chapter 4 we try to unravel how CM and ICM might help *H. oryzae* during infection. Transgenic rice plants overexpressing these genes were generated and further characterized by hormone measurements, RNA-seq and infection experiments. Altogether, results showed that both genes have an effect on several pathways and metabolites involved in plant-pathogen interactions, like phenylpropanoids, ABA and terpenoids.

Taken together, these results will provide us with a better insight of the survival and infection strategies of *H. oryzae*.

Chapter 2. Analysis of the transcriptome of *Hirschmanniella oryzae* to explore potential survival and infection strategies

This chapter is adapted from: Analysis of the transcriptome of *Hirschmanniella oryzae* to explore potential survival strategies and host-nematode interactions. Bauters L., Haegeman A., Kyndt T. and Gheysen G., Molecular Plant Pathology (2014), 15 (4), 352-363

2.1. Summary

The rice root nematode *Hirschmanniella oryzae* is the most abundant PPN in flooded rice fields and is distributed world-wide. Although it is economically less important than sedentary nematodes, it can cause severe yield reductions and economic losses in specific environmental conditions. No transcriptome data for this genus were available until now. 454 sequencing was performed on a mixed life stages population to gain an insight into nematode–plant interactions and nematode survival strategies. The results of two assembly strategies were combined to reduce the redundancy of the data, generating a final dataset of 21,360 contigs. The data were screened for putative plant cell wall-modifying proteins, which facilitate nematode migration through host roots. A β-mannanase, previously not reported in nematodes, was detected in the dataset. The data were screened for putative effector proteins that may alter the host defense mechanism. Two enzymes, CM and ICM, thought to have an effect on the salicyclic acid pathway, were identified. Experimental treatments of *H. oryzae* with artificial seawater showed that late embryogenesis abundant (LEA) proteins and SXP/RAL-2 are induced, suggesting that these proteins are involved in the process of anhydrobiosis. The newly generated data can highlight potential differences between sedentary and migratory nematodes, and will be useful in further studies of host–nematode interactions and the developmental biology of this nematode.

2.2. Introduction

The rice root nematode *Hirschmanniella oryzae* is a migratory endoparasite with *Oryza sativa* as its main host. *Hirschmanniella oryzae* is the most common nematode in flooded rice ecosystems all over the world (Prot and Rahman, 1994). The nematode is adapted to anaerobic conditions and this makes it the most abundant nematode species in these ecosystems (Babatola, 1981; Maung et al., 2010).

Symptoms of damage caused by *H. oryzae* are difficult to interpret. In many cases, there are no visible symptoms above ground, although some chlorosis and growth retardation can appear (Babatola and Bridge, 1979; Ichinohe, 1988; Khuong, 1987). *Hirschmanniella oryzae* prefers to enter the root through lateral roots or root tips, thereby using common invasion sites. It migrates within the aerenchyma of the root, which is well developed in flooded rice,

leaving a trace of necrotic tissue along its path. This necrosis, together with the secondary invasion of microorganisms, causes general browning of rice roots (Babatola and Bridge, 1980). The yield loss as a result of nematode invasion varies between geographical regions and population density. Yield losses of up to more than 30% have been reported (Babatola and Bridge, 1979; Ichinohe, 1988; Prot and Rahman, 1994). Nematodes can survive in wet soil for 7 months without a host and can persist at high population densities in several weeds and food crops (Bridge et al., 2005). *Hirschmanniella oryzae* reproduces sexually and, under favourable conditions, the life cycle can be completed in 33 days, during which the nematode undergoes four moulting stages. All larval and adult life stages can feed on the host (Karakas, 2004).

The majority of nematode sequence data originate from sedentary species, but recent advances in this field have provided more data on migratory nematodes as well (Haegeman et al., 2011b; Jacob et al., 2008; Kikuchi et al., 2011; Nicol et al., 2012). Sedentary species have a huge impact on yield for many important food crops world-wide (Sikora and Fernandez, 2005; Wesemael et al., 2011). In the USA, *Heterodera glycines* alone accounts for almost 30% of the total losses in soybean production (Wrather and Koenning, 2006). However, the economic importance of migratory species, such as *Pratylenchus* spp., *Hirschmanniella* spp. and *Radopholus similis*, which have a broad host range and can have a large impact on several crops should not be ignored (Moens and Perry, 2009).

The expressed sequence tag (EST) data generated in this project have been used to identify secreted putative CWMP and putative effector proteins, secreted molecules which manipulate the host for the benefit of the pathogen. The mechanisms behind the successful infection of a host plant were investigated, as were the molecular tools used by the nematode to cope with detrimental environmental circumstances, such as drought and potential bacterial infection. Until now, no sequence data were available for the genus *Hirschmanniella*, except for some β-1,4-endoglucanases (Rybarczyk-Myd et al., 2012). The generated transcriptome data will provide the scientific community with a new source of information about this genus and migratory nematodes.

2.3. Results

2.3.1. Dataset characteristics

RNA extracted from mixed stage populations of *H. oryzae* was sequenced using a Roche 454 sequencer, generating 450,171 reads; following quality control, 134,205 and 106,911 assembled sequences longer than 150 bp were produced using Newbler and CLC assembly programs, respectively. The CLC software produced more than twice as many contigs as Newbler (48,347 and 23,196 contigs, respectively) (Table 2.1). As CLC software is thought to generate a less redundant assembly, these data were used in further analysis (Kumar and Blaxter, 2010). To allow a greater confidence in the predicted sequences, only those CLC-assembled contigs that showed high similarity with the contigs predicted by Newbler software were retained. This resulted in a batch of 22,321 sequences. To reduce the amount of contaminating sequences, contigs with high similarity to certain plant-pathogenic or soil bacteria, fungi or rice proteins were removed from the dataset. This led to a final set of 21,360 predicted contigs (Table 2.1). The final dataset is enriched in contigs with a relatively high number of reads. Roughly 30% of the contigs consisting of two to five reads were kept in the final dataset, whereas about 80% of the contigs containing more than five reads were retained (Table 2.2).

Table 2.1: Dataset characteristics from the two different assemblies using software programs CLC and Newbler 2.3. The last column contains the characteristics of the final dataset that was used in the different analyses.

	CLC	Newbler	Final dataset
number of reads	450,171	450,171	204,524
average read length	385 nt	385 nt	/
number of contigs	48,347	23,196	21,360
mean contig length	600 nt	549 nt	680 nt
number of singletons	58,564	128,557	/
mean singleton length	374 nt	349 nt	/

Table 2.2: Comparison of the original dataset (CLC) with the final dataset, with regard to the number of reads per contig. The last column shows the percentages of sequences from the original dataset that were retained in the final dataset.

Reads /contig	# CLC contigs	# final contigs	% retained
2 to 5	34,334	9,650	28.11
6 to 10	8,125	6,563	80.78
11 to 20	3,886	3,457	88.96
21 to 50	1,577	1,363	86.43
51 to 100	301	239	79.40
101 to ...	122	88	72.13

2.3.2. Comparison with protein databases and annotation

Almost all sequences, except for 22 contigs, were predicted by ORFpredictor to have an open reading frame (ORF) with a mean length of 144 amino acids; 16,656 of these expected protein sequences started with methionine; 1,356 sequences were predicted to have a putative signal peptide and no transmembrane domain. Among these putative secreted proteins, sequences with an assigned protease function were most abundant. Although certain proteases have been found in the secretome of PPNs, some of them will probably be secreted in the gut to perform a digestive function (Vieira et al., 2011). Also proteins with a lectin domain and transthyretin-like proteins were abundant in this subdataset. Transthyretin-like proteins are members of a family occurring in all types of nematodes. Expression in the gland cells could not be confirmed (Jacob et al., 2007). To our knowledge, only one member was assigned with a function: A transthyretin-like protein from *C. elegans* mediates recognition of apoptotic cells (Wang et al., 2010).

Blastx (bitscore > 50) of all sequences against the SwissProt and Trembl protein databases was used to search for similar sequences, and this resulted in 14,248 significant hits. The resulting identifiers from this search were used as query for functional annotation according to the Gene Ontology (GO) terminology for cellular component, biological process and molecular function. The most abundant GO terms are summarized in Figure 2.1. A total of 153,906 GO terms were assigned to the sequences; 6,473 of these terms were unique. Figure 2.1 shows that most proteins reside in the cytoplasm and the nucleus. Most of the sequences are predicted to have a function in ATP or protein binding. In the ‘biological process’ category, the term ‘oxidation–reduction process’ is overrepresented compared with the other terms in this category.

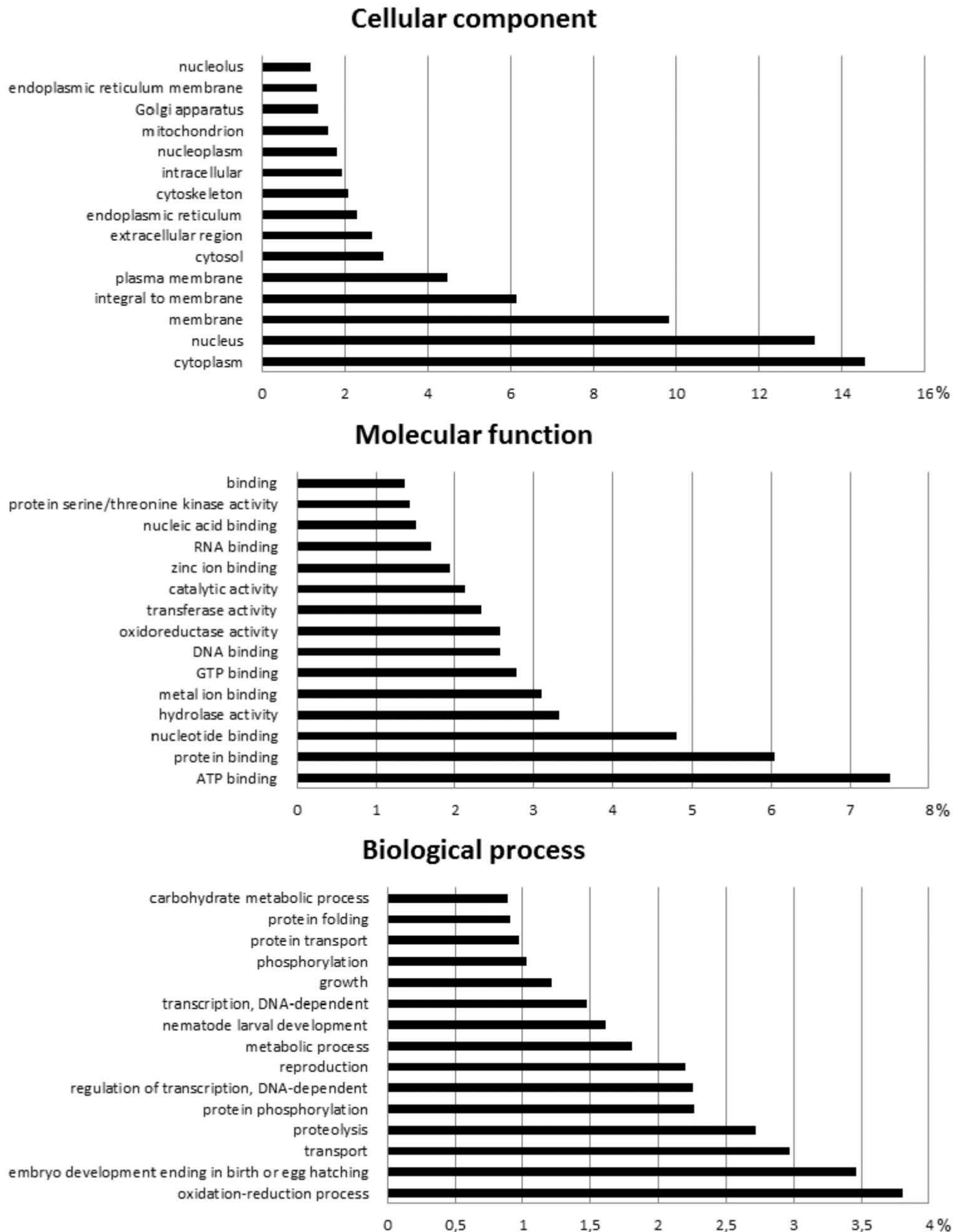

Figure 2.1: Percentage of *Hirschmanniella oryzae* contigs assigned to a certain Gene Ontology (GO) term as predicted by QuickGO from EBI.

HMMscan was used to identify possible Pfam domains. Of the 7,090 sequences without similarity to SwissProt or Trembl databases, 173 were suspected to have a Pfam domain. The 10 most abundant Pfam domains are listed in Table 2.3; 66.7% of the predicted contigs were similar to sequences from SwissProt and Trembl databases. Predicted protein sequences were scanned for the occurrence of Pfam domains. Most of the Pfam domains mentioned in Table 2.3 take part in multiple processes. Protein kinases are involved in a multitude of cellular processes, including development, intercellular communication and differentiation (Manning et al., 2002). The WD-repeat is also implicated in a wide range of functions (Smith et al., 1999), as are the cysteine proteases (Chapman et al., 1997; Grudkowska and Zagdanska, 2004). Other Pfam domains, such as Ras and Arf, are involved in a variety of signaling networks and regulatory pathways (Donaldson and Honda, 2005; Downward, 1998). Cysteine proteases with the Peptidase_C1 domain have very diverse functions; they can even act as manipulators of plant defense if secreted by plant-pathogenic bacteria (Brix et al., 2008; Shindo and Van der Hoorn, 2008). RNA recognition motifs (RRMs) are one of the most abundant protein domains in eukaryotes and are involved in post-transcriptional gene expression processes (Dreyfuss et al., 2002). These Pfam domains, or the corresponding InterPro domains, have been reported previously to be among the most abundant InterPro domains in an assembly of nematode transcriptomes (Parkinson et al., 2004).

Table 2.3: Ten most common protein families with their accession numbers of the Pfam and InterPro databases. The target names and descriptions are specified in the last two columns. The fourth column shows the share of contigs assigned with that Pfam domain.

Pfam	InterPro	Number	Percentage	Target name	Description of target
PF00069	IPR000719	178	1.67	Pkinase	Protein kinase domain
PF07714	IPR001245	135	1.27	Pkinase_Tyr	Protein tyrosine kinase
PF00400	IPR001680	115	1.08	WD40	WD domain, G-beta repeat
PF00071	IPR013753	111	1.04	Ras	Ras family
PF00112	IPR000668	104	0.98	Peptidase_C1	Papain family cysteine protease
PF00153	IPR001993	86	0.81	Mito_carr	Mitochondrial carrier protein
PF00025	IPR006689	84	0.79	Arf	ADP-ribosylation factor family
PF08477	IPR013684	79	0.74	Miro	Miro-like protein
PF00076	IPR000504	71	0.67	RRM_1	RNA recognition motif
PF14259	IPR000505	64	0.60	RRM_6	RNA recognition motif

A total of 52.9% of the sequences showed similarity to the protein sequences of *Caenorhabditis elegans*, 46.6% with *Meloidogyne incognita* and 53.3% with *Bursaphelenchus xylophilus*; 1,323 sequences had homologues in *M. incognita* and/or *B. xylophilus*, but not in *C. elegans* (Figure 2.2). Of these sequences, 78 were predicted to have a putative signal peptide and no transmembrane domain, indicating that these proteins could be involved in plant parasitism. Nine are known to be involved in the infection process: expansin (5), endoglucanase (2), pectate lyase (1) and CM (1). It should be mentioned that excluding *C. elegans* orthologues from this search can have some consequences. It may prevent the discovery of certain proteins involved in parasitism which also have orthologues in *C. elegans*, like ubiquitin extension proteins or SPRY domain containing proteins (Chronis et al., 2013; Rehman et al., 2009). Of the contigs without similarity to the three organisms, 773 were predicted to have a putative secretion signal without a transmembrane domain. Most of these did not show similarities to the SwissProt or Trembl database (585 contigs), or were assigned as an uncharacterized protein (71 contigs).

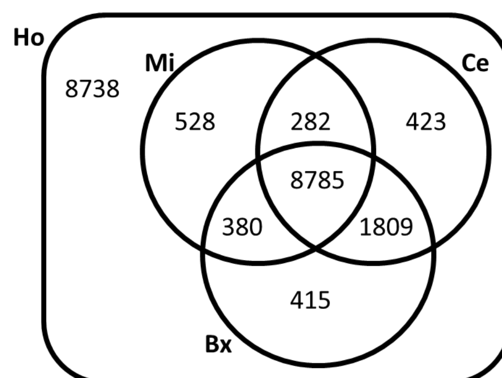

Figure 2.2: Venn diagram showing all 21,360 sequences of *H. oryzae* (Ho) used in this study. Numbers of orthologous sequences indentified in the predicted proteins of *M. incognita* (Mi), *C. elegans* (Ce) and *B. xylophilus* (Bx) are shown.

2.3.3. Most abundant transcripts

To look for transcripts with high abundance, the 100 contigs which contained the greatest number of mapped reads were selected. As a result of normalization of the data, it is not possible to quantify expression absolutely, but the number of reads can provide an idea of the abundance of the transcripts. The normalization does not equalize all transcripts but diminshes the level of the most abundant transcripts. The 100 most abundant contigs are built with an average of 163 reads, compared with an average of 10 reads per contig in the

full dataset. The GO of these 100 contigs showed a bias towards GO terms involving reproduction and development (GO:0009792; GO:0000003; GO:0002119) in the category of 'biological process'. These three terms accounted for 22% of the assigned terms in this category, indicating the importance of these processes during the nematode's life cycle. About 60 of the 100 contigs were annotated as housekeeping genes; 35 contigs were not annotated with a specific function, nine of which were predicted to have a signal peptide. The transcript with the highest abundance consisted of 923 reads and did not show any similarity to other sequences in public databases. The longest possible ORF for this transcript was only 52 amino acids long. The transcript containing the second greatest number of reads (402 reads) was similar to a galactose-binding lectin from *C. elegans*, LEC-8. It has been suggested that this protein is involved in the defense process against bacterial infection (Ideo et al., 2009). Some other highly expressed transcripts code for expansin, a thaumatin-like protein (TLP), a glycoside hydrolase family (GHF) 25 lysozyme and a lysozyme with a destabilase domain. These last three are probably involved in protection against bacterial pathogens (Evans et al., 2008; Zavalova et al., 2006). It has been shown that TLPs are upregulated on bacterial infection in *C. elegans* (Fasseas et al., 2012). Seven TLPs were found in the full dataset, four of which contained a predicted secretion signal. The localization of the expression of one of the TLPs was checked by *in situ* hybridization, showing expression in the pharyngeal region (Figure 2.3 A). The GHF25 lysozyme and the lysozyme with a destabilase domain were blasted (blastp), revealing highest similarity with LYS-8 from *Caenorhabditis briggsae* and ILYS-3 from *C. elegans*, respectively, a protist-type and an invertebratetype lysozyme (Schulenburg and Boehnisch, 2008). Both are known to be upregulated on bacterial infection (Irazoqui et al., 2010; Mallo et al., 2002).

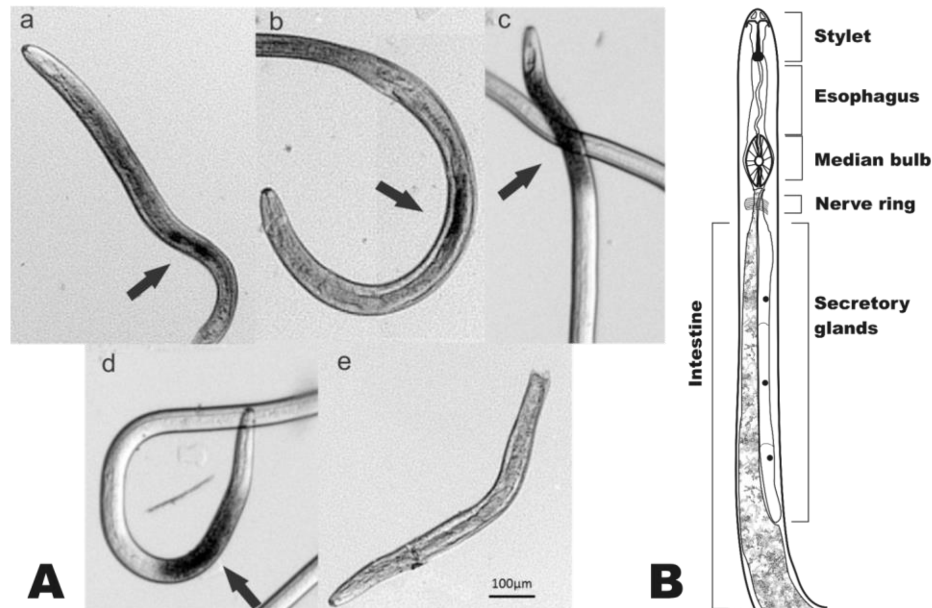

Figure 2.3: (A) Whole-mount in situ hybridization of *Hirschmanniella oryzae* showing the spatial expression of β-mannanase (a), xylanase (GHF30) (b), thaumatin-like protein (c) and chorismate mutase (d). The negative control (e), made with the sense probe for chorismate mutase, showed no signal. Arrows indicate the stained region of the pharyngeal region (c) or pharyngeal gland (a, b, d). (B) Anatomy of the pharyngeal region of *H. oryzae*. In contrast to some other plant-parasitic nematodes (e.g. *Heterodera* spp.), the pharyngeal glands overlap the intestine.

2.3.4. Survival in dry conditions

Hirschmanniella oryzae can survive in dry soils for several months without a host in a state of anhydrobiosis (Mathur and Prasad, 1973). An artificial seawater solution was used to induce anhydrobiosis and mimic drought stress (Feng et al., 2006). Nematodes were able to survive a 24h treatment in 40% artificial seawater solution. When higher concentrations were used, the nematode did not revive in distilled water. Several genes have been reported to play a role in this process in nematodes, including hydrophilic late embryogenesis abundant (LEA) proteins (Browne et al., 2002; Goyal et al., 2005). Six different sequences with similarities to LEA proteins were present in the dataset. All six were hydrophilic and had a negative grand average of hydropathicity (GRAVY) index, with a mean index of −0.817. The expression of the LEA protein with the lowest GRAVY index was investigated by quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) on soaking in artificial seawater solution for 24h. The expression values of two other genes that could be involved in this process were also considered: glutathione peroxidase and *sxp/ral-2* (Tyson et al., 2012). Transcripts encoding LEA and SXP/RAL-2 were highly induced on artificial seawater treatment, with the most significant activation in 30% solution. Glutathione peroxidase was not significantly up- or downregulated at any concentration (Figure 2.4). Expression ratios of both reference

genes varied around unity according to REST 2009 software, indicating that the expression of these genes was not influenced by the treatments. The overall expression ratios were 1.013 and 1.039 for FMRFamide-like neuropeptide 14 and elongation factor 1α, respectively.

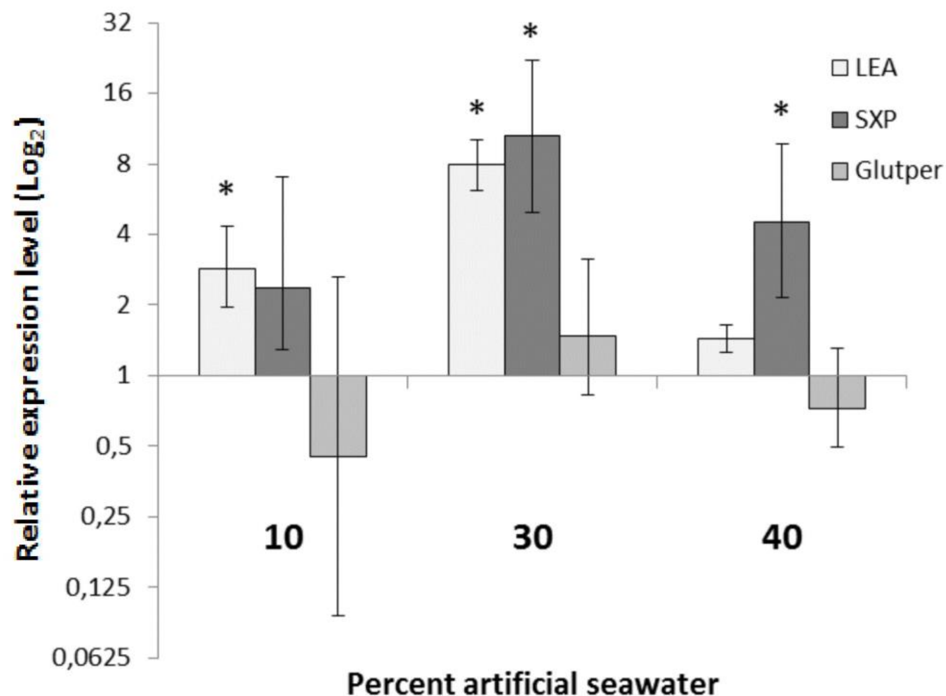

Figure 2.4: Changes in expression level of a late embryogenesis abundant (LEA) protein, *sxp/ral-2* (SXP) and a gluthathione peroxidase (Glutper) from *Hirschmanniella oryzae.* Nematodes were soaked in 10%, 30% or 40% artificial seawater solution for 24h, after which RNA was extracted and the relative expression levels were estimated relative to a 24h soak in distilled water. Each value is the mean of two biological replicates (calculated by taking the average of three technical replicates). Asterisks indicate significant differences ($p<0.05$) between treatments with seawater and distilled water. Bars represent standard errors, calculated with REST2009 software using Taylor's series.

2.3.5. Mining the transcriptome for cell wall-modifying proteins

The blastx output was used to scan the dataset for putative cell wall-modifying proteins. Individual protein sequence similarity searches were performed on the translated ESTs to confirm the results from the blastx output using SwissProt and Trembl databases. Seven different putative cell wall-modifying proteins were discovered (Table 2.4). Expansin is the most abundant sequence in this category, followed by endoglucanase and pectate lyase. The GHF30 xylanase shows similarities to the xylanase from the migratory nematode *Radopholus similis* (Haegeman et al., 2009b). A single β-mannanase (member of GHF5) was also

observed in the transcriptome of *H. oryzae*, as well as genes encoding polygalacturonases and a poly-α-D-galacturonosidase. Most of these proteins contained a putative signal peptide, indicating secretion. The spatial expression pattern of some of these putative CWMPs was tested by performing an *in situ* hybridization. The results of the hybridization of β-mannanase and xylanase (GHF30) are shown in Figure 2.3 A. Both showed staining in the gland cell area, whereas the negative control showed no staining. The pharyngeal gland cells in *Hirschmanniella* species usually overlap the intestine and are unequal in length (Figure 2.3 B).

Table 2.4: Summary of sequences with similarity to cell wall-modifying proteins. The last column indicates whether at least one sequence of a family of enzymes was predicted to have a secretion signal.

Enzyme	Family	#contigs	#reads	SP
Expansin	Beta	8	487	Yes
Beta-1,4-endoglucanase	GHF5	15	136	Yes
Pectate lyase	PL	6	108	Yes
Beta-mannanase	GHF5	1	58	Yes
Polygalacturonase	GHF28	3	13	No
Poly-alpha-D-galacturonosidase	GHF28	1	4	No
Xylanase	GHF30	1	4	Yes

2.3.6. Chorismate mutase and isochorismatase

Next to effector proteins that degrade the plant cell wall, nematodes also secrete proteins that can alter the defense mechanism of the plant. One of these proteins is CM (Lambert et al., 1999). The protein contains a signal peptide and the gland cell spatial expression of the encoding gene was confirmed by *in situ* hybridization (Figure 2.3 A). The protein has a CM type 2 domain (PF01817). A protein sequence similarity search (blastp) using the catalytic domain as query revealed high similarity with CMs from several *Burkholderia* species. A tblastn against the nucleotide or EST database was performed, showing top hits originating from PPN species, both from *Meloidogyne arenaria*. A phylogenetic tree was constructed based on Bayesian inference. CM is in the same clade as other PPNs, with its closest relative a CM from *Pratylenchus coffeae* (Figure 2.5).

Next to CM, two contigs with an ICM domain (PF 00857) were also present in the dataset (ICM1 and ICM2). These sequences did not contain a predicted signal peptide. Sequence similarity searches against the nonredundant protein database revealed that ICM1 is mostly similar to plant-pathogenic bacteria. A tblastn against the EST database at the National Center for Biotechnology Information (NCBI) discovered homologues in other PPNs exclusively. Querying the protein sequence of ICM2 against the nonredundant protein database only revealed similarity to eukaryotic sequences. Most of these sequences were predicted to have a mitochondrial localization in their annotation. ICM2 was compared with nematode ESTs (tblastn) and showed similarity to ESTs derived from nematodes with different lifestyles (plant-parasitic, animal-parasitic and free-living). Both ICM sequences were used to construct a phylogenetic tree (Figure 2.6). ICM2 clusters together with other ICM sequences originating from nematode species, not limited to PPNs. ICM1 homologues in nematodes are restricted to the plant-parasitic species, which could indicate that it is involved in plant parasitism. ICM1 and ICM2 do not show any similarity with each other. No significant similarities were observed between sequences clustered together with ICM1 and sequences from the same organism clustering together with ICM2. A conserved domain search of both ICM sequences revealed that ICM1 has a cysteine hydrolase domain (cd00431, 1.5e-40), whereas ICM2 has an YcaC-related domain (cd01012, 3.59e-54). Both CM and ICM are involved in direct or indirect metabolization of chorismate in plants, a precursor of SA and phenylpropanoids (Dempsey et al., 2011). It is tempting to speculate that CM and ICM could interfere with SA or phenylpropanoid production, thereby altering the defense mechanism of the host. Although ICM was not predicted to have a putative signal peptide, other nematode effectors lacking a signal peptide are secreted into the host tissue as well (Fioretti et al., 2001; Jaubert et al., 2004; Robertson et al., 2000).

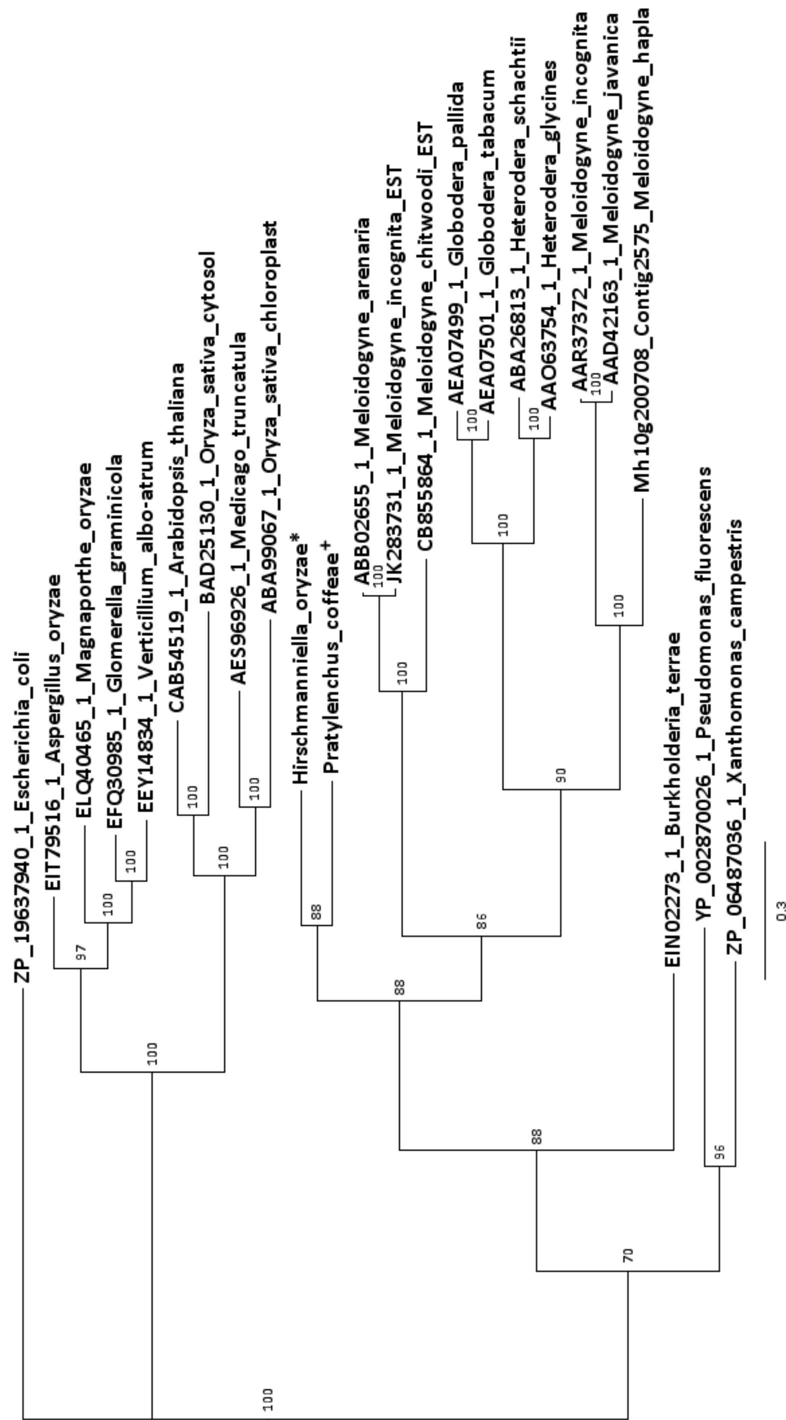

Figure 2.5: Phylogenetic tree of chorismate mutase (CM) constructed with MrBayes software. Posterior probabilities are shown for each node. The closest homologue of the CM of *Hirschmanniella oryzae* comes from *Pratylenchus coffeae*. Nematode sequences cluster together with CM sequences from plant-pathogenic bacteria. Plant sequences are in the same cluster as plant-pathogenic fungi. Taxa are indicated as follows: proteinID_species. An asterisk (*) indicates in-house data. A plus sign (+) indicates data from Haegeman et al. (2011b). The suffix 'EST' indicates a translated EST sequence instead of a protein sequence. The CM sequence of *E. coli* was used as outgroup.

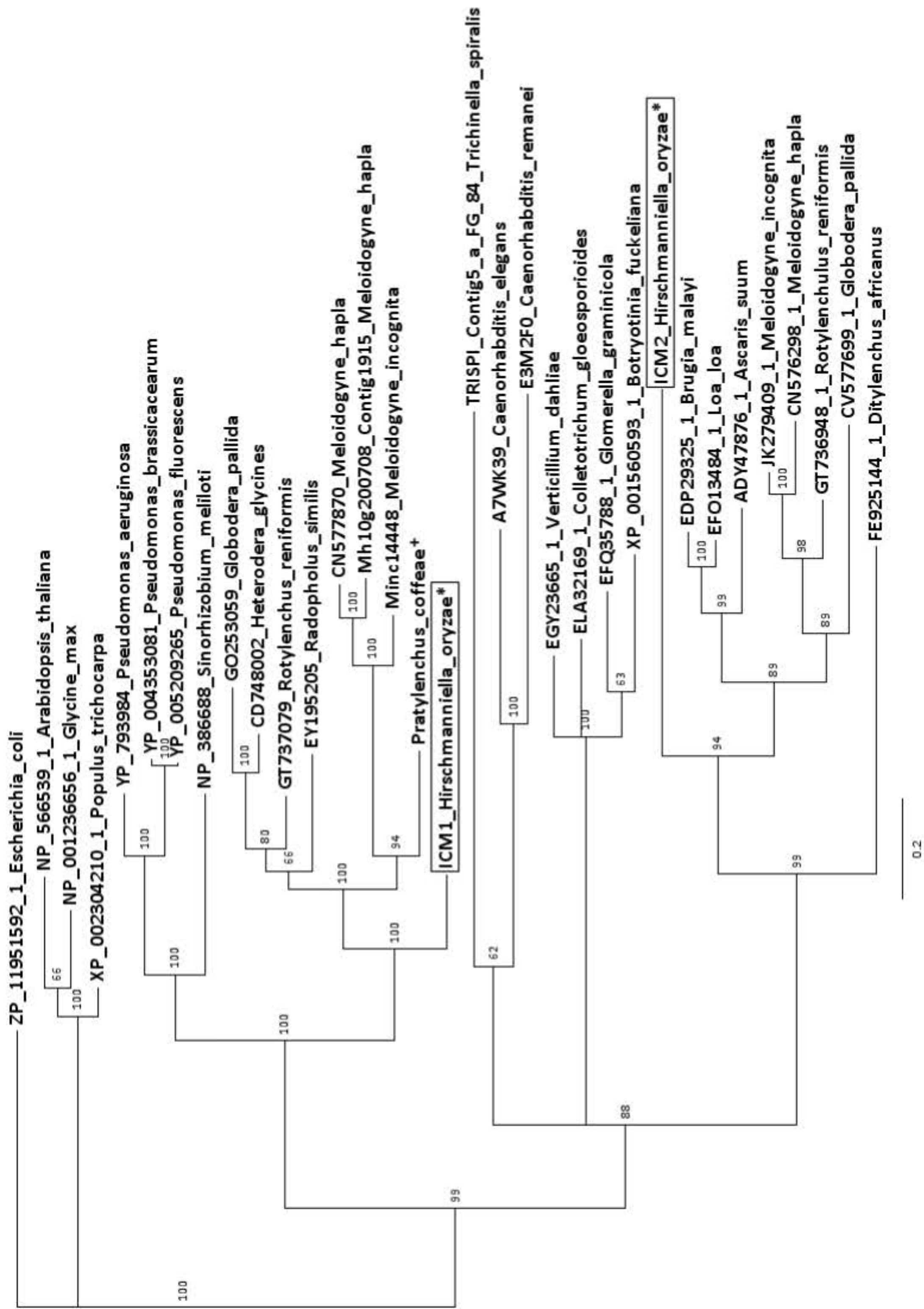

Figure 2.6: Phylogenetic tree of isochorismatase (ICM) constructed with MrBayes software. Posterior probabilities are shown for each node. Homologues in expressed sequence tag (EST) and protein databases on the National Center for Biotechnology Information (NCBI) server were used to construct this tree. Taxa are indicated as follows: proteinID_species. An asterisk (*) indicates in-house data. ICM1 and ICM2 cluster in different branches of the tree. A plus sign (+) indicates data from Haegeman et al. (2011b). The ICM sequence of *E. coli* was used as outgroup.

2.4. Discussion

The transcriptome of *H. oryzae* was studied to gain an insight into survival strategies used by this organism and to investigate the parasitic interaction with its host, *O. sativa*. ESTs were generated using a 454 FLX Titanium Platform on a mixed stages population of *H. oryzae*. 454 sequencing produces longer reads than Illumina sequencing; longer reads are beneficial for transcriptome assembly (Pop and Salzberg, 2008). A total of 450,171 reads, with an average read length of 385 nucleotides, was assembled by a combination of CLC and Newbler software. As there are several different drawbacks to each assembly strategy, we combined the two strategies to have more confidence in each contig. The disadvantage, however, is that several contigs only predicted by one program were discarded during this combined assembly process. It was already shown before that Newbler 2.3 is not able to assemble high contig numbers (Kumar and Blaxter, 2010). The dataset was further reduced by rejecting the sequences with high similarity (bitscore > 200) to possible contaminating sources. By doing so, potential horizontal gene transfer events (Mitreva et al., 2009) might be overlooked. This led us to a final dataset consisting of 21,360 contigs.

To survive, *H. oryzae* must be able to adapt to environmental conditions and must be able to successfully infect a host plant to be capable of feeding and reproducing. During the growing season, rice is flooded and deprived of oxygen. In between growing seasons, however, the field can dry out. In addition, the soil is inhabited by bacteria which may be harmful to nematodes (Costa et al., 2012; Hodgkin et al., 2000; Piskiewicz et al., 2007). *Hirschmanniella oryza*e has been reported to survive in anaerobic conditions (Babatola, 1981). We only detected the presence of a few genes coding for enzymes that are important in anaerobic metabolism pathways, such as fumarate reductase and malate dehydrogenase, but not enough to construct a full pathway (Muller et al., 2012). This is probably caused by the fact that the analyzed nematodes were not harvested under anaerobic conditions.

There is a vast bacterial community thriving in soil and the rhizosphere of rice plants (Lu et al., 2006). Most bacteria are harmless to nematodes. However, some species are nematode pathogens, such as certain *Pasteuria* and *Pseudomonas* species (Siddiqui and Mahmood, 1999). Several antimicrobial peptides, as well as lysozymes and lectins with antibacterial activity, have been described in *C. elegans* (Bogaerts et al., 2010; Irazoqui et al., 2010; Mallo et al., 2002). The top 100 most abundant transcripts mirror the need for *H. oryzae* to protect

itself against microbial invaders. Several proteins with (potential) antimicrobial properties were detected. First, two different types of lysozyme were identified: GHF25 lysozyme (lys25) and a lysozyme with a destabilase domain (dest-lys). Lys25 is upregulated in the intestinal cells of *C. elegans* on pathogen infection (Mallo et al., 2002). Dest-lys is upregulated on bacterial infection in *C. elegans* (Irazoqui et al., 2010), and is also known for its antifungal properties (Yudina et al., 2012; Zavalova et al., 2006). In addition, several lectins are involved in the innate immune system against bacteria. A galactose-binding lectin with strong similarity to LEC-8 of *C. elegans* was the second most abundant transcript in the dataset. *Lec-8* is upregulated on bacterial infection and probably functions as a competitive binding protein for glycolipid molecules that are targeted by the bacterial invader (Ideo et al., 2009; Mallo et al., 2002). The dataset contained several TLPs, one of which was present in the list of most abundant transcripts (113 reads). Spatial analysis by *in situ* hybridization showed this gene to be expressed in the pharyngeal region. In plants, TLPs are classified as pathogenesis-related proteins family 5 and have antifungal properties. In nematodes, they have been reported to be involved in the innate immune system (Fasseas et al., 2012; Golden and Melov, 2004; O'Rourke et al., 2006). Although most antibacterial proteins involved in the innate immune system are expressed in the intestines of the nematode, some are also expressed in the gland cells or pharyngeal region. For instance, LYS-8, an antibacterial lysozyme in *C. elegans*, is upregulated on bacterial infection and its expression has been reported in the pharyngeal region (Mallo et al., 2002). Among several other activities, TLPs also show β-1,3- glucanase activity, implicating that this nematode could secrete this protein as a protection against fungi or to degrade callose in the plant (Liu et al., 2010).

Next to being challenged by parasitic bacteria and/or fungi, another big challenge is surviving long periods of drought. *Hirschmanniella oryzae* is used to a life in flooded rice fields (Prot and Rahman, 1994). Although these rice fields are flooded most of the time, they can dry out in between growing seasons. The nematode has to rapidly adapt to this new situation to overcome the dry period. Several nematodes are known to be able to survive long periods of drought (Reardon et al., 2010; Tyson et al., 2007). They are adapted to these extreme situations by the differential expression of several genes. One of these genes is *lea*, also found in plants in which it accumulates in response to water loss. LEA proteins have been reported in nematodes, where they are strongly induced upon anhydrobiosis (Browne et al., 2002). Other proteins also play a role during this process. In *Panagrolaimus superbus*, an

anhydrobiotic nematode, it was found that SXP/RAL-2 is the most abundant transcript in the transcriptome (Tyson et al., 2012). This led the authors to believe that it is involved in survival during desiccation. Glutathione peroxidases have also been shown to be involved in drought tolerance (Reardon et al., 2010). Anhydrobiosis can be initiated by desiccation and osmotic stress. Nematodes have been shown to respond in a similar way upon both stresses (Tyson et al., 2007). When a state of anhydrobiosis was induced by soaking *H. oryzae* in a diluted solution of artificial seawater for 24 h, thereby mimicking desiccation stress, *sxp/ral-2* and *lea* were both significantly upregulated. Although the upregulation of certain glutathione peroxidases on dehydration of nematodes has been described previously (Reardon et al., 2010), this was not observed for *H. oryzae* upon dehydration. To our knowledge, this is the first time that the upregulation of *sxp/ral-2* upon anhydrobiosis has been described. The SXP/RAL-2 family of proteins is specific to nematodes. It is present in free-living and animal-parasitic nematodes, as well as in PPNs (Tyson et al., 2012). To date, the role of these proteins has not been elucidated. It has been proposed that they are involved in plant parasitism, for instance in feeding cell induction (Tytgat et al., 2005). However, the presence of this family across the phylum may indicate a more general function. The results presented here suggest that SXP/RAL-2 has a role in protection against desiccation. This apparent contradiction can be explained by the fact that there may be several SXP/RAL-2 isoforms within a species, as shown previously for *Globodera rostochiensis* (Jones et al., 2000). These proteins are grouped within the same family, but have a different spatial expression pattern. The expression of two different *sxp/ral-2* genes has been reported in either the amphids or hypodermis of *G. rostochiensis*, and one *sxp/ral-2* gene was expressed in the subventral pharyngeal glands of *M. incognita* (Jones et al., 2000; Tytgat et al., 2005). This could be an indication that diverse members of this family exhibit different functions.

To successfully infect the host plant, the nematode must overcome the cell wall as a structural barrier, which mainly consists of cellulose, hemicelluloses, pectins and structural proteins (Vogel, 2008). To overcome this hurdle, nematodes hold an arsenal of CWMPs thought to have been acquired through horizontal gene transfer from bacteria and/or fungi (Jones et al., 2005). Several cell wall-modifying proteins have already been described in nematodes, such as expansin, β-1,4-endoglucanase, pectate lyase, polygalacturonase and xylanase, which were also found in the *H. oryzae* ESTs (Jaubert et al., 2002b; Kikuchi et al., 2006; Mitreva-Dautova et al., 2006; Qin et al., 2004; Rosso et al., 1999).

A putative new CWMP which has not been reported before in nematodes was identified in the *H. oryzae* ESTs: β-mannanase. Sequence similarity searches revealed one homologous sequence in EST data of the PPN *Xiphinema index*. β-mannanase is possibly involved in hemicellulose degradation. The hemicellulose fraction is more abundant in cell walls of grasses relative to dicots (Vogel, 2008), which could explain the presence of this enzyme in *H. oryzae*, whereas it is absent in the genomes of *M. incognita*, *M. hapla, G. pallida* and *B. xylophilus*. β-Mannanase is important in the degradation of β-(1,4)-linked mannans. It has previously been found in plants, fungi and bacteria and its presence has been confirmed in several plant-pathogenic fungi and bacteria, where it has a role in cell wall degradation (Couturier et al., 2012; Kim et al., 2011; Pham et al., 2010). This is the first report of a β-mannanase encoding gene in nematodes. The presence and activity of this type of β-mannanase have been reported recently in an insect species (Acuna et al., 2012). An *in situ* hybridization showed a spatial expression pattern in the region of the gland cells, ruling out the possibility that this contig originated from bacterial contamination. It might be possible that *H. oryzae* acquired this gene by horizontal gene transfer. A sequence similarity search using blastp against the nonredundant protein database revealed β-mannanase as top hit, originating from *Opitutus terrae*, an obligate anaerobic bacterium which is known to reside in rice paddy soils (Chin et al., 2001). The fact that β-mannanases from both organisms have a high sequence similarity (51% identical residues) and that these two organisms share the same ecosystem, could point to horizontal gene transfer. GHF30 xylanases have been found in PPNs previously, although there is some discussion about the designation of these enzymes to GHF5 or GHF30 (Haegeman et al., 2009b). The presence of all of the enzymes mentioned in this and the previous paragraph shows that *H. oryzae* is well equipped to break through the monocot plant cell wall and migrate through the root. As migratory nematodes must continuously pass cell walls during their whole life cycle, it is not surprising that they possess an arsenal of enzymes to degrade hemicellulose.

Sedentary nematodes secrete effector proteins which aid in the establishment of a specialized feeding site and attenuate the host defense mechanism (Hewezi and Baum, 2013). As migratory nematodes do not form specialized feeding cells and do not stay in one place in the host, one could claim that they are not in need of these specific effector proteins. This used to be a generally accepted idea for necrotrophic fungi, but it was later shown that they are able to manipulate the defense system of the host to their advantage

(Oliver and Solomon, 2010). Therefore, migratory nematodes might also secrete effector proteins to suppress the plant immune system. CM was found in our dataset and in the transcriptome of *Pratylenchus coffeae* (Haegeman et al., 2011b). CM is involved in the shikimate pathway in plants, where it catalyzes the step from chorismate to prephenate. Secretion of CM was suggested by the presence of a putative secretion signal and expression in the region of the pharyngeal gland cells. The secreted CM is probably involved in the deregulation of the SA or phenylpropanoid pathway in the host plant, making it more vulnerable to pathogen attack. This has been shown recently for a secreted CM from *Ustilago maydis*, which lowers the total SA content in infected leaves (Djamei et al., 2011).

In addition to CM, another enzyme involved in the SA pathway in plants was also discovered, ICM. ICM converts isochorismate to 2,3-dihydroxybenzoate and pyruvate, thereby depleting the pool of isochorismate and reducing SA synthesis. ICM has been found in the secretome of phytopathogenic fungi and is thought to have a function in reducing the SA content of the host (El-Bebany et al., 2010; Soanes et al., 2008). Two sequences with similarity to ICM have been found in the dataset. ICM1 clusters together with other PPN sequences in a phylogenetic tree and does not have any homologues in nonparasitic nematodes. Although ICM1 was not predicted to have a putative signal peptide, this observation is an indication that ICM1 might be involved in plant parasitism. ICM2 has homologues in PPNs, as well as free-living nematodes and animal-parasitic nematodes, and has a conserved YcaC-related domain, but does not show similarity to ICM1. A blastp sequence similarity search revealed that it also has putative orthologues in other eukaryotic species, most of which were predicted to have a mitochondrial localization. YcaC-related ICM is probably involved indirectly in energy transduction. It has been shown that it can bind to creatine kinase, which catalyzes the transfer of a phosphate group from phosphocreatine to ADP, yielding ATP and creatine. This is supported by the fact that YcaC-related ICM is mainly localized in mitochondria, where ATP production takes place (Jiang et al., 2008). These results indicate that ICM1 could be an interesting effector protein for the nematode, whereas ICM2 is involved in general cellular functioning.

In conclusion, the transcriptome of *H. oryzae* provides insights into the proteins needed by this nematode to survive in unfavorable environmental conditions. In addition, it augments our understanding of the strategies used to infect the host plant, from CWMPs to putative effector proteins. As the available data for migratory species are scarce compared with the

economically more important cyst and root-knot nematodes, these new data are a convenient source to perform comparative studies.

2.5. Experimental procedures

2.5.1. RNA extraction and sequencing

Juvenile and adult stages of *H. oryzae* obtained from Myanmar were extracted from fresh rice roots using a modified Baermann funnel. RNA was extracted from the nematodes using the RNeasy kit (Qiagen, Hilden, Germany). The RNA was sent to LGC Genomics (Berlin, Germany), where mRNA was isolated and a cDNA library was constructed with the Mint Universal cDNA synthesis kit (Evrogen, Moscow, Russia). The cDNA was normalized using the TRIMMER kit (Evrogen), size selected (>800 bp) and transformed into the vector pDNR-lib. The library was analysed by 454 FLX Titanium sequencing in a run on one-half of a picotitre plate. The raw data have been submitted to the Sequence Read Archive (SRA) database of the NCBI under accession number SRA048498.

2.5.2. Clean up and assembly

The reads were processed with Newbler 2.3 and CLC Genomics workbench 4.0.2 software. Contaminating vector and adaptor sequences were trimmed from the reads and an assembly was performed using standard settings. Singletons and sequences shorter than 150 bp were removed from the dataset. Contigs generated by CLC software were blasted (blastn) against contigs predicted by Newbler 2.3 with a cut-off bitscore of 200.

2.5.3. Blast searches and annotation

The remaining contigs in the dataset were subsequently blasted (blastx, cut-off bitscore of 200) against protein sequences of several plant-pathogenic bacteria and fungi, soil bacteria and rice to remove possible contamination. Predicted proteins from the *O. sativa* genome (version MSU 7.0) were downloaded from the website of the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). The data for the other organisms were downloaded from the NCBI server (http:// www.ncbi.nlm.nih.gov/): *Bacillus* spp., *Pseudomonas fluorescens*, *Rhizobium* spp., *Azospirillum* spp., *Agrobacterium* spp., *Gluconobacter* spp., *Flavobacterium* spp., *Herbaspirillum* spp., *Thiobacillu*s spp.,

Xanthomonas oryzae, *Magnaporthe grisea*, *Magnaporthe salvinii*, *Rhizoctonia solani*, *Cochliobolus miyabeanus*, *Gibberella fujikuroi* and *Ustilaginoidea virens*. The remaining sequences were subjected to a blastx (cut-off bitscore of 50) against the SwissProt and Trembl databases. The output was used for annotation. By using the software application QuickGO from EBI (http:// www.ebi.ac.uk/QuickGO/GAnnotation), GOs were downloaded for the protein identifiers of the blastx top hits. Sequences were compared with (blastx, cut-off bitscore of 50) protein data of *C. elegans*, *M. incognita* and *B. xylophilus* downloaded from Wormbase (http://www.wormbase.org, release WS229), http://www7.inra.fr/meloidogyne_incognita and http:// www.genedb.org/Homepage/Bxylophilus (version 1.2), respectively.

2.5.4. Protein domain search and sequence analysis

Putative proteins were predicted using the OrfPredictor tool (Min et al., 2005). The protein sequences were scanned for the presence of putative signal peptides and the absence of transmembrane domains by SignalP 4.0 using eukaryotes as organism group (Petersen et al., 2011). Pfam HMM motifs were downloaded from the Pfam website (release 26.0). HMMscan (HMMER3.0, hmmer.org) was used to look for possible domains in the contigs with a reporting threshold score of 30. The molecular weight of a protein was calculated using the ProtParam tool from the ExPASy website (http://web.expasy.org/protparam/). To calculate the GRAVY index, the Kyte–Doolittle scale was used (Kyte and Doolittle, 1982). Conserved domains from the Conserved Domain Database (CDD) were retrieved through CD-search in the NCBI server with standard settings.

2.5.5. Dehydration treatment

Nematodes of mixed stages were soaked in an artificial seawater solution or in distilled water as a control. The seawater solution was adapted from Feng et al. (2006). It contained 1,692 mM NaCl, 9 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2.6H_2O$, 25.50 mM $MgSO_4.7H_2O$ and 2.14 mM $NaHCO_3$ dissolved in distilled water. Approximately 500 nematodes were soaked in six-well plates for 24 h in distilled water, 10%, 30% and 40% artificial seawater.

2.5.6. RNA extraction and cDNA synthesis

RNA was extracted with the RNeasy kit (Qiagen). To remove all contaminating DNA, 1 µg of the extracted RNA was treated with 1 µL DNaseI (1 U/µL; Fermentas, Waltham, MA, USA), 0.5 µL RNAsin ribonuclease inhibitor (Promega, Fitchburg, WI, USA) and 3 µL DNaseI buffer (10×, Fermentas) in a total volume of 27 µL. The mixture was incubated at 37 °C for 30 min, after which 3 µL of 50mM ethylenediaminetetraacetic acid (EDTA) was added and incubated for 10 min at 65 °C to stop the reaction. First-strand cDNA synthesis was started by adding 1 µL oligo dT (700 ng/ µL) to 30 µL DNAse-treated RNA and incubating the mixture for 5 min at 70 °C. Afterwards, the following products were added: 10 µL of Goscript 5× reaction buffer, 5 µL of 25mM $MgCl_2$, 2.5 µL of PCR nucleotide mix, 1 µL of Goscript reverse transcriptase (Promega) and 0.5 µL of water to a total volume of 50 µL. The mixture was incubated for 5 min at 25 °C, 2 h at 42 °C and 15 min at 70 °C to stop the reaction.

2.5.7. Q-RT-PCR

The SensiMix SYBR No-ROX kit (Bioline, London, UK) was used to perform Q-RT-PCR. Each reaction contained 10 µL of 2 × SensiMix, 500 nM of each primer and 1 µL of cDNA in a total volume of 20 µL. All reactions were performed in three technical replicates on a Rotor-Gene 3000 (Corbett Life Science, Hilden, Germany) and analyzed with Rotor-Gene 6000 software version 1.7. The PCR conditions were as follows: 10 min of initial denaturation at 95 °C, followed by 45 cycles of 25 s at 95 °C, 25 s at 58 °C and 20 s at 72 °C. The melting curve was generated by gradually increasing the temperature from 72 °C to 95 °C after the last cycle to test the specificity of the amplicon. The data were analysed by REST 2009 software to determine statistically significant differences (Pfaffl et al., 2002). Two reference genes identified in the contigs were used; an FMRFamide-like neuropeptide 14 and an elongation factor 1α. Primers are listed in Appendix B.

2.5.8. Construction of a phylogenetic tree

To construct a phylogenetic tree, homologues of CM or ICM were downloaded from the NCBI server. EST sequences were first translated into protein sequences. The alignment was performed by Clustal Omega on the EBI server with FASTA (Pearson) as output (http://www.ebi.ac.uk/Tools/msa/clustalo/). The output format was converted into nexus format using Readseq (http://www.ebi .ac.uk/Tools/sfc/readseq/). The nexus file was loaded

into MrBayes 3.2.1 to construct a phylogenetic tree using Bayesian inference with a gamma distributed model (Ronquist et al., 2012). The parameters of the likelihood model were set as follows: Nst= 2, Rates= gamma. At least 50,000 generations were performed to achieve a standard deviation below 0.05, which was diagnosed every 1250 generations. The output file was used in FigTree v1.3.1 to visualize the results.

2.5.9. Whole-mount *in situ* hybridization

Genes were cloned in pGEM-T (Promega), using the standard cloning techniques. The primers used for cloning and probe synthesis are listed in Appendix B. Whole-mount *in situ* hybridization was performed on a mixed stages population of *H. oryzae*. Protocol was as described previously by de Boer et al. (1998) with some minor modifications regarding fixation of the nematodes and hybridization temperature. The nematodes were fixed in 2% formaldehyde for 16 h at 4 °C, followed by an additional incubation for 4 h at room temperature. Hybridization was performed at 47 °C. Photographs were taken with a Leica S8AP0 (Wetzlar, Germany) stereomicroscope. Due to the lack of nematodes we were not able to optimize the protocol any further, hence we were unable to produce clearer pictures.

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Chapter 3. Characterization of two putative effector proteins of *Hirschmanniella oryzae*; chorismate mutase and isochorismatase

This chapter was adapted from: Chorismate mutase and isochorismatase, two effectors from the plant-parasitic nematode *Hirschmanniella oryzae* promoting susceptibility by altering secondary metabolism in the host. Bauters L., Kyndt T. Nowak S., Sieprath T., Denil S., Haeck A., Demeestere K., De Meyer T. and Gheysen G. In preparation

3.1. Summary

The rice root nematode *Hirschmanniella oryzae* is the most abundant PPN in flooded rice fields and is distributed world-wide. It can cause severe yield reductions and economic losses in specific environmental conditions. The recently sequenced transcriptome of this nematode provided some more insights into the interactions of this pathogen with its host plant. The transcriptome data was used to identify putative effector proteins. In this chapter the emphasis will be put on two putative effector proteins potentially involved in deregulating the SA and phenylpropanoid pathways in plants. *HoCM*, a gene encoding CM is homologous to genes found in other parasitic nematodes before. Infiltration studies with a GFP fusion construct showed expression of this protein in the cytoplasm of the plant cell. The second putative effector, ICM, is encoded by *HoICM*. To our knowledge this is the first report of a functional ICM characterized in a PPN. The gene and protein structure of both putative effectors was determined. In addition, activity of both proteins was demonstrated. Moreover, experimental evidence demonstrated that both proteins have an effect on the plant defense system when expressed *in planta*. All together, these results indicate that both genes play a role in the infection process of *H. oryzae*, which makes them excellent candidates for further investigation of the rice-nematode interaction.

3.2. Introduction

The migratory rice root rot nematode *Hirschmanniella oryzae* is one of the most abundant PPNs in flooded rice fields. It has been reported all over the world, especially in rice growing regions around the equator. *H. oryzae* is classified as a migratory endoparasite of rice and is adapted to the anaerobic conditions in flooded rice ecosystems (Babatola, 1981). It penetrates the root system through the lateral roots or root tips and migrates through the aerenchyma of the roots, completing its life cycle in about 33 days (Karakas, 2004).

To successfully penetrate and migrate through the roots, *H. oryzae* uses similar strategies to other PPNs. It uses its stylet to pierce the cell wall and also secretes effector proteins to break down the cell wall barrier and decrease the defense system of the host. Endoglucanase was the first cell wall degrading enzyme (CWDE) characterized in PPNs (Smant et al., 1998). Since then, several CWMP have been found in PPNs e.g. pectinases,

xylanases and expansins (Haegeman et al., 2009b; Popeijus et al., 2000; Qin et al., 2004). Numerous effector proteins that have an influence on the defense system of the host have been identified as well in PPNs. Some of them even mimic plant molecules to accomplish their goal (Davis et al., 2008). The recent analysis of the transcriptome of *H. oryzae* enabled us to study potential effector proteins of this species in more detail (Bauters et al., 2013).

SA, a plant hormone, plays an important role as a signaling molecule during the defense reaction upon pathogen infection. SA can be produced through two distinct pathways starting from chorismate, the final product of the shikimate pathway. Several chorismate derived compounds play a role in plant physiology and in the defense system, e.g. SA, auxin, lignin and flavonoids. Disturbing the concentrations of these compounds may affect defense of the plant against pathogen infection (Jones et al., 2007; Nahar et al., 2012). Interestingly, CM and ICM were detected in the transcriptome of *H. oryzae* (Bauters et al., 2013). Both enzymes are potential parasitism genes which are potentially involved in the plant pathway leading to SA and/or phenylpropanoids. CM catalyzes the conversion of chorismate to prephenate. This enzyme is present in plants and bacteria but had not been reported in animals until its discovery in *Meloidogyne javanica* (Lambert et al., 1999). Since then, it has been reported in several other PPNs (Huang et al., 2005b; Jones et al., 2003; Vanholme et al., 2009b). It has been shown that CM, secreted by plant-pathogenic organisms, is able to alter plant cell development by impairing the development of lateral roots and vascular tissue (Doyle and Lambert, 2003). Recent research provided experimental evidence that a secreted CM originating from *Ustilago maydis* reduced SA levels in host plants (Djamei et al., 2011; Doyle and Lambert, 2003). ICM has not been characterized in nematodes before, but it was reported to be present in the genome of *M. hapla* and the transcriptome of *Rotylenchulus reniformis* (Opperman et al., 2008; Wubben et al., 2010). Moreover, it has been found in the secretome of plant-pathogenic fungi, while it is absent in non-pathogenic species (Soanes et al., 2008) and it has been reported to reduce SA content in plants upon infection (Liu et al., 2014). ICM converts isochorismate to 2,3-dihydroxy-2,3-dihydrobenzoate and pyruvate in the presence of water. Isochorismate is an intermediate in the formation of SA, hence ICM is capable of reducing the pool of isochorismate available for formation of SA. Two types of ICM have been reported in *H. oryzae*, but only the protein restricted to the plant-parasitic species will be described here, since the other one is probably involved in general cell metabolism in mitochondria (Bauters et al., 2013).

In this chapter the genetic structure and protein properties of CM (*HoCM*) and ICM (*HoICM*) isolated from *H. oryzae* will be described. Their activity is demonstrated by complementation of *CM* and *ICM* deficient *Escherichia coli* strains, and experimental evidence suggests that both proteins increase susceptibility of the host plant.

3.3. Results

3.3.1. Genetic structure of *HoCM* and *HoICM*

A *CM* and two *ICM* sequences were reported in the transcriptome of *H. oryzae* (Bauters et al., 2013). Primers were designed (Appendix B) to clone the CM sequence (*HoCM*) and the plant parasite-specific ICM sequence (*HoICM*). The two genes were cloned from a cDNA library of *H. oryzae*, resulting in a predicted open reading frame (ORF) of 747 bp and 672 bp respectively. The genes were amplified by PCR from genomic DNA to unravel the exon/intron structure. The fragments were cloned and sequenced. The genomic sequence of *HoCM* was 942 bp long and contained two introns of 72 and 123 bp. The genomic structure of *HoICM* included two introns of 69 and 81 bp, making a total length of 822 bp (Figure 3.1) (sequences can be found in Appendix A). The second exon of *HoICM* lacks the consensus splice site GT/AG, instead it has the non-canonical splice site GT/CG. This non-canonical splice site has been reported in other nematodes before (Ardelli et al., 2006; Vanholme et al., 2009a). Although this splice site can be linked to alternative splicing, no other forms of *HoICM* were detected by PCR on cDNA.

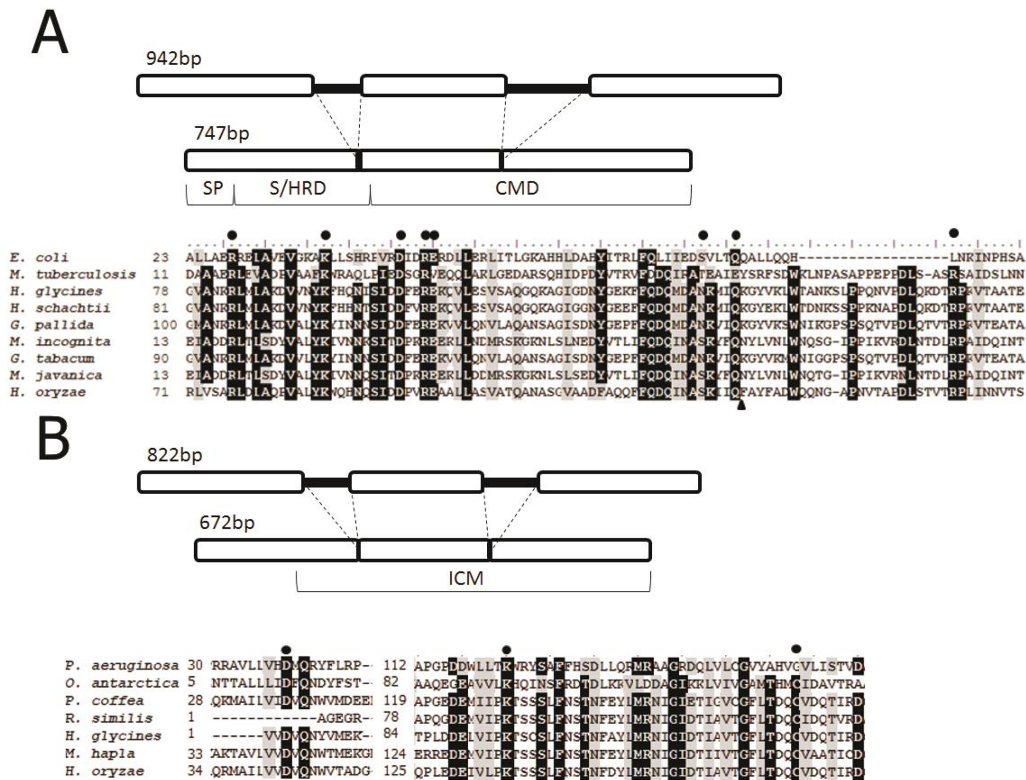


Figure 3.1: Gene structure and protein sequence of *HoCM* and *HoICM*. A: *HoCM*. *HoCM* has a total length of 942 base pairs on genomic level, containing two small introns. The protein consists of three different domains; an N-terminal signal peptide (SP), a serine/histidine-rich domain (S/HRD) and a chorismate mutase domain (CMD) (PF01817). The alignment shows the conserved catalytic residues in several plant-parasitic nematode species. Proteins without signal peptides were used for this alignment. (gene identifiers; *E. coli*: 1311033, *Mycobacterium tuberculosis*: 85544612, *Heterodera glycines*: 49353422, *H. schachtii*: 74422699, *Globodera pallida*: 326786513, *Meloidogyne incognita*: 46198272, *G. tabacum*: 326786515, *M. javanica*: 5353516, *H. oryzae:* KP297892). Eight conserved putative catalytic residues of *M. tuberculosis* and/or *E. coli* are marked with filled dots. A filled triangle shows the position of the second intron. B: *HoICM*. *HoICM* is 822bp long and contains two small introns. It contains an isochorismatase domain (PF00857), but no predicted N-terminal signal peptide. The alignment was made for the full length of the protein, using translated EST sequences for plant-parasitic nematodes: *M. hapla*: CN577870, *H. glycines*: CD748002, *Radopholus similis*: EY195205, *Pratylenchus coffeae*: in-house data and *H. oryzae:* KP297893. Two additional sequences were added of which the isochorismatase gene has been thoroughly investigated: *Oleispira antarctica*: 508729457, *Pseudomonas aeruginosa*: 33357449. The three putative catalytic residues are marked with filled dots, the position of the second intron is marked with a filled triangle.

Previous results showed expression of CM in the pharyngeal gland region of *H. oryzae*, indicating that the protein is most likely secreted by the nematode (Bauters et al., 2013). Several attempts were made to perform an *in situ* hybridization for *HoICM*, all of them were negative or showed ambiguous results. *HoICM* was PCR-amplified from genomic DNA

extracted from a single hand-picked nematode. This experiment was performed three times, each time on an independent single hand-picked nematode. *HoICM* could be amplified in all three experiments, indicating that *H. oryzae* is the true origin. No N-terminal secretion signal was detected for HoICM, targeting the protein to the classical secretory pathway. However, it was predicted to be non-classical secreted based upon a SecretomeP 2.0 score exceeding the threshold of 0.5 (0.566 NN-score) (Bendtsen et al., 2005). This finding corresponds to two ICMs without an N-terminal secretion signal from the plant-pathogenic organisms *Phytophthora sojae* and *Verticillium dahlia* which are also secreted using a non-classical secretory peptide. In this case a secretomeP score of 0.66 and 0.81 was obtained (Liu et al., 2014). PPN-derived EST sequences, homologous to HoICM, were also used in a SecretomeP analysis. Since it was shown that secretion most likely depends on the N-terminal region (Liu et al., 2014), only sequences containing a putative start codon were used in this analysis. This restricted the analysis to sequences of *P. coffeae*, *M. hapla* and *R. reniformis*. Both ICM homologues of *P. coffeae* and *M. hapla* were predicted to be secreted with a SecretomeP score of 0.588 and 0.611 respectively. The sequence of *R. reniformis* did not exceed the threshold (0.385). Although this data points in the direction of possible secretion of ICM by PPNs, it has to be treated with caution since experimental evidence is still lacking.

3.3.2. Protein properties

HoCM and *HoICM* encode proteins of 248 and 223 amino acids respectively. The HoCM protein consists of three domains, the first one being a predicted signal peptide of 20 amino acids. The mass of this mature protein without the signal peptide is 25 kDa with a theoretical isoelectric point (pI) of 7.02. The N-terminal domain has no similarities with other domains in public databases. It consists of 66 amino acids and has two motifs rich in serine and histidine respectively, hence the name serine/histidine-rich domain (S/HRD). The C-terminal part is the region with similarity to the CM type II domain (PF01817) and comprises 162 amino acids (CMD). Catalytic residues have been characterized in CM proteins of *E. coli* and *M. tuberculosis* (Lee et al., 1995; Okvist et al., 2006). The eight conserved catalytic residues are highlighted in Figure 3.1A. All residues are conserved in *H. oryzae*. Seven are conserved in all PPNs used in this alignment. Only serine on position 132 of the mature protein is replaced by asparagine in cyst nematodes.

The HoICM protein consists of 223 amino acids, has a total mass of 25.4 kDa and a theoretical pI of 6.35. It was not predicted to have an N-terminal signal peptide but it has an

ICM domain (PF00857) in the last 188 amino acids. Catalytic residues that have been identified in *O. antarctica* and *P. aeruginosa* are conserved in *H. oryzae* (Goral et al., 2012; Parsons et al., 2003). Sequences of other PPNs were found by performing a tblastn against the EST database on NCBI, providing evidence that *H. oryzae* is not the only PPN expressing this gene. Aligning these sequences showed conservation of the catalytic triad Asp-Lys-Cys in PPNs (Figure 3.1B). A tblastn was also performed against the non-redundant nucleotide database. In this case, only sequences with a prokaryotic origin were found as significant matches. The genomes of *M. incognita* and *M. hapla* also encode ICM proteins that were much bigger (around 400 amino acids) compared to HoICM. This raised the question whether the start codon of *HoICM* was the true start codon. However, the transcriptome of *Pratylenchus coffeae*, one of the closest relatives of *H. oryzae*, also contains an ICM transcript with the same length as *HoICM* (Haegeman et al., 2011b). This transcript contains stop codons, in all three frames, in the 5' terminal region. An ICM from *Rotylenchulus reniformis* was cloned, coding for a protein of 220 amino acids, which is about the same length as HoICM (Wubben et al., 2010). Furthermore, it also has multiple stop codons prior to the start codon. These two observations give us an indication that the putative start codon of *HoICM* is the true start codon.

3.3.3. Protein structure

CMs are classified into two main groups according to their structure: the AroH and AroQ class. Proteins of the rare AroH class, represented by CM of *Bacillus subtillis*, have both α helices and β sheets in their structure (Chook et al., 1993). On the other hand, proteins of the AroQ class are more abundant and are represented by the CM structure of *E. coli*. This class only has α helices in its structure (Lee et al., 1995). The AroQ class is divided into four different subgroups: $AroQ_{\alpha}$, $AroQ_{\beta}$, $AroQ_{\gamma}$ (formerly known as *AroQ) and $AroQ_{\delta}$ (Okvist et al., 2006). Secondary structure predictions showed that HoCM is composed of α-helices and loops, and no β-sheets are present in its structure. This shows that HoCM is a member of the AroQ class. A 3D model was constructed using Phyre² (Protein Homology/AnalogY Recognition Engine V 2.0). The model was constructed using the CM structures of *Mycobacterium tuberculosis* (PDB code 2FP1) and *Yersinia pestis* (PDB code 2GBB) as template. Both proteins are members of the $AroQ_{\gamma}$ subclass. In addition, members of this subclass are known to be exported from the cytoplasm due to an N-terminal signal sequence. Since the S/HRD region of HoCM did not match any of the sequences in the

protein database, only 72% of the residues were modeled with over 90% confidence. The global structure of an $AroQ_{\gamma}$ protein consists of six α-helices connected by loops. The CM domain of HoCM has the same topology predicted with a high confidence. Since the N-terminal region had no homologous sequences in the database it was modelled *ab initio*. This method predicted two additional α-helices in the N-terminal region, but it should be noted that this technique is less reliable compared to homology modelling. The catalytic residues are part of helices 1, 2, 3 and 4, forming the active site of the protein. The predicted tertiary structure of HoCM is shown in Figure 3.2.

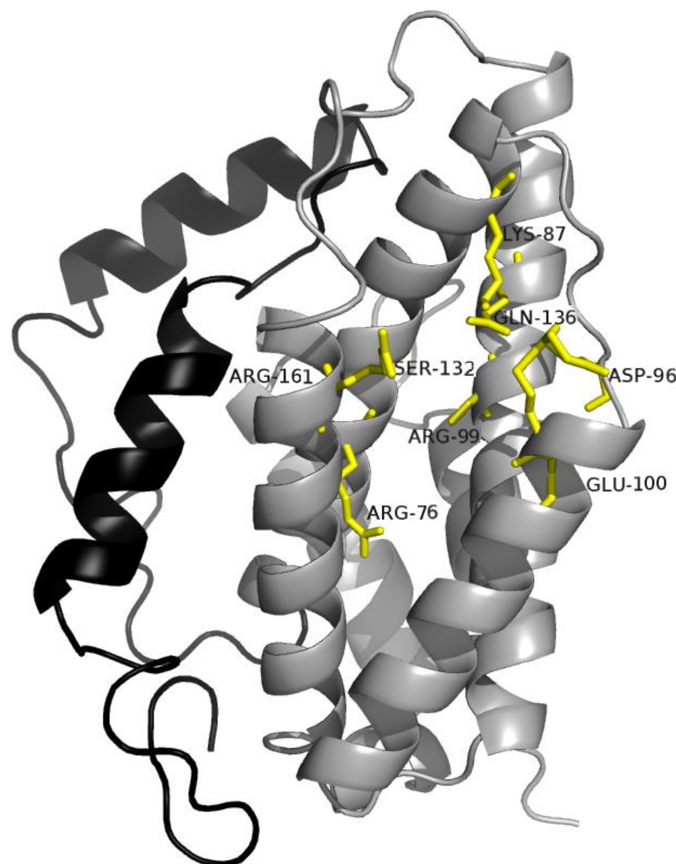


Figure 3.2: Predicted tertiary structure of HoCM. The structure of the mature protein is shown (without secretion signal). It was constructed using homology modeling with CM models of *M. tuberculosis* and *Y. pestis* as template. The C-terminal chorismate mutase domain (grey) consists out of 2 large α-helices in the middle, surrounded by 4 smaller α-helices. The N-terminal part (black) is predicted to have two α-helices. Eight catalytic residues are shown as stick models.

ICM-like hydrolases (ILH) are a large family of enzymes divided into six structural subgroups by the Structural Classification of Proteins (SCOP release 2.03). YecD, N-carbamoylsarcosine amidohydrolase, phenazine biosynthesis protein (PhzD), pyrazinamidase/nicotinamidase, ribonuclease MAR1 and YcaC. These proteins belong to the class of 'alpha-beta-alpha sandwich' according to the CATH structural classification. They adapt a Rossman fold, characterized by a 6 stranded parallel β-sheet flanked by α-helices on each side. The 3D model of HoICM was constructed by using 6 different ICM structures (PDB code: 1NF9, 1NBA, 2FQ1, 3TB4, 3IRV and 3KL2), creating a model of which 95% of the residues were modelled with more than 90% confidence with Phyre^2. The model shows a molecule with a Rossman fold; a 6-stranded parallel β-sheet flanked by 3 big α-helices at one side and 2 at the other. This is different from for instance the PhzD polypeptide where there are 3 α-helices at one side of the 6-stranded β–sheet and one long one at the other side (Parsons et al., 2003). Next to these α-helices, there are 4 putative 3_{10}-helices present as well. The presumed catalytic triad is shown in Figure 3.3. The catalytic center where isochorismate can bind has been described in *Oleispira antarctica* and is conserved in other PPNs (Goral et al., 2012). Computational methods assign HoICM to the structural group of the phenazine biosynthesis proteins with a RMSD (Root Mean Square Deviation) of 0.437 as calculated by a structural alignment in PyMOL 1.6.

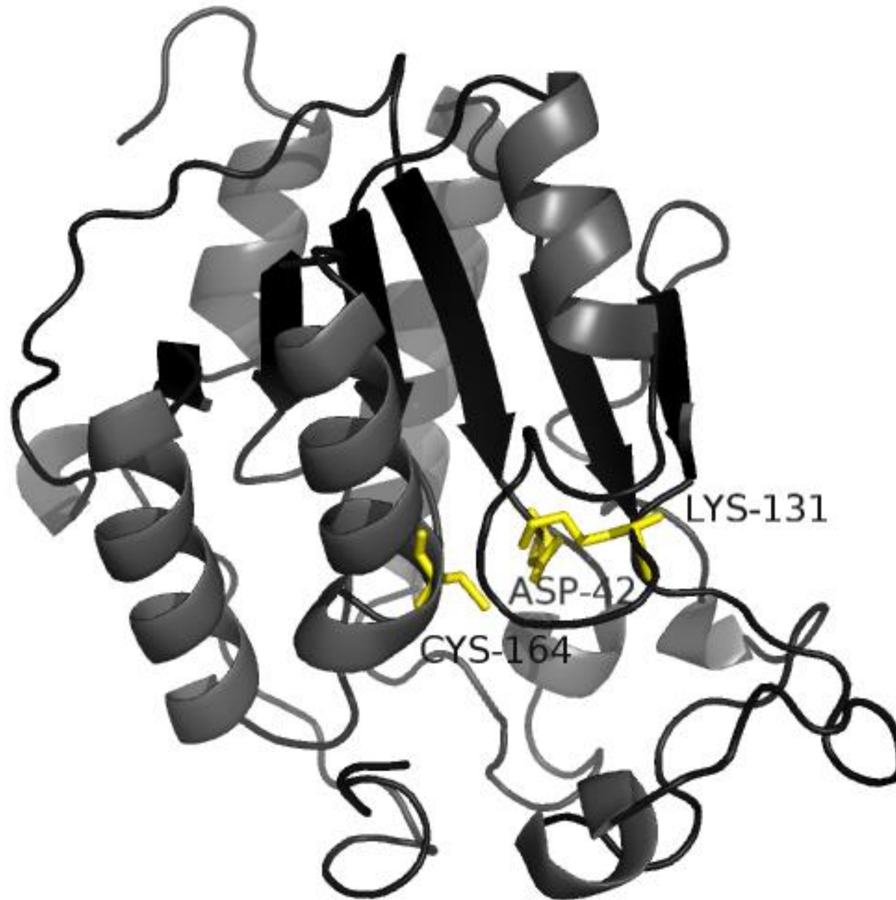


Figure 3.3: Predicted tertiary structure of HoICM. The 6-stranded parallel β-sheet (black) is in the center of the molecule, surrounded by 3 α-helices at one side and 2 at the other side. At the bottom (3) and the top left corner (1) in this view of the molecule, there are 4 3_{10}-helices. The catalytic triad (Cys164- Asp42-Lys131) is labelled.

3.3.4. Activity assay

CM is involved in the shikimate pathway, which is not present in nematodes. This pathway is necessary in bacteria and plants for the biosynthesis of several aromatic amino acids. The activity of HoCM was assessed by a complementation assay. Two constructs were made and expressed in a CM-deficient *E. coli* strain. One construct contained the full *CM* gene without the predicted signal peptide, the second construct only consisted of the catalytic region. They were grown on dropout medium without phenylalanine to test CM-activity. The positive control was *E. coli* containing wild type *B. subtilis* CM (Figure 3.4A). The results confirmed CM-activity of the HoCM protein. The fact that both protein constructs grew equally well shows that the S/HRD region is not necessary for activity. All catalytic residues

are present in the C-terminal region, hence the N-terminal S/HRD region probably has another function, not related to *in vitro* CM-activity.

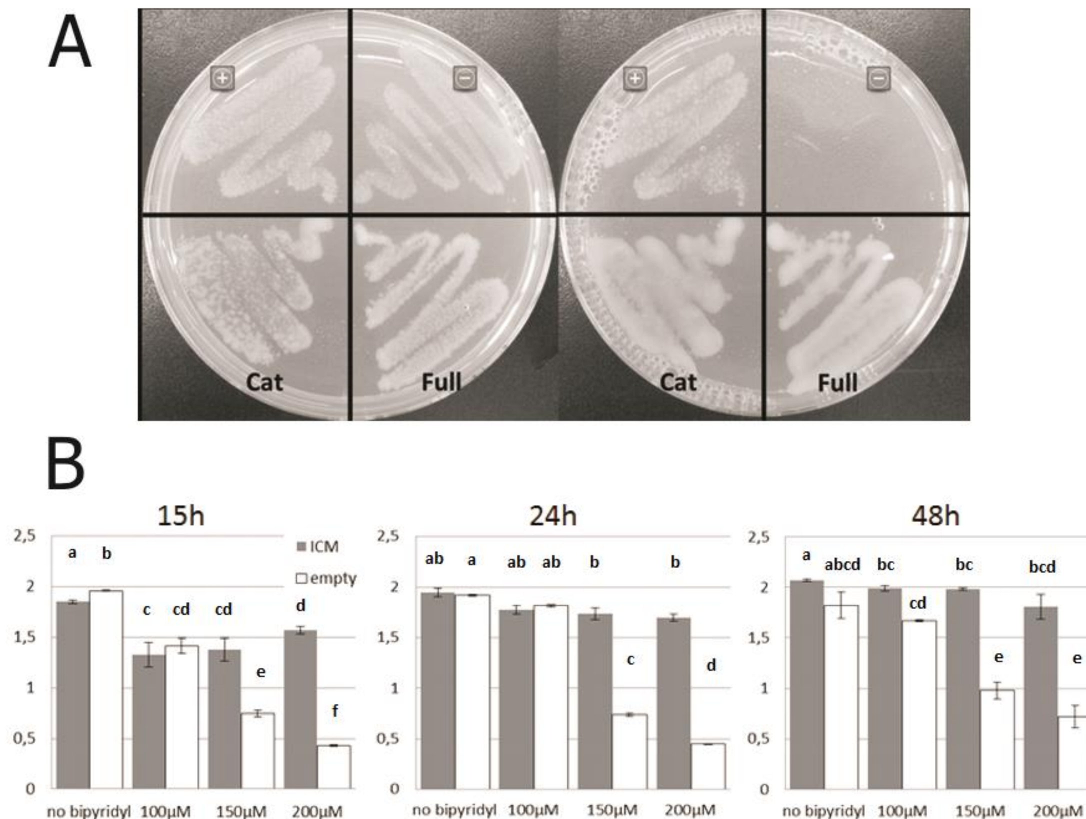


Figure 3.4: Complementation tests of *HoCM* and *HoICM*. A: Complementation assay of the *CM*-deficient *E. coli* strain KA12/pKIMP-UAUC with different constructs of *HoCM*. The left panel shows bacterial growth on M9cY medium supplemented with phenylalanine, the right panel bacterial growth on M9cY medium without additions. +: positive control (*CM* of *B. subtilis*), -: negative control (empty vector pQE30-UA), Cat: catalytic domain of CM, Full: mature CM protein. B: Complementation assay of *entB* mutated *E. coli* AN192. AN192 cells were complemented with an empty vector (pDEST17) (white) or *HoICM* (grey). Cells were grown in liquid medium with different concentrations of bipyridyl to create iron-limitating conditions. Bacterial growth was observed by measuring optical density (y-axis) on three different time points (an average was taken from four bacterial cultures). Treatments were compared with the control (no bipyridyl) and statistically analyzed by a Mann-Whitney test. Each experiment was repeated at least twice. Different letters above the graph indicate the significant differences between the different treatments.

The activity of HoICM was tested using the AN192 *E. coli* strain (Staab and Earhart, 1990). This strain is mutated in *entB*, a gene coding for a protein with ICM activity necessary for the synthesis of the siderophore enterobactin (Ent). Enterobactin is secreted during iron-limiting conditions to chelate Fe^{3+} (Raymond et al., 2003). AN192 cells carrying an expression vector harboring the complete *HoICM* gene were grown under iron-limiting conditions to test the

capability of *HoICM* to complement the *entB* mutation. Cells were allowed to grow for 48 hours in media with three different levels of iron-limiting conditions. Optical density (OD) was used as an indicator of bacterial growth and was measured at three different time points. The results show that AN192 cells harboring *HoICM* are able to grow much better under iron-limiting conditions compared to the empty vector control (Figure 3.4B). After 15 hours there was still a significant difference between the control and the bacteria growing in iron-limiting conditions, but after 24 hours the cells complemented with *HoICM* caught up with the cells under normal conditions. AN192 cells that carried the empty vector were not able to grow well under iron-limiting conditions. Even after 48 hours they could not reach half of the OD-value that *HoICM* complemented cells achieved under the two most stringent conditions (150µM and 200µM bipyridyl). This result proves that HoICM is able to complement the lack of ICM activity in the AN192 strain.

3.3.5. Defense suppression

To test *in planta* activity, an assay was performed to test if these putative effectors were able to suppress the immune system of the plant. Agroinfiltration of putative effectors in *N. benthamiana* leaves has been widely used for this purpose (Postma et al., 2012; Sacco et al., 2009). A hypersensitive response can be initiated by infiltration of *Gp-RBP-1* and *Gpa2*. Both of these constructs were infiltrated together with one of the possible effectors (*HoICM*, *HoCM_CAT* and *HoCM_FULL*) (results not shown). First results indicated that only *HoICM* and *HoCM_FULL* were able to suppress the HR compared to *GFP* (control treatment). Due to the fact that results were not reproducible, a different approach was chosen. *N. benthamiana* leaves were infiltrated with Agrobacteria carrying the effector construct. Afterwards these leaves were infected with *Phytophthora nicotianae*. Susceptibility was scored by measuring the infected area. Figure 3.5 shows a graph with the infected area of the different treatments 72h after infection. Both *HoCM_FULL* and *HoICM* enable the oomycete to infect a larger area after 72h compared to *GFP*. If leaves were infiltrated with only the catalytic domain of CM, there was no significant difference in infection rate.

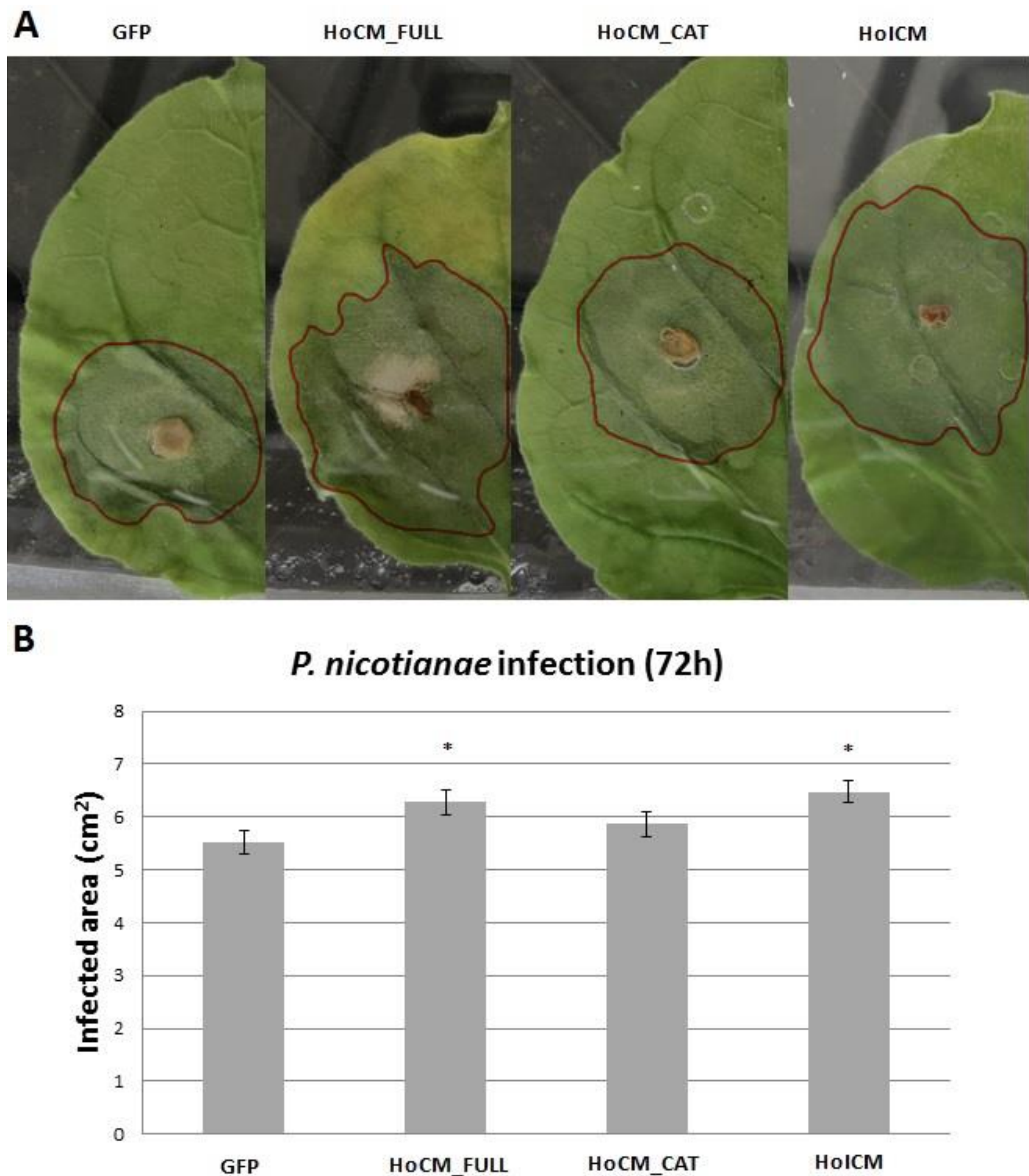
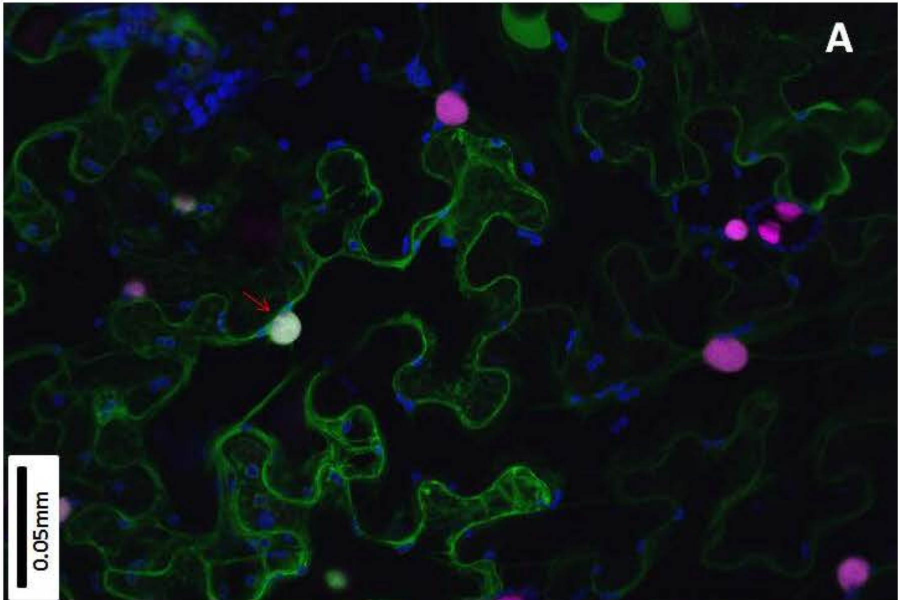


Figure 3.5: (A) Infiltrated leaves infected with *P. nicotianae*. Pictures were taken 72h after infection. Leaves were infiltrated with agrobacteria carrying *GFP* (control, 57 infection spots), *HoCM_FULL* (60 infection spots), *HoCM_CAT* (61 infection spots) or *HoICM* (57 infection spots), 24h prior to inoculation with *P. nicotianae*. (B) Infected area was measured with ImageJ software. Results show that the infected area is significantly bigger in leaves infiltrated with *HoCM_FULL* (p= 0.018) and *HoICM* (p=0.006) (indicated with '*') when compared to the control. No significant reduction or increase could be detected in leaves infiltrated with *HoCM_CAT* (p=0.318).

3.3.6. Localization studies

Localization studies have been performed before on CM and ICM secreted by plant-pathogenic species like *Ustilago maydis*, *Verticilium dahlia* and *Phytophthora sojae* (Djamei et al., 2011; Liu et al., 2014) showing that both proteins reside in the cytoplasm. To check whether the serine/histidine rich region present in HoCM has an effect on localization of the CM protein, agroinfiltration studies were performed. *HoCM_FULL* and *HoCM_CAT* (without the serine/histidine rich region) were cloned into the pK7WGF2 expression vector to fuse GFP to the N-terminal region of the protein. The vector was transferred to *Agrobacterium tumefaciens* GV3101 and infiltrated into leaves of *N. benthamiana* marker lines. Results are shown in Figure 3.6. Both HoCM_FULL and HoCM_CAT are localized in the plant cytoplasm. Since the nucleoli lack the green color, the signal in the nucleus is probably due to passive diffusion of the GFP fusion. When a general *in silico* approach was used to predict subcellular localization for the two constructs, using WoLF PSORT, ambiguous results were obtained (Horton et al., 2007). The sequence of the catalytic region was predicted with a cytosolic localization. However, using the full sequence (without SP), localization scores were too similar to assign a localization prediction with high confidence. *In silico* localization predictions to scan for putative nuclear localization signals (NLS) strengthened the hypothesis of passive diffusion. Three different prediction programs were used to search for putative NLS (cNLS Mapper, NLStradamus and NucPred) (Ba et al., 2009; Brameier et al., 2007; Kosugi et al., 2009). None of them was able to predict a NLS in the protein sequence of HoCM_FULL or HoCM_CAT.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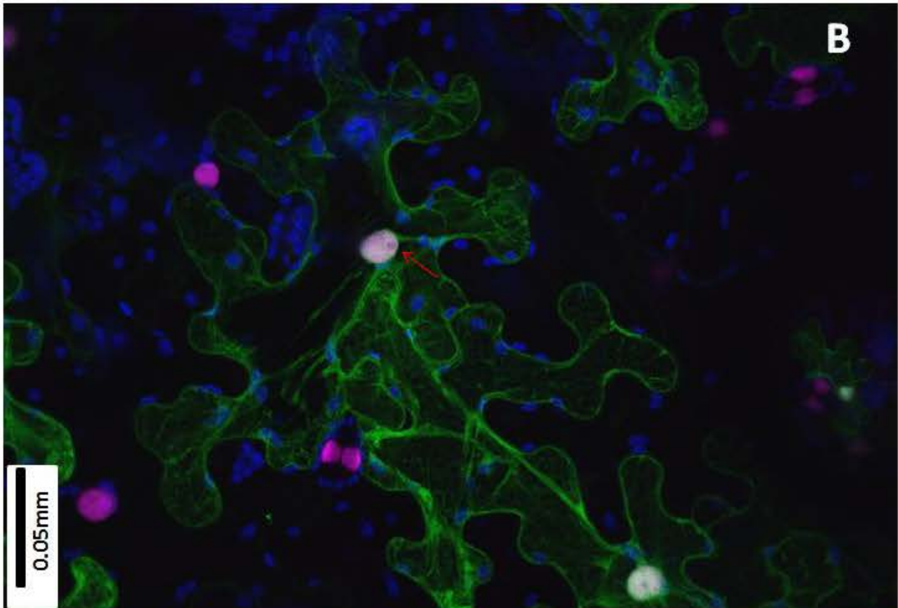
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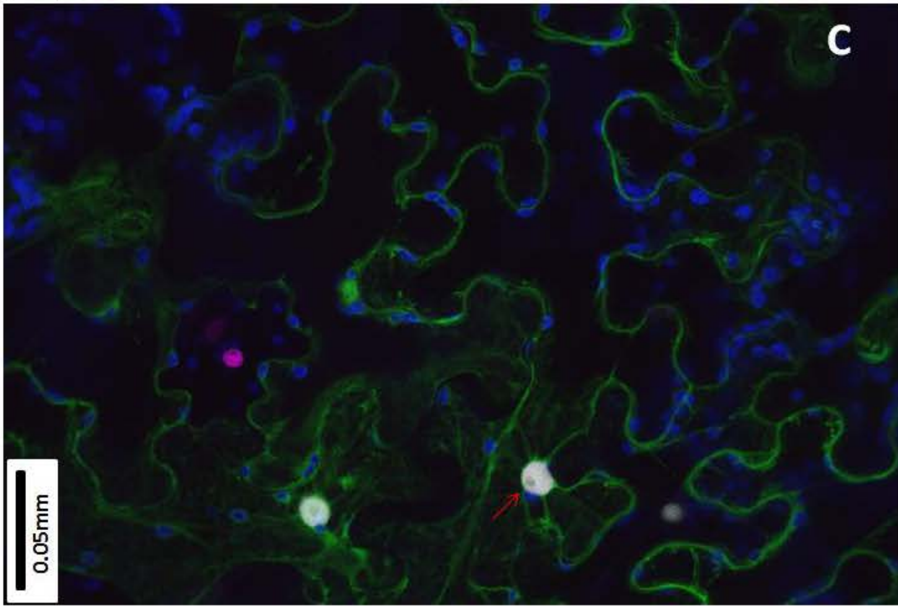
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Figure 3.6: (see previous page) Results of localization studies. Bacteria containing the gene of interest were infiltrated in leaves of transgenic *N. benthamiana* plants (CB157) expressing a nuclear histone marker fused to mRFP. Either free *gfp* was expressed (A) or *HoCM_Full* (B) or *HoCM_CAT* (C) attached to *gfp*. The nucleus of each cell is stained with mRFP and has a purple color. Free GFP localizes in the cytoplasm and in the nucleus by passive diffusion. Both HoCM_Full and HoCM_CAT are localized in the cytoplasm. The signal observed in the nucleus is probably due to passive diffusion. Blue dots are autofluorescence from chlorophyll. A black scale bar indicates 50µm. Red arrows show the position of the nucleoli.

3.4. Discussion

Although CM has been reported before to be present in transcriptomes of migratory PPNs (Bauters et al., 2013; Haegeman et al., 2011b), this is the first time a full length CM has been characterized in one of these species. Chorismate is the final product of the shikimate pathway, which has only been reported in plants and micro-organisms, but not in animals (Herrmann, 1995). Chorismate can be metabolized to the aromatic amino acids phenylalanine and tyrosine through the intermediate prephenate, using CM as a catalyzer. Chorismate can also be metabolized to SA using isochorismate as intermediate (Wildermuth et al., 2001). Furthermore, several other compounds have chorismate as a precursor: e.g. trypthophan, the auxin indole-3-acetic acid (IAA), flavonoids, lignin and phytoalexins. The first report of CM from a nematode was in *M. javanica* (Lambert et al., 1999). It was thought to be involved in the establishment of a feeding site within the host. IAA levels were reported to be lowered by expression of this *CM* in plant roots, which had an influence on root development (Doyle and Lambert, 2003). Since it was reported that IAA is an important factor for feeding site initiation, CM was hypothesized to be a vital element in this process (Goverse et al., 2000).

The CM protein produced by *H. oryzae* consists of three different domains: an N-terminal secretion signal, a serine/histidine rich domain and a catalytic domain with CM-activity. The N-terminal secretion signal together with the expression in the gland cells (Bauters et al., 2013) predicts that this protein is injected into the host. The catalytic domain has been preserved in sequences of other PPNs as well. Catalytic residues, characterized in CM from *E. coli*, are present in all nematode sequences used in the alignment (Figure 3.1 A) except for one residue. Serine at position 152 is conserved in *H. oryzae* and root-knot nematodes, but in cyst nematode CM, it has been replaced by asparagine. In the alignment one can see that

the CM sequences derived from root-knot nematodes lack an N-terminal domain preceding the CM catalytic domain. Vanholme et al. (2009b) proved that this N-terminal sequence was not needed for CM-activity in *H. schachtii*. This region was called the cysteine-rich domain (CR domain) due to the presence of six highly conserved cysteine residues among cyst nematodes. The CM N-terminal domain of cyst nematodes is not similar to the N-terminal domain of HoCM. It is enriched in serine and histidine residues (11 and 13 residues respectively) of which most occur in clusters. Experimental evidence suggests that this S/HRD region is not needed for CM activity *in vitro*. More research is needed to elucidate the function of this N-terminal region. Since it is not needed for enzymatic activity, it is possible that the S/HRD region has a more specific function inside the host cell to aid the nematode in its colonization of the host plant. This hypothesis was supported by the fact that the effect on growth rate of *P. nicotianae* was not significantly elevated when grown on *N. benthamiana* leaves expressing the catalytic domain of *HoCM*, while *P. nicotianae* was able to colonize the tissue faster if the leaf was expressing the complete sequence of *HoCM*. The tertiary structure of the mature protein was determined by homology modelling using CM from *M. tuberculosis* and *Y. pestis* as template. These sequences respectively had 23% and 22% sequence similarity.

ICM was reported in the transcriptome of *H. oryzae* in an earlier study (Bauters et al., 2013) and in *Rotylenchulus reniformis* where an ICM was found with homology to only prokaryotic ICMs (Wubben et al., 2010). This result, together with our blast-results, indicates that this type of ICM was probably acquired by horizontal gene transfer (HGT) from bacteria. This hypothesis was already put forward for the PPN *M. hapla* by Opperman et al. (2008) who discovered a protein encoding gene with an isochorismatase domain. The sequence of ICM does not contain a predicted N-terminal secretion signal, but the fact that the encoding gene is conserved in other PPNs, while being absent in nematodes with a different lifestyle (Bauters et al., 2013), led us to believe it might be involved in plant parasitism. This hypothesis is strengthened by the fact that ICM is found in the secretome and in the culture supernatants of phytopathogenic fungi (El-Bebany et al., 2010; Soanes et al., 2008) although the genes do not encode a signal peptide at the N-terminus of the protein (Liu et al., 2014). This observation indicates that ICMs can be secreted by a non-classical secretory pathway, independent from an N-terminal secretion signal. Furthermore, the authors have shown that the N-terminal region preceding the ICM domain is needed to target the protein to this non-classical secretory pathway. When the N-terminal region preceding the ICM domain was

removed from the full HoICM protein, the SecretomeP 2.0 score dropped below the threshold, indicating that the N-terminal region may have a function in secretion of the protein. A secreted ICM-like protein containing a predicted secretion signal has been found in the necrotrophic fungus *Pyrenophora teres*, so the non-classical type of secretion is not conserved in all plant-parasitic species for ICM (Ismail et al., 2014). The HoICM effector has a conserved catalytic triad (Asp-Lys-Cys) which is also present in ICM proteins of other nematodes and other species. It was reported previously that this catalytic triad is necessary for the protein to perform its function (Liu et al., 2014).

It has been generally assumed that plant resistance against biotrophic pathogens is mediated by SA signaling, while JA and ET signaling pathways are the key players in resistance against necrotrophs (Glazebrook, 2005). Hormone signaling in rice seems to be more complicated. Although antagonistic reactions of SA and JA still exist in rice, both hormones are effective against both hemibiotrophic and necrotrophic rice pathogens (De Vleesschauwer et al., 2014). Root knot nematodes and cyst nematodes keep plant cells alive as their feeding source and are therefore clearly biotrophs. It is difficult to assign *H. oryzae* to either of these groups: biotrophs or necrotrophs. It is known that this migratory nematode can cause big necrotic lesions during its infection of the host (Kyndt et al., 2012a). However, it remains unclear whether *H. oryzae* feeds on this necrotic tissue or just moves to a nearby living cell to feed on. Recent data provided by Nahar et al. (2012) showed that SA signaling is needed for an adequate defense response against *H. oryzae* infection in rice. On the other hand, the SA biosynthesis pathway in rice was also manipulated by *H. oryzae* upon infection (Kyndt et al., 2012b), leading to the assumption that SA signaling is a more important factor during defense upon *H. oryzae* infection.

SA biosynthesis in plants can be carried out through two different pathways, both starting from chorismate as a precursor Figure 3.7. One pathway is a two-step process with isochorismate as an intermediate, catalyzed by the enzymes isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL). The other possibility is a multi-step process through several intermediates, known as the phenylpropanoid pathway (reviewed in Vlot et al., 2009). By secreting the effector proteins CM and ICM into the host tissue, *H. oryzae* can possibly act upon both pathways.

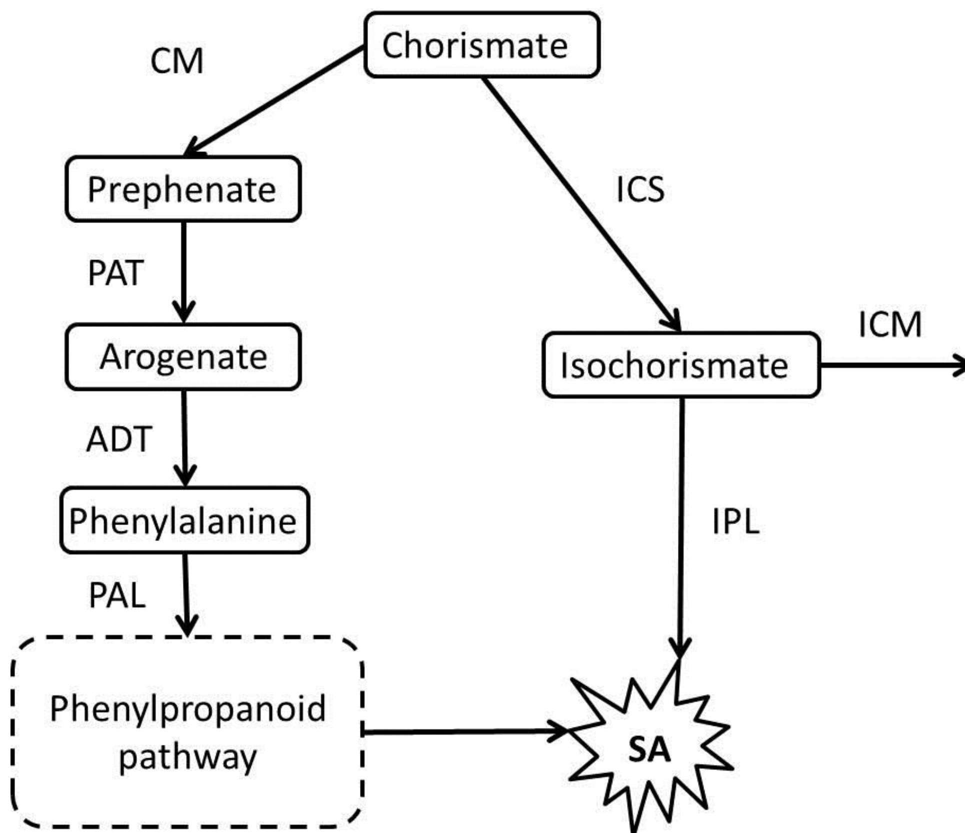

Figure 3.7: Schematical representation of the two possible pathways leading to salicylic acid, starting from chorismate. The right side of the picture shows the two-step pathway to salicylic acid. Isochorismatase can possibly take away isochorismate from this pathway. The left side shows the synthesis of phenylalanine, the starting point of the phenylpropanoid pathway. One of the metabolites of this pathway is salicylic acid. More details about salicylic acid synthesis are given in Chapter 5. Abbreviations: Isochorismate synthase (ICS), isochorismatase (ICM), isochorismate pyruvate lyase (IPL), chorismate mutase (CM), prephenate aminotransferase (PAT), arogenate dhydratase (ADT), phenylalanine ammonia lyase (PAL).

Djamei et al. (2011) have investigated the effect of a secreted CM from *Ustilago maydis* on maize. This research led to the conclusion that the secreted CM was able to diminish SA accumulation upon pathogen infection. When maize tissue was infected with a deletion mutant, the SA accumulation was at least 10-fold higher. Other pathogen species have been shown to secrete this effector as well (Degrassi et al., 2010; Vanholme et al., 2009b). Recently, ICM was shown to be secreted by two fungal pathogens (*Verticillium dahliae* and *Phytophthora sojae*) (Liu et al., 2014). ICM was secreted by an unconventional secretion system and it was able to reduce the SA content at least 3-fold upon transient expression in *N. benthamiana* leaves. This reduction in SA rendered the plant more vulnerable to other pathogens as well.

Here, we reported that the filamentous pathogen *P. nicotianae* was able to infect leaves of *N. benthamiana* faster when these leaves were transiently expressing the effectors CM and ICM. In case of CM, only infiltration with the full CM showed a significant effect. This result indicates that the S/HRD region is needed to suppress the defense system of the host, although it is not needed for *in vitro* activity. The role of this S/HRD domain *in planta* remains to be elucidated. Although the difference looks small, the infected area is 14% and 17% bigger in leaves transiently expressing respectively *HoCM*_full and *HoICM* compared to the control (*GFP*). In addition, while the nematode secretes a whole cocktail of effectors into the host tissue upon infection, only one effector was expressed in the leaf, which might explain the relatively small difference. The small, but significant, difference in growth rate of *P. nicotianae* gives an indication that both effectors are used in attenuating the defense response, but that they are probably just a small part of a complex mechanism.

The enzymatic activity and a model of the 3D protein structure of a nematode secreted CM was described by Vanholme et al. (2009b). They found that cyst nematode CMs have an N-terminal protein domain of unknown function with six conserved cysteine residues, which are probably involved in forming disulphide bonds to increase stability. Interestingly, the catalytic region of HoCM is also preceded by an N-terminal domain, although no sequence similarity was observed with the N-terminal domain from the cyst nematode CM. Instead of conserved cysteine residues, it contains serine and histidine rich regions. Complementation assays have shown that this domain is not involved in enzymatic activity. The effect of this domain on the subcellular localization inside the plant cell was tested by infiltration studies in *N. benthamiana*. Constructs with and without the N-terminal part showed the same localization: in the cytoplasm and the nucleus of the plant cell. No GFP signal was observed in the nucleoli and it has been shown that proteins exceeding 60kDa in size can pass the nuclear pore by passive diffusion in human HeLa cells (Wang and Brattain, 2007). This indicates that the signal in the nucleus is probably due to passive diffusion. Just as *Pratylenchus* species, *H. oryzae* most probably feeds several hours on a single cell, keeping it alive during the process (Zunke, 1990). Whilst feeding, the nematode probably secretes HoCM and HoICM into the cytosol to attenuate the defense system and prevent the cell to undergo apoptosis. Similarly, a secreted CM of the plant pathogenic fungus *Ustilago maydis* is also localized in the plant cytoplasm (Djamei et al., 2011). Our data showed that the S/HRD domain does not influence the subcellular localization of the HoCM protein. Further investigations are necessary to elucidate the function of this domain.

It is tempting to hypothesize that both CM and ICM are secreted by the nematode to deregulate the phenylpropanoid or SA pathway of the host in order to decrease the phenylpropanoid or SA content in the plant. Previous studies have shown that the systemic expression of a rice endogenous ICM and a CM is downregulated upon infection with *H. oryzae* (Kyndt et al., 2014a). This indicates that more chorismate and isochorismate will be available for SA production through the more efficient two-step pathway (Wildermuth et al., 2001). It is probable that the nematode is trying to counteract this by depleting the chorismate and isochorismate pools by secreting HoICM and HoCM. Rice already has high basal levels of SA and evidence suggests that SA does not act as an effective signal molecule to activate many defense genes, but that it rather plays a role in protecting rice plants from oxidative stress (Yang et al., 2004). Nevertheless, Nahar et al. (2012) have shown that SA-deficient *NahG*-lines are considerably more susceptible to *H. oryzae* infection compared to the control rice plants with a normal SA content. Although the role of SA in rice upon pathogen infection remains to be elucidated, these results indicated that SA is a protagonist in the defense response upon infection with *H. oryzae*.

All together, the experimental evidence in this research gives an indication about the activity of the two possible effectors HoCM and HoICM, but the mechanism by which they are able to promote pathogen infection remains elusive and needs further investigation. The possible role of both proteins will be analyzed further in next chapter.

3.5. Experimental procedures

3.5.1. DNA extraction and gene amplification

Nematodes were extracted from infected rice roots by a modified Baermann funnel method. Genomic DNA was extracted from a batch of mixed stages of *H. oryzae* or a single hand-picked nematode as previously described by Bolla et al. (1988), with some minor modifications. Briefly, nematodes were sonicated three times for ten seconds to break cell membranes. Samples were incubated for only 1 hour at 65°C after which DNA was isolated using phenol and chloroform. Isolated DNA and a cDNA bank (Bauters et al., 2013) were used to amplify *HoCM* and *HoICM* by PCR with gene specific primers (Appendix B). PCR fragments were cloned into pGEM-T (Promega) according to the standard T/A protocol and sequenced by the Sanger method (LGC, Germany) to check the insert.

3.5.2. Bioinformatic tools

Several blast programs, available on the NCBI server (http://www.ncbi.nlm.nih.gov/), were used for sequence similarity searches in EST and non-redundant protein databases. Before alignments were made, EST sequences were translated to protein sequences using the Transeq software from EBI (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Alignments were generated with Bioedit (Hall, 1999). Putative N-terminal secretion signals were detected by SignalP 4.1 software (Petersen et al., 2011). Putative unconventional secretion signals were predicted by SecretomeP 2.0 (Bendtsen et al., 2004). EST sequences used in this analysis were: *M. hapla*: CN577870, *R. reniformis*: GT737079 and *P. coffeae*: in house data. Protein domains were identified by searching the Pfam 27.0 database (Punta et al., 2012). The Protein Homology/anologY Recognition Engine V 2.0 (Phyre^2) was used to construct 3D models of HoCM and HoICM (Punta et al., 2012). The output of this homology modelling technique was visualized by PyMOL 1.6 (http://www.pymol.org/).

3.5.3. Complementation assay with *HoCM*

Two *HoCM* fragments (Full and Cat) were amplified by PCR using the full cDNA sequence as template (primer sequences can be found in Appendix B). The first fragment comprised the full sequence of CM without the signal peptide, the fragment containing only the catalytic domain involved base pair 258 to 747. Fragments were cloned into the pQE-30 UA vector (Qiagen) using the standard T/A-cloning protocol. Activity of CM was assayed by transforming the vector into *E. coli* KA12/pKIMP-UAUC cells (Kast et al., 1996) and growing them on M9cY minimal medium (Gamper et al., 2000). Bacterial cells were first grown on regular LB medium with appropriate antibiotics after which replica plates were made on M9cY and M9cY + F. M9cY medium consists of Na_2HPO_4 (6 g/L), KH_2PO_4 (3 g/L), NH_4Cl (1 g/L), NaCl (0.5 g/L), D-(+)-glucose (2 g/L), $MgSO_4$ (1 mm), 4-hydroxybenzoic acid (5 mg/L), 4-aminobenzoic acid (5 mg/L), 2,3-dihydroxybenzoic acid (1.6 mg/L), $CaCl_2$ (0.1 mm), thiamine-HCl (5 mg/L), l-tryptophan (20 mg/L) and L-tyrosine (20 mg/L). M9cY + F plates were additionally supplemented with 20 mg/L of l-phenylalanine. Isopropyl β-d-1-thiogalactopyranoside (IPTG) (133µM) was added to the medium to ensure full induction of the inserted CM sequence. Bacteria were grown at 30°C, 100mg/L carbenicillin and 30mg/L chloramphenicol were added to the plates. KA12/pKIMP-UAUC cells transformed with the Full fragment in another reading frame in pQE-30 UA were used as negative control. Wild-

type *Bacillus subtilis CM* cloned in pMG212H-W transformed in KA12/pKIMP-UAUC cells was used as positive control (Gamper et al., 2000).

3.5.4. Complementation assay with *HoICM*

To test the activity of HoICM, *entB* mutated AN192 *E. coli* cells were used. AN192 cells are unable to grow in iron limiting conditions. *HoICM* was amplified by a two-step PCR starting from cDNA as template to add attB sites to the fragment (primer sequences can be found in Appendix B). The fragment was cloned into the destination vector using the standard Gateway technology protocol (Life Technologies, Carlsbad, CA, USA). In brief: first the fragment was cloned into pENTR221 using BP-clonase after which it was transferred to the expression vector pDEST17 by using LR-clonase. The pDEST17 vector carrying *HoICM* was transformed via heat shock into *E. coli* AN192 cells. *E. coli* AN192 cells transformed with the empty vector were used as negative control. These cells were grown overnight at 37°C in LB medium with 100mg/L carbenicillin. OD was measured at 600nm and equal amounts of bacteria were added to fresh medium containing different concentrations of 2,2’-bipyridyl to create iron-limiting conditions. Concentrations of 2,2’-bipyridyl were 0µM, 100µM, 150µM and 200µM. These different types of media were inoculated with *E. coli* AN192 cells carrying *HoICM* or the empty vector. OD was measured at 600nm at three different time points: 15h, 24h and 48h post inoculation. Two replications were performed in this experiment.

3.5.5. Defense suppression assay

HoICM and *HoCM* constructs were cloned into the pK7WG2 expression vector using standard gateway cloning techniques. *GFP* was used as control. Primers can be found in Appendix B. Vectors with insert were transferred to Agrobacterium strain GV3101 by using triparental mating with *E. coli* containing the pRK2013 plasmid as helper strain. Agrobacteria were grown overnight in LB containing the appropriate antibiotics (100mg/L spectinomycin and 25mg/L gentamycin) at 28°C. Bacteria were centrifuged for 10 min at 3000rpm and dissolved in infiltration buffer (10mM MgCl2, 10mM 2-(N-morpholino)-ethanesulfonic acid (MES), 200µM acetosyringone). OD 600 was set to 0.5 and bacteria were incubated for 3 hours at room temperature. 4 to 6 week old *N. benthamiana* plants were used for agroinfiltration. At least 30 infiltrations were done for each construct. Plants were kept at room temperature for 24 hours before they were infected with *Phytophthora nicotianae*.

P. nicotianae was grown on V8 medium (200mL V8 juice/L, 3g/L $CaCO_3$ and 15g/L agar) for four days in the dark at room temperature. Small agar plugs, with a diameter of 5 mm were made from these plates. Agar plugs were placed in the middle of the infiltrated regions of *N. benthamiana* leaves. The detached leaves were put in petri dishes together with a moist tissue to prevent leaves from drying out. Plates were sealed and incubated at 24°C. Infected area of leaf tissue was measured 72 hours after infection using ImageJ (Schneider et al., 2012). The experiment was performed twice. Anova analysis with "experiment" as an extra variable showed that the size of the infected area was not depending on the experiment, hence both datasets were combined. Data was checked for normal distribution and equality of variance. Both conditions were met and a Student's t-test was performed to compare each treatment with the control (*GFP*).

3.5.6. Subcellular localization studies

HoCM_FULL and *HoCM_CAT* were cloned into the pK7WGF2 expression vector using general Gateway technology (Life technologies). The vectors with insert were transferred to *A. tumefaciens* strain GV3101 by triparental mating and prepared for infiltration as described before. OD600 was set to 0.1 before infiltrating leaves of 4-6 week old transgenic *N. bentha*miana plants (CB157). CB157 plants express a nuclear histone marker (H2B) fused to mRFP, thereby visualizing the nucleus (Martin et al., 2009). Visualization of localization was done 48h post infiltration. Pictures were taken with a Leica Sp2 confocal laser-scanning microscope. GFP was imaged by excitation at 488nm and emission at 505-530nm. Autofluorescence from chlorophyll was captured at 650-700nm. mRFP was excited at a wavelength of 561nm and emission was captured between 580 and 610nm

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Chapter 4. Ectopic expression of nematode derived chorismate mutase and isochorismatase induces changes in secondary metabolism and susceptibility in rice

This chapter was adapted from: Chorismate mutase and isochorismatase, two effectors from the plant-parasitic nematode *Hirschmanniella oryzae* promoting susceptibility by altering secondary metabolism in the host. Bauters L., Kyndt T. Nowak S., Sieprath T., Denil S., Haeck A., Demeestere K., De Meyer T. and Gheysen G. In preparation

4.1. Summary

The migratory PPN *Hirschmanniella oryzae* is well equipped to invade rice roots. It makes use of an extended arsenal of secreted CWMP. Recently, two other potential effectors which could aid the nematode during infection were found in its transcriptome: CM and ICM. Transgenic rice lines were constructed, overexpressing either one of these genes. Overexpression lines were analyzed by RNA-sequencing and hormone measurements. Transgenic lines showed reduced expression of genes involved in the phenylpropanoid pathway and in diterpenoid phytoalexin biosynthesis. Histochemical analysis confirmed the decrease in terpenoid content in roots of the transgenic plants. Hormone measurements on these lines showed an increase in endogenous ABA concentrations in comparison with control plants. In addition, infection experiments showed an increased susceptibility of these lines to nematode infection.

4.2. Introduction

Hirschmanniella oryzae, the rice root rot nematode, is one of the most devastating pathogens in rice growing regions world-wide. Since *H. oryzae* is well adapted to flooding conditions, it is a major problem in flooded rice ecosystems (Babatola, 1981). *H. oryzae* is a migratory nematode, it spends most of its life inside the root, feeding on cells along its path. This can leave a track of necrotic tissue due to secondary infection of rot-inducing micro-organisms (Babatola and Bridge, 1980). To migrate through the root, PPNs are equipped with an arsenal of CWMP that are secreted through the stylet into the host tissue to hydrolyze cell walls (Davis et al., 2011). Sedentary nematodes establish a feeding site, whereby it is important that they remain undetected by the plant, to prevent an adequate defense response. Effectors are secreted by these types of nematodes to alter plant cell metabolisms to their advantage and to attenuate defense mechanisms of the host (Hewezi and Baum, 2013; Jaouannet et al., 2013; Postma et al., 2012). This standoff between attack and defense is often regulated by complex signaling networks (Li et al., 2015). Migratory nematodes stay mobile during their entire lifecycle. They keep on migrating through the root whilst feeding. Due to this lifestyle, they could be able to outrun local plant defenses and secretion of effector proteins influencing plant defense mechanisms might be unnecessary. The same line of thought was used for a long time for necrotrophic fungi compared to

biotrophic fungi. Recent discoveries undermined this hypothesis. Several effector proteins were found in the secretome of necrotrophic fungi that suggest an active attenuation of plant defenses by the pathogen. Examples include ICM, endopolygalacturonase and SIX4 which are secreted into the host tissue by *Stagonospora nodorum*, *Sclerotinia sclerotiorum* and *Fusarium oxysporum* (Soanes et al., 2008; Thatcher et al., 2012; Wang et al., 2009). The same observation was made in migratory nematodes by our and other research groups. Although these species stay mobile, several potential effector proteins which might act on plant defense (e.g. CM and ICM) have been found in the transcriptome of several plant-pathogenic migratory nematodes (Bauters et al., 2013; Haegeman et al., 2011b; Nicol et al., 2012). This implies that migratory nematode species also invest energy in attenuating defense responses of their host.

The focus of this chapter will be on the potential effects of the secretion of ICM and CM by *H. oryzae* into the host tissue and the consequential benefits to the invading nematode. Both proteins act directly or indirectly on chorismate, a known precursor for several secondary metabolites (alkaloids, flavonoids, etc.) and SA. SA is an important phytohormone during defense responses, so interfering with this pathway could have a severe effect on defense. SA is one of the typical immunity related hormones, together with JA and ET it guides the defense system of the host upon pathogen infection.

Two distinct pathways have been reported to synthesize SA from the precursor chorismate. Chorismate can be converted into prephenate, the first step into the phenylpropanoid pathway which leads to SA and other defense metabolites, such as lignin and flavonoids, through a complex, multistep process. Chorismate can also be metabolized to SA with only isochorismate as intermediate. The latter pathway was reported as the main producer of SA upon pathogen infection in Arabidopsis (Vlot et al., 2009). The role of SA during *H. oryzae* infection has not been fully elucidated yet, but the importance of this hormone during nematode infection has been experimentally validated. Infection with *H. oryzae* was reduced when rice plants were sprayed with an SA analog prior to infection (Nahar et al., 2012). In addition, transcriptome studies on systemic tissues after *H. oryzae* infection showed a reduced expression of genes involved in the SA pathway (Kyndt et al., 2012b) as well as the isoprenoid and shikimate pathway (Kyndt et al., 2014a). The secretion of ICM and CM by the nematode into the host tissue might counteract these pathways, thereby reducing resistance of the host. The secretion of CM and ICM by fungal plant-pathogens has already been shown to reduce the SA content in the host, thereby increasing susceptibility (Djamei

et al., 2011; Liu et al., 2014). In this chapter we will try to shed light on the possible role of *HoICM* and *HoCM* in the *H. oryzae* – rice parasitic interaction. RNA-seq and hormone measurements were performed on rice plants overexpressing *HoICM* or *HoCM*. RNA-seq on these transgenic lines revealed that both *HoCM* and *HoICM* are able to interfere with expression of genes regulated by SA and that the activity of several secondary metabolic pathways was altered. Hormone measurements especially showed variation in ABA content in the here-generated transgenic rice plants.

4.3. Results

4.3.1. Overexpression lines

Transgenic rice lines constitutively overexpressing *HoCM_FULL*, *HoCM_CAT* or *HoICM* were generated starting from *O. sativa* cv. Nipponbare. Lines transformed with a vector without insert (empty vector) were used as control. 5 and 2 stable independent lines were obtained for *HoCM_CAT* and *HoCM_FULL* respectively. For *HoICM*, 9 stable independent lines were obtained, but only 3 of them produced enough viable seeds to continue the line and perform further experiments. Presence of the construct was checked on genomic level by PCR with gene-specific primers. Expression was validated by quantitative reverse transcriptase PCR. All lines expressed the constructs. Further experiments were conducted on T1 and T2 plants.

4.3.2. Differentially expressed genes and validation

RNA-sequencing was performed on one line per construct to study transcriptional changes in rice plants overexpressing *HoCM_CAT*, *HoCM_Full* or *HoICM*. Since no clearly different phenotypes were observed between independent lines, lines with the largest amount of seeds were chosen for further analysis. Almost 150 million sequenced RNA fragments were generated. Reads were aligned to the reference genome of *Oryza sativa* cv. Nipponbare (IRGSP-1.0). Only 5.2% of all reads could not be mapped to the rice genome (Table 4.1). Expression profiles of genes were compared between treatments by pathway mapping, Gene Ontology enrichment analysis and statistical analysis of differential gene expression levels.

Table 4.1: Overview of the output of RNA-sequencing.

Sample		Total number of sequence reads	Uniquely mapped reads	Reads mapped to multiple loci	Unmapped reads
Empty	rep.1	13,428,749	11,747,649	868,395	648,609
	rep.2	14,958,403	9,925,189	3,493,130	683,599
	rep.3	15,227,582	11,916,083	1,899,690	846,654
HoCM_Full	rep.1	10,809,981	8,085,457	1,643,525	590,225
	rep.2	14,897,494	12,883,852	943,430	704,651
	rep.3	13,041,032	10,931,807	1,148,182	640,315
HoCM_CAT	rep.1	13,825,284	11,819,619	979,122	789,424
	rep.2	12,350,758	9,332,242	1,768,786	707,698
	rep.3	10,809,639	9,063,044	916,922	597,773
HoICM	rep.1	13,648,763	11,717,531	960,184	700,182
	rep.2	14,460,569	12,562,608	910,609	712,906

Expression levels of all genes in the transgenic lines were compared to those in the line transformed with the empty vector. False discovery rates (FDR) were calculated to investigate differential gene expression in the different transgenic lines. Differentially expressed genes (DEG) (FDR<0.05) were blasted (blastp) against protein data for Viridiplantae available in the SwissProt database for annotation purposes (downloaded 02/2015). The transgenic line overexpressing *HoCM_FULL* had 55 DEGs (13 up, 42 down), when *HoCM_CAT* was expressed 196 DEGs were detected (67 up, 129 down). 105 DEGs (36 up, 69 down) were found in plants overexpressing *HoICM*. Approximately half of these genes had a significant (bit-score >50) hit when blasted against the SwissProt database (Table 4.2). These annotated genes were classified into 6 groups according to their putative function. Several genes with a function in general cell maintenance and development were differentially expressed. More interestingly, genes involved in signaling regulation, stress response and secondary metabolism of phytoalexins, terpenoids and flavonoids were differentially regulated.

A total of 8 DEGs were significantly downregulated over all three constructs when compared to rice plants containing the empty vector. This could be an indication that both proteins

(CM and ICM) influence expression of the same genes, although they have different substrates. Among these genes there were two with a weak similarity to an F-box only protein 7 and to a protein containing an ankyrin-2 type domain. The first one is a negative regulator of NF-κB signaling, which can control apoptosis and immune responses, while the latter is a domain present in NPR1, mediating *PR* gene expression and systemic acquired resistance (Kuiken et al., 2012; Rochon et al., 2006). Plasma membrane ATPase 1 and a polypyrimidine-tract binding protein, two proteins involved in general cell maintenance and development were found to be downregulated in all lines as well. Interestingly, the expression of 2 genes with similarity to a glutathione S-transferase (GST) was reduced in all samples. The activity and expression of GSTs might be dependent on SA signaling in plants (Barba-Espin et al., 2011; Garreton et al., 2002). Geranylgeranyl pyrophosphate synthase, a key enzyme involved in synthesis of isoprenoid metabolites was downregulated in all three samples. Expression of this gene is dependent on SA and JA signaling pathways (Ament et al., 2006). All three constructs also had a reduced expression of a flavin-containing monooxygenase, which might be involved in SA dependent resistance against plant-pathogens (Koch et al., 2006).

Table 4.2: Differentially expressed genes (FDR<0,05) compared to rice plants transformed with the empty vector. Significantly up- or downregulated genes were blasted (blastp) against the SwissProt database. The first and second columns show the UniprotKB identifier and the bit-score (significant hit, bit-score>50), followed by the gene description. The last six columns show the number of genes differentially regulated in the three different constructs. Underlined genes are significantly differentially expressed in all three constructs.

			HoCM_FULL		HoCM_CAT		HoICM	
ID	BITs	Gene Description	Up	Down	Up	Down	Up	Down
Signal Regulation								
C0LGP4	285	Probable LRR receptor-like serine/threonine-protein kinase		1	1			1
C0LGQ4	561	Probable LRR receptor-like serine/threonine-protein kinase			1			
C0LGH2	602	Probable LRR receptor-like serine/threonine-protein kinase				2		
Q8RWZ5	335	G-type lectin S-receptor-like serine/threonine-protein kinase				1		
Q2MHE4	303	Serine/threonine-protein kinase HT1			1			
Q3ECH2	303	Probable receptor-like protein kinase			6	4		
Q9FID6	315	Probable receptor-like protein kinase			2	3		
Q9FID5	331	Probable receptor-like protein kinase				2		
P0C5E2	487	Probable serine/threonine-protein kinase			1			
Q40704	225	MADS-box transcription factor 3			1			
Q9FDW1	251	Transcription factor MYB44						2
Q9XIN0	239	Transcription factor LHW						1
Q9LT67	119	Transcription factor bHLH113						1
Q2HIV9	167	Transcription factor bHLH35						1
Q9ZPS0	57	F-box only protein 7		1		1		1
Q9LDE3	85.5	F-box/kelch-repeat protein					1	
Q9FJJ4	67.4	F-box protein				1		1
Q39096	75.5	Protein EARLY RESPONSIVE TO DEHYDRATION 15						1
Q32SG5	354	Protein RIK						1
Q9C9H7	351	Receptor-like protein 12						2
Q9SZ67	235	Probable WRKY transcription factor 19						1
Q6YYC0	73.6	Zinc finger CCCH domain-containing protein 55		1				
Q8H1D3	585	BTB/POZ domain-containing protein NPY1	1		1			
Q9ZVC9	52.8	Protein FAR1-RELATED SEQUENCE 3	1		1	1		
Q8S1Y9	71.2	Calmodulin-like protein 1			1			

Q94E49	624	Protein kinase PINOID 2			1			
Q9FLV9	561	S-type anion channel SLAH3			1			
Q9LSV3	196	Putative wall-associated receptor kinase-like 16				1		
Q9ZVC2	69.7	Regulatory protein NPR5				1		
General Cell Maintenance and Development								
Q9LHF1	441	Leucine-rich repeat extensin-like protein 4						1
Q8L9S1	96.3	60S ribosomal protein		1				1
B9F058	542	Acyl-[acyl-carrier-protein] desaturase 3, chloroplastic	1					
P22180	1,521	Plasma membrane ATPase 1		1		1		2
Q6ICX4	66.2	Polypyrimidine tract-binding protein homolog 3		1		1		1
Q9M156	60.8	UDP-glycosyltransferase 72B1		1				
Q9ZQ94	396	UDP-glycosyltransferase 73C5		1				
P45129	58.9	Probable periplasmic serine protease do/HhoA-like					1	
Q96TW9	55.5	Probable transporter MCH1					1	
Q6GMH0	69.3	Pre-mRNA-splicing factor 18					1	
Q09686	94	Putative glutamine amidotransferase-like protein					1	
P15268	69.7	Autonomous transposable element EN-1 mosaic protein					1	
O80738	124	Probable alpha,alpha-trehalose-phosphate synthase					1	
Q9FYB5	81.6	Chaperone protein dnaJ 11, chloroplastic					1	
Q8GT20	315	Benzyl alcohol O-benzoyltransferase					1	
Q10359	68.2	Alpha-1,2-galactosyltransferase					1	
B0BN95	68.6	Putative nuclease HARBI1					1	
Q28HE5	95.1	Probable sodium-coupled neutral amino acid transporter 6						1
Q94B38	485	Glucose-6-phosphate/phosphate translocator 2, chloroplastic						3
P16081	1,901	Nitrate reductase [NADH] 1						2
P27968	1,576	Nitrate reductase [NAD(P)H]						1
Q9XI23	920	Boron transporter 4			1			
P27933	907	Alpha-amylase isozyme 3D			1			
P27934	910	Alpha-amylase isozyme 3E			1			
Q0JR25	178	Bowman-Birk type bran trypsin inhibitor			1	1		
A2WK50	386	Bowman-Birk type bran trypsin inhibitor				1		
Q94G86	106	Glucan endo-1,3-beta-D-glucosidase			1			

Q94JJ7	120	Histone H2B.3			1			
Q7GBK0	296	Histone H2B.7			1			
Q6F362	294	Histone H2B.9			2			
Q9FYP9	1,068	Importin subunit alpha-2			1			
Q2S415	912	Leucine--tRNA ligase			1			
Q7XLC6	1,628	Probable potassium transporter 11			1			
B6SFA4	193	Probable helicase MAGATAMA 3			1			
Q54T48	56.2	Probable ubiquitin carboxyl-terminal hydrolase			1			
Q9FKD9	60.5	Putative E3 ubiquitin-protein ligase SINA-like 6			1	1		
Q9SPV4	198	Salicylate O-methyltransferase			1			
Q9LNG5	72.4	Serine/threonine-protein phosphatase 7 long form homolog			1			
Q9SIB2	511	3-ketoacyl-CoA synthase 12				1		
Q01595	114	Cortical cell-delineating protein				1		
Q0J9J6	1,344	Crossover junction endonuclease EME1				1		
Q50EK1	367	Cytochrome P450 716B1				1		
P24465	437	Cytochrome P450 71A1				1		
B1AK53	73.6	Espin				1		
Q63618	75.1	Espin				1		
Q9SWH5	457	Galactoside 2-alpha-L-fucosyltransferase				1		
Q9CPY6	112	Glucose-induced degradation protein 4 homolog				1		
Q0J998	347	Indole-3-acetate O-methyltransferase 1				1		
Q39613	229	Peptidyl-prolyl cis-trans isomerase				1		
Q9UT84	143	Phospholipid scramblase family protein				1		
Q9LJX5	456	Probable mitochondrial adenine nucleotide transporter				1		
Q9SKX5	723	Probable polyamine oxidase 2				1		
Q9URY4	72.0	Putative amidase C869.01				1		
Q6JAH0	552	Putative cis-zeatin O-glucosyltransferase				1		
Q9C7Z9	404	Serine carboxypeptidase-like 18				1		
P17840	84.3	S-locus-specific glycoprotein S13				1		
B7ECS8	204	Putative beta-glucosidase 9					1	
Stress Related								
P46524	251	Dehydrin COR410	1					

Q9FHE1	56.6	Glutathione S-transferase T3		2		2		2
Q9LMA1	382	Probable flavin-containing monooxygenase 1		1		1		1
B9FSC8	129	Putative 12-oxophytodienoate reductase 11					1	
P27337	473	Peroxidase 1					1	
P93184	1,053	Lipoxygenase 2.1, chloroplastic					1	
Q6X4A2	929	CBL-interacting protein kinase 31						1
Q943E6	301	16.9 kDa class I heat shock protein 2				1		
Q5VRY1	333	18.0 kDa class II heat shock protein				1		
Cell Wall Related Proteins								
Q336T5	562	Expansin-B3		1				
Q9LI65	275	WAT1-related protein		1		1		
Q5NAT0	1,307	Endoglucanase 2			1			
Phytoalexins/Terpenoids/Flavonoids								
Q94ID7	109	Geranylgeranyl pyrophosphate synthase, chloroplastic		1		1		1
C5YHI2	489	Beta-sesquiphellandrene synthase					1	
O23731	418	Chalcone synthase 8						1
P42390	52.8	Indole-3-glycerol phosphate lyase, chloroplastic				1		1
Q9SEV0	201	Anthocyanidin reductase				2		
Q6YTF1	577	Ent-cassadiene C11-alpha-hydroxylase 2				1		
Q6Z5I0	718	Ent-copalyl diphosphate synthase 2				1		
Unclassified								
Q8C8R3	50.1	Ankyrin-2		1		2		1
A4IGM4	132	Hydroxysteroid 11-beta-dehydrogenase 1-like protein		1		1		
Q8GZ63	524	Pentatricopeptide repeat-containing protein				1		
		Uncharacterized		1	1			1
		Nohit	9	25	30	72	17	29

The reliability of the RNA-seq data was tested by validating the expression of a randomly chosen set of differentially expressed genes, by Q-RT-PCR. Results are visualized in Figure 4.1. All but one of the tested genes were significantly up- or downregulated. Eleven of the genes confirmed RNA-seq results. Only one gene showed a different differential expression pattern: the gene coding for histone H2B.3 was significantly upregulated according to the RNA-seq data ($Log_2FC=1.6$), while it was downregulated according to the Q-RT-PCR data. The expression of two genes, coding for glutathione S-transferase and a receptor-like protein kinase, was so strongly downregulated that it was undetectable by Q-RT-PCR in the transgenic lines. These results indicate the reliability of the generated RNA-sequencing data.

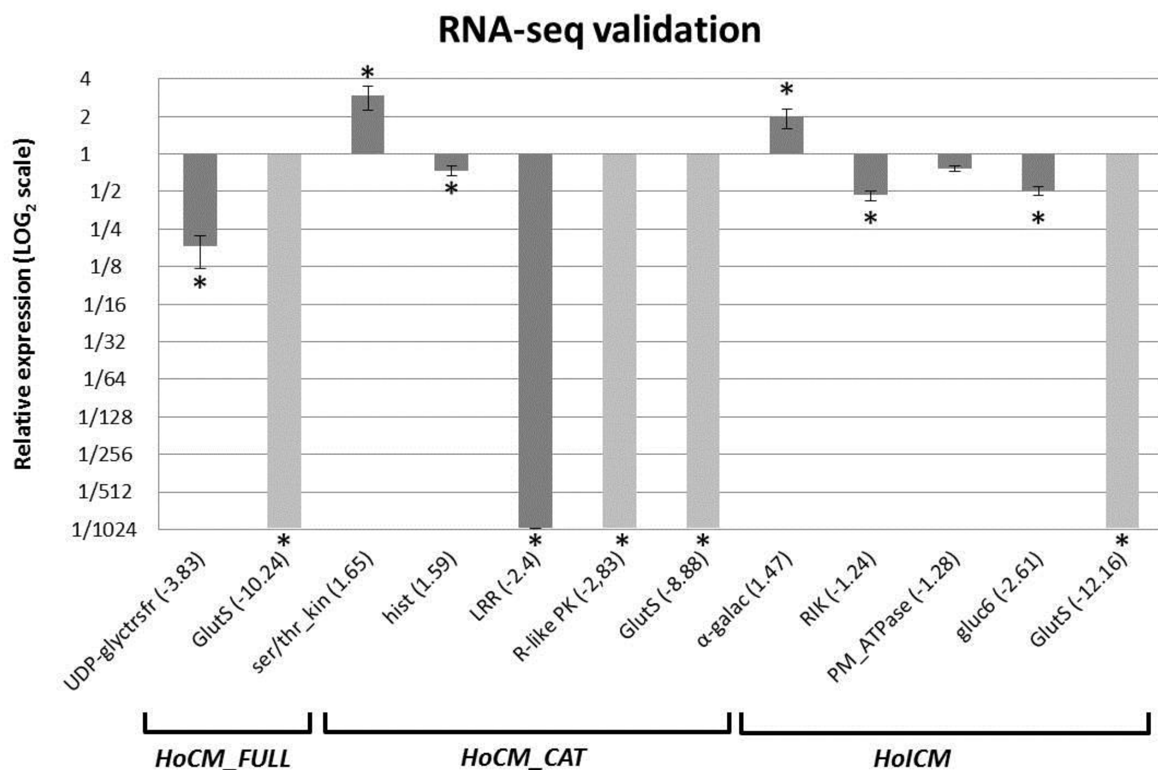

Figure 4.1: Validation of RNA-seq results. Expression values were calculated compared to expression in the empty vector control (expression level set at 1). Normalization was done with two reference genes (*EIF5C* and *EXP NARCAI*). Expression values are plotted on a Log_2-scale. Expression values are a mean of two biological replicates (calculated by taking the average of three technical replicates). Statistical analysis was performed with REST 2009 software (an asterisk (*) indicates a significant difference). Light grey bars indicate genes of which expression could not be detected in the respective overexpression line. UDP-glyctrsfr: UDP-glycosyltransferase, GlutS: Glutathione S-transferase, ser/thr_kin: Serine/threonine-protein kinase, hist: Histone H2B.3, LRR: LRR receptor-like serine/threonine-protein kinase, R-like PK: Receptor-like protein kinase, α-galac: α-1,2-galactosyltransferase, RIK: Protein RIK, PM_ATPase: Plasma membrane ATPase, gluc6: Glucose-6-phosphate/phosphate translocator 2. In between brackets the log_2FC according to RNA-sequencing is given. Bars represent standard errors, calculated with REST2009 software using Taylor's series.

4.3.3. Gene ontology enrichment

A parametric analysis of gene set enrichment (PAGE) was conducted to look for differential quantitation of gene ontology terms. The Log_2 fold changes of all genes were used as input for this analysis. All levels of GO in the category "Biological Process" were considered, with a significance level of 0.05 and a Hochberg multi-test adjustment method. GO terms with a calculated Z-score higher than 4 (absolute value) for at least one of the three constructs are visualized in Figure 4.2. All transgenic rice plants are repressed in their "secondary metabolic process" GO. Looking at the child terms of this GO term, the repression was due to a reduction in the GO term "Phenylpropanoid metabolic process". The GO term "Lignin catabolic process" is reduced in *HoCM_FULL* and *HoICM* overexpressing plants. "Diterpene phytoalexin metabolic process" is suppressed in plants overexpressing HoCM. Rice plants expressing any of the *HoCM* constructs are also repressed in the general "Defense response", while all three lines are reduced in "Oxidation reduction".

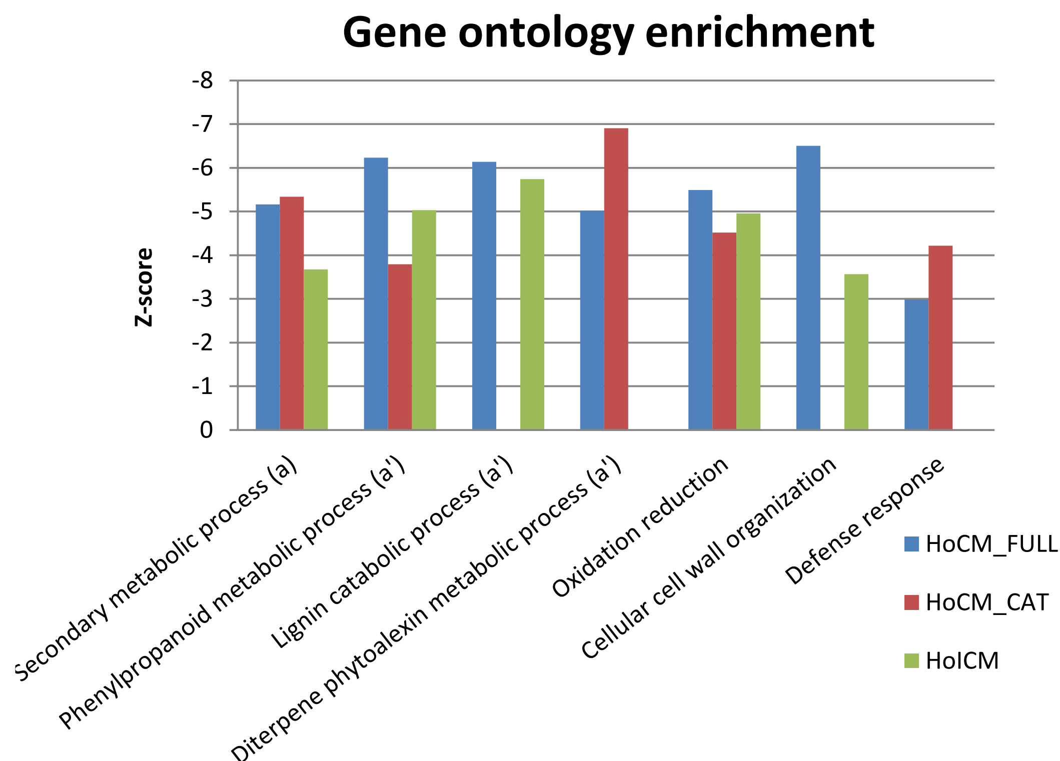

Figure 4.2: Gene ontology term enrichment analysis, showing the significantly enriched GO-terms in the category "Biological process". GO terms for which at least one of the three constructs showed significant difference with an absolute Z-score (y-axis) value of 4 are shown in the graph. a and a' indicate an ancestor term (a) with its child terms (a'). All GO terms represented in this graph are significantly less abundant in the transgenic lines (Z-score<0) compared to control plants (empty vector line).

4.3.4. Terpenoid visualization

Since GO analysis predicted changes in terpenoid metabolism, terpenoid molecules in roots of transgenic rice plants were visualized with antimony trichloride, which generates a brown color in presence of terpenoids. Two week-old rice roots were stained in an antimony trichloride solution. Results can be seen in Figure 4.3A. It is clear from these pictures that rice roots overexpressing nematode derived CM or ICM contain less terpenoids compared to rice roots transformed with an empty vector. For statistical analysis, at least three roots of each treatment were stained, and the images were converted to black and white pictures to measure grey scales (Figure 4.3B). All transgenic lines have a significant higher grey scale value, indicating that they contain significantly less terpenoids.

4.3.1. Pathway mapping

Differences in gene expression levels of all rice genes were also studied by looking at changes in metabolic pathways in plants, using MapMan. Significant differential pathway activities were detected by performing a Wilcoxon rank sum test on the log_2 fold changes with a Benjamini Hochberg correction. When focusing on the general cellular response of the plant cell, a significant difference was found in the two *HoCM* constructs in the category “biotic stress”, while this group of genes was not differentially expressed in *HoICM* plants. Looking into more detail, this downregulation is mostly due to differentially expressed genes involved in signaling (for instance receptor kinases and MAP kinases). When a log_2FC cut off of 0.5 was applied, a total of 197 and 204 genes involved in biotic stress were downregulated in plants overexpressing *HoCM_FULL* and *HoCM_CAT* respectively (162 in *HoICM* overexpressing plants). When looking at upregulated genes involved in biotic stress, 66 and 89 genes have a log_2FC over 0.5 in *HoCM_Full* and *HoCM_CAT* plants respectively (86 in *HoICM* plants). All significantly affected pathways are presented in Figure 4.4. Except for “protein synthesis” all three constructs show similar results regarding inhibition or activation of pathways. This representation shows that the “signaling” and “transport” pathways are suppressed in all transgenic lines in comparison with the control plants, while “RNA” is upregulated. Both “cell wall cellulose synthesis” and “cell wall arabinogalactan proteins (AGP)” pathways are downregulated in *HoICM* and *HoCM_Full* plants

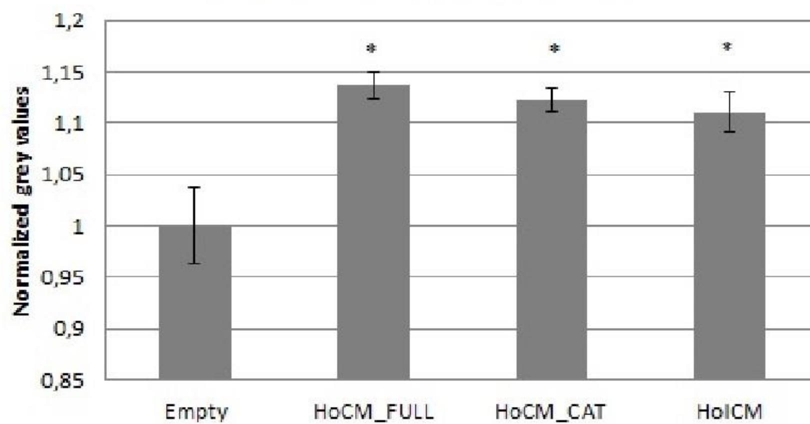

Figure 4.3: Terpenoid analysis. A: rice roots were stained with an antimony trichloride solution to visualize terpenoids (brown color). B: Statistical analysis was performed by a Mann-Whitney test on calculated grey-scale values, normalized by grey-scale values of the control (empty vector). All transgenic lines contained significantly less terpenoids compared to the empty vector control (indicated with asterisks). Error bars represent the standard error of the mean.

Figure 4.4: Visualization of differentially regulated genes/pathways. Figure was based on results obtained by the PageMan application in MapMan. Significance was calculated with a Wilcoxon test. Obtained p-values were adjusted according to Benjamini and Hochberg (1995). Red color indicates upregulation, blue color indicates downregulation. Only significantly different expressed genes/pathways are shown.

4.3.2. Hormone measurement

Root and shoot tissue of three week old plants was used to quantify concentrations of ABA, IAA, JA and SA using high performance liquid chromatography followed by mass spectrometry analysis. Results (Figure 4.5) indicated that levels of ABA are elevated in shoots of all generated overexpression lines. Auxin levels are elevated in shoots and roots of *HoCM_CAT* and *HoICM* overexpressing lines in comparison with control plants, although this difference is only significant in shoots of *HoCM_CAT* plants, probably due to a high degree of biological variation for this hormone in the experimental setup. Endogenous SA levels are very high in rice shoots, but no differences were observed when comparing transgenic with control plants. SA levels in rice roots are generally much lower compared to SA levels in shoots. There is a slight reduction in SA levels in roots of *HoICM* overexpressing plants versus control plants, but this difference was not significant ($p=0.071$). Endogenous levels of JA did not change in shoots or roots upon expression of *HoCM* or *HoICM*.

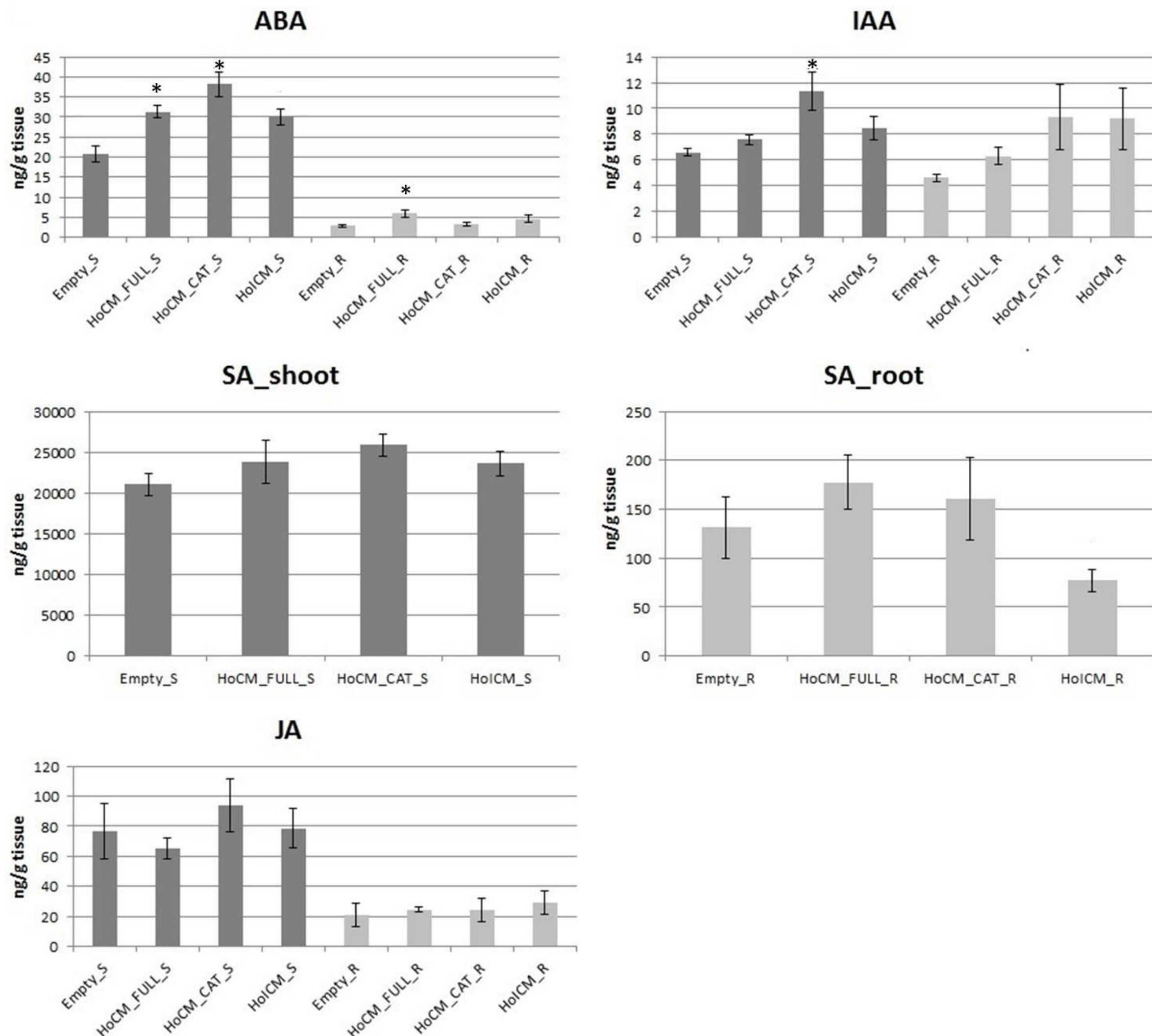

Figure 4.5: Results of hormone measurements on three week old plants. Quantities are expressed as ng hormone per g of tissue (y-axis). Measurements were performed on shoot (S, dark grey bars) and root (R, light grey bars) tissue for abscisic acid (ABA), indole-3-acetic acid (IAA), salicylic acid (SA) and jasmonic acid (JA). Two lines for each construct were treated as independent repeats of each separate construct. Data was analyzed with the non-parametric Mann-Whitney test comparing each construct with the empty vector control. Significant differences ($p<0.05$) are indicated with an asterisk (*). Error bars represent the standard error of the mean.

4.3.3. Infection assay

Given the observed variations in hormone content and changes in defense regulation on transcriptional level of *HoICM* and *HoCM* overexpressing plants, resistance against nematode infection was tested. Since culturing *H. oryzae* under lab conditions is problematic, we opted to use the rice RKN *M. graminicola* instead, another important rice

root pathogen, to asses susceptibility of the transformants. In this assay only plants overexpressing *HoICM* and *HoCM_FULL* were used to compare with the empty vector control. Nematodes were allowed to infect the roots for two weeks, after which roots were stained with acid fuchsin. Susceptibility was measured by counting the number of galls per root system. As seen in Figure 4.6, plants overexpressing *HoCM_FULL* are more susceptible to RKN infection, showing almost twice as many galls compared to the control. Results for plants overexpressing *HoICM* are ambiguous. The first experiment showed more galls on these plants compared to the empty vector control, but when the same experiment was repeated, no significant differences were detected. Ectopic expression of *HoCM_FULL* in rice plants clearly has an effect on resistance against RKNs, while the effect of expressing *HoICM* on resistance needs further investigation.

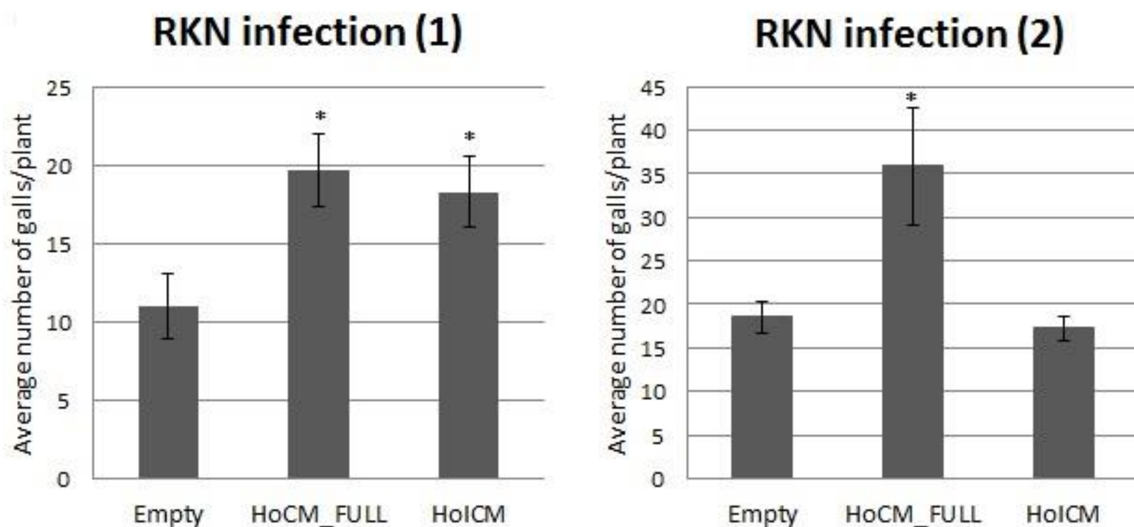

Figure 4.6: Effect of overexpression of *HoICM* and *HoCM_Full* on susceptibility against the RKN *M. graminicola*. Bars represent the average number of galls of 8 infected plants per treatment. Asterisks (*) indicate values significantly different from the control (Empty vector) according to a non-parametric Mann Whitney test (p<0.05). Error bars represent the standard error of the mean. Graphs show independent repetitions of the same experiment.

4.4. Discussion

Hirschmanniella oryzae migrates through the root tissue of rice plants, feeding on cells along its path. Recently CM and ICM were detected in the transcriptome of this nematode (Bauters et al., 2013). CM has been characterized in sedentary nematodes before and reported to be

present in the transcriptome of other migratory species as well (Bekal et al., 2003; Doyle and Lambert, 2003; Haegeman et al., 2011b; Jones et al., 2003; Nicol et al., 2012; Vanholme et al., 2009b). ICM has not been detected in nematodes before, but homologues were found in PPN specific EST sequences available in the Genbank database (Bauters et al., 2013).

To test the effect of HoCM and HoICM on the host plant, overexpression lines were generated for three different constructs: *HoICM*, *HoCM_FULL* and *HoCM_CAT*. RNA-seq was performed to investigate changes in gene expression levels Gene ontology enrichment analyses uncovered significant reductions in several GO terms. Especially “secondary metabolic process” and its child term “phenylpropanoid metabolic process” were reduced in all three transgenic lines. It is not surprising that this GO term is affected since both ICM and CM act upon chorismate (or chorismate derived metabolites), which is the starting point of the phenylpropanoid pathway (Dempsey et al., 2011). Within the “phenylpropanoid metabolic process” GO term also lignin catabolism is affected in *HoICM* and *HoCM_FULL* overexpressing plants. Genes involved in “Diterpenoid phytoalexin metabolic process” are generally inhibited in *HoCM_FULL* and *HoCM_CAT* overexpressing plants. Diterpenoid phytoalexins (DPs) have several functions in plants. Their antimicrobial properties have been well documented. DPs accumulate in infection spots were necrotic tissue starts to develop (Huffaker et al., 2011). They are responsible for inhibition of spore germination and mycelial growth of invading fungi (Koga et al., 1997; Sekido and Akatsuka, 1987). Transgenic lines deficient in *OsCPS4*, a phytoalexin biosynthetic gene, showed increased susceptibility to rice blast fungus (Toyomasu et al., 2014). DPs are also secreted in the rhizosphere of the plant, thereby inhibiting growth of surrounding dicotyledonous plants (Toyomasu et al., 2014). Although information about the role of DPs during nematode infection is lacking, their involvement in response to attacks of herbivorous insect species has been described DPs accumulated in maize upon infection with the European corn borer and in rice infested with the white-backed planthopper (Dafoe et al., 2011; Kanno et al., 2012). Next to defense against insects, DPs are also associated with abiotic stress responses. DPs accumulated upon drought and salinity in maize roots and rendered them more tolerant to these types of stress (Vaughan et al., 2015).

Terpenoid precursors can be derived from either the mevalonate (MVA) pathway in the cytoplasm or the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in plastids (Vranova et al., 2013). DPs are predicted to be synthesized from geranylgeranyl pyrophosphate (GGPP) derived from the plastidial MEP pathway (Schmelz et al., 2014). Looking at significantly

differentially expressed genes in the transgenic lines overexpressing HoCM_FULL, HoCM_CAT or HoICM, a gene similar to a GGPP synthase (GGPPS) was downregulated in all three lines. This particular GGPPS is homologous to a GGPPS from the rubber tree (UniProt id Q94ID7), which is localized in the chloroplast (Takaya et al., 2003). Chloroplastic GGPPS is not only involved in synthesis of defense-related compounds, but it is also related to growth and seed yield (Tata et al., 2015). Some terpenoids might be involved in nematode resistance by accumulation in lesions near the infection site (Veech, 1982), so a reduced terpenoid content can influence nematode susceptibility. In addition, silencing of a GGPPS encoding gene in *Nicotiana attenuata* led to reduced levels of hydroxygeranyllinalool diterpenoid glycosides and rendered the plants susceptible to the tobacco hornworm (Jassbi et al., 2008). Relative terpenoid content of rice roots was estimated using antimony trichloride staining, proving a strong and significant reduction in terpenoid content in *HoCM* and *HoICM* overexpressing plants. The mechanism by which overexpression of *HoCM* or *HoICM* influences the diterpenoid phytoalexin biosynthesis pathway remains unclear. It was shown before that the phenylpropanoid and terpenoid pathway are tightly regulated, and that metabolites produced by one pathway can affect the other (Xie et al., 2008)

Plant hormones are known to regulate the secondary metabolism of plants by complex positive and negative interactions (Schmelz et al., 2014). It is plausible that changes in concentration or ratios of hormones have an influence on the DP pathway. Therefore, hormone measurements were performed on rice plants overexpressing *HoICM*, *HoCM_FULL* or *HoCM_CAT*. A significant increase in ABA was observed in *HoCM* overexpressing lines. The downregulation of arabinogalactan proteins (AGPs) in the cell wall could have been caused by this increase in ABA. AGPs have been associated with plant development and reproduction, but also with plant defense (Nguema-Ona et al., 2013). Expression of AGP encoding genes is reduced by increasing ABA concentrations (Johnson et al., 2003). Rises in ABA levels have been associated with inhibition of the terpenoid phytoalexins lubimin and rishitin (Henfling et al., 1980), and the latter was shown to have nematicidal activities (Alphey et al., 1988). Furthermore, ABA is able to decrease expression of *PAL* genes, which are responsible for one of the first steps in the phenylpropanoid pathway. So the rise in ABA can be (partially) responsible for the alterations in the phenylpropanoid and diterpenoid pathways (Audenaert et al., 2002; Ward et al., 1989).

The rise in ABA is remarkable since a gene involved in ABA biosynthesis (*GGPPS*) was downregulated in all three constructs. GGPPS metabolizes the end products of the MVA and

MEP pathways to geranylgeranyl pyrophosphate (Vranova et al., 2013). This compound can be metabolized further to carotenoids, which are the precursors of ABA (Nambara and Marion-Poll, 2005). *GGPPS* are present in small gene families in the plant genome. For instance, *A. thaliana* has 12 members and maize has 5 (Lange and Ghassemian, 2003; Zhou et al., 2015). These GGPPS catalyze the same reaction, but can have different localized expression patterns (Vranova et al., 2013). Different GGPPS enzymes might guide the metabolic fluxes originating from GGPP. This hypothesis was experimentally confirmed by the fact that silencing *GGPPS* in tobacco resulted in a reduction of diterpenoid glycosides while the carotenoid concentrations remained unchanged (Jassbi et al., 2008). Overexpression of a sunflower *GGPPS* in tobacco led to a reduction in carotenoid content (Tata et al., 2015). In contrast, if a *GGPPS* originating from *Gentiana lutea* was used, carotenoid content increased (Ji et al., 2009). These results imply that the metabolic flux originating from GPP is dependent on the GGPPS used to synthesize GPP, which means that it is possible that silencing one GGPPS gene reduces terpenoid content, while levels of other GPP derived metabolites remain unaffected.

A non-significant reduction in SA concentration was observed in roots overexpressing *HoICM*. This result is not that peculiar since it is believed that the ICS-pathway accounts for the bulk of SA production (Vlot et al., 2009). It is probable that HoICM depletes the isochorismate pool available for SA production, which explains the reduction in root SA content. The fact that no differences were detected in shoot SA content could be due to the extremely high endogenous SA content in rice shoots compared to roots and shoot tissues of other plants (Djamei et al., 2011; Liu et al., 2014). The effect of overexpressing a single gene might be obsolete if endogenous levels are already very high. Levels of JA were unaffected for all three constructs. Auxin levels were elevated in shoots of *HoCM_CAT* overexpressing plants. Mapman analysis showed that these plants are also enriched in transcripts originating from auxin-responsive genes, which confirms the higher AUX content in this sample.

Hormonal regulation in plants is a complex network whereby hormones are subjected to intensive crosstalk between each other. Hormone signaling during defense responses is dependent on the type of pathogen but also the type of plant. SA and ABA are known to work antagonistically (Jiang et al., 2010; Xu et al., 2013). It is possible that overexpression of *HoCM* or *HoICM* slightly influences SA biosynthesis, thereby reducing any inhibitory constraints that SA has on ABA signaling (Meguro and Sato, 2014).

Mapman analysis revealed that genes involved in the phenylpropanoid pathway are mostly downregulated in plants overexpressing *HoCM_FULL* or *HoCM_CAT*. These genes include for instance genes encoding phenylammonia lyase (PAL) and 4-coumarate-CoA ligase. In contrast to our observations, Djamei and colleagues (2011) reported an increase in phenylpropanoid biosynthesis products in plants infected with *U. maydis*, which indicates that chorismate is directed into the phenylpropanoid pathway. The secreted CM from *U. maydis* has been shown to be able to bind plant endogenous CMs, but no reports on reduction or increase of activity have been reported to date (Djamei et al., 2011). Further research is needed to unravel the underlying mechanism and elucidate the relation between HoCM and endogenous plant CMs.

Infecting plants with *M. graminicola* showed that ectopic expression of *HoCM* was able to induce susceptibility in rice. This data has to be treated with caution. Rice plants were infected with RKNs instead of *H. oryzae*. Both species are able to infect rice roots. Although *M. graminicola* is a sedentairy nematode, it has a migratory phase. *CM* is predominantly expressed during the migratory phase of RKNs (Huang et al., 2005b), underlining its importance during migration. During the migratory phase, RKNs migrate in between the cells, while *H. oryzae* moves intercellularly. The difference in migration and the presence of a sedentairy stage in RKNs can affect the outcome of the infection experiment. Hence, infections with *H. oryzae* are needed to complete the story. The mechanism promoting susceptibility remains elusive. The reduction in certain isoprenoid and phenylpropanoid components, or the decrease in SA/ABA content or any combination of these factors might be responsible for this observation. Results of infection experiments of *HoICM* overexpressing plants are ambiguous and need further attention before conclusions can be drawn.

The obtained results show that the expression of *HoCM* and *HoICM* in plant tissue has an effect on several secondary metabolic pathways and hormone concentrations in the plant, but the mechanism by which these two genes are able to achieve these changes remains elusive and is awaiting further study.

4.5. Experimental procedures

4.5.1. Vector construction and rice transformation.

cDNA sequences of HoICM and HoCM were amplified by PCR on a cDNA library (Bauters et al., 2013). Primer sequences are provided in Appendix B. Expression vectors were created for three different constructs: *HoICM* (containing the complete open reading frame), *HoCM* without secretion signal (HoCM_FULL) and only the catalytic domain of *HoCM* (*HoCM_CAT*). Vectors were created by standard Gateway^R cloning (Life Technologies). Vector pMBb7Fm21GW-UBIL was used to overexpress these genes in rice, driven by the maize ubiquitin promotor. The vector was obtained from Plant Systems Biology (VIB, Belgium). Cloned sequences as well as position in the vector were verified by Sanger sequencing (LGC, Germany). Expression vectors were introduced into *A. tumefaciens* (strain EHA105) using triparental mating. Selection was done on YEB agar plates (5g/L beef extract, 5g/L peptone, 1g/L yeast extract, 5g/L sucrose) containing spectinomycin (100µg/mL) and rifampicin (25µg/mL). Rice seeds of cv. Nipponbare (GSOR-100) used for transformations, were obtained from the Genetic Stocks Oryza Collection (Washington DC, USA). Rice transformation was performed with a protocol adapted from Paine et al. (2005), Zhang et al. (1997) and Hiei et al. (1994). Control lines were generated by introducing the empty vector (without insert) into rice plants. Successfully transformed calli were selected using 80mg/L glufosinate (Sigma-Aldrich, St. Louis, MO, USA) in the medium. Once shoots were regenerated, plantlets were transferred to soil and incubated at 27°C under a 16/8h light/dark regime. Leaf samples were taken and DNA was extracted to check for the presence of the gene of interest by PCR using gene specific primers (Appendix B). DNA was extracted by heating a leaf sample at 95°C for 15 minutes in 400µl extraction buffer (200mM TrisHCl, 250mM NaCl, 25mM EDTA and 0.5% SDS). Afterwards 50µl chloroform was added. The sample was centrifuged and 200µl isopropanol was added to 200µl of the upper aquatic phase to precipitate the DNA.

4.5.2. RNA-extraction, sequencing and validation

Rice plants were grown under greenhouse conditions where a temperature of 30°C was maintained. Leaf samples were taken when rice was in flowering stage. Leaf samples were taken from three plants of a line transformed with the empty vector, and three plants of a

line overexpressing *HoCM_FULL* or *HoCM_CAT*. Due to limited seed availability, the line of which most seeds were available per transformant was chosen. Because of technical reasons only 2 independent replications were used for *HoICM* overexpressing plants. Tissue was homogenized using liquid nitrogen. Subsequently RNA was extracted using the Nucleospin RNA extraction kit (Machery-Nagel, Düren, Germany) according to the manufacturers' guidelines. An extra DNAse treatment with DNAse I (Thermo Scientific, Waltham, MA, USA) was incorporated in the protocol. At least 4µg RNA of each sample was sent for RNA sequencing (Microsynth, Balgach, Switzerland). An mRNA library was constructed using the Illumina TruSeq RNA sample preparation kit. The library was sequenced using a NextSeq platform (Illumina). To validate RNA-seq results, a set of genes was selected randomly to perform Q-RT-PCR. RNA was extracted with the RNAeasy Plant Mini kit (Qiagen) according to manufacturer's protocol. cDNA was synthesized as described in 2.5.6. Q-RT-PCR was performed as described before (2.5.7), but here *EIF5c* and *EXP NARCAI* were used as reference to normalize expression results. Primers can be found in Appendix B.

4.5.3. Mapping and gene-expression profiling

Reads from all libraries were mapped to the *O. sativa* ssp. *japonica* reference genome (IRGSP-1.0) using the Spliced Transcripts Alignment to a Reference (STAR) software with standard settings (Dobin et al., 2013). Expression of transcripts per sample was quantified as the sum of all reads mapped to those respective exons. Differential gene expression was assessed using the R-package "EdgeR" version 3.8.5 (Robinson et al., 2010). Data was analyzed using a generalized linear model (GLM) approach. To filter out technical variations influencing relative expression values, data was normalized using normalization factors calculated by the Trimmed Median of M-values method with standard settings as implemented in the EdgeR package (Robinson and Oshlack, 2010). The proposed GLM was fitted to the design matrix using the Cox-Reid profile-adjusted likelihood method (McCarthy et al., 2012). Differential gene expression levels (log_2 fold change) were assessed by looking at false discovery rates (FDR) calculated from p-values corrected by the Benjamini and Hochberg algrorithm (Benjamini and Hochberg, 1995). FDR-scores were used to detect differentially expressed genes (DEGs) (FDR<0.05). In other analyses (Gene Ontology enrichment and metabolic pathway analysis) the fold change (Log_2FC) was used.

4.5.4. Gene ontology enrichment and pathway analysis

GO enrichment analysis was performed using agriGO (Du et al., 2010). Gene identifiers with their corresponding Log_2FC were used as input in a Parametric Analysis of Gene Set Enrichment (PAGE) (Kim and Volsky, 2005). The Hochberg multi-test adjustment method was performed and a significance level of 0.05 was used. Visualization of expression values onto metabolic pathways was achieved by Mapman (Thimm et al., 2004). A Wilcoxon Rank Sum test was performed with Benjamini Hochberg correction to find statistical differences in expression of these pathways.

4.5.5. Nematode infection assay

The *M. graminicola* culture, originally isolated in the Philippines was maintained on *O. sativa* cv. Nipponbare in potting soil at 27°C (16/8h light regime). Nematodes were extracted from rice roots using a modified Baerman funnel technique. Rice seeds were first germinated on wet tissue paper at 27°C in the dark for three days. Germinated seedlings were transferred to PVC tubes containing SAP (Sand and Absorbent Polymer) substrate (Reversat et al., 1999). Two week old plants were infected with about 200 nematodes/plant. Two weeks after infection, nematode susceptibility was assessed by counting the number of galls per root. Galls were visualized by boiling roots in 0.8% acetic acid and 0.013% acid fuchsin for 3 minutes after which they were washed under running tap water and destained in acid glycerol.

4.5.6. Hormone measurements

Rice seeds were germinated and put into PVC tubes containing SAP (as described in 4.5.5). Rice plants were grown in a plant incubator at 30°C (16/8h light cycle) for 3 weeks after which root and shoot tissue was harvested separately. Plant material was homogenized by grinding it in liquid nitrogen. Two independent lines were used per construct (only one line for the empty vector control). Three individual plants of each line were used, each time 100mg of root and shoot tissue was weighed. Extraction of the homogenized plant material was performed at -80°C using the modified Bieleski solvent. After filtration and evaporation, chromatographic separation was performed on a U-HPLC system (Thermo Fisher Scientific) equipped with a Nucleodur C18 column (50 x 2 mm; 1.8 µm d_p) and using a mobile phase gradient consisting of acidified methanol and water. Mass spectrometric analysis was carried

out in selected-ion monitoring (SIM) mode with a Q Exactive™ Orbitrap mass spectrometer (Thermo Scientific), operating in both positive and negative electrospray ionization mode at a resolution of 70.000 full width at half maximum.

4.5.7. Histochemical analysis

Rice seeds were sterilized by washing them with 70% ethanol for 5 minutes followed by washing in a 5% bleach solution for 30 minutes. Sterilized seeds were sowed on MS medium (Murashige and Skoog) in square petri-dishes. Plants were incubated at 30°C for two weeks (16/8 light/dark regime). Visualization was performed in a saturated solution of antimony trichloride solution in 40% perchloric acid (Mace et al., 1989). Roots were carefully removed from the plate and incubated for 2 minutes in the staining solution, after which images were taken of the full root with a PowerShot SX40 HS camera (Canon, Tokyo, Japan). Staining solution was preheated to about 50°C to prevent precipitation of antimony trichloride. For a global quantitative measurement of staining intensity, images were transformed to 8-bit black and white pictures. Grey values were normalized by dividing them by the average grey value of the control. Results were analyzed with the non-parametric Kruskall-Wallis test. Only three plants were used as control (empty vector), six plants were used for each overexpression line (one line per construct).

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Chapter 5. General discussion and perspectives

5.1. Introduction

Hirschmanniella oryzae is a widely-spread nematode in rice growing regions world-wide, especially where rice is cultivated under flooding conditions. Although intensive research has been conducted regarding occurrence, reproduction and characteristics of this nematode, molecular research lags behind (Babatola and Bridge, 1979; Babatola and Bridge, 1980; Karakas, 2004; Maung et al., 2010). The research that is described in this thesis was initiated by analyzing the transcriptome of *H. oryzae* (Bauters et al., 2013). The transcriptome data provided us with the first molecular insights into *H. oryzae*. Although genome-analysis is a well-established molecular tool, transcriptome analysis is still worthwhile to perform. More and more genomes of PPNs have become available, providing an excellent basis for further molecular analyses (Abad et al., 2008; Cotton et al., 2014; Opperman et al., 2008). But although these data offer insights into presence and absence of genes, but in most cases it lacks the ability to give information regarding their expression. This is where transcriptomics and genomics join forces. Genomic studies give an overview of the gene content of an entire organism, which can be used as a giant framework to analyze transcriptome data, providing gene expression status of the organism in a specific state or environment. An example of the combination of both techniques is the recent genome project of *G. pallida* (Cotton et al., 2014).

Of course, transcriptomics has its disadvantages. For instance, the nature of the generated reads is often dependent on environmental circumstances and developmental stages of the organism under investigation upon RNA-extraction. However, when the ultimate goal is to study an organism in a specific developmental stage or environment, transcriptomics is the preferred technique. In our research, a 2-day protocol was used to extract nematodes from rice roots, and as a result it is likely that the expression of certain parasitism genes was reduced during extraction, rendering them undetectable in a transcriptome analysis. Therefore, a future whole genome analysis is needed to fill the gaps.

The generated transcriptome data was used to generate an image of the plant-nematode relationship, the association between *H. oryzae* and *O. sativa* was brought to a molecular level. Plant-invading organisms need to deal with several lines of defense of the plant. These lines of defense can have a structural nature, like the plant cell wall, or a more biochemical nature. Both structural and biochemical defenses can be naturally present or induced upon infection. *H. oryzae* is able to take these hurdles and to successfully infect its host. To attain this, it secretes an arsenal of effectors into the host tissue to hydrolyze the cell wall and to reduce plant defense systems.

5.2. Walls are crumbling

The first barrier that needs to be breached is the plant cell wall. Several secreted CWMP have been characterized in PPNs. But even when the cell wall is breached, cell wall-associated molecules can still activate plant defense, thereby activating ROS production, cell wall fortification and production of defense compounds. These subjects have been thoroughly reviewed by Davis et al. (2011) and Hématy et al. (2009). In this general discussion we will focus on enzymes that have not been described yet, or that differ in *H. oryzae* compared to other PPNs.

Hemicellulose is an important element of the plant cell wall; it is the noncellulosic polysaccharide fraction that includes xyloglucan, mannan, and xylans. Regarding hemicellulose content, the composition of grass cell walls differs from dicotyledonous cell walls. The total hemicellulose fraction is larger in grasses compared to dicotyledonous species (Vogel, 2008). Since *H.oryzae* has to find its way through cell walls of rice during almost its entire life cycle, it is not that remarkable that it is equipped with an arsenal of cell wall degrading enzymes to tackle this hurdle. Examples are for instance polygalacturonase and xylanase. Interestingly, *H. oryzae* is the first nematode in which a β-mannanase has been found and of which the expression in the pharyngeal glands was confirmed by *in situ* hybridization. Β-mannanase is used to degrade mannan structures in the hemicellulose fraction, which are polysaccharides consisting of a backbone of β-1,4-linked mannose residues, supplemented with side chains of α-1,6-linked galactose residues (Moreira and Filho, 2008). β-Mannanase cleaves the β-1,4 internal linkages between mannose residues randomly, creating shorter mannan structures. Although no biochemical data is available regarding activity of mannanases originating from nematodes, this enzyme might aid the nematode in degrading the hemicellulose fraction of the cell wall. However, in *M. graminicola*, another important nematode infecting rice roots, β-mannanase has not been reported so far, nor in any other PPNs that infect dicotyledonous hosts. Only *Xiphinema index*, an important pathogen in vine yards, expresses this enzyme, since a homolog was detected in the available EST data (Demangeat et al., 2005). β-Mannanases have been found in the secretome of several plant-pathogenic fungi (Arfi et al., 2013; Gübitz et al., 1996). Intriguingly, although cell walls of grasses contain more hemicellulose compared to dicotyledonous species, the mannan fraction is only minor (Vogel, 2008; Zhang et al., 2012).

The cell wall forms an ingenious dynamic defense system. Damage molecules associated with cell wall degradation can act as elicitors of defense response. A clear example is the interaction of the WAK1 receptor with pectin or pectin fragments (oligogalacturonides) in

Arabidopsis, which can induce a transient defense response, including accumulation of reactive oxygen species and pathogenesis-related proteins (Denoux et al., 2008; Kohorn and Kohorn, 2012). The question arises which other types of cell wall associated oligosaccharides can function as DAMPs to trigger a defense response.

Mannan polysaccharides, which give structure to cell walls as part of the hemicellulose fraction and function as storage of non-starch carbohydrates in endosperm walls of seeds (Lopes and Larkins, 1993), have been suggested to play a role as signaling molecules during plant growth and development (Liepman et al., 2007). Recently a possible additional role for mannan oligosaccharides was discovered. Cocoa plants sprayed with a phosphorylated mannan oligosaccharide-based product showed increased resistance against the witches' broom disease caused by *Moniliopthora perniciosa* (Pereira et al., 2013). In addition, phosphomannans, present in fungal cell walls, are recognized by pathogenesis-related (PR) protein 5 from tobacco, thereby rendering the plant more resistant (Ibeas et al., 2000). These results imply that mannan oligosaccharides are recognized by the plant, possibly as DAMPs/PAMPs, leading to activation of the plant defense system. If the β-mannanase secreted by *H. oryzae* would help in hydrolyzing cell wall structures, it can generate loose mannan oligosaccharides, which might function as DAMPs. Since *H. oryzae* is a migratory nematode it is able to outrun this proposed local defense. This might be the reason why no homologue of β-mannanase could be found in any of the available RKN or CN genomes. RKNs and CNs have a long sedentary phase during their lifecycle, thereby being unable to outrun local defense responses. However, sedentary nematodes do secrete pectate lyases, which could potentially trigger defense responses by production of oligogalacturonides, the breakdown products of pectin. Fact is that these sedentary species only express pectate lyases during their migratory phase, while expression is abolished when entering a sedentary state (Huang et al., 2005a). So while the nematode is most vulnerable, unable to outrun defense responses, it keeps a low profile to remain undetected. The potential role of mannan oligosaccharides as defense inducer during nematode infection has not been investigated before and might be an interesting topic for future research.

Polygalacturonase (PG) and poly-α-galacturonosidase both are involved in pectin degradation, with the difference that the first randomly hydrolyses (1,4)-α-D-galacturonidic linkages in pectate, while the latter releases digalacturonate from the end of the pectic acid molecule. Experimental evidence showed that both enzymes are needed for full virulence of the plant-pathogenic bacteria *Ralstonia solanacearum* (Huang and Allen, 1997). PGs have been described before in PPNs, but to our knowledge this is the first time a galacturonosidase was found in a nematode (Jaubert et al., 2002a). When this sequence was

used as query to search the *M. incognita* genome, 4 significant matches were found, 2 of which contained a predicted signal peptide. All were annotated as polygalacturonase, but when compared with the non-redundant protein database, almost all most similar sequences were annotated as a poly-galacturonosidase protein of *R. solanacearum*. These results indicate that the presence of poly-galacturonosidases in nematodes is not restricted to *H. oryzae*. Whether these proteins possess real galacturonosidase activity or are just annotated as such needs to be investigated.

PGs can be divided into exo- and endo-polygalacturonases. The first type cleaves monomeric products from the terminal part of a pectin molecule while the latter cleaves randomly, generating oligogalacturonides (OGAs) (Jayani et al., 2005). Experimental evidence showed that spraying plants with OGAs can increase resistance against pathogens (van Aubel et al., 2013). Oligogalacturonides with a polymerization degree between 9 and 15 can activate local plant defenses (Bruce and West, 1982; Roby et al., 1985). Plants have evolved to anticipate to this phenomenon and produce polygalacturonase-inhibiting proteins (PGIP). PGIPs inhibit the activity of PGs, thereby increasing the retention time of produced OGAs, which in turn will have an impact on the severity of the defense response (Gomathi and Gnanamanickam, 2004). While the secretion of endo-PG into plant tissue will have a higher impact on the structure of a pectin molecule due to the rapid decrease in average chain length, they will also generate elicitor-active OGAs (ten Have et al., 2002). It would be beneficial for the nematode to secrete exo-PGs, thereby preventing OGA generation while degrading pectin polymers. PGs found in PPNs so far have been annotated as exo-PGs, although no biochemical evidence for this assignation exists to date. Looking into more detail by constructing a phylogenetic tree, nematode PGs cluster together with bacterial endo-PGs (Jaubert et al., 2002a). So the question remains in which manner nematodes cleave pectin molecules in plant cell walls. If PPNs also have poly-α-galacturonosidases in their secretome, they would be able to degrade pectin polymers without generating OGA elicitor molecules. Additional research and biochemical characterization is needed to determine the type of PGs present in nematode secretions.

Another peculiar protein family that could be involved in parasitism is SXP/RAL-2. Our results suggest that nematodes utilize members of this protein family to arm themselves against severe environmental circumstances like drought (Bauters et al., 2013). The gene encoding an SXP/RAL-2 family member used in this research was similar to SXP/RAL-2 originating from *M. incognita* (Tytgat et al., 2005). The encoding gene was upregulated in *H. oryzae* when desiccation stress was mimicked. Another member of this family is the most abundant transcript present in the transcriptome of the anhydrobiotic nematode *Panagrolaimus*

superbus (Tyson et al., 2012). The SXP/RAL-2 family is present in several species all over the phylum Nematoda, regardless of their life style. The omnipresence suggests a general function for SXP/RAL-2 proteins. The precise role has not been elucidated yet. Since some of them are expressed in the pharyngeal glands, it has been suggested that these proteins could play a role in parasitism as well (Tytgat et al., 2005). The possible role during plant parasitism was not investigated for the SXP/RAL-2 protein used in this research. Previous experiments have shown that SXP/RAL-2 proteins are present in the secretome of several PPNs, but that they are not all secreted through the pharyngeal glands. Expression of this gene was also detected in the hypodermis and in the amphids (Jones et al., 2000; Tytgat et al., 2005). This apparent discrepancy in localization of expression might denote difference in function. In this research one *SXP/RAL-2* gene was chosen randomly to measure differences in expression upon dehydration treatment. Expression levels of this transcript were not tested during other conditions (e.g. during infection) nor were expression levels of any other transcripts with similarity to *SXP/RAL-2*. Hence we cannot rule out the possibility that SXP/RAL-2 family proteins produced by *H. oryzae* are involved in parasitism as well. It would be interesting to test spatial expression patterns of genes in this family and try to link this spatial expression to involvement in the anhydrobiotic process. Recently the 3D protein structure of an SXP/RAL-2 family member from the animal parasitic nematode *Anisakis simplex* was elucidated, showing similarity with the Ca^{2+} binding protein calmodulin. Experimental evidence suggested that SXP/RAL-2 was able to bind Mg^{2+}, not Ca^{2+} (Garcia-Mayoral et al., 2014). These results shed a bit more light on a possible function for this protein. Since various research groups have published different results regarding localization of expression and accompanying proposed functions, more research is needed to elucidate the biological role of each family member.

5.3. Knock out defense

Once the cell wall has been penetrated, nematodes still have to overcome a range of elicited defense responses to be able to successfully colonize the host plant. Transcriptomic data of *H. oryzae* showed the presence of two putative effector genes (*HoCM* and *HoICM*) that were originally hypothesized to have an effect on SA production inside host cells (Bekal et al., 2003; Huang et al., 2005b; Jones et al., 2003) The discovery of such effector proteins in migratory nematodes was unexpected, but indicates that these species also invest energy in trying to attenuate local defense responses of the host.

HoCM codes for a CM type II protein, which can be found in the secretome of other PPNs as well (Bekal et al., 2003; Huang et al., 2005b; Jones et al., 2003; Lambert et al., 1999). Catalytic residues which have been characterized in *E. coli* are conserved in these nematode CM sequences (Bauters et al., 2013; Lee et al., 1995). It is interesting to note that there is a clear difference between CM proteins secreted by cyst nematodes and the ones secreted by root-knot nematodes. CN CM proteins contain an additional N-terminal region which is absent in RKNs. This extra region contains about 70 amino acids and has 6 highly conserved cysteine residues (Vanholme et al., 2009b). No homologies were found for this N-terminal region in proteins other than the secreted CMs of CNs. The catalytic region of HoCM is also preceded by an N-terminal domain, which is completely different from the N-terminal region in CNs. Although the conserved cysteine residues reported by Vanholme et al. (2009b) were not present, two serine and histidine rich islands were found, hence the name serine/histidine rich region (S/HRD). Histidine rich regions have been associated with metal binding properties in several proteins (Paksi et al., 2008; Williams et al., 2000), but none of the elucidated 3D CM protein structures have a metal ion as ligand. Both CNs and *H. oryzae* do not need the N-terminal part of the protein for enzymatic activity (Bauters et al., 2013; Vanholme et al., 2009b). These observations leave us with two unanswered questions: i) Why is the N-terminal part absent in RKN CMs? ii) What is the function of this domain if not needed for enzymatic activity?

Subcellular localization was tested with a construct containing the full gene without secretion signal and a construct containing only the catalytic domain. Expression of both proteins was localized in the cytoplasm and in the nucleus. The latter location is probably due to passive diffusion since nucleoli were not stained (see Chapter 3). The outcome of this experiment suggests that the N-terminal region is not needed to guide the protein to a specific part of the plant cell. It is known that CM proteins of eukaryotes and most bacterial species form dimeric proteins (Lee et al., 1995). The question arises whether the N-terminal region is needed for dimerization. The absence of the N-terminal domain in RKNs makes it difficult to introduce this hypothesis, unless both types of CMs have a different function *in planta*. The latter statement is unlikely since both RKNs and CNs have the same lifestyle, which is completely different from the lifestyle of *H. oryzae*. Yeast two-hybrid (Y2H) assays could be performed to investigate whether HoCM is able to bind to plant CMs to regulate their function as was already shown in the maize-*U. maydis* interaction (Djamei et al., 2011). If an interaction partner in plants can be found, it would be interesting to test whether the S/HRD region is needed for this interaction, in particular the histidine and serine islands. Infection experiments with *P. nicotianae* showed that expression of *HoCM_FULL*, but not *HoCM_CAT*, increases susceptibility in infiltrated *N. benthamiana* leaves. However, RNA-seq

results showed that both *HoCM_FULL* and *HoCM_CAT* overexpressing plants have more downregulated genes grouped in the “biotic stress” category, independent from the presence of the N-terminal region. Since these results seem contradicting, more research is needed to elucidate the role of this domain and its absence in RKNs.

CM acts upon chorismate, metabolizing it to prephenate, which is processed to arogenate by prephenate aminotransferase (PAT). Arogenate in its turn is the precursor of phenylalanine and tyrosine, two aromatic amino acids. This process is compartmentalized and takes place in the chloroplast (Rippert et al., 2009). Next to plastidic CMs, CMs with a cytosolic localization have been reported in plants as well (Eberhard et al., 1996b; Eberhard et al., 1996a). This is peculiar since chorismate is compartmentalized within the chloroplast where it is synthesized by the shikimate pathway. Eberhard and collegues (1996b) found three CMs in *A. thaliana* of which one had a predicted cytosolic location. All types of CM were expressed in root and shoot tissue of *A. thaliana*. Both plastidic CMs were allosterically regulated by tryptophan (Trp), phenylalanine (Phe) and tyrosine (Tyr) concentrations and can be induced by pathogen infection, while the activity of cytosolic CM (CM*) remains unaffected by all these conditions (Eberhard et al., 1996b; Lillo et al., 2008). Both cytosolic and plastidic CMs have been predicted in *O. sativa* as well (Schenck et al., 2015). Sommer et al. (1998) showed the presence of chorismate in the cytosol of tobacco plants. This observation proves that chorismate is transported from the chloroplast into the cytosol by a yet unknown mechanism (Widhalm and Dudareva, 2015). The cytosolic chorismate could function as substrate for CM*. Since no other post-chorismate portions of the pathway have been discovered in the cytosol, the significance of CM* is still open for debate (Rippert et al., 2009).

Recent discoveries regarding this topic have changed the hypothesis that Phe and Tyr are exclusively synthesized in the chloroplasts in plants. Plants where the chloroplastic pathway to Phe was silenced were still able to synthesize Phe. In addition, the compound phenylpyruvate was detected in relatively high concentrations in these plants (Maeda et al., 2010). These observations suggest that Phe can be synthesized in an alternative pathway using phenylpyruvate as intermediate, which is common in most microorganisms (Maeda and Dudareva, 2012). Enzymes responsible for cytosolic Phe and Tyr synthesis have recently been detected in plants. Starting from prephenate, phenylpyruvate and 4-hydroxyphenylpyruvate are metabolized to Phe and Tyr respectively (by prephenate dehydratase (PDT) and prephenate dehydrogenase (PDH)) (Figure 5.1) (Schenck et al., 2015; Yoo et al., 2013). Thus, recent progress in this field has proven the existence of a cytosolic Phe/Tyr biosynthesis pathway next to the common chloroplastic pathway. The precise role

of this alternative pathway remains elusive since it seems less efficient in producing Phe and Tyr. To date, this alternative route has not been reported in rice. However, a rice gene encoding arogenate dehydratase (ADT) was shown to have prephenate dehydratase activity as well (Yamada et al., 2008). Although arogenate is the preferred substrate for this enzyme, the presence of PDT activity is an indication that there is an alternative Phe production pathway in rice as well. To our knowledge, no other enzymes involved in the cytosolic Phe/Tyr biosynthesis pathway have been identified in rice so far.

It was shown that a secreted CM from *U. maydis* was able to bind cytosolic CM from maize, without impairing its function (Djamei et al., 2011). When HoCM is secreted in host tissue it probably metabolizes cytosolic chorismate, thereby it might increase the flow of chorismate from chloroplast to cytosol. This would mean that less chorismate is available in the chloroplast to go through to the phenylpropanoid pathway (red arrow in Figure 5.1). The fact that genes involved in the phenylpropanoid metabolism in plants are generally repressed in transgenic plants overexpressing *HoCM* strongly confirms this hypothesis. In contrast, metabolomic studies conducted by Djamei et al. (2011) showed increased levels of certain phenylpropanoids (like for instance coumaroyl- and caffeoylquinate and syringine) when plant tissue was infected with a wild type pathogen compared to a CM-deficient pathogen. In addition, the same research reported reduced levels of SA due to the secretion of CM into the plant tissue during infection, an observation which was not seen in *HoCM* overexpressing plants. The apparent contraditions between our results and the data provided by Djamei et al. (2011), might be due to some differences in the used plant species and in experimental setup. Although both investigated host plants are monocotyledonous species (*Z. mays* and *O. sativa*), there is a huge difference in their endogenous SA levels. While SA quantities of approximately 100 ng/g of fresh weight are observed in maize, the concentration in rice leaf tissue is about 200-fold higher. High endogenous SA concentrations might make it impossible to detect small differences due to expression of *HoCM*. Furthermore, the data of Djamei et al. (2011) is based upon hormone measurements after infection with a wild type and CM-deficient pathogen, while non-infected overexpression lines were analyzed in our research. Infection of host plants will induce defense responses, including SA accumulation (Loake and Grant, 2007). It is imaginable that the effect on SA synthesis will be more profound once the pathway is induced to produce more SA upon pathogen infection. The same reasoning may be applied for the contradictory observations regarding the phenylpropanoid pathway. Another, more plausible, explanation is that the regulation of the phenylpropanoid pathway is more complicated than expected. Expression of a bacterial CM in Arabidopsis showed increased levels of certain secondary metabolites derived from Phe, as well as reductions of other Phe-derived secondary metabolites (Tzin et al., 2009). These

results illustrate the complexity of the phenylpropanoid pathway and the need for further investigation to unravel the effects of CM on this pathway and to clarify these seemingly contradictory results. Since it is known that the regulation of the phenylpropanoid pathway is complicated and that chorismate availability is not a limiting factor in some reactions, the effect on the whole arsenal of secondary metabolites during infection should be investigated by metabolomic studies of the overexpression lines (Maeda and Dudareva, 2012; Viitanen et al., 2004).

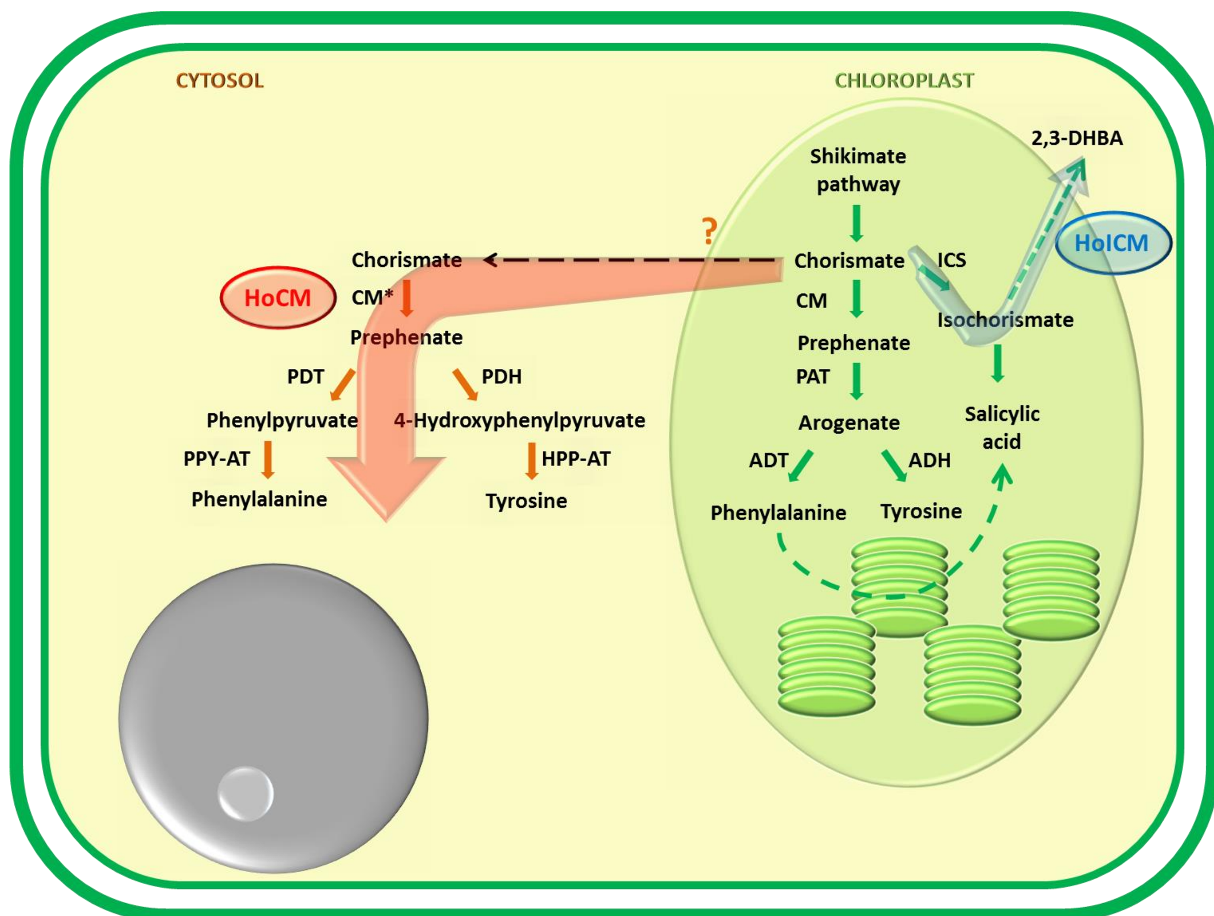

Figure 5.1: Chorismate production by the shikimate pathway takes place in the chloroplast. Arogenate production is catalyzed from chorismate by chorismate mutase (CM) and prephenate aminotransferase. Arogenate is metabolized into phenylalanine or tyrosine by arogenate dehydratase (ADT) and arogenate dehydrogenase (ADH) in plastids. In some plants an alternative cytosolis Phe/Tyr synthesis pathway exists, visualized in the schedule. Enzymes catalyzing these cytosolic reactions are: cytosolic CM (CM*), prephenate dehydratase (PDT), prephenate dehydrogenase (PDH), phenylpyruvate aminotransferase (PPY-AT) and hydroxyphenylpyruvate aminotransferase (HPP-AT). Chorismate can also be metabolized to isochorismate by isochorismate synthase (ICS), which in its turn is a precursor for salicylic acid or 2,3-DHBA. The current hypothesis is that CM originating from *H. oryzae* (HoCM) will direct the chorismate flow into the cytosol (red arrow) and that nematode derived isochorismatase (HoICM) will direct the flow of isochorismate to 2,3-DHBA instead of salicylic acid.

CM secreted by plant pathogens has shown to aid in the infection process (Djamei et al., 2011; Lambert et al., 2005). Curiously, when a *CM* gene derived from *A. thaliana* was overexpressed in soybean, it conferred resistance against *H. glycines* (Matthews et al., 2014). Noteworthy, the CM used by Matthews and colleagues was localized in the chloroplasts, highlighting the fundamental difference between plastidic and cytosolic CMs. While the first confers resistance, the latter promotes susceptibility, probably by extracting CM from the plastid which would otherwise be available for the phenylpropanoid pathway or SA production. To date, no complete cytosolic pathway leading to Phe or Tyr has been found in rice, so the precise function of cytosolic chorismate needs further investigation.

There might still be another explanation to the secretion of CM. Phenylalanine is an essential amino acid, hence nematodes are obliged to acquire this nutrient from their host. This raises the question whether secreted CM might aid in increasing the concentration of essential amino acids, rather than attenuating defense responses. Secreted CM might help in guiding the chorismate flow to aromatic aminoacids. It was already shown that phenylalanine is highly enriched in syncytia, but it is not known if CM is responsible for this metabolic change (Hofmann et al., 2010).

Isochorismate is synthesized in the chloroplast. HoICM and other secreted ICMs are not predicted to have a chloroplast targeting signal (Liu et al., 2014). This could mean that isochorismate is transported into the cytosol by a yet unknown transport mechanism or by diffusion (Verberne et al., 2000). Secreted ICM from the plant pathogens *P. sojae* and *V. dahlia* are able to hydrolyze plant isochorismate to 2,3-dihydro-2,3-dihydroxybenzoate (DDHB), the precursor for 2,3-dihydroxybenzoate (2,3-DHBA) (Liu et al., 2014). Research performed on Arabidopsis showed that the bulk of produced 2,3-DHBA was derived from isochorismate, but it can also use SA as precursor (Bartsch et al., 2010). Although 2,3-DHBA is a very weak inducer of PR-genes (Abad et al., 1988; Bartsch et al., 2010), its role in defense is probably negligible since high concentrations of this compound in combination with low SA content promote susceptibility instead of resistance (Zhang et al., 2013). It is more probable that 2,3-DHBA is involved in limiting adverse effects caused by high SA concentrations (van Hulten et al., 2006; van Leeuwen et al., 2007). Seeing the fact that *HoICM* overexpression lines show significantly reduced root SA levels, ICM might draw isochorismate from the chloroplast to metabolize it to 2,3-DHBA, thereby reducing the isochorismate pool available for SA production (Figure 5.1). The fact that the difference is not significant could be due to large biological variation between samples. Optimization by using pooled samples and more replicates could reduce variation and increase statistical power.

Although several attempts were made to show the spatial expression pattern of ICM by *in situ* hybridization, no clear results were obtained. The reason why we could not visualize expression *in situ* might be because *ICM* is expressed at very low levels, or only when the nematode is burrowing its way through the root tissue. To increase the share of *ICM* transcripts, whole pharyngeal gland cells could be isolated to extract RNA, making it easier to detect expression of *ICM* (Huang et al., 2003; Maier et al., 2013). In this way it might be possible to confirm expression in the gland cells. However, there are several findings that support a role for *HoICM* in parasitism: i) The *ICM* gene from *H. oryzae* has homologues in PPNs exclusively, not in nematodes with a different lifestyle. ii) ICM is present in the secretome of several plant-parasitic fungi, where it has a possible role in virulence (Soanes et al., 2008). iii) ICM was shown to be secreted by *V. dahliae* and *P. sojae* into the host tissue, where it functions as an effector to render the host more susceptible to invading pathogens. iv) In addition, no conventional secretion signal was needed to target the protein for secretion into the host tissue by *V. dahlia* and *P. sojae* (Liu et al., 2014).

Since *H. oryzae* is a root pathogen, one could argue that it can not have an effect on SA biosynthesis which takes place in chloroplasts in leaf tissue. To our knowledge, no research groups have clearly demonstrated SA production in roots. However, there are several indications that SA is synthesized in root tissue as well. The production of chorismate and the aromatic aminoacids phenylalanine and tyrosine takes place in roots and shoots (Leuschner and Schultz, 1991; Staehr et al., 2014). In addition, ICS is expressed in roots and responds to pathogen infection (Ji et al., 2015) and both chloroplastic and cytosolic CMs are highly expressed in *A. thaliana* roots (Eberhard et al., 1996b). Additional research is needed to demonstrate SA production in plant roots and to elucidate the type of plastid involved in this process.

A rather unexpected observation was the increase in ABA content in shoots of overexpression lines. Except for compartmentalization in the chloroplast (Nambara and Marion-Poll, 2005), to date, no experimental evidence is available directly linking chorismate to the ABA biosynthesis pathway, implying an indirect effect of overexpressing *HoCM* or *HoICM* on ABA content. The increase in ABA is probably related to the SA-ABA antagonistic relation (Jiang et al., 2010; Xu et al., 2013). An additional hypothesis is that the fluctuations in phenylpropanoid synthesis, caused by the observed changes in gene expression of this pathway, have an influence on ABA content. It has been reported that certain phenylpropanoids can affect ABA induced changes, probably by accelerating ABA metabolism (Ray and Laloraya, 1984; Sharma et al., 2003). Further research is needed to

confirm and explain the rise in ABA content and to determine probable shifts in SA/ABA ratio upon pathogen infection.

The rise in ABA is even more peculiar when taken into account that the concentration of terpenoids, synthesized by the same pathway is lowered in *HoCM* and *HoICM* overexpression lines. Figure 5.2 shows a simplified diagram of the terpenoid biosynthesis starting from the end products of the cytosolic MVA pathway or the plastidic MEP pathway. Several terpenoid subcategories are presented in this figure. It is possible that the reduced expression of one geranylgeranyl pyrophosphate synthase (GGPPS) gene (see Chapter 4) leads to an increase in GGPP precursors. In turn, the increased concentration of the precursors could be used by other rice endogenous GGPPS to guide the synthesized GGPP into the carotenoid synthesis pathway, leading to the production of ABA. This could explain the increase in ABA in the transgenic lines. To our knowledge, there is no known direct link between this pathway and the pathways were CM and ICM are involved in. Further research is needed to elucidate the mechanism by which *HoCM* and *HoICM* can affect ABA and terpenoid concentrations.

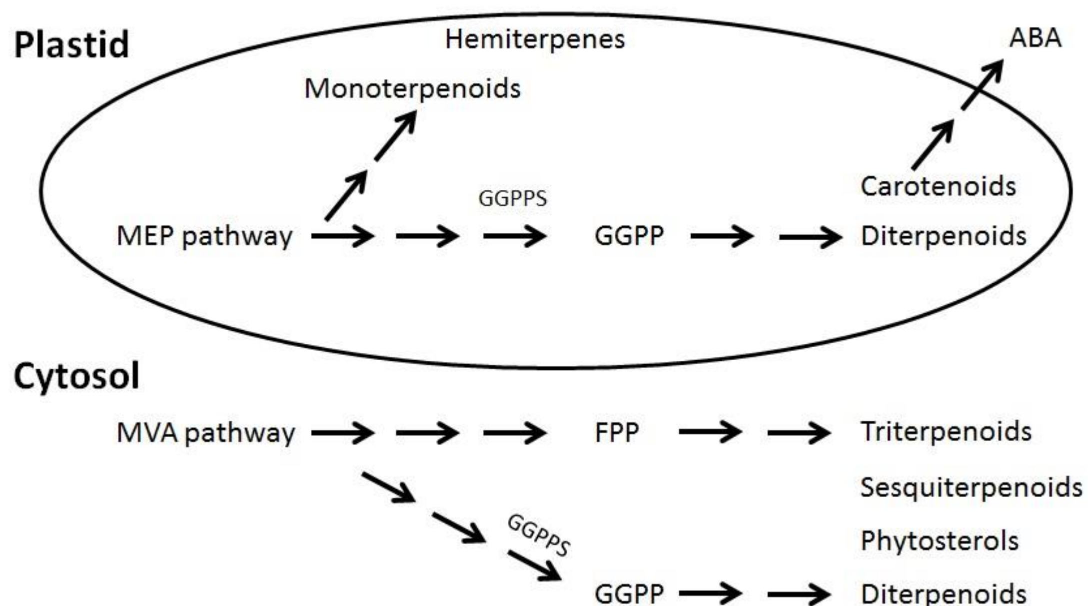

Figure 5.2: Simplified diagram of terpenoid biosynthesis in plants indicating some importing subcategories in the terpenoid metabolites. MEP: 2-C-methyl-D-erythritol 4-phosphate, ABA: abscisic acid, MVA: mevalonate, FPP: farnesyl pyrophosphate, GGPP: geranylgeranyl pyrophosphate.

Infection experiments with *M. graminicola* showed that plants expressing *HoCM* were more sensitive to nematode infection. First results of infection experiments on *HoICM* overexpressing plants indicate that HoICM also plays a role in rendering the plant more susceptible. Further experiments are needed to confirm this. The mechanism by which a higher sensitivity is achieved remains elusive, making it an interesting subject for further investigation. ABA is a promoter of nematode susceptibility while SA, phenylpropanoids and terpenoids promote resistance (Ahuja et al., 2012; Nahar et al., 2012; Veech, 1982; Wuyts et al., 2006). Whether the rise in ABA or a decrease in SA, phenylpropanoids or terpenoids is responsible for the increase in susceptibility remains a question, but it gives enough food for thought for future research.

5.4. Perspectives

In this thesis, a molecular study was performed on the root rot nematode *Hirschmanniella oryzae*, an important pathogen of rice. Transcriptomic data provided us with a new information source to investigate this nematode of which almost nothing was known on molecular level. In this research, the newly generated data was predominantly used to browse for genes important during the parasitic relationship with its host. However, this is just the tip of the iceberg. The same information can be used to gain more insight into the lifecycle of this nematode, the potential of using RNAi to study gene function, the presence of metabolic pathways, the differences and similarities with other nematodes, etc. These data, combined with data of *P. coffeae*, *P. thornei, D. africanus* and *R. similis* are an excellent starting point to make a comparison between migratory nematodes and their sedentary counterparts (Haegeman et al., 2009a; Haegeman et al., 2011b; Jacob et al., 2008; Nicol et al., 2012).

Although the focus in this research was on CM and ICM and their effect on the host, another interesting discovery was made during this research. A transcript homologous to β-mannanase was found in the transcriptome of *H. oryzae*, but was not reported in other nematodes before. Biochemical activity assays could show if the protein has true mannanase activity, indicating whether it might play a role in cell wall digestion. Additional studies like silencing the gene with RNAi, or overexpression assays could help in elucidating the potential role of β-mannanase during infection.

Nematode secreted CWMP have been extensively studied in other PPNs and were also recovered from the transcriptome of *H. oryzae* (Bauters et al., 2013; Davis et al., 2011). In this thesis we mainly focused on effector genes that could have an effect on plant defense, in particular *HoCM* and *HoICM*. The results obtained here differ from results obtained by Djamei et al. (2011) and Liu et al. (2014) who studied secreted CM and ICM of fungal pathogens, especially focusing on the effect of these proteins on the SA content of the plant. The fundamental difference here is that we looked at SA content of uninfected plants expressing *HoCM* or *HoICM*, while Djamei et al. (2011) and Liu et al. (2014) infected wild type plants with pathogens with a mutated *CM* or *ICM* gene. It is possible that the difference in SA content is more pronounced when the SA synthesis machinery is induced for massive production upon pathogen infection. To test this, hormone measurements should be conducted on infected plants to test the effect of HoCM and HoICM on a pathogen-induced pathway. Alternatively, *HoCM* or *HoICM* can be expressed in *CM*- or *ICM*-deficient fungal strains to see whether this complementation increases infectivity or has an effect on SA content in the host plant. In this case it would be feasible to use a different host that has lower endogenous SA levels (for instance Arabidopsis, maize or soy), so the effect on SA content is more distinguishable. Results of these experiments, combined with hormone measurements on infected tissue of *HoCM* or *HoICM* overexpressing plants will provide us with a better insight of the biological role of these two proteins. In addition, metabolomics of the lines overexpressing HoCM or HoICM can shed some light on the flows in which chorismate is channeled in *HoCM* or *HoICM* plants.

The reduction in terpenoid content is rather peculiar since the functions of CM or ICM cannot be directly linked to this effect. Terpenoids are considered the most diverse group of metabolites with over 50,000 different molecules identified in existing organisms (Thulasiram et al., 2007). They have a complex biosynthetic pathway, starting from farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP) originating from the cytosolic MVA pathway or plastidic MEP pathway (Vranova et al., 2013). One GGPP synthase gene which is silenced in all three transgenic lines (overexpressing *HoCM_FULL*, *HoCM_CAT* or *HoICM*) might be responsible for this drop in terpenoid content. It would be interesting to analyze rice plants mutated in this gene, regarding terpenoid content and nematode susceptibility. Unfortunately, to our knowledge, no mutants in this specific gene (LOC_Os05g14580) have been reported so far. Metabolomic analyses on the three

transgenic lines could reveal whether the reduction in terpenoid content is due to a general drop in terpenoid concentration or a reduction in a specific category of terpenoids.

Summary

PPNs are a major pest in several food crops worldwide and cause severe yield losses. In the past, most attention has gone to sedentary PPNs, a type of nematodes that forms specialized feeding structures inside the host root. With the recent sequencing of the transcriptome of *Hirschmanniella oryzae*, *Radopholus similis* and several *Pratylenchus* spp., more and more data has become available for migratory species as well. In the past, nematode research mainly focused on phylogeny, occurrence, behavior, host range and agricultural impact. The last few decades, nematode research has gradually shifted to a more molecular approach due to new emerging molecular techniques.

PPNs are armed with a stylet, a short protrusible spear-like structure, to help in puncturing cell walls. This characteristic distinguishes them from nematodes with a different lifestyle. To infect their host, PPNs use their stylet, but they also secrete a cocktail of effector proteins that have a role in cell wall digestion or defense suppression. In this research we will focus on one specific migratory PPN, *H. oryzae*, and the close relationship with its host, *Oryza sativa*.

H. oryzae is most abundant in flooded rice fields in rice growing regions worldwide. To survive in these extreme conditions the nematode should be adapted to an anaerobic environment. In addition, nematodes can go into a state of anhydrobiosis to bridge the period in between growing seasons when the fields can dry out completely. Several adaptations, discussed in Chapter 2, enable this nematode to thrive in such ecosystems. To survive, PPNs have to be able to successfully penetrate the host roots. Having a stylet alone is not sufficient to overcome the formidable barrier that is the cell wall. This is why nematodes secrete a bunch of CWMP to break down this obstacle. In most cases, these proteins are synthesized in the pharyngeal glands and secreted trough the stylet. CWMP have been intensively discussed and characterized in other nematodes and were found in the transcriptome of *H. oryzae* as well. Interestingly, the expression of β-mannanase, a putative cell wall modifying protein that has not been found in nematodes before, was reported in the pharyngeal glands. In addition, a characteristic N-terminal signal peptide targeted the protein for probable secretion in the host tissue (Chapter 2). The precise function of this protein during nematode infection needs to be elucidated, but it probably

involves degradation of the mannan fraction of the hemicellulose portion in the plant cell wall.

Penetration of the cell wall is just a first battle won by the nematode, but the defense system of the host is not defeated yet. Once the invading pathogen is detected, the host is capable of activating a multitude of ingenious defense responses to counteract the pathogen. The defense network is tightly regulated by an interacting mechanism of phytohormones. Most important defense-related hormones are ABA, SA and JA. Remarkably, two enzymes acting directly or indirectly on chorismate, the precursor of SA, were found in the transcriptome of *H. oryzae*. One of them, CM, has been reported previously in other PPNs but the precise role and in particular the mechanism by which it aids nematodes during infection is still under debate. The presence of an N-terminal signal peptide and its expression in the pharyngeal glands confirmed secretion in the host tissue (Chapter 2). The second gene encoded ICM. The protein lacked an N-terminal signal targeting it for secretion, and several attempts to locate expression of this gene in the pharyngeal glands failed. The fact that this type of ICM is specific to PPNs and that its non-conventional secretion was shown in other plant-parasitic organisms strengthens the hypothesis that it is secreted by *H. oryzae* into the root tissue.

Both genes were further characterized to reveal the intron-exon structure. The 3D structure of both proteins was predicted by homology modelling. Since there are no sequence similarities available for a peculiar N-terminal serine/histidine rich domain of CM, this part was modelled *ab initio*. Both modeled protein structures are discussed in Chapter 3. Protein activity was assessed by using CM or ICM deficient *E. coli* strains. Results showed that both proteins were functionally active and that the serine/histidine rich region of CM was not needed for activity. Once the activity of both proteins was confirmed, the next step was to try to elucidate their biological role *in planta*.

Since SA is an important signaling hormone during defense reactions, it is imaginable that secreted HoCM and HoICM have an effect on resistance against biotic stress. Several methods were applied to test this hypothesis. Leaves of *N. benthamiana*, infiltrated with either *HoCM* or *HoICM* showed an increased susceptibility for *P. nicotianae* (Chapter 3). In addition, rice plants overexpressing *HoCM* or *HoICM* were more vulnerable to nematode infection (Chapter 4). These tests show that both proteins aid in diminishing plant defenses during infection. To search for the mechanism by which these proteins are able to establish

these effects, overexpression lines were further investigated. RNA-seq analysis revealed alterations in the phenylpropanoid pathway and terpenoid biosynthesis pathway. The latter was confirmed by histochemical staining of terpenoids in rice roots. Roots overexpressing *HoCM* or *HoICM* showed almost no staining compared to the control (rice plants transformed with an empty vector). Hormone measurements revealed elevated levels of ABA. Intriguingly, SA levels remained unchanged, except in roots of *HoICM* overexpressing plants. The mechanism by which HoCM or HoICM is able to mingle with ABA levels and terpenoid concentrations remains elusive for the moment, but it is an interesting subject for further investigation.

In this thesis, we provided evidence of the role of HoCM and HoICM in attenuating defense responses. But now the challenge remains to further elucidate the mechanisms by which these proteins are able to do so, and more important, investigate how this knowledge can be used to develop a more sustainable agriculture.

Samenvatting

Plantparasitaire nematoden worden wereldwijd beschouwd als een belangrijke plaag op verschillende gewassen. In het verleden werd er vooral veel aandacht besteed aan de sedentaire soorten, een type nematoden dat speciale voedingscellen aanmaakt in de plant om te overleven. Met de sequenering van de transcriptomen van *Hirschmanniella oryzae*, *Radopholus similis* en verschillende *Pratylenchus* soorten, kwam er ineens een overvloed aan data beschikbaar met betrekking tot migratorische plantparasitaire nematoden. Vroeger werd er vooral veel aandacht besteed aan fylogenie, voorkomen, “host range” en economische impact van schade veroorzaakt door nematoden. De laatste decennia is het onderzoek naar nematoden langzaam maar zeker de moleculaire weg opgegaan door het ter beschikking komen en het op punt stellen van nieuwe, meer geavanceerde technieken.

Plantparasitaire nematoden hebben een stylet, een korte intrekbare speer die gebruikt wordt om celwanden te doorboren. De aanwezigheid van dit item onderscheidt hen van nematoden met een andere levensstijl. Om de waardplant te infecteren, maken nematoden naast hun stylet ook nog gebruik van een een arsenaal van effectoreiwitten die uitgescheiden worden via het stylet in het plantenweefsel. Deze eiwitten spelen een rol in het verteren van de celwand of het inhiberen van de verdediging van de plant. In deze thesis leggen we de nadruk of één specifieke migratorische nematode, namelijk *H. oryzae*, en de interactie ervan met de waardplant *Oryza sativa*.

H. oryzae is vooral aanwezig in rijstvelden die continu onder water staan. Om onder deze extreme omstandigheden te kunnen overleven moet de nematode enigszins aangepast zijn aan een anaeroob milieu. *H. oryzae* kan ook in een staat van anhydrobiose gaan om zich te beschermen tegen periodes van droogte die voorkomen tussen twee groeiseizoenen. Verschillende aanpassingen, beschreven in hoofdstuk 2, stellen *H. oryzae* in staat om goed te gedijen in deze ecosystemen. Om te overleven moeten plantparasitaire nematoden de celwand van de cellen van de waardplant kunnen afbreken. Enkel en alleen met hun stylet lukt dit niet, daarom secreteren ze ook een arsenaal aan celwand modificerende eiwitten die helpen om dit obstakel te overwinnen. In de meeste gevallen worden deze eiwitten aangemaakt in de faryngeale klieren en uitgescheiden via het stylet in het plantweefsel. In deze studie bespreken we enkele interessante celwand modificerende eiwitten die nog niet beschreven zijn in plantparasitaire nematoden, maar wel aanwezig zijn in het transcriptoom

van *H. oryzae*. De expressie van β-mannanase, een mogelijk celwand modificerend eiwit, is aangetoond in de faryngeale klieren van *H. oryzae*. De expressie in de klieren en de aanwezigheid van een N-terminaal signaalpeptide bevestigen de hypothese dat dit eiwit gesecreteerd wordt in het plantenweefsel. De exacte functie van dit eiwit dient nog onderzocht te worden, maar het helpt waarschijnlijk bij het afbreken van de mannan-fractie in het hemicellulose gedeelte van de celwand.

Indien de nematode erin slaagt om de celwand te penetreren, is dit slechts de eerste hindernis die overwonnen wordt. Waardplanten zijn niet zo kwetsbaar als op het eerste zicht lijkt. Wanneer de plant een pathogeen detecteert, wordt er een heel scala aan verdedigingsresponsen geactiveerd om de verdere invasie van de pathogeen te verhinderen. Dit netwerk van responsen wordt gereguleerd door interagerende mechanismen van plantenhormonen. De belangrijkste verdedigings-gerelateerde hormonen zijn abscicinezuur, salicylzuur en jasmijnzuur. In het transcriptoom van *H. oryzae* zijn twee genen gevonden die direct of indirect inwerken op chorismaat, de precursor van salicylzuur. Deze twee genen coderen voor chorismaat mutase (HoCM) en isochorismatase (HoICM). De aanwezigheid van chorismaat mutase in secretomen van andere plantparasitaire nematoden werd reeds aangetoond, maar de exacte rol van dit eiwit en het werkingsmechanisme *in planta* moet nog opgehelderd worden. Aangezien de expressie van dit gen werd aangetoond in de faryngeale klieren en door het feit dat er ook een N-terminaal signaalpeptide aanwezig is, mogen we aannemen dat het mature eiwit door de nematode in het plantenweefsel wordt gesecreteerd. Isochorismatase daarentegen, heeft geen N-terminaal signaalpeptide en ondanks meerdere pogingen kon de expressie van dit gen niet aangetoond worden in de faryngeale klieren. Het feit dat dit gen specifiek is voor plantparasitaire nematoden en dat homologen van dit eiwit op een non-conventionele manier gesecreteerd worden door andere plantparasitaire organismen, is een indicatie dat *H.* oryzae dit eiwit ook secreteert in het plantenweefsel.

Beide genen werden verder gekarakteriseerd door de opheldering van de exon-intron structuur. Ook de 3D structuur van beide eiwitten werd gemodelleerd. Aangezien er geen gelijkaardige sequenties beschikbaar zijn voor het serine/histidine rijke N-terminale gedeelte van chorismaat mutase, werd dit gedeelte *ab initio* gemodelleerd. Beide structuren worden beschreven in hoofdstuk 3. De actitiveit van beide eiwitten werd nagegaan met behulp van chorismaat mutase of isochorismatase deficiënte *E. coli* cellen. Resultaten van deze proefopzet wezen uit dat beide eiwitten actief zijn en dat het N-terminale gedeelte van

chorismaat mutase overbodig is voor activiteit. De volgende stap in het onderzoeksproces is het ontrafelen van de biologische rol van beide eiwitten *in planta*.

Aangezien salicylzuur een belangrijk signaalhormoon is gedurende het verdedigingsmechanisme van de plant, is het niet onmogelijk dat beide eiwitten een effect hebben op resistentie tegen biotische stress. Verschillende methodes werden uitgeprobeerd om deze hypothese te testen. Bladeren van *N. benthamiana*, geïnfilteerd met *HoCM* of *HoICM* vertoonden een hogere gevoeligheid voor *P. nicotianae* (hoofdstuk 3). Ook rijstplanten die deze genen tot expressie brachten bleken gevoeliger te zijn voor infectie met nematoden. Deze testen tonen aan dat beide eiwitten in staat zijn om de verdediging van de plant te verminderen. Om het mechanisme te onderzoeken waarmee ze dit effect kunnen bewerkstelligen, werd er een RNA-seq analyse uitgevoerd op de transgene planten die *HoCM* of *HoICM* tot expressie brachten (hoofdstuk 4). Deze analyse bracht aan het licht dat HoCM en HoICM erin slaagden om veranderingen teweeg te brengen in de biosynthese van phenylpropanoiden en terpenoiden. De verlaagde concentratie aan terpenoiden werd bevestigd door een histochemische kleuring. Rijstwortels die *HoCM* of *HoICM* tot overexpressie brachten, vertoonden bijna geen kleuring van terpenoiden vergeleken met de controle. Hormoonmetingen op deze planten brachten een stijging in abscicinezuur aan het licht. Een daling in salicylzuur bleef uit, enkel in wortels die *HoICM* tot overexpressie brachten werd er een daling gedetecteerd in de salicylzuurconcentratie. Het mechanisme waarbij HoCM en HoICM in staat zijn om de concentratie aan abscicinezuur en terpenoiden te veranderen blijft momenteel een vraagteken, maar het biedt wel een interessant onderwerp voor verder onderzoek.

In deze thesis hebben we de rol besproken van HoCM en HoICM bij het verzachten van de verdedigingsmechanismen van de plant. De uitdaging die nu nog voor ons ligt is het ontrafelen van het mechanisme waarmee deze eiwitten dit effect kunnen bewerkstelligen, en hoe we deze kennis kunnen gebruiken om in de toekomst een meer duurzame landbouw te ontwikkelen.

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Dankwoord

Een doctoraat, mijn doctoraat (lees: een werk van velen, maar met mijn naam op de cover), een werk van lange adem waar ik met heel veel plezier aan gewerkt heb, zelf tijdens de moeilijke momenten. Zes jaar labowerk, zes jaar opzoekwerk, zes jaar met hoogtepunten en tegenvallers, frustraties en heuglijke momenten, allemaal gebundeld in dit kleine boekje. Zoveel werk, en toch begin je hier te lezen, helemaal op het einde, bij het dankwoord. Het enige stukje in dit manuscript dat geen labowerk of voorafgaand opzoekwerk vereiste. Dat is een beetje zoals naar de bioscoop gaan om enkel de aftiteling van een film mee te pikken. Geen enkel probleem, je kan nog altijd je fout recht zetten en niemand die het door zal hebben. Gewoon beginnen lezen bij de eerste pagina en dan kom je hier vanzelf terug. Geen zorgen, ik zal een tekentje zetten zodat je weet tot hoever je al gelezen hebt in dit dankwoord, dan hoef je niet opnieuw beginnen lezen. Je kan straks gewoon verder lezen vanaf het bolletje. ●

Alles goed gelezen? Zeker? Toch even testen met een klein vraagje! Hoe groot zijn de voedingscellen die *Hirschmanniella oryzae* aanmaakt in de wortel?

Het juiste antwoord kan je hieronder vinden.

H. *oryzae* is een migratorische nematode en maakt helemaal geen voedingscellen aan.

Indien je de vraag juist beantwoord hebt, heb je toch al minstens een deeltje van dit werk gelezen, of had je al voldoende achtergrondkennis. Als er één eigenschap is die je verder ontwikkelt tijdens het werken aan een doctoraat, naast zelfstandig werken, is het wel kritisch denken. Daardoor weet ik ook wel dat de meeste mensen gewoon zullen doorlezen. Maar wat de meeste mensen niet doorhebben, is dat het vrij goed opvalt als je dit boekje ineens ondersteboven houdt om onmiddellijk het juiste antwoord te lezen! AHA!

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vol tanden staan terwijl de andere drie zullen zeggen: “Ah, is dat niet de lanbouwingenieur van vroeger?”. Dus ook ik koos voor deze opleiding. In mijn laatste jaar kreeg ik de kans om mijn thesis te doen op de vakgroep Moleculaire Biotechnologie, met Lieve als promotor. Hier kreeg ik dan een eerste voorsmaakje over hoe uitdagend en boeiend wetenschappelijk onderzoek kan zijn. Mede dankzij de uitstekende sfeer binnen de groep ben ik hier uiteindelijk nog zes jaar blijven plakken om een doctoraat af te werken.

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Waar te beginnen bij een dankwoord? Misschien bij het begin, en dan chronologisch proberen verder werken? Eigenlijk werd de basis voor dit doctoraat al zeven jaar geleden gelegd, tijdens mijn thesis. Gedurende die thesis en de eerste jaren van mijn doctoraat waren er constant drie personen die mij ruggesteunden op wetenschappelijk niveau. In de eerste plaats **Lieve**, zonder wie dit project niet mogelijk geweest zou zijn. Je gaf me de mogelijkheid om aan het labo te blijven na mijn thesis, om zo een doctoraat te kunnen behalen. Mede dankzij jouw verbeteringen is mijn doctoraat geworden wat het nu is. Ik stond er altijd van verbaasd hoe je zelfs de kleinste fouten kon halen uit elke tekst die ik je doorstuurde, tot zelfs het aantal spaties toe. Maar wat ik het meest apprecieer is je “open deur” mentaliteit. Of je nu gehaast was om op tijd naar een vergadering te vertrekken, of druk bezig was met projecten, je deur stond altijd open. Op elk moment kon ik binnen wandelen om raad te vragen, hetgeen altijd een zeker gevoel van gerustgesteldheid bood. Toen ik hier begon, **Tina**, was je nog post-doc, maar ondertussen heb je al een felbegeerde prof-positie kunnen bemachtigen. Ik stelde het altijd enorm op prijs dat je, ondanks je overvolle agenda, altijd wel een gaatje kon vinden om een tekst kritisch na te lezen, constructief te discussiëren of me een duwtje in de juiste richting te geven. De laatste van de “grote drie” heeft ons ondertussen al verlaten om een nieuwe carrière uit te bouwen aan het ILVO. **Annelies**, het eerste jaar heb ik je waarschijnlijk meer dan eens verveeld met al mijn vragen over programmeren in Perl, maar ik waardeer het enorm dat je altijd met

evenveel geduld een antwoord hebt helpen vinden. Je was er mede voor verantwoordelijk dat ik uiteindelijk de bio-informatica microbe te pakken heb gekregen. Samen hebben we dan uiteindelijk nog een extra bachelor diploma in de bio-informatica weten te behalen. Niet alleen je wetenschappelijke input mis ik nu, maar ook de sfeer en dagelijkse "vertellingskes". Zeker toen **Birger** er nog was, was de sfeer veel levendiger in het buro. Birger is iemand met hetzelfde gevoel voor humor als mij. In die tijd kon ik nog streken uithalen en was er twijfel over de schuldige, nu komt iedereen jammer genoeg onmiddellijk bij mij uit.

Na het vertrek van de oude garde (waar ik mijzelf ook stilletjes toe reken) werd de sfeer in het buro terug opgekrikt door de komst van enkele nieuwkomers zoals Mei, Silke en Jasmien. **Mei** was someone who could brighten the mood with her smile or one of her witty remarks. **Silke**, bedankt dat ik altijd op je hulp kan rekenen en bedankt voor de discussies en de steun. **Jasmien**, jouw onderzoek is totaal verschillend van dat van de rest van de groep, maar toch wou ik je graag bedanken voor de praatjes die af en toe voor de nodige afleiding konden zorgen. **Henok** & **Ruben**, twee kerels die altijd tijd vrij hadden voor een grapje, flauwe woordspeling of wat onschuldig kattenkwaad. Ruben en **Diana** die altijd wel tijd over hadden om een feestje te organiseren en een origineel en grappig gedicht te schrijven als uitnodiging. Ook bedankt aan de voltallige **nema-groep**, voor jullie wetenschappelijke input, de ideeën die jullie me gaven tijdens jullie presentaties en de hulp in het labo.

Naast de leden van het nema-team die bezig zijn aan een doctoraat hebben we natuurlijk nog heel wat andere mensen die ik een gemeend dankjewel verschuldigd ben. Ik hoop dat ik jullie de voorbijgaande jaren al duidelijk gemaakt heb hoezeer ik jullie hulp apprecieer, maar voor het geval dit niet duidelijk was, vermeld ik het hier nog eens. Eerst en vooral zou ik de mensen op het secretariaat nog eens in de bloemetjes willen zetten. Dag in dag uit stonden **Fien** en **Sofie** klaar om het ons als student wat makkelijker te maken. Bedankt om alles altijd zo snel mogelijk te regelen als ik weeral eens wat laat met een bestelling op de proppen kwam. Bedankt voor alle antwoorden op mijn vragen, ook al hadden jullie de handen vol. Bedankt om zoveel te regelen voor mij tijdens het jaar en nu voor mijn doctoraat en vooral bedankt om dit altijd te doen met een lach. Dan hebben we ook nog **Geert**, waarbij ik altijd terecht kon voor computer-gerelateerde problemen of wat tips & tricks voor het gebruik van de server. Die tips & tricks hebben me echt wel sneller vooruit geholpen voor al mijn bio-informatica toepassingen.

Aangezien ik in sommige periodes vrij druk bezig was met de practica, kon ik nu en dan op wat hulp rekenen van **Lien** om het labowerk wat sneller vooruit te laten gaan. Hiertoe kon ik vooral rekenen op je expertise van Q-RT-PCR en infectietesten met nematoden. Je draaide er ook je hand niet voor om om kleinere dingen af te werken in het labo als ik mij terug moest focussen op de practica, bedankt hiervoor. Naast een hulp in het labo zag ik je ook een beetje als mijn wandelende encyclopedie, je was een haast onuitputtelijke bron van informatie over experimenten en protocols. Ik had je ook beloofd om een volledige pagina aan je te wijden in mijn dankwoord, en aangezien ik altijd mijn beloftes probeer na te komen... Eigenlijk was het nog helemaal zo moeilijk niet om een pagina vol te schrijven!

Naast Lien wil ik ook **Isabel** bedanken. Je bent nog niet zo lang aan het werk binnen onze groep, maar toch heb je je al helemaal kunnen inwerken en je enthousiasme werkt aanstekelijk.

Naast de nema hebben we ook nog andere groepen, **nano**, **csi** en **glyco** waarvan het onmogelijk is om alle leden op te noemen in dit dankwoord, want ik vrees dat het nu al wat langdradig aan het worden is. Toch zou ik graag minstens enkele mensen bij naam noemen. Om te beginnen **Tom**, die altijd klaar stond om wat uitleg te geven over de microscoop en om bij te springen bij de practica voor de tweede bachelor. Bij de glyco's hebben we dan **Annelies**, **Nausicaä en Jonas**, die altijd wel tijd maakten voor een praatje, een grapje of wat goede raad. De twee nieuwkomers **Jeroen** en **Sofie** zorgden voor nog wat meer leven in de brouwerij, wat ten zeerste geapprecieerd werd. Dan hebben we nog de recent afgesplitste immunogroep waar **Annelien** en **Sarah** de harde kern vormen. Bedankt voor de afleiding wanneer ik die eens nodig had en de bemoedigende woorden in mijn schrijffase.

Inderdaad **Lore**, je naam is nog niet voorbij gekomen, maar kijk, een aparte alinea helemaal voor jou alleen. Aangezien er door de jaren heen alleen maar practica bijkwamen, kon ik wel wat hulp gebruiken. Vier jaar lang hebben we samen aan de practica gesleuteld, ze samen gegeven of ze onder elkaar verdeeld. Bedankt voor alle hulp en steun. Ook bedankt dat je al vier jaar hebt kunnen leven met mijn flauwe humor, ook al was je daar meestal het onderwerp van.

Als laatste zou ik dan ook nog graag mijn familie bedanken, die altijd en onvoorwaardelijk klaar stonden om mij te helpen met eender wat. In het bijzonder dan de mama. Aangezien ze niet kon helpen bij het schrijfwerk, het nalezen of het labowerk, probeerde ze dit te compenseren met eten, gratis taxidiensten en bezorgde telefoontjes. Eigenlijk vermoed ik dat het niet ik was die de meeste stress had in de eindfase van mijn doctoraat... Bedankt aan de hele familie om te luisteren wanneer ik het nodig had, maar vooral (en andere studenten zullen dit begrijpen) om er niet altijd naar te vragen als ik er zelf niets over vertelde.

Dan rest er mij enkel nog één belangrijk iemand om te bedanken, **Kaat**; vriendin, vrouw en manusje-van-alles. Bedankt om geduldig te zijn in de periodes waarin ik het druk had en weinig tijd voor je had. Ook al kon je me niet veel helpen, je kon me kalmeren en geruststellen, gewoon door er te zijn. Mijn teksten nalezen kon je wel, en dat deed je dan

ook met volle overgave. Geen enkele schrijffout of onduidelijke zinsconstructie ontsnapte aan je haviksoog. Om dit even te staven: ik had met opzet drie dt-fouten in dit dankwoord geschreven, Kaat heeft ze er alle vijf uitgehaald!

Zes jaar doctoreren, ’t was een toffe reis. Bedankt aan alle reisgezellen en heel veel succes aan diegenen die hun bestemming nog niet bereikt hebben!

Curriculum vitae

Personal information

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Education

2010 to 2012 — Bachelor in biomedical laboratory technology, option bio-informatics @home opleiding Howest, Rijselstraat 5, 8200 Brugge

2004 to 2009 — Bioscience engineer in cell and gene biotechnology
Ghent University, Faculty of Bioscience Engineering
Coupure links 653, 9000 Ghent
MSc thesis: RNAi van parasitismegenen en huishoudgenen bij plantenparasitaire migratorische nematoden (Promoter: Prof. Dr. G. Gheysen, Co-promoter: Dr. ir. Tina Kyndt)

1998 to 2004 — High school, science-mathematics
Sint-Hendriks en Zusters Maricolen Instituut
Guido Gezellelaan 105, 9800 Deinze

Professional experience

October 2009-present: Assistant at the Laboratory Molecular Genetics
Department of Molecular Biotechnology, Faculty of Bioscience Engineering Ghent University, Coupure Links 653, 9000 Gent

- Assistance to education of Prof. Dr. G. Gheysen (responsible/assisting for/in the practical exercises of “Molecular techniques in nematology”, “General techniques in nematology”, “Biochemical analysis techniques”, “Plant biotechnology”, “Molecular phytopathology”, “Biochemistry and molecular biology”, “Gene technology and plant biotechnology” and “Gene technology and molecular diagnostics”)

- Doctoral research topic: How the plant-parasitic nematode *Hirschmanniella oryzae* is able to subdue the defense system of rice; a molecular analysis (Promoter: Prof. Dr. G. Gheysen)

Publications

A1:

Prentice K., Pertry I., Christiaens O., **Bauters L.**, Bailey A., Niblet C., Ghislain M., Gheysen G., Smagghe G. (2015).Transcriptome analysis and systemic RNAi response in the African sweetpotato weevil (*Cylas puncticollis*, Coleoptera, Brentidae), Plos One, 10(1), e0115336

Kyndt T., Denil S., **Bauters L.**, Van Criekinge W., De Meyer T. (2014). Systemic suppression of the shoot metabolism upon rice root nematode infection. Plos One, 9(9), e106858

Bauters L., Haegeman A., Kyndt T., Gheysen G. (2014). Analysis of the transcriptome of *Hirschmanniella oryzae* to explore potential survival strategies and host-nematode interactions. Molecular Plant Pathology, 15(4), 352-363.

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

Kyndt T., Denil S., Haegeman A., Trooskens G., **Bauters L.**, Van Criekinge W., De Meyer T., Gheysen G. (2012). Transcriptional reprogramming by root knot and migratory nematode infection in rice. New Phytologist, 196(3), 887-900.

A2:

Nsengimana J., **Bauters L.**, Haegeman A., Gheysen G. (2013). Silencing of Mg-pat-10 and Mg-unc-87 in the plant parasitic nematode *Meloidogyne graminicola* using siRNAs. Agriculture, 3(3), 567-678.

Conferences: oral and poster presentations

- COST Action 872: Exploiting genomics to understand plant-nematode interaction (24-27/05/2010, Lisbon, Portugal), attended
- 63th International Symposium on Crop Protection, in association with COST 872 final meeting (24/05/2011, Ghent, Belgium), oral presentation: **Bauters L**., Haegeman A., Gheysen G., Analysis of the transcriptome of *Hirschmanniella oryzae* to gain further insight into the plant-nematode interaction
- 31st International Symposium of the European Society of Nematologists (23-27/11/2012, Adana, Turkey), oral presentation: **Bauters L.**, Haegeman A., Kyndt T.,

Gheysen G., Analysis of the transcriptome of *Hirschmanniella oryzae* to explore potential survival strategies and host-nematode interactions

- 6th Effectome Meeting (25-27/09/2013, Lauret, France), poster: **Bauters L.**, Haegeman A., Kyndt T., Gheysen G., A secreted chorismate mutase from *Hirschmanniella oryzae*
- 2nd Annual Conference of the SUSTAIN Action, Pathogen-Informed strategies for Sustainable Broad-Spectrum Crop Resistance (15-17/10/2014, Zakopane, Poland), oral presentation: **Bauters L.**, Kyndt T., Gheysen G., Chorismate mutase and isochorismatase as possible effectors of the migratory nematode *Hirschmanniella oryzae*

Other meetings

- RNAi training school (12-15/04/2010, Belfast, United Kingdom)

Tutor of Master Dissertations

- Gil Luypaert: "Moleculaire analyse van parasitismegenen bij de plantenparasitaire nematode *Hirschmanniella oryzae*", Academic year 2010-2011
- Homan Regmi:"Cloning and expression analysis of rice genes involved in infection with *Hirschmanniella oryzae*", Academic year 2010-2011
- Jorden van de Walle: "Studie van effectoren van *Hirschmanniella oryzae* die interageren met de salicylzuurpathway van rijst" Academic year 2012-2013
- Eline Van Vlasselaer: "Ontrafelen van de interacties tussen rijst en de plant-parasitaire nematode *Hirschmanniella oryzae*", Academic year 2013-2014

PR-activities

Assisted in several PR-related events like" Flemish Science Week", "Biotechnology day", "open day of Faculty of Bioscience Engineering" and introduction of the department to bachelor students from Antwerp University

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Appendix A

>DNA HoCM
ATGTGCAATTTGATTTGCGCACTTTGTTTTGCCATTCTTCTGATTTTCGTTGCCGAAAGCAAGCATTCA
TCGTCATCCTCCAAGAGCAGCGAGAGCAGCCATCAGAATGGCCAGACCGCCAGCAAATTCTGCCGTT
TTATAGCAATGGAATATGCAAATGGAGAAGGAGGAGCAGAACATCATCATCATCGGCATCGGCATC
ATCACCAACATCATCATGGCAGCAACAGAACAACAACAACGGATCTGCAAGCGG*GTATGCATTTACT*
*GTATGAATGAATGAATGATGCGAGATGTGCATCCATCCGCAATCATCCACAATTGCCAG*TGATGGAA
GTGATCCGGCTGGTAAGTGCCCGTCTCGATTTGGCCCAACCGGTTGCGCTGTACAAATGGCAACACA
ACCAGTCCATTGATGACCCGGTGCGTGAGGCCGCACTGTTGGCCAGCGTTGCGACACAGGCAAATG
CATCCGGAGTGGCGGCGGATTTTGCGCAACAATTCTTCCAGGATCAGATCAACGCCAGCAAAATCAT
TCAA*GTCATTTAATTAACAACAGAAAATGAATGCCTTTTAGAATATTTCGGCAACAAAATCCAAATTG*
*CTGAACTTATTTTGTTGGAGCAAATTGTCGAATCAATTCCCCCCAAATTGTTTTTCCAG*TTTGCCTACTT
CGCTGATTGGCAACAGAATGGGGCACCGAATGTGACAGCGCCCGACCTCAGCACCGTTACGCGTCC
GTTGATCAACAATGTGACCAGCGAATTGGTGCAAGCGTTGGCGCCAATCCAATCAATTCGCTTTGAG
CGCAAATGTCCATTCGATGTGGCAATCGGATTCGCGCAGTTGTTTGGCACGCAACAAATCAACACGA
ATCCGGAGGCGGATCGTGCCAAAGCACTTTGCGTGGCATTGGCTCACATTTGTGCTCCCATCAATTG
A

* Underlined text in italics represents intron sequences.

>cDNA HoCM
ATGTGCAATTTGATTTGCGCACTTTGTTTTGCCATTCTTCTGATTTTCGTTGCCGAAAGCAAGCATTCA
TCGTCATCCTCCAAGAGCAGCGAGAGCAGCCATCAGAATGGCCAGACCGCCAGCAAATTCTGCCGTT
TTATAGCAATGGAATATGCAAATGGAGAAGGAGGAGCAGAACATCATCATCATCGGCATCGGCATC
ATCACCAACATCATCATGGCAGCAACAGAACAACAACAACGGATCTGCAAGCGGTG**ATGGAAGTGA**
TCCGGCTGGTAAGTGCCCGTCTCGATTTGGCCCAACCGGTTGCGCTGTACAAATGGCAACACAACC
AGTCCATTGATGACCCGGTGCGTGAGGCCGCACTGTTGGCCAGCGTTGCGACACAGGCAAATGCA
TCCGGAGTGgCGGCGGATTTTGCGCAACAATTCTTCCAGGATCAGATCAACGCCAGCAAAATCATT
CAATTTGCCTACTTCGCTGATTGGCAACAGAATGGGGCACCGAATGTGACAGCGCCCGACCTCAGC
ACCGTTACGCGTCCGTTGATCAACAATGTGACCAGCGAATTGGTGCAAGCGTTGGCGCCAATCCAA
TCAATTCGCTTTGAGCGCAAATGTCCATTCGATGTGGCAATCGGATTCGCGCAGTTGTTTGGCACGC
AACAAATCAACACGAATCCGGAGGCGGATCGTGCCAAAGCACTTTGCGTGGCATTGGCTCACATTT
GTGCTCCCATCAATTGA

* Underlined text represents sequence coding for the predicted N-terminal signal peptide. Bold text shows the sequence that was used to design the HoCM_CAT construct.

>Protein HoCM
MCNLICALCFAILLIFVAESKHSSSSSKSSESSHQNGQTASKFCRFIAMEYANGEGGAEHHHHRHRHHH
QHHHGSNRTTTTDLQAV**MEVIRLVSARLDLAQPVALYKWQHNQSIDDPVREAALLASVATQANAS**
GVAADFAQQFFQDQINASKIIQFAYFADWQQNGAPNVTAPDLSTVTRPLINNVTSELVQALAPIQSI
RFERKCPFDVAIGFAQLFGTQQINTNPEADRAKALCVALAHICAPIN

* Underlined text represents the predicted N-terminal signal peptide. Bold text shows the catalytic region used in the HoCM_CAT construct.

>DNA HoICM
ATGGAATTTCTGAAAAAAAGCAATCACAGTCTCAAACCGCATCCGGATGCCCGTCCACGTGACGTTT
ATCCGCAAGGGAGAATCGAACGACTGGTTGCGCAGCGAATGGCAATTCTGGTTGTGGACGTGCAGA
ATTGGGTGACAGCTGATGGACGCACGGAATTCCATGCAAAAGTGCGCGAACAAACAATCCCAAACA
TTCGCCGGCTGTTGGCGGTGGGGCGAGAGCATGGCATCGAG*GTATATACATAATATGCCTTTCCCGC*
*ATTCATTTTCAATTCCTTCAATTCCCAAACAACGTTTCCGCAG*ATTGTGTTCACAACAATTGAGAATTTA
ACGCGCGATGGGCGCGATCGCAGTCTGGACTACAAGCTGTCCAATTTCTTCGTGCCAAAAGGCTCCT
GGGATGCCAAAGTGGTGGAGGAATTGCAGCCGTTGGAGGACGAAATTGTGTTGCCGAAAACGTCGT
CCAGCCTGTTCAATTCAACCAATTTCGATTATTTG*GTTGGTTAAACAGTGATAAAACACGCTGATTGG*
*CAGCATATTTCCCATTTGTAAATTTGCTTTTTCCTCTTCCCCTTTTCG*ATGCGCAACATTGGAATCGATA
CGATTGCAGTCACCGGCTTCCTCACCGACCAATGCGTGGACCAAACAATTCGGGACGGAGCAGACAA
AGGCTATCGCATGATTTGTGTAACAGATTGTTGTGCAACAGTCAGTGAGGAACGGCATGCGGCGGC
ATTGAATGCAATCAAGGGGTATTGCCGCATGGAGAGCACCAAAACGCTGATTGAAGCAATCCGACA
CGACATTAACACATAG

* Underlined text in italics represents intron sequences.

>cDNA HoICM
ATGGAATTTCTGAAAAAAAGCAATCACAGTCTCAAACCGCATCCGGATGCCCGTCCACGTGACGTTT
ATCCGCAAGGGAGAATCGAACGACTGGTTGCGCAGCGAATGGCAATTCTGGTTGTGGACGTGCAGA
ATTGGGTGACAGCTGATGGACGCACGGAATTCCATGCAAAAGTGCGCGAACAAACAATCCCAAACA
TTCGCCGGCTGTTGGCGGTGGGGCGAGAGCATGGCATCGAGATTGTGTTCACAACAATTGAGAATTT
AACGCGCGATGGGCGCGATCGCAGTCTGGACTACAAGCTGTCCAATTTCTTCGTGCCAAAAGGCTCC
TGGGATGCCAAAGTGGTGGAGGAATTGCAGCCGTTGGAGGACGAAATTGTGTTGCCGAAAACGTCG
TCCAGCCTGTTCAATTCAACCAATTTCGATTATTTGATGCGCAACATTGGAATCGATACGATTGCAGT
CACCGGCTTCCTCACCGACCAATGCGTGGACCAAACAATTCGGGACGGAGCAGACAAAGGCTATCG
CATGATTTGTGTAACAGATTGTTGTGCAACAGTCAGTGAGGAACGGCATGCGGCGGCATTGAATGC
AATCAAGGGGTATTGCCGCATGGAGAGCACCAAAACGCTGATTGAAGCAATCCGACACGACATTAA
CACATAG

>Protein HoICM
MEFLKKSNHSLKPHPDARPRDVYPQGRIERLVAQRMAILVVDVQNWVTADGRTEFHAKVREQTIPNI
RRLLAVGREHGIEIVFTTIENLTRDGRDRSLDYKLSNFFVPKGSWDAKVVEELQPLEDEIVLPKTSSSLFNS
TNFDYLMRNIGIDTIAVTGFLTDQCVDQTIRDGADKGYRMICVTDCCATVSEERHAAALNAIKGYCRM
ESTKTLIEAIRHDINT

Appendix B

Primer	Sequence (5'->3')	Application	Target
Bmanis_F Bmanis_R	TGAACCTGATGAACGAATGG GTGTAGCCCTTGCTCTTTGC	probe for in situ hybridization	β-mannanase
xylan30is_F xylan30is_R	ATACAACGCTTATGTGCTGTGG TCAGTTGGACACGAATGTGG	probe for in situ hybridization	xylanase (GHF30)
CMis_F CMis_R	GCAACACAACCAGTCCATTG CTCCGGATTCGTGTTGATTT	probe for in situ hybridization	chorismate mutase
Thauis_F Thauis_R	GCCGAATTTGGTTTGAAGAA ATAATTCGTTGGTCCGCAAG	probe for in situ hybridization	thaumatin-like protein
flp14F flp14R	CAGCCACACAAGGATTGACC CAAACTCGTGTTTCCTCTTGC	Q-RT-PCR reference gene	FMRFamide-like neuropeptide 14
ElFacGamF ElFacGamR	TCTCTGGAGTTTGGGTTTGG CCTTTGGATCCAGTTTCTTCC	Q-RT-PCR reference gene	elongation factor 1-ϒ
SXP2F2 SXP2R2	GGATTCGGCAGAAGATTTGG CAAATGCACAATGCCTTCC	Q-RT-PCR	SXP/RAL-2
leaCF leaCR	TGCTGTGGATGATGATTTGG GGGAGGATGTGAAAATTGAGC	Q-RT-PCR	LEA protein
glutperF glutperR	TGACATCACGTGGAATTTCG GCTGCTGCTGTTCTTCTCG	Q-RT-PCR	glutathione peroxidase
HoCMfull_F HoCMfull_R	ATGTGCAATTTGATTTGCG TCAATTGATGGGAGCACAA	cloning full gene	chorismate mutase
HoCMnoSP_F HoCMcat_F	CATTCATCGTCATCCTCCA ATGGAAGTGATCCGGCTG	cloning withouth SP cloning catalytic domain	
OligodT	TTTTTTTTTTTTTTTTTTTTTTTTT	cDNA synthesis	
HoICM_F	ATGGAATTTCTGAAAAAAAGCAA	cloning full gene	isochorismatase

HoICM_R	CTATGTGTTAATGTCGTGTCGGA		
HoICM_F_attb HoICM_R_attb	AAAAAGCAGGCTTCACCATGGAATTTCTGAAAAAAAGCA AGAAAGCTGGGTTCTATGTGTTAATGTCGTGTCGGA	attaching attb sites to target	isochorismatase
SP6 T7	ATTTAGGTGACACTATAGAATACTCAAGC TAATACGACTCACTATAGGGCGAATTGG	used for sequencing targets in pGEM-T	
pQE_F pQE_R	CCCGAAAAGTGCCACCTG AGTTCTGAGGTCATTACTGG	used for sequencing targets in pQE-30 UA	
attb1 attb2	ACAAGTTTGTACAAAAAAGCAGGCT ACCACTTTGTACAAGAAAGCTGGGT	complete attb sites for Gateway cloning	
HoCMfull_F_attb HoCMcat_F_attb HoCMfull_R_attb	AAAAAGCAGGCTTCACCATGCATTCATCGTCATCCTCCA AAAAAGCAGGCTTCACCATGGAAGTGATCCGGCTG AGAAAGCTGGGTTTCAATTGATGGGAGCACAA	attaching attb sites to target	chorismate mutase
EXP_NARCAI_F EXP_NARCAI_R	AGGAACATGGAGAAGAACAAGG CAGAGGTGGTGCAGATGAAA	Q-RT-PCR reference gene	expressed protein (LOC_Os07g02340)
EIF5C_F EIF5C_R	CACGTTACGGTGACACCTTTT GACGCTCTCCTTCTTCCTCAG	Q-RT-PCR reference gene	expressed protein (LOC_Os11g21990)
08g08130_1_F 08g08130_1_R	TCCCTCCACCTTTTGATGG TGTAAATAGGCGCACCAACC	Q-RT-PCR	Glutathione S-transferase (LOC_Os08g08130)
10g09990_2_F 10g09990_2_R	GGATACGTCTCGGAGTTTTCG AAGGCAGGATCTGCTTCG	Q-RT-PCR	UDP-glycosyltransferase (LOC_Os10g09990)
01g04409_2_F 01g04409_2_R	CTTGGTGATGGTGGTTTTGG AAGGTTCTGGTGCAGTAGGC	Q-RT-PCR	serine/threonine-protein kinase (LOC_Os01g04409)
01g05610_2_F 01g05610_2_R	AATTCCTCTGTCACTCGTTGG CTCAGGGCAACCAATTAACC	Q-RT-PCR	histone H2B.3 (LOC_Os01g05610)
04g52606_2_F 04g52606_2_R	CTTGAGCTCCGTGGAGTAGG GACACAGTTTGGAAGAACATGC	Q-RT-PCR	LRR receptor-like serine/threonine-protein kinase (LOC_Os04g52606)
01g02830_2_F 01g02830_2_R	TTTGCATTGTTGGATTGTGG CACAGAAGAATGGCCTTGG	Q-RT-PCR	receptor-like protein kinase (LOC_Os01g02830)
07g0417800_2_F	GTTGGTGGATGAACAGTTGG	Q-RT-PCR	α-1,2-galactosyltransferase

07g0417800_2_R	ATCTCCCCCTTTGAGCTAGG		(LOC_Os07g23494)
03g11520_1_F 03g11520_1_R	GGACGTGAGTTCAGTCTCACC TCGTCTCATCGATGTTGTCC	Q-RT-PCR	protein RIK (LOC_Os03g11520)
07g09340_2_F 07g09340_2_R	CCCCCATCTATCTTGTGTCC GATTCAGATGGCCAAGAACC	Q-RT-PCR	plasma membrane ATPase (LOC_Os07g09340)
07g33954_1_F 07g33954_1_R	GTCATCCTCCTCCCTTTCG CGATGCTGAATGTCAGTGG	Q-RT-PCR	glucose-6-phosphate/phosphate translocator 2 (LOC_Os07g33954)
QPCR_HoICM_F QPCR_HoICM_R	TCGATACGATTGCAGTCACC ATGCGATAGCCTTTGTCTGC	Q-RT-PCR	isochorismatase
QPCR_HoICM_F QPCR_HoICM_R	GCTGATTGGCAACAGAATGG AAGCGAATTGATTGGATTGG	Q-RT-PCR	chorismate mutase