TO THE MEMORIES OF MY GRANDPARENTS

稻花香里说丰年，听取蛙声一片。

The fragrance from the paddy fields is promising them a bumper year,

the croaks from the acres of frogs are pleasing their ears.

*Night Trip in Yellow Sand Road*

Xin Qiji (1140-1207)

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# Analysis of rice response and improvement of defence to the root-knot nematode *Meloidogyne graminicola*

Hongli Ji

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

**Titel van het doctoraatsproefschrift in het Nederlands:**

Analyse van de rijstreactie en verbetering van de afweer tegen de wortelknobbelaal *Meloidogyne graminicola*

**Cover illustration:** The front cover symbolizes the content of this thesis; the balance between the nematode, rice plant and giant cells resemble a yin-yang symbol. Designed by Henok Yimer, Gisele Herren and Ruben Verbeek.

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# Contents

# List of Abbreviations

| | |
|---|---|
| 2-DDG | 2-deoxy-D-glucose |
| ABA | abscisic acid |
| ABRE | ABA-responsive elements |
| ACC | amino cyclopropane carboxylate |
| ACO | aminocyclopropane-1-carboxylate oxidase gene |
| ANOVA | analysis of variance |
| AOA | aminooxyacetic acid |
| AOS | allene oxide synthase |
| Avr | avirulence |
| BABA | beta-aminobutyric acid |
| BR | brassinosteroids |
| BTH | benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester |
| cDNA | copy DNA |
| DG | dorsal gland |
| DIECA | ammonium diethyldithiocarbamic acid |
| Dpi | days post inoculation |
| dsRNA | double stranded RNA |
| EFNs | extratrafloral nectarines |
| EGase | beta-1, 4-endoglucanase |
| ERF | ethylene-responsive factor |
| ET | ethylene |
| ETI | effector-triggered immunity |
| ETS | effector-triggered susceptibility |
| ETYA | 5, 8, 11, 14-eicosatetraynoic acid |
| FACs | fatty acid–amino acid conjugates |

| | |
|---|---|
| FLS2 | FLAGELLIN-SENSING 2 |
| F-primer | forward primer |
| FW | fresh weight |
| GFP | green fluorescent protein |
| GHF5 | glycosyl hydrolase family 5 |
| GO | gene ontology |
| GUS | β-glucuronidase |
| $H_2O_2$ | hydrogen peroxide |
| HGT | horizontal gene transfer |
| HIPVs | herbivore-induced plant volatiles |
| Hpi | hours post inoculation |
| HR | hypersensitive response |
| ICS | isochorismate synthase |
| INA | dichloroisonicotinic acid |
| INA | 2, 6- dichloroisonicotinic acid |
| IR | induced resistance |
| ISR | induced systemic resistance |
| J2 | second stage juvenile |
| JA | jasmonic acid |
| KDa | kilo dalton |
| L-AOPP | L-2-aminooxy-3-phenylpropionic acid |
| LCM | laser capture microdissection |
| LRR-RLK | leucine-rich repeat receptor-like kinase |
| M (A) PK | mitogen-activated protein kinase |
| MAMP | microbial-associated molecular pattern |
| MeJA | methyl jasmonate |
| mRNA | messenger RNA |
| MW | molecular weight |

| | |
|---|---|
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NahG | salicylate hydroxylase |
| NCED | nine-cis epoxycaretenoid dioxygenase |
| NFS | nematode feeding site |
| NH1 | NPR1 homolog 1 |
| NPR1 | non-expresser of PR proteins 1 |
| OX | overexpression |
| PAL | phenylalanine ammonia lyase |
| PAMP | pathogen-associated molecular pattern |
| PCR | polymerase chain reaction |
| PDF1.2 | plant defensin 1.2 |
| PGPF | growth-promoting fungi |
| PGPR | growth-promoting rhizobacteria endophytic |
| PIs | proteinase inhibitors |
| PPN | plant parasitic nematode |
| PR | pathogenesis-related |
| PRR | pattern recognition receptor |
| PTI | PAMP- triggered immunity |
| qRT-PCR | quantitative reverse transcription-polymerase chain reaction |
| R-gene | resistance gene |
| RKN | root-knot nematode |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| ROS | reactive oxygen species |
| R-primer | reverse primer |
| RT-PCR | reverse-transcription polymerase chain reaction |
| SA | salicylic acid |
| SAR | systemic acquired resistance |

| | |
|---|---|
| SVG | subventral gland |
| TF | transcription factor |
| TMV | tobacco mosaic virus |
| WIR | wound induced resistance |
| WT | wild type |

# CHAPTER 1

# General introduction

## 1.1 Nematodes and plant parasitic nematodes

### 1.1.1 General introduction to nematodes

"If all matter in the universe except the nematodes were swept away, our world would still be dimly recognizable. We would find mountains, rivers, lakes and oceans represented by a film of nematodes as well as the location of towns and various plants and animals since there would be a corresponding massing of certain nematodes" ----Cobb, 1915

There are nearly 20,000 described nematode species classified in the phylum Nematoda (round worms), they are thought to have evolved from simple animals some 400 million years before the "Cambrian explosion" (Poinar Jr, 1983). Nematodes are multicellular animals, in size they range from 0.3 mm to over 8 m, but most of nematodes are microscopic, and they are invisible by naked eyes. Nematodes are extremely abundant and diverse, only insects exceed their diversity. They are evolutionarily related to insects as they also undergo four molts from juvenile to the adult phase. Most nematodes are free-living and feed on bacteria, fungi or protozoa, some are animal parasites, and some are plant parasitic nematodes (PPNs, around 15% of the described species). The dynamics, activity and different feeding types of free living nematodes are good environmental indicators to detect for environmental disturbance and also contribute to food web interactions and ecosystem processes, such as the cycling of nitrogen and carbon. In addition, free-living nematodes help microbial colonization of substrates and mineralization of the soil nutrients. Animal parasitic nematodes were first described in ancient Chinese scientific literature as early as 2700 B.C. (Maggenti, 1981). *Ascaris lumbricoides* is the largest intestinal roundworm infection of humans worldwide and causes a disease known as ascariasis. The first described plant parasitic nematodes (PPN) were discovered in wheat seeds by Needham (1744). Although PPN feed on all parts of the plant, including roots, stems, leaves, flowers and seeds, most of them attack roots. They pierce plant cell walls with a specialized spear called stylet and withdraw the cell nutrient as food. PPN are recognized as major agricultural pathogens as they can cause plant damage ranging from negligible injury to total destruction of plant materials (Fig 1.1). The severity of plant injury resulting from nematode activity depends on several factors such as the combination of plant and nematode species, soil types, and culture practices etc.

### 1.1.2 Plant parasitic nematodes

The infraorder Tylenchomorpha comprises the largest and economically most important group of plant parasitic nematodes.

Based on the parasitic strategies, PPN can be classified into 2 major groups: Ectoparasitic nematodes and endoparasitic nematodes, each group can be further divided into migratory or (semi) sedentary plant parasitic nematodes according to their feeding strategies.

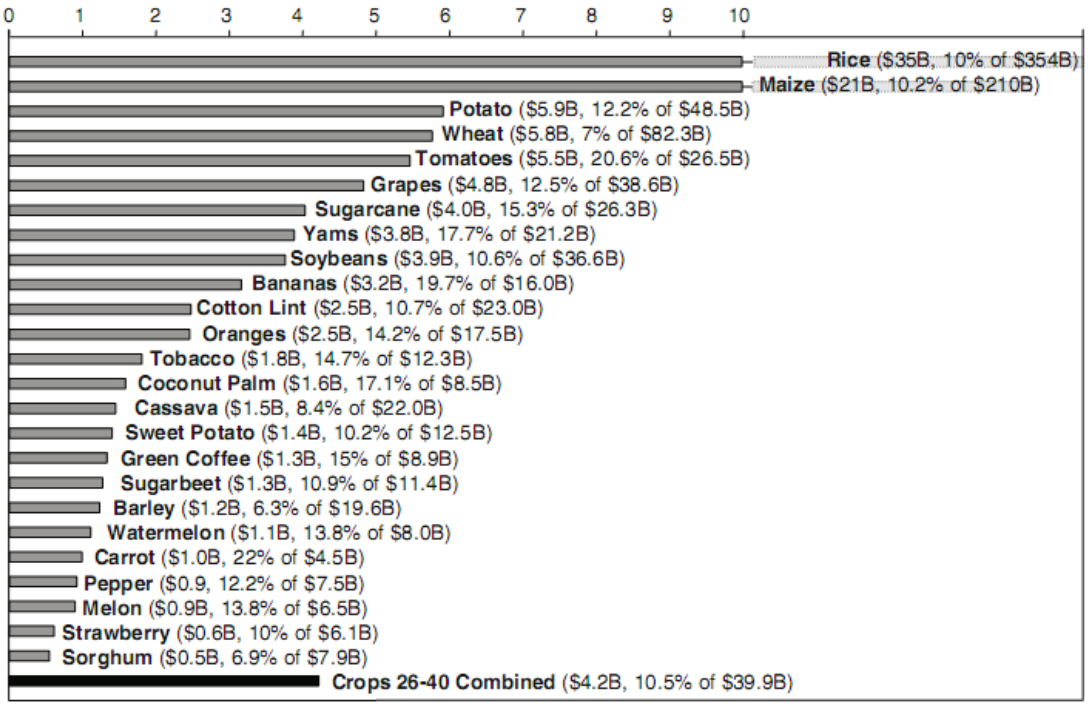


Fig 1.1 Estimation of potential global nematode damage in billions of dollars based on a 1987 yield loss survey (Sasser & Freckman, 1987). Reproduced from McCarter (2009).

**Ectoparasitic nematodes**

An evolutionary study of PPN based on ribosomal DNA indicated that within the Tylenchids, migratory ectoparasitic feeding is an ancestral trait for all major PPN (Bert *et al.*, 2011). Ectoparasitic nematodes remain outside of the plant tissue during all their life stages and use their stylet to puncture plant cells and feed on the cytoplasm. The longer the stylet, the deeper the nematode can feed. For example, *Tylenchorhynchus* only feeds on root hairs or/and epidermis cells. The damage caused by ectoparasitic nematodes is usually limited to necrosis of the cells. However, some of them, such as Longidoridae and Trichodoridae, are vectors for plant viruses (Decraemer *et al.*, 2006).

**Migratory endoparasitic nematodes**

Species of migratory plant endoparasitic nematodes are included in three nematode families, Pratylenchidae, Anguinidae, and Aphelenchoididae. Species of Pratylenchidae inhabit primarily cortical cells in roots and other below-ground tissues, whereas species in the other two

families parasitize the above- ground parts of plants. All life stages of migratory plant endoparasitic nematodes can be found in the soil or in plant tissues. They feed and reproduce during migration between or through plant cells. All plant endoparasitic nematodes secrete enzymes to degrade host tissues, some of them induce hormone imbalance that causes swelling of the tissue, and some induce lesions. *Bursaphelenchus xylophilus* is a migratory endoparasitic nematode that feeds on living trees as well as on fungi, which supports the hypothesis that plant feeding nematodes evolved from those feeding on fungi. Next to direct damage by migratory endoparasitic nematodes, they can also act as a vector for bacterial or fungal pathogens, and increase plant susceptibility to other pathogens (Moens & Perry, 2009, Jones *et al.*, 2005).

**Sedentary endoparasitic nematodes**

The most evolved and damaging nematodes in the world have a sedentary endoparasitic life style. This type of feeding has evolved three times independently, in which, false root-knot nematodes (*Nacobbus*) and the cyst-forming nematodes (*Heterodera* spp. and *Globodera* spp.) most likely evolved from migratory ectoparasitic nematodes, while root-knot nematodes (*Meloidogyne* spp.) appear to have evolved from migratory endoparasitic nematodes (Bert *et al*., 2011). Sedentary nematodes are obligate biotrophs, i.e., they can only feed on living plant cells.

Sedentary nematodes invade root tissues soon after hatching and then establish a permanent, stationary feeding location. Females do not move from that site for the rest of their life. The host range of root-knot nematodes encompasses more than 3,000 plant species and it is the economically most important genus; the cyst nematodes are the second major group of important plant-parasitic nematodes (Karssen *et al.*, 2006, Turner *et al.*, 2006). Second stage juveniles (J2) of both the root-knot and cyst nematodes are attracted to plant roots, and accumulate at the region of cell elongation just behind the root tip. After penetration, J2 of root-knot nematode migrate intercellularly to the root tip and turn around when they get to the root apex. Subsequently, J2 move back up in the vascular cylinder over a short distance, where they become sessile in the vascular tissue of the zone of elongation. However, J2 of cyst nematodes move intracellularly through cortical cells until they reach the pericycle and proceed to select a suitable feeding site. Both of them penetrate the roots and migrate by the combination of physical damage via thrusting of the stylet and breakdown of the cell wall by secreting degrading enzymes (Turner *et al*., 2006, Karssen *et al*., 2006, Gheysen *et al.*, 2006). A root-knot nematode selects 2–12 cells to initiate its feeding sites, those cells enlarge and are converted into multinucleate cells through synchronous nuclear divisions without cell division, and the feeding sites formed by root-knot nematodes are called giant cells (Fig 1.2A). Hyper-

plasia and hypertrophy of the surrounding cells lead to the formation of the typical root gall (Jones & Payne, 1978, Gheysen *et al*., 2006). Unlike root-knot nematodes, a cyst nematode selects one plant cell, plasmodesmata are widened by plant enzymes, then the cell walls are opened and adjacent protoplasts fuse, hundreds of adjacent cells incorporate to form the feeding sites for cyst nematodes: syncytium (Fig 1.2B, Gheysen & Fenoll, 2002). The mechanism of feeding site formation is not fully understood, however, it is clear that secretions from pharyngeal glands of nematodes play an important role in this process. For instance, the effector 19C07 from *Heterodera schachtii* was reported to interact with *Arabidospsis* auxin influx transporter LAX3 (Lee *et al.*, 2011), and manipulate auxin influx in syncytia, thereby facilitating their development (Davis *et al.*, 2000, Gheysen & Fenoll, 2002, Vanholme *et al.*, 2004, Gheysen *et al*., 2006, Hamamouch *et al.*, 2012).

After establishing a suitable feeding site, J2 undertake further feeding. Then they stop feeding and molt three times to undergo morphological changes to become pyriform-shaped adults (Fig 1.3B). A female root-knot nematode secretes a gelatinous matrix into which she extrudes a large number of eggs. The life cycle of a mitotic parthenogenic root-knot nematode is illustrated in Figure 1. 4.

Cyst nematodes do feed during J3 and J4 stages. The adult female of cyst nematodes is characterized by the tanning and drying (cutinization) of the body wall following fertilization and production of embryonated eggs (Karssen *et al*., 2006, Turner *et al*., 2006).

Above-ground symptoms of infection with sedentary endoparasitic nematodes include suppression of shoot growth, nutritional deficiencies such as chlorosis, temporary wilting and reduction of crop yields. In addition, infection by root-knot nematodes and cyst nematodes increases host susceptibility to other pathogens and nematodes (Bridge *et al.*, 2005).

Nematode under study: *Meloidogyne graminicola*

Rice is the most widely consumed staple food for a large part of the world's human population, especially in Asia. However, pests and diseases are serious problems in rice production. The soil borne diseases, especially those caused by plant parasitic nematodes, are becoming more economically significant since water saving systems have been increasingly implemented (De Waele & Elsen, 2007). More than 200 species of plant-parasitic nematodes have been reported to be associated with rice (Prot, 1994). Among these, the rice root-knot nematode, *Meloidogyne graminicola* is globally considered as the major nematode causing problems in rice production systems. *M. graminicola* was first described as a new species infecting grasses and oats in the USA in 1968 (Golden & Birchfield, 1968). Since then, it has been reported to have a broad host range including *Oryza sativa*, *Alopecurus sp*., *Avena sativa*, *Beta vulgaris*,

*Brachiaria mutica*, *Brassica juncea*, *Sphenoclea zeylanica*, *Spinacia Oleracea* and *Triticum aestivum* etc. (Dutta *et al.*, 2011). It has been found mainly on rice in South and Southeast Asia but also in South Africa, United States, Colombia, and Brazil (De Waele & Elsen, 2007). In recent years, it has become a pathogen in wheat in Nepal, India, Pakistan and Bangladesh in the rice-wheat production system (Pokharel *et al.*, 2007, Culman *et al.*, 2006, Sharma-Poudyal *et al.*, 2003, Sharma, 2001).

Second stage juveniles of *M. graminicola* cannot invade rice roots when the soil is under flooded conditions, but after draining off water, it can quickly infect host tissues (De Waele & Elsen, 2007). Yield damage of *M. graminicola* is greater under non-flooded, upland conditions with less water than in lowland conditions. Under simulated upland conditions, yield losses from *M. graminicola* ranged from 20% to 80% (Plowright & Bridge, 1990, Prot & Matias, 1995, Tandingan *et al.*, 1996), and in simulations of intermittently flooded rice, yield losses due to *M. graminicola* ranged from 11% to 73% (Soriano *et al.*, 2000).

Like all root-knot nematodes, the J2 of *M. graminicola* are attracted to the roots and penetrate the roots just behind the root tip. When J2 settle down and start to feed, giant cells (Fig 1.2A) are formed as a food source for the nematodes, meanwhile, neighboring cells start to divide and give rise to a typical hook-like gall in the root tips (Fig 1.3A), which is known as root-knots. J2 moult three times to become swollen females, which lay their eggs in a gelatinous matrix inside the roots (Fig 1.3B). In most of the cases, females go through parthenogenesis without fertilization by males (Dutta *et al.*, 2012). Completion of the life cycle from J2 to the second-generation J2 at 29/26 °C (day/night) under non-flooded conditions was 20 days (Fernandez *et al*., 2013).

The seriously impaired roots caused by *M. graminicola* infection lead to poor absorption of water and nutrients from the soil, and as a consequence, plant growth is stunted, leaves are chlorotic and plants mature early. When studying the effect of the soil type on *M. graminicola* infection, Soriano *et al.* (2000) found that nematodes in sandy loam soil caused more severe damage to plant growth than in clay loam soil.

There are not many options to control *M. graminicola*, continuous flooding appears to be effective (Kinh *et al.*, 1982), when the rice crop is flooded early and kept flooded until a late stage of development (Garg *et al.*, 1995, Soriano *et al*., 2000). By amending the soil with application of mass culture of two nematophagous fungi (*Arthrobotrys oligospora* and *Dactylaria eudermata*), Singh *et al.* ( 2007 ) found that the number of root galls to be reduced by 86.9% and 81.1%, and number of females by 94.2% and 91.7%, respectively. Resistant cultivars hold out the most promising and effective control for root-knot nematodes, and *Oryza*

*glaberrima* and *Oryza longistaminata* have proved to be resistant to *M. graminicola* (Soriano *et al.*, 1999, Plowright *et al.*, 1999).

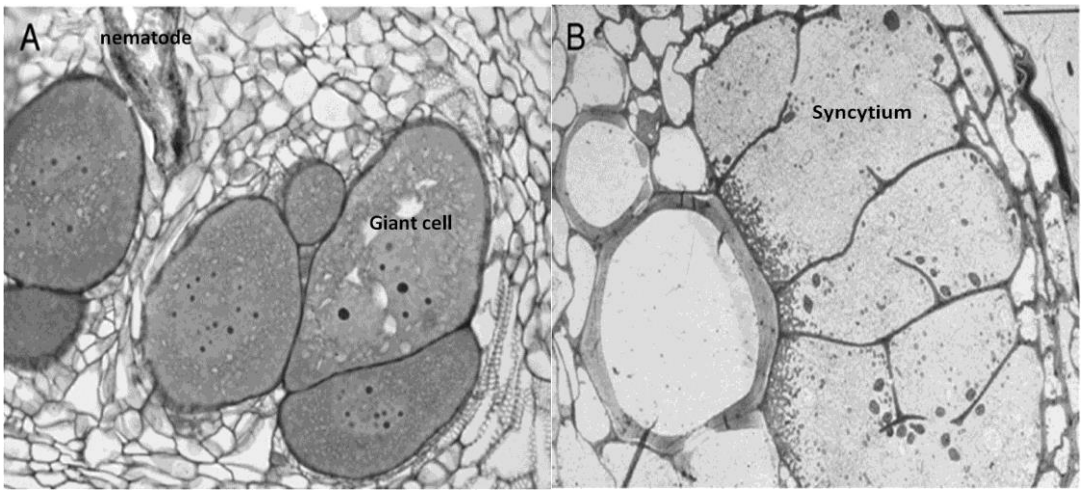


Fig 1.2 Nematode feeding sites (NFS). (A) Giant cells in rice roots caused by the rice root-knot nematode. (B) Syncytium in potato roots caused by the potato cyst nematode. Reproduced from (Haegeman *et al.*, 2012).

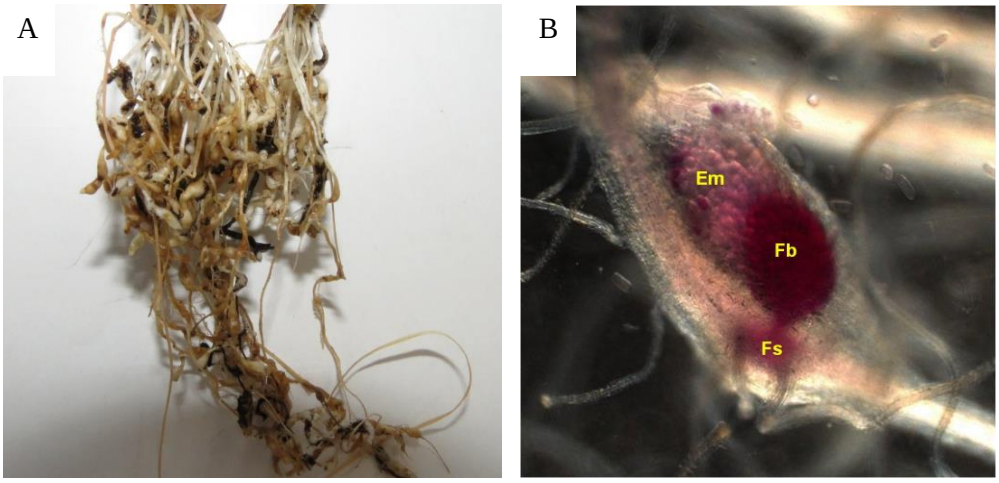


Fig 1.3 (A) Rice roots infected by *M. graminicola*. (B) Females of *M. graminicola* inside galls. Em: egg mass; Fb: Female body; Fs: Feeding site

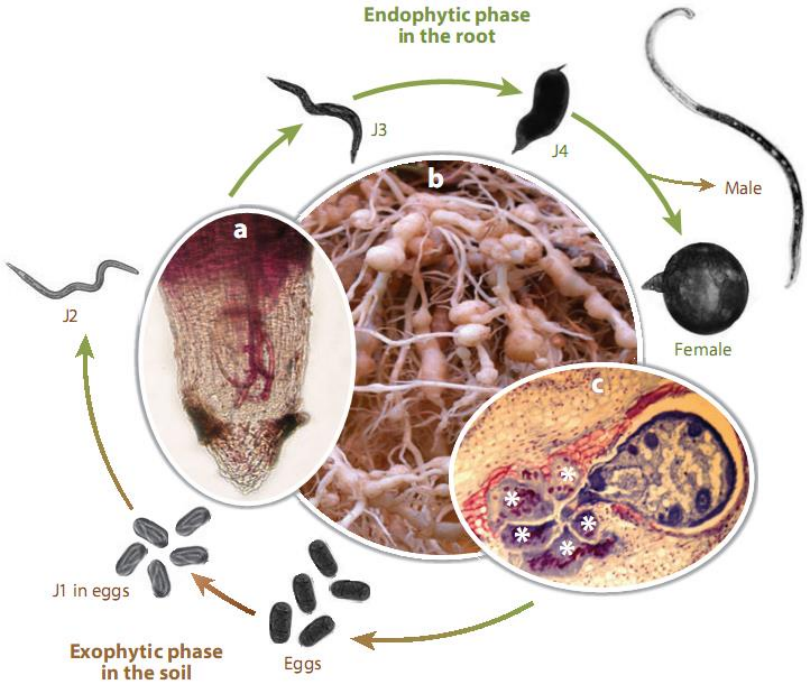


Fig 1.4 Life cycle of a mitotic parthenogenetic root-knot nematode. (a) Longitudinal section of a root tip showing second-stage juveniles (J2) (stained with acid fuschin) turning around at the root meristem to migrate into the vascular cylinder. (b) Typical symptoms (i.e. galls) on tomato roots. (c) Longitudinal section of an infested root showing a mature female and five giant cells (∗) constituting the nematode feeding site. Reproduced from Castagnone-Sereno *et al.* (2013).

### 1.1.3 Management and control of plant parasitic nematodes

#### Prevention

Prevention should be the first line of management. This strategy keeps nematode populations from infected sites away from clean sites. It involves certification of plant propagation materials, restricting nematode dissemination, cleaning machinery and irrigation water (Perry & Moens, 2006).

#### Chemical control

Chemical control consists of two broad methods: fumigants and non-fumigant nematicides. Fumigants perform best control in soils where there is adequate moisture. The high efficacy is related to their high volatility at ambient temperatures. The gas diffuses through the spaces of soil particles, and nematodes in between the spaces are killed. Non-fumigant nematicides are relatively easy to apply, the products are as granules or liquids, and they can be directly applied to the soil surface. The active ingredients are then released and spreaded by rainfall or irrigation. However, all nematicides are eventually degraded and when they are flushed

through the soil, it causes problems to groundwater or other soil organisms. In addition, some of them are also toxic to humans, and are expensive to develop. Because of the high and non-specific toxicity, and the public concerns about food safety and the adverse impact on the environment, the use of nematicides is becoming more and more restricted (Haydock *et al.*, 2013).

**Cultural control**

Cultural control of nematode problems encompasses crop rotation, fallow, multiple cropping, soil amendment, flooding, weed control, and use of natural enemies (Perry & Moens, 2006) .

**Plant resistance**

Resistant plants are defined as "plants that can reduce pathogen growth and do not have the ability to allow pathogen reproduction" (Gururani *et al*., 2008). Resistant varieties can be obtained by crossing naturally existing resistance or suitable genetic materials into commercial varieties. Or by genetic engineering which directly integrate a desired trait (i.e. resistance gene) into another plant genome by using biotechnology.

## 1.2 Plant innate immune system

Plants are subjected to attack by an armada of different microbial pathogens and parasites. During evolution, plants have developed a powerful immune system to arm with their attackers. The initial obstacle for the phytopathogens is plant pre-existing defence, such as structural barriers, including the plant cell wall, spines, wax on cell surface; or antimicrobial toxins. Once attackers are able to overcome the constitutive defence layers, they still face plant innate immunity which is triggered by two levels of microbial recognition. The first branch of plant immunity occurs at the plant cell surface. Pathogen- or Microbial-associated molecular patterns (PAMPs/MAMPs) such as fungal chitin, bacterial flagellin or bacterial lipopolysaccharides are conserved molecular signatures in many microbes. They are perceived by plant transmembrane pattern recognition receptors (PRRs), resulting in activation of multiple downstream defence signaling events to achieve a basal level of resistance, this immunity is called PAMP-triggered immunity (PTI) (Fig 1.5, Chisholm *et al.*, 2006, Spoel & Dong, 2012). PTI leads to physical isolation of the pathogens through producing ion fluxes, callose, phenolics and active oxygen production, as well as activation of a downstream mitogen activated protein kinase cascade, transcriptional changes and production of pathogenesis-related (PR) proteins and phytoalexins (Spoel & Dong, 2012). The best characterized PAMP in plants is the bacterial elicitor flagellin, which is recognized in the plant model *Arabidopsis thaliana* by leucine-rich repeat receptor-like kinases (LRR-RLK), FLAGELLIN-SENSING 2 (FLS2).

FLS2 directly binds to flg22 and interacts with brassinosteroid receptor BRI1-associated receptor kinase 1 (BAK1) to trigger the basal defence signaling (Chinchilla *et al.*, 2007, Zipfel, 2008). In the model monocot rice (*Oryza sativa*), the rice homologue OsFLS2 has been demonstrated to act as a functional fagellin receptor (Takai *et al.*, 2008), and a variety of different PAMPS have been shown to be active in rice, including bacterial lipopolysaccharides (Desaki *et al.*, 2006) and chitin (Kishimoto *et al.*, 2010).

During evolution, adapted pathogens acquired the ability to secrete effectors directly into the plant cell to circumvent PTI. In turn, through co-evolution with pathogens, plants have developed intracellular immune receptors known as resistance (R) proteins that can directly or indirectly recognize pathogen effector molecules which are termed avirulence (Avr) proteins, resulting in activating effector triggered immunity (ETI, Fig 1.5, Jones & Dangl, 2006, Spoel & Dong, 2012). ETI is a faster and stronger version of PTI that typically associates with programmed cell death of the infected cells, this hypersensitive response (HR) limits pathogen to the initial infected point and restricts access to water and nutrients. ETI also leads to the production of antimicrobial molecules, and massive generation of ROS, leading to local resistance to the pathogen (Greenberg & Yao, 2004, Glazebrook, 2005). Most R genes encode nucleotide-binding leucine-rich repeat (NB-LRR) proteins (Collier & Moffett, 2009). NB-LRR proteins can recognize diverse pathogen effectors and activate similar local and systemic defence responses, which involves hormone networks, ROS-generation, and gene expression. LRR-mediated disease resistance is effective against obligate biotrophs, and hemibiotrophic pathogens but not against necrotrophs ( Fig 1.5, Jones & Dangl, 2006 ).

There are two ways that effectors can be recognized by R genes, direct recognition and indirect recognition (Jones & Dangl, 2006, Spoel & Dong, 2012). Direct recognition happens if one effector is recognized by a corresponding NB-LRR protein. For instance, Amrita, a *Magnaporthe grisea* effector, is recognized directly by the rice NBS-LRR protein PITA and this induces a Pi-ta-mediated defence response, such as cell death to stop the spread of *M. grisea* (Jia *et al.*, 2000). However, for many R genes in plant genomes, this gene for gene model cannot explain the broad immune capacity of plants; therefore, the "guard hypothesis" was proposed and proven (Jones & Dangl, 2006). Several R proteins recognize type III effectors indirectly, as they "guard" self-proteins which are perturbed or modified by pathogen effectors, and then R protein activation is triggered (Jones & Dangl, 2006 ). The best studied R proteins guarded cellular target is the *Arabidopsis thaliana* protein RPM1-INTERACTING PROTEIN4 (RIN4). Without pathogen challenge, R proteins will detect unmodified RIN4, and will maintain in an inactive state. Once a pathogen such as *Pseudomonas syringae* attacks, and injects effector molecules into the plant cell, the RIN4 will be phosphorylated by a certain

kinase, the modified RIN4 will be detected by R proteins and this leads to their activation and triggers effector-triggered immunity (McHale *et al.*, 2006).

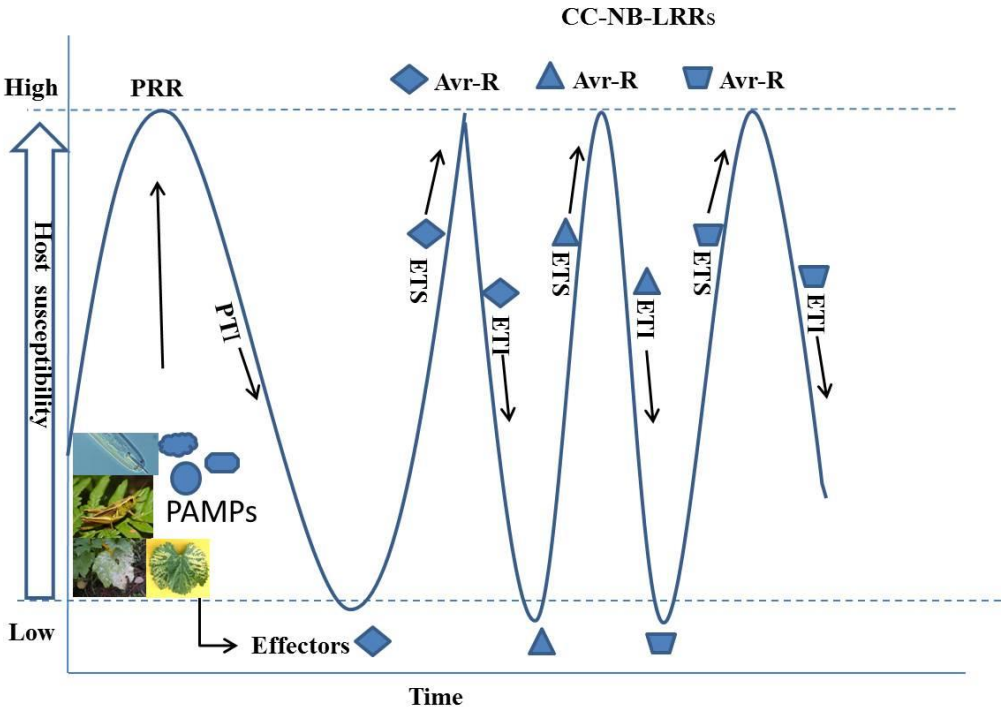


Fig 1.5 A zig-zag model illustrates the quantitative output of plant immune system. Adapted from Jones and Dangl (2006). PRR: Pattern recognition receptor, CC-NB-LRR: Coiled-coil-nucleotide-binding site-leucine-rich repeat.

Nematode resistance genes

A few nematode resistance genes have been mapped and cloned from agronomically important crops like potato, tomato, beet, pepper, soybean and cereals. Some examples are illustrated in this section.

The first cyst nematode resistance gene identified was *Gpa2* from *Solanum tuberosum* (Van Der Vossen *et al.*, 2000). This NBS-LRR gene contributes resistance of potato to the cyst nematode *Globodera pallida*. Additionally, another R gene *Hero* against potato cyst nematode from tomato confers high level of resistance to another potato cyst nematode *Globodera rostochiensis* and partial resistance to *Globodera pallida*.

The first root-knot nematode resistance gene *Mi1* was identified from tomato, there are three homologues, *Mi-1.1, Mi-1.2* and one pseudogene (Rossi *et al.*, 1998, Vos *et al.*, 1998). *Mi-1.2* belongs to an NBS-LRR super family of R genes and not only exhibits resistance to root-knot nematodes but also to potato aphid and whitefly *Bemisia tabaci* (Rossi *et al.*, 1998, Vos *et al.*, 1998, Nombela *et al.*, 2003, Goggin *et al.*, 2006). In addition, an *Mi-1* gene homolog *Rpi-blb2*

gene from *Solanum bulbocastanum* confers a broad spectrum resistance against *Phytophthora infestans* in potato (Vossen *et al.*, 2005).

There are two major types of resistance responses in the incompatible plant-nematode interactions related to R genes. The first type is characterized by a rapidly induced hypersensitive response in the early stage of feeding site formation accompanied by the accumulation of phenylpropanoid compounds, necrosis, and subsequently feeding site development will be arrested (Table 1.1A, Bakker *et al.*, 2006).

Another type is HR-independent response, which is characterized by degeneration of feeding cells at the late stages of the infection process. At the initial stage, there is no difference in feeding site morphology between compatible and incompatible interactions, however, during a later stage, the feeding cells in the resistant plants contain less dense cytoplasm and more vacuoles compared to the feeding cells in susceptible plants. Moreover, there are also no clear connections with surrounding cells, in the end, feeding cells become necrotic and degenerate, resulting in less developed females (Table 1.1B, Bakker *et al*., 2006).

The resistance mechanism of some R genes against cyst and root-knot nematode infection is summarized in Table1.1, adapted from Bakker *et al*. (2006).

Table 1.1A Overview of the reported mode of actions of plant resistance genes against nematodes. Those R genes rapidly induce HR responses in the early stage of feeding site formation.

| Host plant | Nematode | R gene | Reference |
|---|---|---|---|
| Tomato | *M. incognita* | *Mi1* | (Paulson & Webster, 1972) (Riggs & Winstead, 1959) |
| Potato | *G. rostochiensis* | *H1* | (Rice *et al.*, 1985) |
| Soybean | *H. glycines* | ? | (Mahalingam & Skorupska, 1996) |
| Soybean | *H. glycines* | ? | (Endo, 1991) |
| Pepper | *M. incognita* | *Me3* | (Bleve-Zacheo *et al.*, 1995) |
| *Arabidopsis* | *H. glycines* | Non-host | (Grundler *et al.*, 1997) |
| Wheat | *H. avenae* | ? | (Bleve-Zacheo *et al*., 1995) |

Table 1.1B Overview of the reported mode of actions of plant resistance genes against nematodes. Those R genes induce the degeneration of feeding cells at the late stages of the infection process, and they do not induce HR responses.

| Host plant | Nematode | R gene | Reference |
|---|---|---|---|
| Wheat | *H. avenae* | ? | (Williams & Fisher, 1993) |
| Barley | *H. avenae* | *Ha2, Ha3* | (Seah *et al.*, 2000) |

| | | | |
|---|---|---|---|
| Sugar beet | *H. schachtii* | *Hs1pro−1* | (Yu, 1984) |
| Pepper | *M. incognita* | *Me1* | (Bleve-Zacheo *et al.*, 1998) |
| Common bean | *H. glycines* | ? | (Becker *et al.*, 1999) |
| Tobacco | *M. incognita, M. acrita* | ? | (Powell, 1962) |
| Potato | *G. pallida* | *Gpa2* | (Goverse *et al.*, 2001) |
| Potato | *G. pallida, G. rostochiensis* | *Grp1* | (Rice *et al.*,1987) |

## 1.3 Molecular aspects of nematode and plant interactions

### 1.3.1 Nematode genes involved in parasitism

The compounds that are secreted by a nematode into the host tissues in order to promote parasitism and subsequently alter plant physiology are considered as nematode effectors. They have evolved specifically or may have been modified from other successful pathogens (Davis *et al.*, 2000). The main source of the effectors is from one dorsal gland (DG) and two subventral glands (SVG), which are the transcriptionally active pharyngeal glands (pharyngeal gland: large secretory cell, having enzymatic functions and variable secretory activities). The secretions enter the nematode stylet, and are injected into plant cells in order to establish parasitism. Although effector proteins are mainly produced from the pharyngeal glands, proteins from amphids (amphids: invaginations of cuticle in the anterior of nematodes, chemosensory organs) and epidermis also play a role in the parasitic process (Jones *et al.*, 2000, Semblat *et al.*, 2001). Cuticle associated molecules may be essential for nematodes to avoid host defence, and the compounds secreted by amphids may be involved in perception of environmental stimuli, or may act as signaling molecules during the interaction with host plants (Curtis, 2007, Perry, 1996, Semblat *et al.*, 2001).

Degradation of the plant cell wall by nematodes during penetration and migration

The plant cell wall represents a formidable barrier to plant parasitic nematodes, both for those that the bodies remain outside cells (ectoparasite) or those that need to penetrate and migrate in plant roots (endoparasite). It is a thick and rigid structure; the major carbohydrates making up the plant primary cell wall are cellulose, hemicellulose and pectin. The nematodes can disrupt the plant cell wall with their stylet in combination with pharyngeal gland secretions to mediate penetration and migration through plant tissues (Sijmons *et al.*, 1994, Hussey *et al.*, 1998). Lots of evidence demonstrates that nematodes secrete cell wall degrading enzymes to assist in this process. Smant *et al*. (1998) identified two beta-1, 4-endoglucanases (EGase) which belong to family 5 of the glycosyl hydrolases from two cyst nematode species: *Globodera rostochiensis* and *Heterodera glycines,* respectively. *In situ* hybridization showed the four EGases are expressed in the SVG. This was the first example of non-symbiotic degradation of cellulose in plant cell wall by an animal (Smant *et al.*, 1998). The cyst nematode

EGase was remarkably similar to bacterial members of glycosyl hydrolase family 5 (GHF5) (Henrissat & Bairoch, 1996), which was the first evidence of genes acquired through horizontal gene transfer (HGT) from prokaryotes to eukaryotes (Davis *et al*., 2000, Keen & Roberts, 1998, Smant *et al*., 1998, Gogarten *et al.*, 2002, Jones *et al*., 2005, Haegeman *et al.*, 2011). Since then, many endoglucanase genes from other PPN species have been identified (Davis *et al.*, 2008, Haegeman *et al.* 2012, Davis *et al.*, 2011). Most of those endoglucanases belong to GHF5. Interestingly, the endoglucanases present in *Bursaphelenchus xylophilus* belong to GHF45, which show highest homology to fungal sequences. This is logic because these endoglucanases could have been obtained from fungi by HGT since *B. xylophilus* is a fungal feeder (Kikuchi *et al*., 2004).

Besides endoglucanases, other cell wall degrading enzymes that act on other sugar polymers as well as other proteins without any hydrolytic activity, including expansin and cellulose binding proteins, have also been identified in plant parasitic nematodes, a more detailed description of those proteins from different PPN are can be found in Davis *et al*. (2011) and Haegeman *et al*. (2012).

Effectors involved in feeding site formation and host defence suppression

While many parasitism genes that are expressed in the subventral glands (SVG) produce proteins that assist in penetration and migration, the dorsal gland (DG) cell is more active during and after establishing the feeding cells. Many stylet secretions from cyst and root-knot nematodes contain effectors that are active during the sedentary phase of parasitism, either for feeding site induction or for suppressing host defence. They are produced in the DG or in both DG and SVG. In the following paragraphs, some effectors involved in feeding site formation and host defence suppression are being described as examples.

Among pharyngeal gland genes from the root-knot nematode *Meloidogyne javanica*, one clone that codes for a potentially secreted chorismate mutase (CM) was characterized (Lambert *et al.*, 1999). Later on, CM was found to be produced by a broad range of PPN as an effector. CM is a key regulatory enzyme in the shikimate pathway in plants and bacteria, and except it, there are no other enzymes from this pathway present in PPNs (Haegeman *et al*., 2012). The authors hypothesized that CM from nematodes might play an essential role in nematode parasitism on plants, however, it is still not clear how. *Meloidogyne javanica* chorismate mutase 1 (MjCM-1) has been shown to lower indole-3-acetic acid (IAA) by causing a competition for the common substrate chorismate, resulting in an alteration of chorismate-derived metabolites and plant cell development (Doyle & Lambert, 2003). Recently, it was found that Cmu1, a constitutively active chorismate mutase secreted by *Ustilago maydis* is taken up into the cytosol of its host maize cells and competes with the endogenous SA bio-

synthesis pathways for chorismate. This results in limiting defence responses triggered by salicylic acid. Therefore, it was suggested that chorismate mutase could modulate auxin levels or salicylic acid mediated defences of the host (Djamei *et al.*, 2011).

Secretory peptides that shared homology to a C-terminal motif of the CLAVATA3/ESR-related (CLE) family in *Arabidopsis thaliana* were identified from the parasitic stage of soybean cyst nematode *Heterodera glycines* (Wang *et al.*, 2001, Olsen & Skriver, 2003, Gao *et al.*, 2003), and more recently from *Heterodera schachtii* and the potato cyst nematode *Globodera rostochiensis* (Wang *et al.*, 2011, Lu *et al.*, 2009, Patel *et al.*, 2008). Expression of the peptide from *H. glycines* in *Arabidopsis thaliana* can rescue the *A. thaliana* (*At*) *clv3-1* mutant. The intriguing similarity between host and parasitic sequences could be an example of adaptive molecular mimicry, although the biological function of these peptides mimics in feeding cell formation is unclear at present. CLAVATA3 (CLV3) is known to be involved in cell-fate determination in meristem formation, and those peptides possibly trigger developmental changes necessary for syncytium formation (Olsen & Skriver, 2003, Wang *et al.*, 2005, Replogle *et al.*, 2011, Replogle *et al.*, 2013, Mitchum *et al.*, 2008). A root-knot nematode gene, *16D10*, which is expressed in the subventral gland cells, encodes a 13 amino acid secretory peptide that has some homology to the C-terminal CLE motif of plant CLE peptides (Huang *et al.*, 2003). However, it cannot rescue *clv3* mutant phenotypes in *A. thaliana* (Huang *et al.*, 2006b). Transgenic plants with RNA silencing against this peptide exhibited a broad root-knot nematode resistance (Huang *et al.*, 2006a). However, the real function in parasitism is still unknown. Cyst and root-knot nematodes probably target similar plant processes to initiate and maintain feeding sites. Nevertheless, they apply different molecular means to accomplish their goals, as illustrated in the case for CLE peptides (Mitchum *et al.*, 2013).

Being biotrophic pathogens, sedentary nematodes have to keep their feeding sites alive and functional for all the developing stages. In order to achieve this successful interaction, root-knot and cyst nematodes need to suppress host defence. To date, no nematode proteins or cuticle compounds triggering PTI have been identified. *Mi-CRT* (CRT: calreticulin) from *M. incognita* was demonstrated to suppress PTI. *Mi-CRT* overexpression in *A. thaliana* suppressed the induction of defence marker genes and callose deposition after treatment with the pathogen-associated molecular pattern elf18 (Jaouannet *et al.*, 2013). At present, there are few nematode effectors that have been linked experimentally to ETI. The best example of nematode effectors linked to ETI is the Gp-RBP-1 SPRYSEC gene from *Globodera pallida* (Sacco *et al.*, 2009). Transient co-expression of *Gp-RBP-1* together with the nematode resistance gene *Gpa2* (CC-NB-LRR) triggered HR response in plant leaves (Smant & Jones, 2011, Rehman *et al.*, 2009). Another example is the root-knot nematode gene *Mi-Cg1* which appears to be involved in triggering an immune response in tomato plants carrying the *Mi-1.2*

resistance gene (Goggin *et al*., 2006, Gleason *et al*., 2008). Researchers found that continuously culturing an avirulent strain of *M. javanica* carrying *Cg1* on resistant tomato carrying *Mi-1.2* would result in the loss of *Cg1* and *M. javanica* becoming virulent. Silencing the *Cg1* gene in the avirulent strain by RNA interference conferred virulence to this strain on tomato carrying *Mi-1.2*, thus, implying a role of *Cg1* in triggering *Mi-1* mediated resistance. The mechanism behind the interaction of Cg1 with the Mi-1.2 protein is currently unknown (Hewezi & Baum, 2013, Gleason *et al*., 2008).

Through the approach of transcriptomic and genomic sequence mining of nematodes, large panels of candidate effectors have been identified, some of them used to protect the nematodes during migration, some involved in ubiquitination, and some targeting plant signaling pathways (Mitchum *et al*., 2013, Haegeman *et al*., 2012, Smant & Jones, 2011, Quentin *et al.*, 2013, Gheysen & Mitchum, 2011). Transcriptomic studies of the pharyngeal glands combined with genome studies will truly expand our understanding of effector repertoires and their variability among genera, species and pathotypes. The approaches that allow high-throughput interactor screens, such as yeast two-hybrid analyses, *in planta* bimolecular fluorescence complementation (BiFC), will help to study the function of those effectors in parasitism ( Mitchum *et al*., 2013).

### 1.3.2 Plant genes involved in feeding site formation

As biotrophic pathogens root-knot and cyst nematodes induce remarkable reprogramming of (un)differentiated root cells during their feeding site formation, induction and suppression of host genes seem to be essential to establish this long-term interaction with plants.

The role of plant hormones in nematode feeding sites

Many genes differentially expressed in response to nematodes are involved in the hormone pathways. Plant hormones are not only involved in feeding site formation, but also in plant defence. Biotrophs need to manipulate the balance between their own feeding site development and suppression of plant defence. The major hormonal changes occurring in nematode feeding site (NFS) and surrounding tissues deduced from the expression of hormone biosynthesis and degradation genes, and hormone responsive genes were summarized minutely by Kyndt *et al.* (2013a). In the following paragraphs, some examples are described.

Previous studies have revealed that auxin accumulated in galls and feeding cells of root-knot and cyst nematodes (Karczmarek *et al.*, 2004, Grunewald *et al.*, 2009). Further examination of the role of auxin in feeding cell development by using auxin transport and signaling mutants has shown that there is significantly less infection by both root-knot and cyst nematodes in those mutants compared to control plants (Grunewald *et al.*, 2009, Goverse & Bird, 2011).

These results suggest that auxin plays an important role in NFS formation and maintenance, and further assists nematode development.

Ethylene (ET) is significantly attenuated in the gall tissue and feeding sites at early time points (Kyndt *et al.*, 2013a). However, functional analysis of *Arabidopsis* transgenics over-producing ET resulted in more cyst nematodes in plant roots, and histological analysis showed the enlargement of the syncytia in ET-overproducing plants (Wubben *et al.*, 2001, Goverse *et al.*, 2000).

As seen in Figure 1.6, the defence related hormone salicylic acid (SA) was strongly attenuated in 3 days after inoculation (dai) galls in rice (Kyndt *et al.*, 2012a) and in *Arabidopsis* (Barcala *et al.*, 2010). This may be due to root-knot nematodes strongly suppressing plant defence. However, in the case of cyst nematodes, the SA pathway genes were up-regulated in the surrounding tissue of syncytia at early time points, the damage to the cells during cyst nematode intracellular migration probably contributes to the up-regulation of defence related genes.

The role of other hormones involved in NFS has been illustrated very minutely in the following reviews: Kyndt *et al*. (2013a) and Goverse & Bird (2011).

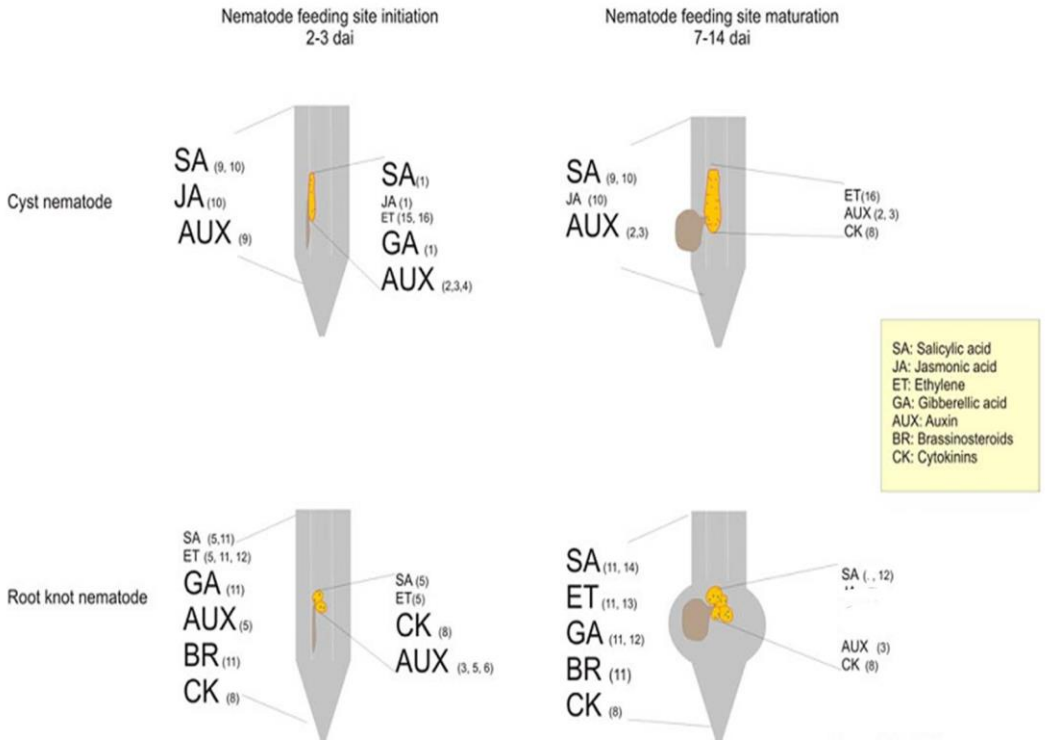


Fig.1.6 Schematical representation of the transcriptional changes in plant hormone pathways upon nematode infection in a compatible interaction with plant roots and in isolated nematode feeding sites. Activation or repression is shown in comparison with the corresponding uninfected root tissue, and is

illustrated as big or small lettering, respectively. For hormones not indicated on the graph the data are contradictory, or no data are available. Reproduced from Kyndt *et al*. (2013a). Reference: (1) (Ithal *et al*., 2007a) (2) (Goverse *et al*., 2000) (3) (KARCZMAREK *et al*., 2004) (4) (Grunewald *et al*., 2009) (5) (Barcala *et al*., 2010) (6) (Hutangura *et al*., 1999) (8) (Lohar *et al*., 2004) (9) (Ithal *et al*., 2007b) (10) (Alkharouf *et al*., 2006) (11) (Kyndt *et al*., 2012a) (12) (Jammes *et al*., 2005) (13) (Glazer *et al*., 1985) (14) (Ibrahim *et al*., 2011) (15) (Hermsmeier *et al*., 2000) (16) (Ali *et al*., 2013)

Plant cell cycle genes in nematode induced feeding sites

Root-knot and cyst nematodes feed for a long period from the feeding sites they formed, which have high metabolic activity. As seen by a dense cytoplasm, multiple enlarged nuclei, and small vacuoles. Activation of the cell cycle has been demonstrated to be crucial for normal feeding site development and subsequent nematode growth and reproduction (de Almeida Engler *et al*., 1999, Van de Cappelle *et al*., 2008). Functional analysis of endoreduplication related genes demonstrated their strong implication in endoreduplication process taking place in giant cells and syncytia. A reduction in feeding-cell size, severely impaired nematode maturation and reproduction were observed in plants in which the cell endocycle was inhibited. Not only endoreduplication, but also mitosis plays an important role in feeding cell formation. Mitosis occurs in giant cells for getting abundant nuclei and also in surrounding cells prior to incorporation into syncytia. When mitosis was blocked by oryzalin, giant cells were less dense compared to untreated ones and a large number of nematodes failed to mature. Similarly, treated syncytia were narrow compared to untreated ones and with slightly fewer mature females. Manipulation of the precise balance between mitosis and endoreduplication in feeding cells is crucial for nematode survival and reproduction (de Almeida Engler & Gheysen, 2013).

Besides cell cycle activation, plant cytoskeleton remodeling also occurs in feeding site formation, it has been discussed by de Almeida Engler & Favery (2011), Kyndt *et al*. (2013a), and Caillaud *et al.* (2008).

A global view on the changes in gene expression in feeding sites

To study the alteration of plant organ development such as changes in cell cycle, hormone regulation, and cell wall architecture by nematode infection, and to get a global view of transcriptome changes during feeding cell formation in compatible or incompatible interactions, tools including cDNA subtraction, promoter–β-glucuronidase fusions, *in situ* hybridizations, (*in situ*) reverse-transcription polymerase chain reaction (RT-PCR), and microarrays have been exploited (Gheysen *et al*., 2006).

However, as giant cells and syncytia make up only a small fraction of the root cells, the analysis of gene expression only at the feeding cells is required. Laser capture microdissection (LCM) which originally was developed as an effective tool for the isolation of individual cells in mammalian systems (Bonner *et al.*, 1997, Emmert-Buck *et al.*, 1996), allows to isolate and clone mRNA populations from individual target cells within complex tissues. This technique coupled with microarray was successfully employed for capturing giant cells and syncytia. Data generated by this combination provide us a deep understanding of the plant genes truly involved in feeding site formation or maintenance (Klink *et al.*, 2007, Ithal *et al*., 2007b, Portillo *et al.*, 2009, Fosu-Nyarko *et al.*, 2009, Barcala *et al*., 2010, Portillo *et al.*, 2013, Klink *et al.*, 2010, Damiani *et al.*, 2012).

More recently, the deep sequencing technology RNAseq was successfully applied to study the transcriptome reprogramming in rice roots caused by nematodes (Kyndt *et al*., 2012a). RNAseq allows identification of novel transcripts which are not covered in microarrays. Through analyzing the local transcriptional changes in rice roots upon *Meloidogyne graminicola* and *Hirschmanniella oryzae* (root rot nematode) infection, the authors found genes involved in metabolic pathways and nutrient transport to be enhanced in the induced root gall. However, genes participating in local defence were suppressed. For example, the antimicrobial peptide OSTHI7 was strongly down-regulated in 3dai galls. The migratory *Hirschmanniella oryzae*, on the other hand, induce programmed cell death and oxidative stress, and obstruct the normal metabolic activity of the root. The different cellular responses in plants to the two distinct feeding behavioral nematodes provide an unprecedented insight into the compatible interaction between nematodes and plant roots (Kyndt *et al*., 2012a).

## 1.4 Induced resistance

After plants are exposed to certain biotic or abiotic stimuli, they can express an enhanced defensive capacity to subsequent pathogen infections, and this resistance is commonly referred to as induced resistance (IR). This enhanced state of resistance is effective against a broad range of pathogens and parasites, including fungi, bacteria, viruses, nematodes, parasitic plants, and even insect herbivores (Choudhary *et al.*, 2007).

Induced resistance is a form of resistance caused by activating the host's own genetically programmed defence pathways, leading to a fast and strong response upon pathogen infection (Hammerschmidt, 2007). Defence responses can be activated, or primed for rapid activation, in locally infected sites or in systemic tissues. Induced resistance can be split broadly into systemic acquired resistance (SAR), induced systemic resistance (ISR), BABA-induced resistance (BABA-IR) and wound induced resistance (WIR).

### 1.4.1 Systemic Acquired Resistance

The first detailed description of systemic acquired resistance was tobacco mosaic virus (TMV) induced resistance in tobacco (Ross, 1961). A zone surrounding TMV caused lesions on a hypersensitive host became highly resistance to subsequent inoculation of the same leaf and even extended to distant upper leaves, resulting in much smaller, and occasionally fewer lesions. This resistance is referred to as systemic acquired resistance (SAR, Ross, 1961). Subsequent work has shown that systemic induction of disease resistance in plants by necrotizing pathogens is a general phenomenon, and occurs in many di- and mono-cotyledonous species (Hammerschmidt, 1995, Sticher *et al.*, 1997).

SAR refers to the enhanced resistance that occurs against a broad and distinctive spectrum of pathogens, and is triggered by pathogen- induced localized necrosis. The inducing necrosis is either part of the HR or a symptom of disease (Durrant & Dong, 2004, Maleck & Dietrich, 1999). Within a few hours of the localized necrosis, the plant begins to express a subset of pathogenesis related genes both locally and systemically, and the signal molecule salicylic acid plays an important role in this process (Antoniw & White, 1980, Van Loon, 1985, Gaffney *et al.*, 1993).

The SAR state is activated by many microbes that cause tissue necrosis but it can also be induced by exogenous application of SA or its functional analogs INA (2,6-dichloroisonicotinic acid), BTH (benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester) (Ryals *et al*., 1996, Sticher *et al*., 1997). Both INA and BTH induce a broad spectrum resistance and PR gene expression in the same way as biological inducers of SAR, they interact with the SAR signal pathway either at or downstream of the site of SA, since both of them induce SAR in SA deficient plants (Shah, 2003, Delaney, 2010).

Signals in Systemic Acquired Resistance

SAR has been studied extensively in the two dicot models tobacco and *Arabidopsis*. The onset of SAR is accompanied by the increased accumulation of the signaling hormone salicylic acid (Yalpani *et al.*, 1991). The first insight that SA might participate in SAR came from experiments by White. By exogenous injection of acetylsalicylic acid (aspirin) or SA into tobacco leaves, they reported induced resistance to subsequent infection by TMV and accumulation of PR proteins (White, 1979). Since then, SA has been proven as an essential signal for SAR across a range of plants, although the mechanisms by which SA induced SAR might differ (Conrath *et al.*, 2006, Durrant & Dong, 2004). Further support for the importance of SA for SAR came from studies with mutants and transgenic plants that exhibit altered levels of SA. Removal of SA in transgenic tobacco and *Arabidopsis* plants that produce the bacterial SA-degrading enzyme salicylate hydroxylase (NahG) which converts SA to catechol results in

more susceptibility to subsequent disease challenge and these plants are unable to mount an SAR response after biological induction (Gaffney *et al*., 1993, Ryals *et al*., 1996). NONEXPRESSER OF PR GENES 1 (NPR1) is a transcription factor activator that is present in the cytosol in an oligomeric form in the absence of SA, when SA accumulates in the cytosol, NPR1 is monomerized and enters the nucleus to interact with transcription factors, leading to PR gene activation (Mou *et al.*, 2003). Its expression is likely mediated by WRKY transcription factors, as mutation of the WRKY binding sites (W-boxes) in the *NPR1* promoter abolished its expression (Yu *et al*., 2001). Plants overexpressing *NPR1* did not contain enhanced SA levels or increased PR gene expression. However, more rapid or higher induction of PR genes was observed in these over-expressing lines after pathogen attack (Cao *et al.*, 1998, Friedrich *et al.*, 2001).

SAR was first detected 2–3 days after primary virus infection and reached a maximum after 7 days, demonstrating that plants require time to generate, transport and deliver the long-distance signal that induces resistance in the upper leaves (Ross, 1961). Recently, Park and co-workers provided evidence that methyl salicylic acid (MeSA), rather than SA, functions as the critical mobile signal by grafting experiments in tobacco. MeSA esterase, which converts MeSA back into SA, is required for signal perception in systemic tissues (Park *et al.*, 2007). However, although *A. thaliana* mutants that lack salicylic acid methyltransferases failed to accumulate MeSA, they could still activate SAR, and it has been found that most of the MeSA produced by *A.thaliana* during infection is evaporating as a volatile (Attaran *et al.*, 2009). Hence, there must be other molecules also serving as SAR signals. Indeed, besides MeSA, several other mobile SAR signals have been discovered in *Arabidopsis*, such as glycerol-3-phosphate (Chanda *et al.*, 2011), azelaic acid (Jung *et al.*, 2009), dehydroabietinal (Chaturvedi *et al.*, 2012). Those diverse signals are highly condition dependent, the network between those signals is considered as a safety mechanism to prevent cost-intensive immune response in different defence systems (Dempsey & Klessig, 2012).

Compared with dicots, the knowledge of SAR in monocots is scarce. In rice, five *NPR1*-like genes have been identified, among which *OsNPR1* (also called *OsNH1*) is the closest *AtNPR1* homolog (Yuan *et al*., 2007). Over-expression of endogenous *OsNPR1* resulted in an enhanced resistance to *Xanthomonas oryzae* pv. *oryzae* and the blast fungus *Magnaporthe oryzae* in rice (Yuan *et al.*, 2007). Transgenic rice plants expressing *NahG* increased the susceptibility against *Magnaporthe grisea* (Yang *et al.*, 2004), although PR gene expression profiles were not changed. Rice has high basal levels of free SA that are only weakly responsive to pathogen attack, however, SA plays an important role to modulate redox balance and protects rice plants from oxidative damage caused by aging, pathogen attack, or abiotic stress (Yang *et al*., 2004).

The WRKY family of transcription factors has been suggested to play an essential role in controlling defence gene regulation through the W-box motif in their promoter regions (Rushton *et al.*, 1996, Eulgem *et al.*, 1999, Maleck *et al.*, 2000). Rice has a large WKRY transcription factor family; OsWRK45 has been shown to be transcriptionally up-regulated after SA and BTH treatments. BTH induced resistance against *Xanthomonas oryzae* pv. o*ryzae* and *Magnaporthe oryzae* but is compromised in *OsWRKY45* knockdown rice. *OsWRKY45* over-expressing rice plants show an excellent resistance to both of these pathogens, although not to the sheath blight pathogen *Rhizoctonia solani* (Shimono *et al.*, 2012, Shimono *et al.*, 2007, Takatsuji *et al.*, 2010). Meanwhile, most genes up-regulated by BTH treatment are regulated by OsWRKY45 and many are directly associated with plant defence (Takatsuji *et al.*, 2010). All the results suggest that OsWRKY45 plays an important role in SA mediated plant defence. Epistasis analysis revealed that WRKY45 is involved in a signaling pathway downstream of SA but independent of OsNPR1 in the SA conduit (Shimono *et al.*, 2007).

Application of SA against plant-parasitic nematodes

Many studies have been carried out to investigate the role of SA against plant-parasitic nematodes. Foliar application of BTH to glasshouse *Cabernet sauvignon* grapevines caused a significant reduction in mature females and egg deposition in root-knot nematode species (Owen *et al.*, 2002). Similarly, foliar spray of SA to okra and cowpea 24h before inoculation with *M. incognita* reduced nematode infestation (Nandi *et al.*, 2003), and the same result was also observed upon the foliar application to tomato against *M. incognita* (Zinovieva *et al.*, 2011). Moreover, soil-drench with SA markedly reduced *M. incognita* reproduction (less than 50% that of untreated plants) on susceptible tomato roots, and this treatment caused a long-lasting induction of plant defence as even the infestation by the second generation of the nematode was inhibited (Molinari & Baser, 2010). It has also been shown that SA plays a critical role in the defence response mediated by the *Mi-1* root-knot nematode resistance gene. Tomato plants carrying the *Mi-1* gene and expressing the *NahG* gene, partially lost the resistance against root-knot nematodes (Branch *et al.*, 2004) and aphids (Li *et al.*, 2006b). When supplied with BTH, the resistance was completely restored (Branch *et al.*, 2004). Exogenous application of BTH on the shoots of *Oryza sativa* L. cv. Nipponbare induced a systemic defence in rice against root-knot nematode *M. graminicola*, and *NahG* transgenic rice plants had slightly more galls compared to wild type (Nahar *et al.*, 2011). Similarly, INA and BTH on tomato only induced minor defence responses against *M. javanica*, with slightly lower gall numbers observed.

Besides conferring resistance to root-knot nematodes, evidence has been found that SA and related chemicals can also confer resistance to cyst nematodes. Kempster *et al.* (2001) found

that applying SA and BTH induced clover resistance against *Heterodera trifolli*. Similarly, Wubben *et al*. (2008) found that SA-deficient mutants exhibited increased susceptibility to *H. schachtti*, and SA-treated wild type *Arabidopsis* showed decreased *H. schachtii* infection.

PR proteins

Pathogenesis-related (PR) proteins are small proteins with antimicrobial activities, found to be inducible by infection with various types of pathogens in many plant families. Most PRs are induced in various tissues in response to the signaling compounds salicylic acid, jasmonic acid, ethylene or abscisic acid. Abiotic stresses can also elicit defence-related protein induction, as in the case of osmotic stress, cold stress, or wounding (Horvath *et al*., 1998, Ponstein *et al*., 1994, Xu *et al*., 1994). Some PRs have been found to be expressed in a developmentally controlled, organ-specific manner in healthy plants (Van Loon *et al*., 2006).

PRs have a dual cellular localization – vacuolar and apoplastic. Most acidic PR proteins are located in the intercellular spaces, whereas basic PR proteins are predominantly located in the vacuole (Van Loon *et al*., 2006).

### 1.4.2 Induced Systemic Resistance

Colonization of plant roots by some soil microbes, such as plant growth-promoting rhizobacteria (PGPR) or endophytic fungi (PGPF), can directly promote plant growth by competition for nutrients with soil-borne pathogens, making nutrients available for the plant, improving plant nutrient uptake and photosynthesis (Spaepen *et al.*, 2009, Trillas & Segarra, 2009), via repression of soil-borne pathogens by the production of siderophores or antibiotics and improving plant stress tolerance (De Vleesschauwer & Höfte, 2009). These beneficial microorganisms can indirectly reduce plant disease through activating the plant to better defend itself, termed as induced systemic resistance (ISR) (Kloepper *et al.*, 1992, Van Loon *et al.*, 1998).

ISR was first described in 1991. Van Peer *et al*. (1991) discovered that resistance can be induced by the rhizobacterium *Pseudomonas* sp. strain WCS417r against *Fusarium* spp. in carnation (Van Peer *et al.*, 1991), and simultaneously, Wei *et al.* (1991) reported that some selected PGPRs were shown against the same fungus in leaves of cucumber. Meanwhile, another research group independently demonstrated that rhizosphere *Pseudomonas* spp. could also induce disease resistance in the common bean against the halo blight bacterial pathogen (Alstrom, 1991).

Initially, ISR was known to be triggered by PGPR and particularly by *Pseudomonas* spp. and *Bacillus* spp. (Van Wees *et al.*, 2008, Kloepper *et al.*, 2004). Recent studies have shown that ISR can also be elicited by mycorrhizal fungi (Trillas & Segarra, 2009, Pozo & Azcon-

Aguilar, 2007). ISR has been reported in many different plant–pathogen systems (Shoresh *et al.*, 2010, Van Oosten *et al.*, 2008, Van Loon *et al.*, 1998, Zehnder *et al.*, 1997). Various beneficial microorganisms are known to induce ISR in monocots (Balmer *et al.*, 2013). The potential resistance induced by PGPR in monocots depends on the host-PGPR combination and on the type of attackers. For example, *Psedomonas aeruginosa* 7NSK2 and *Serratia plymuthica* IC1270 induce resistance against *Magnaporthe oryzae* in rice, but they enhance disease severity caused by *Rhizoctonia solani* (De Vleesschauwer *et al.*, 2006).

Recognition

It is plausible that activation of ISR is based on host recognition by the beneficial microbes through microbe-associated molecular patterns (MAMPs). These compounds include cell surface molecules, such as flagellin and lipopolysaccharides, or secreted metabolites like siderophores, antibiotics, biosurfactants and even volatile organic compounds (De Vleesschauwer & Höfte, 2009, Van Wees *et al.*, 2008). In some cases, plant immunity is triggered by certain MAMPs but in other cases, the same MAMPs are not recognized. For instance, in tomato the *P. putida* WCS358 siderophore pseudobactin 358 triggers systemic resistance, but Tn5 transposon mutant defective in biosynthesis of the fluorescent siderophore pseudobactin does not. In bean, however, the same mutant is as effective as the wild-type strain (Meziane *et al.*, 2005).

There are diverse MAMPs that have been identified as ISR elicitors in monocots as well. For example, siderophores and antibiotics produced by *Pseudomonas* strains, such as pseudobactins and pyocyanin, are important defence elicitors in rice against *M. oryzae* (De Vleesschauwer *et al.*, 2008). The literature about the various MAMPs identified in the established monocot ISR pathosystems has been reviewed comprehensively by Balmer *et al.* (2013).

Signaling pathways involved in Induced Systemic Resistance

MAMPs of beneficial microbes are recognized by the plant immunity system and ultimately result in an enhanced defensive capacity of the plant. Although the signaling pathway is initiated in roots, the effects are also present in the aerial part. This suggests an extensive coordination between the plant and the beneficial organism and signaling routes involved in ISR would play an essential role (Knoester *et al.*, 1999, Pieterse *et al.*, 1998). In the past decade, research on the defence-signaling pathways that are activated by beneficial microorganisms revealed that ethylene and jasmonic acid (JA) are often the central players in the regulation of ISR (De Vleesschauwer & Höfte, 2009, Van Wees *et al.*, 2008). ISR triggered by PGPR and PGPF was shown to be blocked in JA-and/or ethylene signaling mutants of *Arabidopsis*

(Hossain *et al.*, 2008, Korolev *et al.*, 2008, Stein *et al.*, 2008). For instance, both the JA-response mutant *jar1* and the ET-response mutant *etr1* were incapable to develop ISR against *P. syringae* pv. tomato upon colonization of the roots by WCS417r bacteria (Pieterse *et al.*, 1998). Surprisingly, the endogenous levels of JA or ET do not appear to alter during induction of resistance by WCS417r in *Arabidopsis* wild type (Pieterse *et al.*, 2000). In addition, JA- and ET-regulated genes were not up-regulated either (Van Wees *et al.*, 1999). Nevertheless, JA- and ET-responsive gene expression is enhanced after pathogen infection relative to non-induced plants (Pozo *et al.*, 2008). Moreover, in ISR-expressing plants the capacity to convert 1-aminocyclopropane-1-carboxylate (ACC) to ethylene was significantly enhanced, providing a greater potential to produce ethylene upon pathogen attack (Hase *et al.*, 2003, Pieterse *et al.*, 2000). Therefore, the sensitivity to JA and ET is likely to be boosted as a result of ISR. WCS374r-elicited ISR against *M. oryzae* in rice was completely abolished in an ethylene-insensitive *OsEIN2* antisense line and JA-deficient mutant *hebiba*. However, it is maintained in *NahG* transformants, suggesting that in the rice- *Magnaporthe oryzae* pathosystem, WCS374r-mediated ISR derives primarily from JA/ET-driven effects (De Vleesschauwer *et al.*, 2008).

Although WCS417r-mediated ISR was maintained in SA non-accumulating *Arabidopsis NahG* transformants (Pieterse *et al.*, 1996, Van Wees *et al.*, 1997), and ISR was suggested to be mediated by an SA-independent resistance mechanism, it is becoming increasingly clear that not all rhizobacteria-triggered ISR is mediated by JA/ET. Several examples of PGPR and PGPF that trigger ISR in an SA-dependent manner have been documented as well (Van Wees *et al.*, 2008, Van Loon *et al.*, 2006, De Meyer *et al.*, 1999, Audenaert *et al.*, 2002, Ryu *et al.*, 2003, Barriuso *et al.*, 2008). Moreover, some PGPR or PGPF induced ISR in monocots is also depending on SA (Molitor *et al.*, 2011, Balmer *et al.*, 2013).

More and more examples of PGPR and PGPF induced ISR in different plant species have been shown to be depending on NPR1, with ISR abolished in *npr1* mutants (Van Loon & Bakker, 2006, Pieterse *et al.*, 1998, Van Wees *et al.*, 2008). Hence, NPR1 was supposed to play a pivotal role in reaching the induced state not only in SAR but also in ISR.

Transcriptome analysis of *Arabidopsis* root tissue during colonization by *P. fluorescens* WCS417 revealed a differential expression of 94 genes locally in the roots (Verhagen *et al.*, 2004). Among those genes, the MYB family transcription factor, *MYB72* appeared to be specifically up-regulated in the roots upon recognition and plays an important role in ISR onset not only induced by beneficial bacteria but also by beneficial fungi (Verhagen *et al.*, 2004, Segarra *et al.*, 2009, Van der Ent *et al.*, 2008). T-DNA knockout mutants *myb72-1* and *myb72-2* are incapable of mounting ISR against a set of pathogens, indicating that MYB72 is

essential to establish broad-spectrum ISR. However, *MYB72* over-expressors do not show enhanced levels of disease resistance (Van der Ent *et al*., 2008), demonstrating that it is not sufficient for ISR induction, probably acting in concert with other signaling components. Yeast two-hybrid analysis revealed that MYB72 physically interacts in vitro with the ETHYLENE INSENSITIVE3 (EIN3)-LIKE3 transcription factor EIL3, linking MYB72function to regulation of the ethylene-dependent signaling pathway (Van der Ent *et al*., 2008).

Transcriptome analysis of ISR-expressing leaves in plants of which the roots were treated with WCS417 or other beneficial microbes revealed that the systemic changes in gene expression are either relatively mild or not directly induced (Verhagen *et al*., 2004, Van Wees *et al*., 2008). However, subsequent infection with a pathogen led to an augmented expression of a large number of genes in ISR expressing plants compared to control plants (Van Wees *et al*., 1999, Verhagen *et al*., 2004, Cartieaux *et al.*, 2008, Ahn *et al.*, 2007). This enhanced defensive capacity without direct induction of defence responses in the absence of pathogens is called priming (Conrath *et al.*, 2002, Conrath *et al*., 2006). Primed plants have an enhanced capacity to rapidly and effectively mount defence responses to biotic and abiotic stress (Conrath *et al*., 2006).

In *Arabidopsis*, rhizobacteria-mediated ISR is often associated with priming for enhanced expression of JA/ET regulated genes and reinforcement of the cell wall at the site of pathogen attack (Van Oosten *et al*., 2008, Ahn *et al*., 2007, Verhagen *et al*., 2004, Van der Ent *et al*., 2008, Cartieaux *et al*., 2008, Van Wees *et al*., 1999). MYC2, a well-known player in JA regulated signaling (Lorenzo *et al.*, 2004), was found to be an important regulator of priming during ISR induced by different microbes (Pozo *et al*., 2008). Research has shown that MYC2 transcription factor binding sites are over-represented in the promoters of priming-responsive genes in ISR-expressing plants and the *myc2* mutant was unable of mounting WCS417-ISR against *P. syringae* and *Hyaloperonospora arabidopsidis* (Pozo *et al*., 2008).

In addition, an enhanced deposition of callose-rich papillae is observed upon infection by the oomycete *H. arabidopsidis* in WCS417-pretreated plants (Van der Ent *et al*., 2008). Besides forming a physical barrier, defence molecules such as $H_2O_2$, phenylalanine ammonia-lyase (PAL), phenolics, and various proteins and glycoproteins with hydrolytic and antifungal properties, as well as diverse secondary metabolic products are also accumulating at the sites of infection after pre-treatment with PGPR (Chen *et al.*, 2000, Van Loon *et al*., 1998).

A model for ISR is illustrated in Figure 1.7

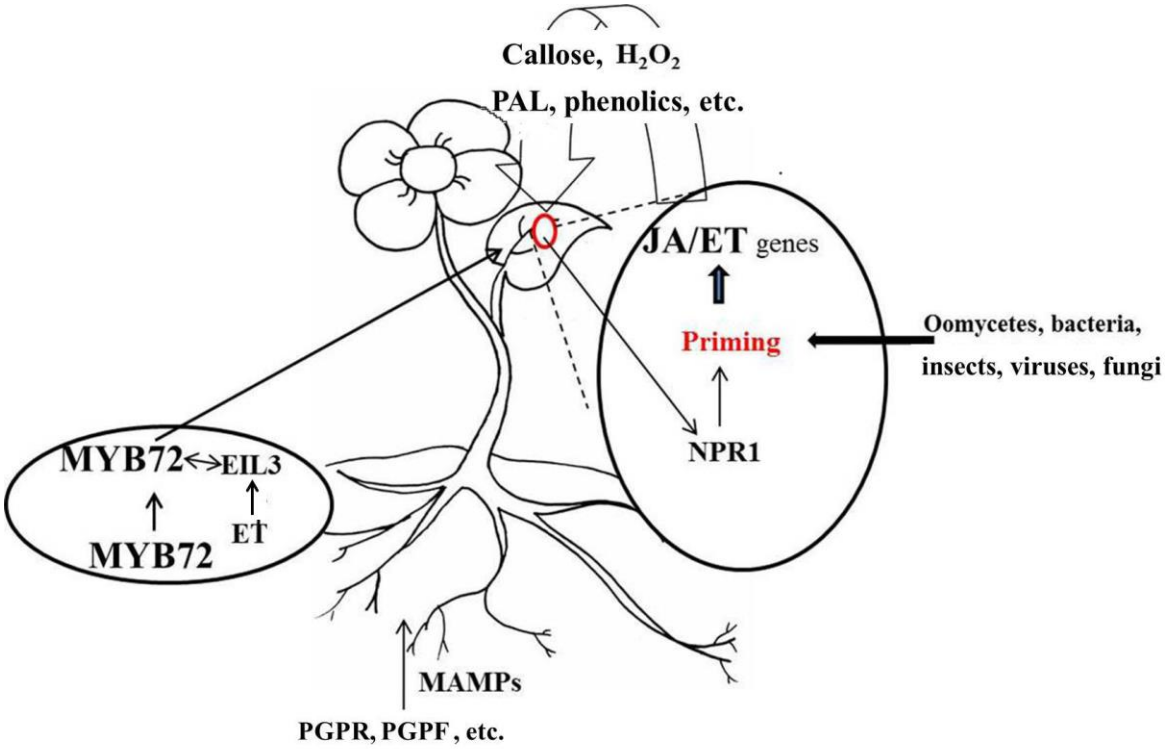


Fig 1.7 Model for the ISR signaling pathway. Recognition of MAMPs of beneficial rhizosphere-colonizing microorganisms leads to a local activation of the transcription factor MYB72 in the roots. Subsequently, MYB72 putatively interacts with the transcription factor EIL3. Downstream of, or in parallel with MYB72/EIL3, a so far unidentified ET signaling component is required in the roots for the onset of ISR in the leaves. The ISR signal transduction cascade requires NPR1, probably in the systemic tissue. Systemically, induction of ISR is associated with priming for enhanced expression of a set of JA-responsive and/or ET-responsive genes and increased formation of callose-containing papillae at the site of attempted pathogen entry. Attack by pathogens or insects, as depicted on the right side of the figure, activates defence responses in the plant, which is accelerated in ISR-primed plants. Adapted from Van Wees *et al*. (2008).

Application of PGPR, PGPF and ISR signaling molecules against plant parasitic nematodes

Plant-parasitic nematodes co-exist in the rhizosphere with biologically diverse bacterial or fungal communities. The best studied PGPR to affect parasitic nematode infection on plants are *Bacillus* spp., *Pseudomonas fluorescens* and *Rhizobium etli* (Tian *et al.*, 2007, Racke & Sikora, 1992, Sikora, 1992). The mechanisms of PGPR to reduce the plant parasitic nematode population include direct toxicity by production of antibiotics, competition for space and nutrients, alteration of root exudate patterns, production of repellent substances to affect nematode attraction and promote plant growth (Sikora *et al.*, 2007).

The first report of the involvement of rhizobacteria-mediated ISR in nematode control was by Hasky-Günther and Sikora (1995), who reported the involvement of ISR as a mechanism in

the control of *G. pallida* by *Bacillus Sphaericus* B43 and *Rhizobium etli* G12 on potato. Later on, many research groups studied ISR by PGPR using the split-root system. When PGPR was applied on one side of the roots, there was a lower number of juveniles penetrating the other side of the roots as well as a lower rate of reproduction (Hasky-Günther & Sikora, 1995, Hasky-Günther *et al.*, 1998, Mahdy *et al.*, 2001, Mahdy *et al.*, 2000, Schafer, 2007). These effects may be due to the PGPR fortifying the physical and mechanical strength of the host cell wall, or the accumulation of defence chemicals (Van Loon *et al.*, 1998, Siddiqui & Mahmood, 1999, Ramamoorthy *et al.*, 2001).

Some commercial biofertilizers, such as Equity, BioYield, and AgBlen, are mainly composed by different PGPRs. Greenhouse studies found that those biofertilizers induce significant reductions in *M. incognita* eggs per gram of root, juvenile nematodes and galls per plant on tomato (Sikora *et al.*, 2007, Burkett-Cadena *et al*., 2008).

Arbuscular mycorrhizal (AM) fungi are also well known PGPF to control plant parasitic nematodes. They can decrease nematode infestation as well as reproduction (Pinochet *et al.*, 1996, Siddiqui & Mahmood, 1999). They show similar modes of action as PGPR, such as competition in the rhizosphere at infection sites in the host, direct toxicity, alteration of root exudates to repel nematodes and induction of plant systemic resistance (Hol & Cook, 2005, De La Peña *et al.*, 2006, Schafer, 2007).

Here we present some representative examples associated with PGPR/PGPF and ISR signal molecules showing induced plant resistance against plant parasitic nematodes (Table 1.2).

Table 1.2 Summary of the defence mechanisms associated with PGPR/PGFR and JA/ET.

| **Resistance-inducing agent** | **Host plant** | **PPN** | **Reference** |
|---|---|---|---|
| ***Agrobacterium radiobacter*** | Potato | *Globodera pallida* | (Hackenberg *et al.*, 1999) |
| ***Bacillus sphaericus* B43** ***Agrobacterium radiobacter G12*** | Potato | *Globodera pallida* | (Hasky-Günther *et al.*,1998) |
| ***Serratia marcescens*** | Tomato | *Meloidogyne incognita* | (Almaghrabi *et al*, 2012) |
| ***Bacillus amyloliquefaciens*** | Tomato | *Meloidogyne incognita* | (Burkett-Cadena *et al*.,2008) |
| ***Bacillus subtillis*** | Tomato | *Meloidogyne incognita* | (Adam *et al.*, 2014) |
| ***Serratia marcescens*** | Tomato | *Meloidogyne incognita* | (Almaghrabi *et al*.,2013) |
| ***Pseudomonas fluorescens*** | Sugar beet | *Heterodera schachtii* | (Oostendorp & Sikora,1989) |
| ***Pseudomonas fluorescens*** | Rice | *Hirschmanniella oryzae* | (Swarnakumari *et al.*, 1999) |
| ***Pseudomonas aeruginosa IE-6S+ & P. fluorescensCHA0*** | Tomato | *Meloidogyne javanica* | (Siddiqui & Shaukat, 2003) |
| ***Rhizobium etli, Bacillus sphaericus B43*** | Potato | *Globodera pallida* *Meloidogyne incognita* | (Siddiqui & Shaukat, 2003) |

| | | | |
|---|---|---|---|
| ***Bacillus cereus S18*** | Tomato | *Meloidogyne incognita*<br>*Meloidogyne javanica* | (Reitz *et al.*, 2000) |
| ***Rhizobium etli G12*** | Vegetables | *Meloidogyne incognita*<br>*/M.javanica/M.arenaria* | (Mahdy *et al.*, 2001) |
| ***Rhizobium etli G12*** | Sugarbeet | *Heterodera schachtii* | (Mahdy *et al.*, 2001) |
| ***Rhizobium etli G12*** | Potato | *Globodera pallida* | (Mahdy *et al.*, 2001) |
| ***Pseudomonas fluorescens Pf1*** | Tomato | *Meloidogyne incognita* | (Santhi & Sivakumar, 1995) |
| ***Pseudomonas chitinolytica*** | Tomato | *Meloidogyne javanica* | (Spiegel *et al.*, 1991) |
| ***Rhizobium etli G12*** | Potato | *Globodera pallida* | (Reitz *et al.*, 2000) |
| ***Glomus versiforme*** | Grapevine | *Meloidogyne incognita* | (Li *et al.*, 2006a) |
| ***Scutellospora castanea***<br>***Glomus spp.*** | *Ammophila arenaria* | *Pratylenchus penetrans* | (De La Peña *et al.*, 2006) |
| ***Glomus intraradices*** | Grapevine | *Xiphinema index* | (Hao *et al.*, 2012) |
| ***Glomus mosseae*** | Banana | *Radopholus similis* | (Elsen *et al.*, 2003) |
| ***Glomus coronatum*** | Tomato | *Meloidogyne incognita* | (Diedhiou *et al.*, 2003) |
| ***Scutellospora heterogama*** | Sweet passion fruit | *Meloidogyne incognita* | (Anjos *et al.*, 2010) |
| ***Glomus mosseae*** | Tomato | *Meloidogyne incognita*<br>*Pratylenchus penetrans* | (Vos *et al.*, 2012) |
| **JA** | Tomato | *Meloidogyne incognita*<br>*Meloidogyne arenaria* | (Fujimoto *et al.*, 2011) |
| **MeJA/ET** | Rice | *Meloidogyne graminicola* | (Nahar *et al.*, 2011) |

Priming for enhanced defences and its potential in agriculture

More and more research has found induced resistance responses to be frequently associated with a primed state in which the plants are able to respond more rapidly and/or more robustly when re-exposed to biotic or abiotic stress. This unique physiological state is called "priming" (Conrath, 2011, Conrath *et al*., 2006, Conrath *et al*., 2002). Priming can be elicited by beneficial microorgamisms, necrotizing attackers, herbivores, MAMPs, pathogen-derived effectors, and many various natural and synthetic compounds (Heil & Bueno, 2007, Frost *et al*., 2008, Conrath *et al*., 2006).

Although the molecular basis of cell priming is unclear, recent research has suggested that dormant mitogen-activated protein kinases accumulate in primed plants and this phenomenon was linked to enhanced *PAL1* and *PR1* defence gene activation (Beckers *et al*., 2009). Moreover, defence related transcription factors (Segarra *et al*., 2009) and secondary metabolites such as azelaic acid (Jung *et al*., 2009) and volatile organic compounds (Van der Ent *et al*., 2009) are all potentially crucial in the priming mechanism.

The continuous activation of defence reactions was shown to reduce plant fitness such as growth and fruit or seed set under pathogen-free conditions (Goellner & Conrath, 2008). The trade-off dilemma between disease resistance and costs of defence activation can probably be overcome by priming. Compared to direct induction of defence, primed plants causes less fitness costs (van Hulten *et al*., 2006). When attacked by pathogens, it has even been demon-

strated to provide plants with a fitness benefit under conditions of pathogen attack (van Hulten *et al*., 2006, Walters & Fountaine, 2009, Walters *et al*., 2008). Hence, priming offers a smart, effective, and realistic option for effective plant protection, and provides broad-spectrum resistance. It could be a valuable tool for sustainable crop protection.

### 1.4.3 BABA-Induced Resistance

In addition to induced resistance by the above-described biological agents, there are various chemicals that can biologically mimic the induced resistance phenomena. Many of those chemicals are endogenous plant compounds, or functional analogues thereof, such as SA, BTH, INA, JA, MeJA and azelaic acid. An exception to this rule is β-aminobutyric acid (BABA), a non-protein amino acid that rarely occurs in nature (Jakab *et al.*, 2001, Cohen, 2002). The only report of BABA in connection to plants describes its presence in root exudates of tomato plants grown in solarized soil (Gamliel & Katan, 1992). BABA is a potent inducer of resistance in plants with a broad-spectrum activity. It is not only effective against biotic stress, but also against some types of abiotic stress (Cohen, 2002, Jakab *et al*., 2001, Jakab *et al.*, 2005, Zimmerli *et al.*, 2008, Pastor *et al.*, 2013). BABA is a racemic mixture of the R and S enantiomers, but only the R form is effective in inducing plant resistance. BABA has been tested repeatedly *in vitro* on many plant pathogens, where it has been shown that it is not directly toxic to microorgamisms (Cohen, 2002, Jakab *et al*., 2001). *In vivo* toxicity can also be ruled out, since experiments using $C^{14}$-labeled BABA clearly demonstrated that the substance is not metabolized by the plant and stays active (Cohen & Gisi, 1994). Intriguingly, BABA has an instantaneous effect even when applied post-infection, and still provides effective disease control. This feature bears a significant advantage over BTH since BTH can only work prior to the presence of the disease. Moreover, a curative effect of BABA application has also been reported in some pathosystems (Oostendorp *et al.*, 2001). Therefore, BABA seems to have a high potential to play a role in sustainable disease management in the field. Indeed, several successful evidences come from greenhouse and field tests on the efficacy of control against different pathogens by foliar application or soil drench of BABA or BABA in combination with fungicides (Reuveni *et al.*, 2001, Andreu *et al.*, 2006, Altamiranda *et al.*, 2008). BABA has been shown to move systemically through the plant (Walters *et al.*, 2005, Cohen, 2002, Jakab *et al*., 2001). In *Arabidopsis*, BABA was much better tolerated when applied to the roots than sprayed on the leaves. Spraying BABA at higher concentrations on the leaves induced necrosis in tobacco (Siegrist *et al.*, 2000, Cohen & Gisi, 1994). Nevertheless, Cohen and Gisi (1994) commented on the fact that only 36% of the applied BABA was taken up by the roots, in contrast to a 90% uptake through cut ends of petioles. They proposed that (i) the roots are partially impermeable to BABA; (ii) there is a limitation due to the transport capacity of a transporter; (iii) there exists a competition between amino acids and BABA for the

same transporter. BABA was found to induce the stress-induced morphogenic response (SIMR) which is also observed when plants are exposed to high concentrations of amino acids. However, this BABA-mediated SIMR was inhibited by L-glutamine. The authors suggested the possibility that BABA shares a common transporter with L-glutamine, and BABA may be thus inhibited to translocate into the cell in the presence of excess levels of L-glutamine. This hypothesis still needs to be proven (Wu *et al.*, 2010).

The remarkably wide range of effectiveness of BABA-induced resistance suggests that multiple resistance responses are involved. Some research has shown BABA-induced resistance in certain pathosystems is independent of the known defence signaling cascades, such as SA, ET, or JA, suggesting an additional mechanism of protection. However, BABA-induced resistance, just like SAR and ISR, is frequently associated with priming for various pathogen induced defence responses (Van der Ent *et al*., 2009, Cohen, 2002, Conrath, 2011, Conrath *et al*., 2006, Conrath *et al*., 2002). In the following sections, BABA-induced resistance to plant pathogens, insects, and abiotic stresses, as well as the mode of actions in some selected pathosystems will be summarized (Table 1.3). BABA-induced resistance against plant parasitic nematodes will be reviewed as well. Finally, the mode of action of BABA induced resistance according to the established studies will be discussed in a more detail.

BABA-induced resistance against plant parasitic nematodes

A number of studies have demonstrated that BABA-IR can provide control against nematodes. The first study of BABA-IR against PPN was carried out by Oka *et al* (1999). Application of BABA to tomato plants, either as a soil drench or a foliar spray, reduced root-galling and numbers of eggs produced by *M. javanica*, decreased the number of second stage juveniles penetrating plant roots and the size of adults. In addition, BABA treated giant cells were small and vacuolated. BABA also induced resistance against *M. javanica* by post-treatment after nematode inoculation. Soil drench of 0.25mM BABA to tomato plants together with *M. javanica* inoculation also reduced galls, egg masses and reproduction rate. Moreover, seeds treatment with BABA (0.25mM) plus later soil drench enhanced BABA-IR compared to soil drench alone (Fatemy *et al.*, 2012). Treated seeds alone induced resistance against *M. javanica* as well (Mutar & Fattah, 2013). Treatment with 40mM BABA on tomato seeds for 30, 60 and 120 minutes all caused significant reduction in nematode infestation and a lower average of the root gall index (Mutar & Fattah, 2013). The effect of BABA in the development and reproduction of another root-knot nematode *M. chitwoodi* was also assessed in tomato plants. Conversely, foliar application of 20mM BABA two days before inoculation with *M. chitwoodi* was not effective in reducing nematode penetration or reproduction in tomato plants. The authors discussed that the contrasting data may be due to differences in the method of

delivering the treatments and the concentrations used, and would also depend on the host and the pathogen (dos Santos *et al.*, 2013). Sahebani & Hadavi (2009) showed BABA treated tomato plants generate reactive oxygen species (ROS) and the enzymes related to their metabolism, the maximum level of ROS was observed at five days after BABA treatment. Additionally, BABA treated cucumber roots induced high level of $H_2O_2$ and total phenolic compounds, together with enhanced activities of peroxidase, polyphenol oxidase and catalase compared to non-treated roots (Sahebani *et al.*, 2011). Therefore, ROS production and phenolic compound accumulation in BABA treated roots may contribute to BABA-IR against nematodes in tomato and cucumber. Pre-treatment with BABA as a soil drench of 2mM significantly reduced the number of galls and egg masses induced by *M. javanica* in mung bean plants (Ahmed *et al.*, 2009). BABA-induced resistance was shown not only to be functional in dicots but also in monocots. On wheat, foliar sprays and soil drenches with BABA induced resistance against the cyst nematodes *H. avenae* and *H. latipons*, as well as root-knot nematode *Meloidogyne sp*. (Oka & Cohen, 2001). On pineapple, foliar sprays of 20mM BABA were effective against *M. javanica* (Chinnasri *et al.*, 2006).

Table 1.3 Pathosystems in which local and systemic resistance were induced by BABA.

| Plant | Protection against | Mode of action | Reference |
|---|---|---|---|
| ***Arabidopsis*** | *Alternaria brassicicola* | Accumulation of Abscisic acid (ABA), ABI1 gene and callose deposition. | (Flors *et al.*, 2008) |
| | *Alternaria brassicicola*<br>*Plectosphaerella cucumerina* | Soil drench: ABA dependent callose accumulation but JA, ET, SA independent. | (Ton &Mauch‐Mani, 2004) |
| | *Plectosphaerella cucumerina* | Soil drench: Callose accumulation and primed plants induction indolic metabolite (indole-3-carboxylic acid). | (Gamir *et al.*, 2012) |
| | *Pseudomonas syringae*<br>*Hyaloperonospora Arabidopsis* | Soil drench: Primed plants showed faster and higher accumulation of transcripts of defence-related genes in the SA pathway. Primed state is transferred to descendants. | (Slaughter *et al.*, 2012) |
| | *Pst* DC3000 | Soil drench: Direct and primed up-regulation of SA responsive genes. Inhibits *Arabidopsis* response to the bacterial effector coronatine. | (Tsai *et al.*, 2011) |
| | Drought stress tolerance | Soil drench: Primed plants enhanced ABA accumulation and accelerated stress gene expression, stomatal closure. | (Jakab *et al.*, 2005) |
| | Thermotolerance | Soil drench: BABA medicated accumulation of ABA transcription factors but no activation of ABA response genes, acts via activation of HSP101. Ethylene, SA independent. | (Zimmerli *et al.*, 2008) |
| | *Botrytis cinerea* | Soil drench: Accumulation of PR1 but not PDF1.2. Inactive in plants with impaired SAR pathway but still active in plants impaired in JA and ET pathways. | (Zimmerli *et al.*, 2001) |
| | *Peronospora parasitica* | Soil drench: Callose deposition, formation of trailing necrosis and hypersensitive response, independent of SAR, JA/ET signaling pathway. | (Zimmerli *et al.*, 2000) |
| | *Pst* DC 3000 | Soil drench: Dependent on SAR pathway but not JA/ET. | |
| | Potassium stress | Seedlings soaking: Enhancing low $K^+$ stress tolerance by increasing $K^+$ uptake partially via modulation of $K^+$ uptake genes. | (Cao *et al.*, 2008) |

| | | | |
|---|---|---|---|
| | Cadmium stress | Seedlings soaking: Against cadmium stress via glutathione dependent pathway. | (Cao *et al*., 2008) |
| **Potato** | *Phytophthora infestans* | Spray: cDNA-AFLP showed transcript derived fragments involved in signaling, cell wall strengthening and synthesis of antimicrobial compounds increased compared to non-induced plants. | (Li *et al.*, 2009) |
| | | Soil drench: BABA-IR requires SA but not oxylipins. | (Eschen-Lippold *et al.*, 2010) |
| | | Spray: Increase phenol and phytoalexin content. | (Olivieri *et al.*, 2009) |
| | *Fusarium sulphureum* | Scrawled BABA on potato slices: BABA increased the activities of peroxidase (POD), polyphenoloxidase (PPO) and phenylalanine ammonialyase (PAL), and accumulated lignin, flavonoids and phenolics in slices. | (Yin *et al.*, 2010) |
| **Tomato** | *Phytophthora infestans* | Spray: Accumulation of PR proteins, β-1,3 glucanase and chitinase. | (Cohen *et al*., 1994) |
| | *Botrytis cinerea* | Seed treatment: Priming defence. | (Worrall *et al.*, 2012) |
| | *Clavibacter michiganensis* ssp. | Spray: Higher activity of PAL, peroxidase and accumulation of $H_2O_2$. | (Baysal *et al.*, 2005) |
| | *Pst* DC 3000 | Spray: Synergistic effect with salt stress through induced $H_2O_2$ generation and guaiacol peroxidase activity. | (Baysal *et al.*, 2007) |
| | *Meloidogyne javanica* | Spray or soil drench: small and vacuolated giant cells. | (Oka *et a.,* 1999) |
| | | Seed treatment plus soil drench: Significantly reduced the numbers of galls and egg masses compared to control. No study on resistance mechanisms. | (Fatemy *et al*., 2012) |
| | | Seed treatment alone: Significant reduction in nematode infestation and a lower average of the root gall index. No study on resistance mechanisms. | (Mutar & Fattah, 2013) |
| | | Soil drench: Induction of ROS and accumulation of phenolic compounds | Sahebani & Hadavi (2009) |
| **Tobacco** | Tobacco Mosaic Virus | Spray: Cell death, HR, superoxide and hydrogen peroxide, a local and systemic increase of SA and *PR-1a*, NahG transgenic plants donot induce resistance after BABA application. | (Siegrist *et al*., 2000) |
| | Potassium deficiency | Spray: Increased $K^+$ uptake partially via ROS-dependent mechanism. | (Jiang *et al.*, 2012) |
| **Cucumber** | *Pseudoperonospora cubensis* | Leaf disc soaking: Accumulation of callose and cell death, production of reactive oxygen intermediates. Degenerated | (Walz & Simon, 2009) |

| | | | |
|---|---|---|---|
| | & *Colletotrichum lagenarium*<br><br>*Meloidogyne javanica* | primary hyphae.<br><br>Soil drench: Induction of phenolic compounds. | <br><br>(Sahebani *et al*., 2011) |
| ***Brassica juncea*** | *Alternaria brassicae* | Spray: Enhanced expression of PR proteins but independent of SA and JA accumulation. | (Kamble & Bhargava, 2007) |
| **Pepper** | *Phytophthora capsici* | Spray: Accumulation of β-1,3-glucanase, chitinase isoforms and SA.<br>Spray: Formation of electron-dense wall appositions, degeneration of hyphal mitochondrial structure. | (Hwang *et al.*, 1997)<br>(Lee *et al.*, 2000) |
| **Pea** | *Uromyces pisi* | Spray: Increased phenolic contents.<br><br>Spray: 2-DE analysis found BABA-IR via phenolic biosynthesis pathway. | (Barilli *et al.*, 2010)<br><br>(Barilli *et al.*, 2012) |
| **Wheat** | Desiccation | Soil drench: Triggers ABA accumulation, leads to stomata closure, reduced ROS and increased antioxidant defence enzymes. | (Du *et al.*, 2012) |
| **Citrus** | *Diaphorina citri*<br><br>*Penicillium digitatum* | Soil drench & spray: Primed plants accumulate higher PR2 (beta-1, 3-glucanase).<br><br>Apply on fruit peel surface: high concentration direct antifungal activity, induction of chitinase and PAL activities. | (Tiwari *et al.*, 2013)<br><br>(Porat *et al.*, 2001) |
| **Lettuce** | *Bremia lactucae* | Spray: Induced rapid encasement of pathogen with callose and accumulation of $H_2O_2$ in penetrated cells.<br><br>Post infection application (dpi) soil drench and spray: 1dpi application induced HR, 2dpi: encasement of haustoria with callose, 3 and 4dpi: $H_2O_2$ accumulation. | (Cohen *et al.*, 2010)<br><br>(Cohen *et al.*, 2011) |

| | | | |
|---|---|---|---|
| **Grapevine** | *Plasmopara viticola* | Spray: ROS production contributes to BABA-IR.<br><br>Soil drench: Primed accumulation of stilbene phytoalexins and increase in transcripts of genes involved in phenylpropanoid pathway.<br><br>Soil drench: Induced callose deposition, lignin formation around infection sites, defence mechanisms depending on phenylpropanoids and JA pathway. | (Dubreuil-Maurizi *et al.*, 2010)<br><br>(Slaughter *et al.*, 2008)<br><br>(Hamiduzzaman *et al.*, 2005) |
| **Apple** | *Erwinia amylovora* | Spray: Induced free SA level. | (Hassan &Buchenauer, 2007) |
| **Mung bean** | *Meloidogyne javanica* | Soil drench: Significantly reduced number of galls and egg masses. No study on resistance mechanisms. | (Ahmed *et al*., 2009) |
| **Wheat** | *Heterodera avenae*<br>*Heterodera latipons*<br>*Meloidogyne* sp. | Soil drench or foliar spray: Significantly reduced the number of cysts and egg masses. No study on resistance mechanisms. | (Oka & Cohen, 2001) |
| **pineapple** | *Meloidogyne javanica* | Foliar spray: Reduced egg masses production up to 64% compared to control. No study on resistance mechanisms. | (Chinnasri *et al.*, 2006) |

Mode of action of BABA-induced resistance

The mechanism of BABA-IR is diverse. BABA induces a variety of physical and biochemical defence mechanisms as well as defence signaling pathways (Table 1.3). The differences in the mode of action are largely depending on host-pathogen interactions. It appears that the mechanism of BABA-IR is pathosystem-specific (Cohen, 2002, Jakab *et al*., 2001).

Treatment with BABA has been reported to lead to induction of pathogenesis-related proteins (Cohen, 2002, Jakab *et al*., 2001). However, the accumulation of PR-proteins varies depending on the host-pathogen system and the mode of application. For example, it seems that solanaceous plants (i.e. tomato, pepper, and tobacco) may respond to BABA by accumulating of PR proteins without pathogen challenge, whereas in crucifers (i.e. *Arabidopsis* and Cauliflower), PR protein accumulation is only induced after pathogen infection (Cohen, 2002). Cohen & Gisi (1994) demonstrated that the accumulation of PR proteins without pathogen challenge was only found when BABA was sprayed on tobacco leaves, but neither with soil drench nor stem injection. However, all the application methods resulted in BABA-IR resistance in tobacco against *Peronospora tabacina,* indicating there are other mechanisms beyond PR protein accumulation that are responsible for BABA-IR in this tobacco- *Peronospora tabacina* interaction. Indeed, except PR protein accumulation, foliar spray of BABA sometimes induces the formation of small necrotic spots, which resembles the spots initiated by microbes during hypersensitive response (HR). Localized necrosis leads to the formation of reactive oxygen species, lipid peroxidation, phytoalexins, induction of callose and lignin around the lesions, which will contribute to BABA-induced resistance against pathogens (Cohen, 2002).

In tobacco, BABA was no longer able to protect *NahG* transgenic plants against TMV (Siegrist *et al*., 2000), which indicates that BABA induced resistance against TMV in tobacco is SA-dependent. However, *NahG* tobacco plants challenged with downy mildew showed no difference in protection by BABA compared to wild type plants (Cohen, 2001). In addition, in *Arabidopsis*, priming by BABA against the bacterial pathogen *Pseudomonas syringae* pv. Tomato DC3000 and the fungal pathogen *Botrytis cinerea* depends on an intact SA signaling pathway, but is independent of a functioning JA/ET pathway, as in the *Arabidopsis-Botrytis cinerea* interaction, *PR-1* was strongly potentiated but not JA marker gene *PDF1.2* (Zimmerli *et al*., 2000, Zimmerli *et al*., 2001). Accordingly, Tsai *et al*. (2011) demonstrated that BABA-IR against Pst DC3000 on *Arabidopsis* induced either direct or primed up-regulation of SA responsive genes, and partially relies on suppression of the plant response to the bacterial effector coronatine (Tsai *et al*., 2011). In accordance with the above-mentioned observations, a quantitative PCR based genome-wide screen for BABA responsive transcription factor (TF)

genes revealed an enhanced expression of SA-regulatory TFs, such as WRKYs in BABA-IR of *Arabidopsis* against *Pst* DC3000 (Van der Ent *et al*., 2009). Yet, BABA-IR against the oomycete pathogen *Hyaloperonospora parasitica* was expressed in *Arabidopsis* genotypes impaired in either the SA, ET, or JA signaling pathway (Zimmerli *et al*., 2000). Moreover, BABA-IR against two necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina* were unaffected in *Arabidopsis* mutants impaired in camalexin synthesis, JA sensitivity, ethylene sensitivity and SA signaling (Ton & Mauch-Mani, 2004). Cytological investigations at sites of attempted penetration by those pathogens demonstrated the augmentation of callose-rich papillae (Zimmerli *et al*., 2000, Ton & Mauch-Mani, 2004). The correlation of primed deposition of callose and BABA-IR was revealed in experiments with the callose synthesis inhibitor 2-deoxy-D-glucose (2-DDG). 2-DDG significantly reduces BABA-induced protection against *A. brassicicola*, suggesting a critical role for callose in BABA-induced protection against this necrotrophic pathogen (Ton & Mauch-Mani, 2004). Moreover, the callose synthesis mutant *pmr4-1* failed to express BABA-IR against *P. cucumerina.* Hence, callose seems crucial for the BABA-IR protection against these two nectrophic pathogens (Ton & Mauch-Mani, 2004). The possibility of an involvement of abscisic acid (ABA) in BABA-IR of callose formation was investigated by two *Arabidopsis* ABA signaling mutants, *aba5-1* and *abi4-1*. Priming for enhanced papillae formation by BABA after *P. cucumerina* infection was absent in the mutants. In addition, exogenous application of ABA mimicked the effect of BABA with respect to increased formation of callose-rich papillae and resistance to fungal ingress, indicating that ABA could be involved in the enhancement of callose deposition upon infection and BABA-IR in this pathosystem (Ton & Mauch-Mani, 2004). This hypothesis is further substantiated by experiments from the characterization of mutants impaired in BABA-induced sterility (*ibs*) (Ton *et al*., 2005). *Arabidopsis* becomes female-sterile when treated with high doses of BABA due to the induction of high levels of callose in the ovules (Jakab *et al*., 2001). Three *ibs* mutants were identified in a mutagenesis screen. *ibs2* and *ibs3* mutants were found to be reduced in the ability to express BABA-IR against *H. parasitica* and salt stress (Ton *et al*., 2005). Isolation and subsequent characterization of the two T-DNA–tagged *ibs* mutants found that the *ibs2* mutant carries a T-DNA insertion in the 5'-untranslated region of the *AtSAC1b* gene encoding a polyphosphoinositide phosphatase (PI), mutant *ibs3* is affected in the regulation of the *ABA1* gene encoding the ABA biosynthetic enzyme zeaxanthin epoxidase. These findings further strengthen the conclusion that PI- and ABA-dependent signaling regulate BABA-mediated priming for augmented callose deposition (Ton *et al*., 2005). However, BABA-IR in grapevine (*Vitis vinifera*) against downy mildew (*Plasmopara viticola*) through the potentiation of callose formation seems dependent on JA signaling, but not ABA (Hamiduzzaman *et al*., 2005).

These characteristics of BABA-IR suggest that BABA not only mimics SAR-related priming by potentiating SA-inducible defences, but also primes pathogen-induced deposition of callose-containing papillae, BABA-IR requires intact biosynthesis and perception of ABA or JA (Ton & Mauch-Mani, 2004, Hamiduzzaman *et al*., 2005, Van der Ent *et al*., 2009). Moreover, treatment with the priming agent BABA triggers a faster stomatal closure upon abiotic stress conditions (Jakab *et al*., 2005). All the observations suggest the mechanism of BABA-IR is diverse, depending on the specific plant and microbe interaction, as well as on environmental factors.

### 1.4.4 Wound-Induced Resistance

Upon mechanical damage or leaf-attack by herbivores, plants also activate their defences in un-infested leaves (Orians, 2005), an effect that is referred to as wound- induced resistance (WIR). Cell signalings of WIR are generated after perception of extracellular signals from herbivores such as fatty acid–amino acid conjugates (FACs), subsequently, these initial cues are transmitted within the plant by signal transduction pathways that include calcium ion fluxes, and phosphorylation cascades, then lead to release of linolenic acid from the chloroplast cell membrane in the end (Howe & Jander, 2008). They are subsequently regulated by jasmonates (Howe & Jander, 2008) that accumulate both locally and systemically in response to wounding (Glauser *et al.*, 2008). Systemin, a plant peptide hormone, is thought to be representing the primary wound signal in some solanaceaous plants. It is a 18-amino acid peptide derived from a 200-amino acid precursor prosystemin that has been originally discovered in tomato (Pearce *et al.*, 1991). This peptide acts at or near the local site of wounding, increasing JA-synthesis above the threshold that is required for the systemic response in tomato (Ryan & Pearce, 2003). Hence, systemin is thought to locally potentiate the synthesis of JA, whose recognition in distal leaves is linked to octadecanoid signaling (Ryan & Pearce, 2003). In addition to release direct defence-related compounds after wounding, such as phytoalexins, plants also rely on indirect protection which is afforded by herbivore-induced plant volatiles and extrafloral nectar that attract natural enemies of the herbivore (Kessler & Baldwin, 2002). Herbivore-induced plant volatiles (HIPVs) are commonly emitted from plants after herbivore attack which include terpenes, green leafy volatiles, ethylene, and other volatile organic compounds (Arimura *et al.*, 2005). HIPVs were reported to attract natural enemies and repel ovipositioning of herbivores, and activation or priming of intra- plant defence signaling events. Moreover, HIPVs have been demonstrated to prime nearby plants for enhanced direct and indirect defence responses in both laboratory and field conditions (Dicke *et al.*, 2009, Kessler & Halitschke, 2007). HIPVs not only work above ground but also below ground, for example, HIPVs may attract the specialist parasitoids of root-feeding larvae and entomopathogenic nematodes (*Heterohabditis megidis*) (Van Tol *et al.*, 2001, Neveu *et al.*, 2002). Extrafloral

nectarines (EFNs) are specialized nectar-secreting organs, which become active after herbivore attack, and increase nectar secretion which mainly comprise of carbohydrates and proteins (Bentley, 1977). These secretions are believed to function as an indirect anti-herbivore defence by attracting predators of those herbivores.

Events associated with induced resistance phenomenon in plants described in this section are summarized in Figure 1.8.

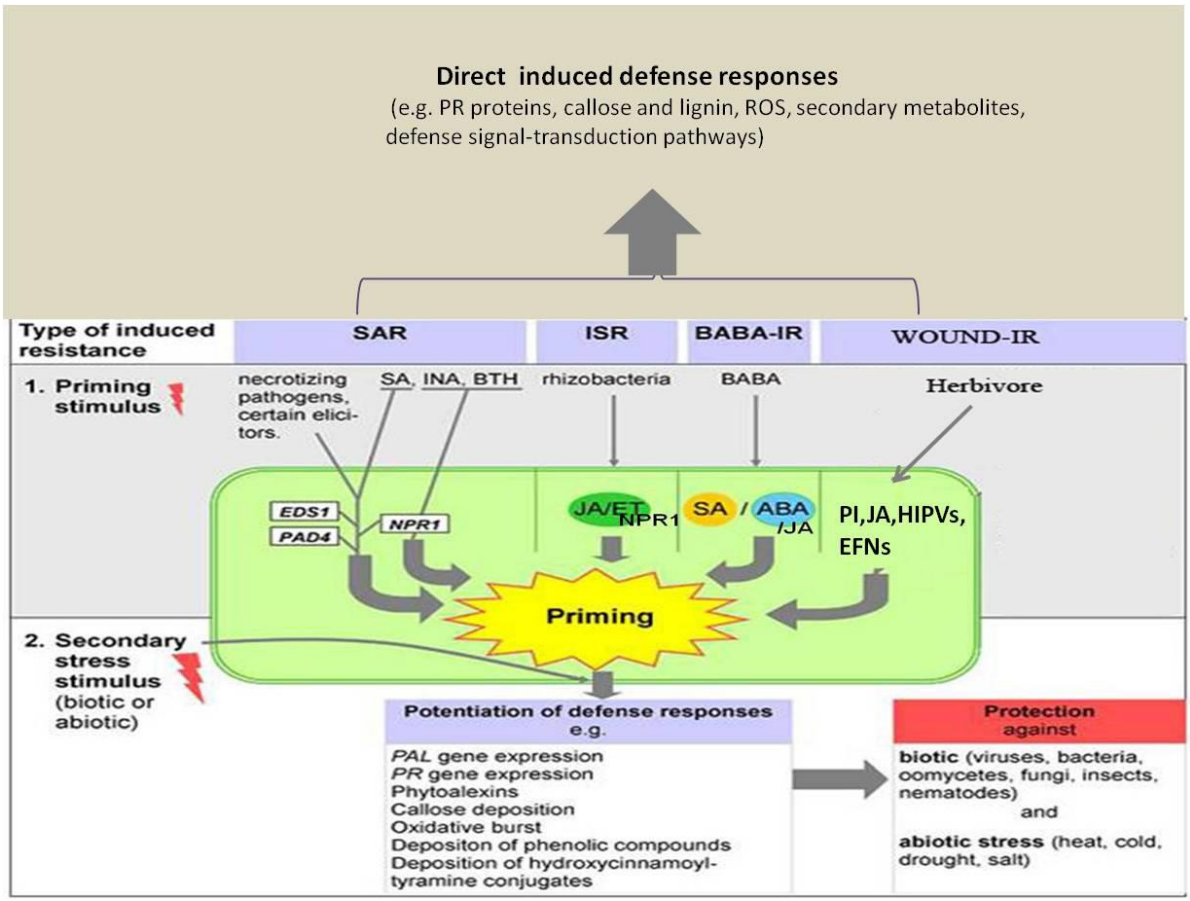


Fig1.8 Induced resistance phenomena in plants. Modified from Goellner & Conrath (2008).

## 1.5 Engineering of disease resistant plants

Traditionally, plant resistance is achieved by conventional plant breeding, which means new varieties of plants are bred by combining the favorable traits from parent plants but exclude their undesirable traits by back-crossing to one of the parental plant over a number of generations. Conventional breeding, however, does not allow insertion of exogenous genes from unrelated species, and is in general time-consuming. A large number of pathogenic strains have outpaced the development of new resistant plant varieties using conventional plant breeding strategies. Genetic engineering has been promoted for the past three decades as a solution for these problems. Genetic engineering is a technology that introduces new genetic

elements directly into a cell's genome. The key difference between genetic engineering and conventional breeding is that instead of randomly mixing genes in conventional breeding, genetic engineering is done by directly inserting a specific gene which is associated with a desirable trait into a new plant variety. A well-known example is the worldwide grown biotech Bt (*Bacillus thuringiensis*) corn, which contains genes from a soil bacterium, *Bacillus thuringiensis* and provides resistance against the European corn borer (*Ostrinia nubilalis*) (Ostlie *et al.*, 1997).

Introducing R genes is at the frontier of engineering disease resistance, as a strategy in creating durable disease resistance. Compared with traditional breeding this not only reduces the time, but it also allows multiple R genes to be transferred at the same time and introduced into sexually incompatible species, which was previously impossible by breeding (Gilbert *et al.*, 2006).

The constitutive production of antimicrobial proteins in transgenic plants, including the pathogenesis-related (PR) proteins, provided a new approach for the first generation transgenic disease resistant plants. The effect of these proteins is direct, furthermore, in most cases, only a single gene is necessary to produce the antimicrobial agent (Collinge *et al.*, 2010). Overexpression of an intracellular chitinase and an extracellular β-1,3 glucanase together resulted in a synergistic effect in reducing disease better than when deployed singly, as in carrot (Melchers & Stuiver, 2000), tobacco (Zhu *et al.*, 1994), and tomato (Jongedijk *et al.*, 1995). Other PR or antimicrobial proteins, for instance, osmotin and thaumatin- like proteins, thionins and defensins, expressed in various transgenic plants have also been shown to delay disease development or reduce disease severity (Chan *et al.*, 2005, Thomma *et al.*, 2002, Bohlmann & Broekaert, 1994).

The increasing knowledge on immunity-related signal transduction pathways provides new opportunities to manipulate plant disease resistance. Genes encoding key signaling components such as hormones, defence modulators and transcription factors can be introduced into target plants, where they can activate a complete battery of defence responses and could provide broad-spectrum resistance (Mourgues *et al.*, 1998, Stuiver & Custers, 2001). A typical example for the potential use of these genes is *NPR1*. Over-expression of *NPR1* enhances disease resistance levels against a broad range of pathogens in *Arabidopsis*, without any adverse plant phenotypes (Cao *et al*., 1998). Over-expression of *Arabidopsis NPR1* in wheat led to resistance against *Fusarium graminearum* (Makandar *et al.*, 2006), furthermore, when introducing the same gene into carrot plants, it enhanced resistance against a broad range of pathogens including biotrophic and nectrophic fungi and a foliar bacterial pathogen (Wally *et*

*al.*, 2009). Transgenic rice over-producing its own *OsNPR1* acquired high levels of resistance to *Xanthomonas oryzae* pv. *oryzae* (Chern *et al.*, 2005).

### 1.5.1 Engineering plants for nematode resistance

Plant-nematode parasitism is one of the most destructive and uncontrollable biotic stress on crops, and the effect on agriculture is severe (Fig 1.1). Management of nematode parasitism is therefore imperative. Integrated use of chemicals, resistant crop varieties and cultural and biological practices provide the most successful management strategies. However, these approaches have left much to be desired. Chemical control dependent approaches are under heavy international regulation as they have many environmental and health concerns, and they furthermore impose an additional financial burden on growers. Crop rotation as a strategy also has a limited utility against parasitic species with a cosmopolitan host range, such as the root-knot nematode, which may potentially parasitize up to 3,000 plant species (Abad *et al.*, 2003). Therefore, the most cost effective and sustainable strategy for limiting crop damage by plant parasitic nematodes is the use of resistant plants. Naturally occurring resistance has been exploited successfully in a number of crop species by conventional breeding. However, due to the many drawbacks caused by conventional breeding described above, biotechnology- derived crops with nematode resistance will be a promising alternative approach as an integrated management strategy to control plant parasitic nematodes (Atkinson *et al.*, 2003, Thomas *et al.*, 2006). There have been some excellent reviews about engineered nematode resistance over the past years (Atkinson *et al*., 2003, Thomas *et al*., 2006).

There are essentially three approaches for engineering resistance against plant-parasitic nematodes: transgenic expression of natural resistance genes; targeting and disruption of the nematodes, and attenuating feeding site formation (Thomas *et al*., 2006).

<u>Transgenic expression of natural resistance</u>

Transgenic expression of natural R genes into susceptible but agronomically important crops is a promising approach to generate nematode resistant plants. Many R genes have been identified and used in conventional breeding programmes for a number of crops (Table1.1). With genetic engineering, it is possible to rapidly transfer these genes to elite cultivars or other plant species (McDowell & Woffenden, 2003).

To date, however, there has been limited and variable success with transgenic expression of R genes against nematodes. It seems that transgenic resistance is much more likely to result after intra-specific transfers than after inter-specific ones (Fuller *et al.*, 2008). For example, transfer of the NBS-LRR gene *Hero* into a susceptible tomato cultivar conferred comparable resistance to *Globodera* species to that seen in an introgressed *Hero* line. Nevertheless, no

significant resistance was achieved in transgenic potato expressing the same construct (Sobczak *et al.*, 2005). Attempts to transfer *Mi-1.2* mediated root-knot nematode resistance from tomato to tobacco have been unsuccessful (Rossi *et al*., 1998). Recently, however, the tomato *Mi1* gene has been introduced into a root-knot nematode susceptible cultivar of lettuce (*Lactuca sativa*) resulting in transgenic lines resistant to *M. incognita* (Zhang *et al.*, 2010).

Potential disadvantages of transgenic R-genes include limitations on the range of nematode species (a small subset of parasites is recognized), limited R genes available and the likelihood of rapid resistance breaking and selection of virulent species or pathotypes (Starr *et al.*, 2002). Such a situation occurred in the United Kingdom where the widespread use of potato cultivars carrying the *H1* resistance gene successfully controlled *G. rostochiensis* in potato but led to an increase in the prevalence of another potato cyst nematode *G. pallida* (Lilley *et al.*, 2011).

Disruption of nematode target genes

**Antifeedant/Nematicidal Proteins**

The most extensively studied approach for engineering resistance against plant parasitic nematodes is the expression of proteinase inhibitors (PIs) in plant roots. Proteinase inhibitors are present in a range of proteinase classes and are widely expressed throughout the plant kingdom, they are often induced after wounding and herbivore attacks. Most of the naturally occurring PIs have been shown to be detrimental to feeding nematodes, reducing their growth and fecundity (Lilley *et al*., 2011). Correspondingly, proteinase genes and their digestive activity have been identified in plant parasitic nematodes (da Rocha Fragoso *et al.*, 2009, Fragoso *et al.*, 2005). Therefore, inhibition of protease activity might be expected to have a broad effect across many plant-parasitic nematodes including both migratory and sedentary parasites (McCarter, 2009). Cysteine proteinase inhibitors, termed cystatins, have received the most attention due to their successful applications. Multiple studies have demonstrated that transgenic expression of a modified version of a rice cystatin , Oc-1ΔD86, can interfere with nematode reproduction (Atkinson *et al*., 2003, Lilley *et al*., 2011). In tomato hairy roots, expression of Oc-1ΔD86 using the cauliflower mosaic virus promoter resulted in significantly smaller female *G. pallida* after 6 weeks of infection when compared to control roots (Urwin *et al.*, 1995). Oc-1ΔD86 expressed in transgenic *Arabidopsis* plants considerably reduced the size of female *H. schachtii* and *M. incognita* relative to controls, with growth arrested prior to egg-deposition (Urwin *et al.*, 1997). Potato plants expressing Oc-1ΔD86 from the cauliflower mosaic virus promoter which were challenged with potato cyst nematode *Globodera pallida* in a field trial resulted in a decrease in cyst number by 55–70% (Urwin *et al*., 1995). Transgenic plants of four elite African rice varieties constitutively expressing the modified

OcIΔD86 displayed 55% resistance to *M. incognita* (Vain *et al.*, 1998). Interestingly, full resistance to *G. pallida* was achieved in the United Kingdom field by introduction of Oc-IΔD86 into natural partially resistant potato cultivars (Urwin *et al.*, 2003). One key feature of cystatins for successful nematode control is that they are relatively small proteins (~11 kD), which will not be excluded by the feeding tube of sedentary endoparasitic nematodes that has been reported to restrict the size of molecules entering the intestine (Böckenhoff & Grundler, 1994).

**Silencing essential nematode genes**

Host-generated RNA interference (RNAi) targeting a nematode gene displays an efficient strategy to silence essential genes and hence to control the infection (Gheysen & Vanholme, 2007, Lilley *et al.*, 2007). Although it is not clear if the target transcript suppression observed arises from ingestion of plant derived siRNAs or dsRNA that is subsequently processed by the nematode, some proof-of-concept studies have shown extremely promising results. The first study of host delivered RNAi was in tobacco plants expressing dsRNA of *Meloidogyne* genes encoding a splicing factor and a component of a chromatin remodeling complex (Yadav *et al.*, 2006). Transgenic plants expressing both hairpin (a sequence of RNA that makes a tight hairpin turn) constructs displayed much fewer nematodes and galls and lacked detectable transcripts for the targeted genes in the nematodes (Yadav *et al.*, 2006). A high level of resistance to root-knot nematodes was also achieved by targeting a parasitism gene *16D10* expressed in the subventral gland cells of *M. incognita* in *Arabidopsis* (Huang *et al.*, 2006a). Transgenic *Arabidopsis* revealed a 63–90% reduction in the number of galls, an overall decrease in gall size and a corresponding reduction in total egg production, compared to control vector-transformed lines. Importantly, the high degree of homology between the 16D10 sequences of different *Meloidogyne* species led to broad-range resistance against *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*.

A number of studies have now demonstrated that hairpin RNAs expressed in the model plant *Arabidopsis* can only partially reduce transcript abundance of targeted parasitism genes of *H. schachtii* (Patel *et al.*, 2008, Patel *et al.*, 2010, Sindhu *et al.*, 2009). Furthermore, a large variation in the infection results were observed by different research groups between and even within transgenic lines (Sindhu *et al.*, 2009, Kyndt *et al.*, 2013b). One of the possible reasons is due to the promoter methylation in transgenic plants when plants detect exogenous genes "invading' (Kyndt *et al.*, 2013b).

Disruption of sensory function

Lectins, or agglutinins, are ubiquitous proteins present in both plants and animals that recognize and reversibly bind specific mono- or oligosaccharides (Van Damme *et al.*, 1998). Expression of snowdrop lectin (GNA) directed by the CaMV35S promoter in potato, oilseed rape and *Arabidopsis* confers partial resistance to cyst and root-knot nematodes (Burrows *et al.*, 1998, Burrows & De Waele, 1997, Ripoll *et al.*, 2003). It is thought that the lectin may bind glycoproteins in the amphidial secretions and interfere with the nematode sensory perception and its ability to establish feeding cells (Thomas *et al*., 2006).

Targeting the nematode feeding site

In case of endoparasitic nematodes, an alternative strategy to target the nematode directly is to disrupt the nematode feeding sites. Transcriptional analysis of feeding sites provides key insights into the physiological process and cellular structure in feeding sites, which will offer an advanced knowledge for engineering plants targeting nematode feeding sites. However, this approach would require a promoter that would become active specifically in NFS (i.e. a cell death gene, or a phytotoxic gene), it would be active in NFS but would have minimal or no overlap in other cell tissue. Fortunately, more and more plants with fully sequenced genome, well developed microarrays or RNA-seq techniques, and successfully identified feeding site expression transcripts, will offer the wealth of resources for identifying promoters that are much more active in NFS than in other plant tissues (Atkinson *et al*., 2003, McCarter, 2009, Thomas *et al*., 2006).

## 1.6 Plant thionins

After contact with pathogens, plants produce many biological active compounds, including small peptides, which present direct antimicrobial properties against pathogens. Those compounds are called antimicrobial peptides. They are considered to play an important role in plant defence (Garc á-Olmedo*et al.*, 1998, Castro & Fontes, 2005). Among plant antimicrobial peptides, thionins were the first whose activities against plant pathogens were studied *in vitro* (Garcia-Olmedo *et al*., 1992, De Caleya *et al.*, 1972).

Plant thionins are included in the pathogenesis-related proteins as the PR-13 group (Epple *et al.*, 1995). They are small (about 5KDa), cysteine-rich, and usually basic. They are found in monocots (grains), eudicots including dicotyledonous (mistletoes and *Pyrularia pubera*), and rosids (Stec, 2006). Most thionins present toxic effects against bacteria, fungi, yeast and animal cells (Bohlmann and Apel, 1991, Florack & Stiekema, 1994, Bohlmann 1994).

### 1.6.1 Biochemical properties of thionins

The mature thionins are generally 45-47 amino acids long and can be classified into at least five types (I-V). Type I thionins (purothionins) were initially identified from wheat grain endosperm. They have 45 amino acid residues, eight of which are in the central disulfide loop, they are highly basic and contain no negatively charged residues (Garc á-Olmedo *et al.*, 1998). The second type is represented by thionins identified from leaves of *Pyrularia pubera* and of barley (Vernon *et al.*, 1985, Bohlmann & Apel, 1987, Gausing, 1987). Type II thioins have four disulphide bridges at the same positions as type I thionins, but they are less basic and with some negatively charged residues (Garcia-Olmedo *et al.*, 1992). Type III thionins were isolated from stems and leaves of mistletoe species (*Loranthaceae*). They have three disulphide bridges and contain fewer basic residues. Their sequences have 46 residues and nine of them are in the central disulphide loop (Samuelsson & Pettersson, 1970, Romagnoli *et al.*, 2003, Pal *et al.*, 2008, Bhave & Methuku, 2011). The type IV thionin, crambin, isolated from seeds of *Crambe abyssinica*, is 46 amino acids long, with an overall neutral charge and three disulphide bonds (Vanetten *et al*., 1965, Bhave & Methuku, 2011). Type V thionins are quite divergent, the second and eight cysteines of type I thionins are missing in this type and thus, a new bridge is formed between the unmatched cysteines. Type V thionins are also neutral, they have been identified in a cDNA library derived from developing kernels of wheat and *Aegilops* species (Castagnaro *et al.*, 1992 , Castagnaro *et al*.,1995).

The three-dimensional structure of thionins has been studied in both crystallized form and in solution (Hendrickson & Teeter, 1981, Brunger *et al.*, 1987). The three dimensional structure of type I, III and IV are similar in shape, and it resembles the Greek letter γ. It is comprised of two antiparallel α-helices in the long arm and two short β strands (β-sheet). The groove between the α-helices and β-sheets contains the Tyr13 residue. The plasma membrane interaction with Tyr13 may be associated with cell content leakage, which is the main mechanism of the toxicity against yeast, mammal, fungal and bacterial cells (Carrasco, 1981).

### 1.6.2 Antimicrobial activities of thionins

The toxicity of thionins to bacteria, yeast, fungi (De Caleya *et al.*, 1972), cultured mammalian cells (Nakanishi *et al.*, 1979, Carrasco *et al.*, 1981), and insect larvae (Kramer *et al.*, 1979) has been investigated for several decades. Toxicity was observed when the barley endosperm thionin was injected intravenously or intraperitoneally to mice, guinea pigs and rabbits, but not upon oral administration (Coulson *et al.*, 1942, Garc á-Olmedo *et al*., 1989). Insect larvae were sensitive to different endosperm thionins when the proteins were administered through the hemocoel but not when incorporated in the food (Kramer *et al*., 1979).

The necessity of the Tyr13-residue for toxicity was verified in crambin. Crambin is non-toxic, and Tyr13 is not there but instead, it is Phe13, which proves the importance of Tyr13 in toxicity (Stec, 2006). Pore or water channel formation or disturbance of membrane organization is hypothesized as the causes of cell lysis, and the conserved Cys are also needed for toxicity and stability of the peptides (Thevissen *et al.*, 1996, Oard, 2011, Majewski & Stec, 2010, Hughes *et al.*, 2000, Llanos *et al.*, 2006).

A study of the effects of α-hordothionin on the membranes of *Fusarium culmorum* indicated that thionins caused $Ca^{2+}$ influx and $K^{+}$ efflux in the fungal hyphae (Thevissen *et al.*, 1996). The toxic effect not only alters the balance of $Ca^{2+}$ and $K^{+}$, it also induces the linkage of phosphate ions, nucleotides, amino acids, and inhibits the incorporation of sugars (Garcia-Olmedo *et al.*, 1992, Bohlmann & Apel, 1991). Apart from alteration of membrane permeability, Carrasco *et al*. (1981) demonstrated that the barley endosperm thionins inhibit the synthesis of DNA, RNA and proteins in cultured mammalian cells.

### 1.6.3 Implication of thionins in plant defence

Plant thionins are synthesized as preproteins consisting of a signal sequence, a thionin domain and an acidic domain. Thionins are secreted into vacuoles, protein bodies, extracellular spaces, and the plant cell wall (Bohlmann and Apel, 1991). Several reports on transgenic plants expressing thionin genes have shown that those transgenic plants were more resistant against a wide range of fungal or bacterial pathogens.

Overexpression of a hordothionin gene from barley in tobacco conferred resistance to *Pseudomonas syringae* (Carmona *et al*., 1993), and enhanced resistance to *Fusarium oxysporum* was observed in transgenic *Arabidopsis thaliana* plants that overproduced its endogenous thionin *THI2.1* (Epple *et al*., 1998). Iwai *et al.* (2002) showed that heterogeneous expression of an oat cell wall-bound thionin gene in rice made the plants more resistant to two major seed transmitted phytopathogenic bacteria, *Bukholderia plantarii* and *Bukholderia glumae*. Transgenic tobacco expressing a β-hordothionin gene showed resistance to *Botrytis cinerea* and *Pseudomonas solanacearum* (Charity *et al*., 2005). Tomato plants expressing an *Arabidopsis* thionin *THI2.1* showed enhanced resistance to bacterial wilt and *Fusarium* wilt diseases. Overexpression of a thionin gene from barley in sweet potato gave resistance to black rot disease caused by *Ceratocystis fimbriata* (Muramoto *et al*., 2012). Moreover, enhanced resistance to gray mold (*Botrytis cinerea*) was observed in transgenic potato plants expressing thionin genes isolated from *Brassicaceae* species (Hoshikawa *et al.*, 2012). However, no studies on transgenic plants with RNA interference or knock-out of thionin genes have been reported.

Plant thionin genes are not only induced by pathogens and wounding (Ebrahim-Nesbat *et al.*, 1989, Ebrahim-Nesbat *et al.*, 1993, Epple *et al.*, 1995, Lee *et al.*, 2000, Vignutelli *et al.*, 1998), they are also induced by chemicals or phytohormones. Barley leaf thionin genes can be induced by jasmonic acid (Andresen *et al*, 1992), INA (Wasternack *et al.*, 1994), and salicylate (Kogel *et al.*, 1995). However, *Arabidopsis* thionin gene *THI2.1* was only induced by methyl jasmonate and INA but not by salicylate (Epple *et al*., 1995, Holger *et al*., 1998, Alberto *et al*., 1998). A pepper thionin gene, *Cathion1* was strongly induced by treatment with ethephon or methyl jasmonate, weakly by salicylic acid and benzothiadiazole (Lee *et al*., 2000). Kitanaga *et al.* (2006) found that rice thionin gene expression was positively regulated by jasmonic acid, gibberellins and brassinosteroids. A microarray data of rice cDNA showed that *OsTHI2* was up-regulated in 30-day-old calli after a 3-day-treatment with either 50 µM abscisic acid or 50 µM gibberellin acid (Yazaki Junshi *et al*., 2003).

A few researchers have studied the spatial expression patterns of thionin genes. Barley thionin genes are mainly expressed in seed endosperm (Ponz *et a*l., 1983) and leaves (Holger *et al*., 1987). *Arabidopsis THI2.1* is constitutively expressed at very high levels in flowers and siliques. A weak *GUS* expression was seen in the apical region and young leaf primordia in 12 and 13-day-old seedlings. However, no *GUS* expression was detectable in 9-day-old seedlings (Alberto *et al*., 1998). Transcripts of endogenous rice thionin genes (several homologous genes) were found to be abundant in coleoptiles (4-day-old) and rare in roots, but not detectable at all in leaf blade and panicle (2-month-old) using northern blot analysis (Iwai *et al*., 2002).

The expression pattern of different rice thionin genes in rice tissues, as obtained by Genevestigator analysis (Hruz *et al*., 2008), is presented in addendum Figure A1. The distribution of thionin gene expression in rice is quite diverse, and they are expressed in different tissues. Generally, rice thionin genes from chromosome 6 are mainly expressed in root tips, radicles, roots and coleoptiles.

# CHAPTER 2

## Problem statement and thesis outline

## 2.1 Problem statement

Rice is the staple food of over half the world's population. It is the predominant dietary energy source for 17 countries in Asia and the Pacific, 9 countries in North and South America and 8 countries in Africa (Gelman & Choi, 2008). More than 75% of the rice production comes from irrigated lowland (Tuong and Bouman 2003). However, fresh water is becoming a precious commodity due to the increasing consumption from household, industry, and agriculture (Molden *et al*., 2007). Therefore, scientists are now seeking new rice production systems that can cope with water scarcity. Aerobic rice is a new production system where adapted rice varieties are grown in non-puddled, non-flooded, and non-saturated fields (Tuong and Bouman, 2003). Aerobic rice genotypes can reduce water requirements for rice production as much as 50% in comparison with lowland rice (Huaqi *et al*. 2002). However, if continuously cultivated with aerobic rice, a yield reduction has been observed. The rice root-knot nematode *Meloidogyne graminicola* is considered one of the possible causes of these yield reductions (De Waele *et al*., 2013). The use of nematicides is becoming more and more restricted due to the environmental concerns. Hence, plant resistance or tolerance to *M. graminicola* damage is essential. Although resistance to *M. graminicola* has been identified in *Oryza longistaminata* and *Oryza glaberrima* (Soriano *et al*., 1999, Plowright *et al*.,1999), it is taking time to transfer these resistance properties into cultivated *O. sativa* by conventional breeding (Bridge *et al*., 2005). Genetic engineering has many advantages to the traditional breeding, it can introduce a broader and more diverse range of genes into one plant species, and generally, it has minimal effect on beneficial soil microbes (Wally & Punja, 2010, Gurr & Rushton, 2005, Liu *et al*., 2005). On the other hand, plants can exhibit a fast and strong defence response upon pathogen infection when they are appropriately stimulated by inducing agents (Hammerschmidt, 2007). This induced resistance responses can be activated or primed not only in locally infected sites but also can establish immunity in systemic tissues (Van der Ent *et al*., 2008). However, in contrast to knowledge on foliar pathogens and dicots, still relatively little is known about the defence responses of monocots, such as rice, against plant parasitic nematodes. Exploring rice-nematode interaction provides an opportunity to study the defence mechanisms in the root system of this model monocot plant. Furthermore, increasingly available *in silico* data offer a holistic approach to elucidate a large set of differentially expressed genes participating in plant response upon invasion by nematodes. Such genes can eventually serve in the development of resistant rice cultivars by genetic engineering or other breeding methods. From a practical point of view, genetic engineering of plants for disease control, induced resistance by inducing agents, and analysis of transcriptome changes in nematode feeding sites or galls, will supply effective and advanced approaches in plant defence research in the interaction between root-knot nematodes and rice.

## 2.2 Objectives and thesis outline

We sought to provide primary evidence for improvement of defence to the root-knot nematode *Meloidogyne graminicola* in rice. In particular, the objectives of this work were:

(i) To assess whether overexpression of rice endogenous thionin genes can enhance plant defence against root-knot nematode *M. graminicola* and to study the regulation of rice thionin gene expression by defence-related hormones.

(ii) To evaluate whether BABA can trigger defence in rice, a monocot system, against the root-knot nematode *M. graminicola* and gain insights into the mechanisms of this kind of induced resistance.

(iii) To advance our knowledge of the transcriptome in giant cells formed by *M. graminicola* in rice roots at different time points and to compare the transcriptional changes in giant cells and in galls.

The first research part of this work, chapter 3 is dedicated to the role of rice thionins in modulating rice defence against *M. graminicola*. The role of plant thionin genes was explored extensively in past decades, however, the role of thionin genes against plant parasitic nematodes has never been investigated. In this chapter, rice thionin genes were functionally tested for their putative role in rice resistance against the root-knot nematode *M. graminicola.* In addition, the interaction between thionin genes and hormone signal pathways was analyzed as well.

In the second part of this work, covering chapter 4, the focus is shifted to the phenomenon of BABA-induced resistance in rice. Although many studies have shown BABA-IR against a wide range of plant parasitic nematodes, very few researches were conducted on analyzing the mechanisms behind BABA-IR against nematodes, especially using monocots as hosts. The effectiveness of BABA-IR against the root-knot nematode *M. graminicola* and the associated mechanisms using different biosynthesis and signaling mutants were investigated. Furthermore, a histochemical and microscopical investigation of the defence reactions in BABA-IR was performed.

In the third part of this work, chapter 5, the transcriptome changes in giant cells induced by *M. graminicola* in rice roots was performed, by using RNAseq after laser capture microdissection of these cells. Knowledge of genes involved in feeding site formation will provide an alternative approach in plant defence against sedentary nematodes. The possible roles of some differentially expressed genes were addressed in this chapter.

Finally, in chapter 6, I briefly recapitulate the results and discuss the applications of this work and future prospects.

# CHAPTER 3

## The role of thionins in the interaction between

## *Meloidogyne graminicola* and *Oryza sativa*

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Manuscript in preparation

## Abstract

Thionins are pathogenesis-related proteins that are thought to be involved in plant defence. However, rice thionin genes are strongly suppressed in galls induced by the root-knot nematode *Meloidogyne graminicola*. Here, we present data of an in-depth analysis of the expression pattern of different rice thionin genes in galls at different time points after inoculation. Moreover, the expression of rice thionin genes was observed to be differently induced by defence-related hormones. Transgenic lines of *Oryza sativa* cv. Nipponbare overproducing endogenous *OsTHI7* results in decreased susceptibility to *M. graminicola* and enhanced tolerance to *Pythium graminicola* infection.

## 3.1 Introduction

In nature, plants are attacked by a diverse range of pathogens and herbivores, which cause major yield reduction. However, plants have evolved sophisticated strategies to protect themselves from disease and herbivore feeding. As soon as a pathogen is recognized, plants initiate rapid defence responses, activating signaling pathways and accumulating pathogenesis- related (PR) proteins (Goodman & Novacky, 1994, Fritig *et al.*, 1998, Durrant & Dong, 2004). Most of those PR proteins having antimicrobial activity are of low molecular weight (5KDa-75KDa), basic or acidic and highly resistant to proteases. PR proteins generally have a dual cellular localization, apoplastic or vacuolar or both (Edreva, 2005, Dixon *et al.*, 1991). To date, at least 17 distinct families of PR proteins have been recognized based on their serological relatedness, biological activities, and amino acid sequences (Van Loon *et al.*, 2006). In rice, only *PR1*, *PR8* and *PR10*, have been reported to be induced following bacterial and fungal infection (Mitsuhara *et al.*, 2008, Kim *et al.*, 2008, Park *et al.*, 2004). Some PR genes are regulated differently depending on the pathogen species (Chen & Ronald, 2011). The family of PR13, known as thionins, is a group of anti-microbial peptides. They are generally 45-47 amino acids long, usually basic, cysteine-rich with a molecular weight of about 5KDa. Thionins are synthesized as much larger preproproteins, including a signal peptide, a basic mature thionin domain and a long acidic domain. The 6-8 cysteines in mature thionin form disulphide bridges (Bohlmann & Apel, 1991, Stec, 2006). A major feature of thionins is their wide range of toxic effects on different biological systems. Most studied thionins are toxic to bacteria, fungi, yeast, and various mammalian cell types *in vitro*. The toxic effects seem to be based on destruction of the organization of cell membranes by the interaction with negatively charged phospholipids. This causes a disturbance of the $Ca^{2+}$ or $K^{+}$ balance, and results in leakage of proteins, nucleotides, and other components (Bohlmann & Apel, 1991, Terras *et al.*, 1995, Thevissen *et al.*, 1996). In addition to the damage on pathogen membranes, Garcia-Olmedo *et al.* (1983) demonstrated an inhibitory effect of thionins on microbial protein syn-

thesis. Thionin genes are not only induced by pathogen attack in plants, but are also regulated by chemical inducers, such as heavy metals (Fischer *et al.*, 1989), jasmonic acid or methyl jasmonate (Andresen *et al.*, 1992, Epple *et al.*, 1995, Vignutelli *et al.*, 1998, Lee *et al.*, 2000, Kitanaga *et al.*, 2006), 2,6-dichloroisonicotinic acid (Wasternack *et al.*, 1994), salicylic acid (Kogel *et al.*, 1995, Lee *et al*., 2000), ethylene (Lee *et al*., 2000), gibberellin acid and brassinolide (Kitanaga *et al*., 2006). Further support for a role of thionins as defence peptides comes from studies where exogenous or endogenous thionin genes are overexpressed in plants. Overexpression of plant thionin genes leads to enhanced resistance against a broad range of bacterial and fungal pathogens (Bohlmann & Broekaert, 1994, Garc á Olmedo *et al.*, 1998, Epple *et al.*, 1997, Iwai *et al.*, 2002, Holtorf *et al.*, 1998, Chan *et al.*, 2005, Hoshikawa *et al.*, 2012, Muramoto *et al.*, 2012, Shirasawa-Seo *et al.*, 2002). Although plants possess protective mechanisms against their pathogens, their defensive network is not infallible. To successfully infect a plant, pathogens have evolved virulence molecules. For example, effectors secreted from pathogens can overcome, manipulate or suppress host defence and help to establish compatible interactions (Van Loon *et al*., 2006, Nomura *et al.*, 2005). *Oryza sativa* is susceptible to a variety of pathogens, and among which are plant parasitic nematodes. Based on scientific and economic importance, a list of the top 10 plant parasitic nematodes was put forward, and root-knot nematodes (RKN) were in the first position (Jones *et al.*, 2013). The rice root-knot nematode *Meloidogyne graminicola* is becoming the most damaging nematode since the rice production from traditional paddy system has shifted to water-saving systems (Tandingan *et al.*, 1996, Soriano *et al.*, 2000, Bridge *et al.*, 2005, De Waele & Elsen, 2007, Dangal *et al*., 2009). Yield losses of up to 87% have been reported (Jones *et al*., 2013). Infective second stage juveniles (J2) penetrate the roots and migrate intercellularly towards the root apex, and then they make a U-turn and move upwards in the vascular bundles to the differentiation zone, where they settle down and initiate permanent feeding sites. These large, multinucleated, metabolically active giant cells are the nutrient source from which the nematode feeds for the remainder of its sedentary life (Gheysen & Mitchum, 2011, Kyndt *et al.*, 2013a). Due to hyperplasy and hypertrophy of the surrounding cells, galls are appearing, typically hook-formed in the case of *M. graminicola*. During the infection process, root-knot nematodes secrete a cocktail of compounds, containing effectors and other chemicals, in order to establish a successful compatible interaction (Gheysen & Mitchum, 2011, Kyndt *et al*., 2013a). A previous transcriptome study of 3 days post inoculation (dpi) and 7dpi galls upon *M. graminicola* infection in rice revealed that, instead of up-regulation as could be expected for PR proteins upon pathogen infection, most rice thionin genes on chromosome 6 were down-regulated in galls (Kyndt *et al.*, 2012a, Table 3.1). Moreover, thionin genes on chromosome 6 were also attenuated in roots at 2dpi after *Pythium graminicola* infection (De Vleesschauwer *et al*., unpublished data). This might be the consequence of *M. graminicola* or

*Pythium graminicola* suppression of plant defence, indicating that rice thionins on chromosome 6 might be resistance factors against *M. graminicola* and *Pythium graminicola*. To test this hypothesis, we first examined the expression pattern of three rice thionin genes from chromosome 6 in different developmental stages of galls by qRT-PCR as well as by promoter-GUS analyses. Subsequently, *OsTHI7* was overexpressed in *O. sativa* cv. Nipponbare to test its effect on *M. graminicola* and *Pythium graminicola*. Finally, the regulation of three rice thionin genes in response to plant defence related hormones was studied by qRT-PCR and promoter-GUS analyses.

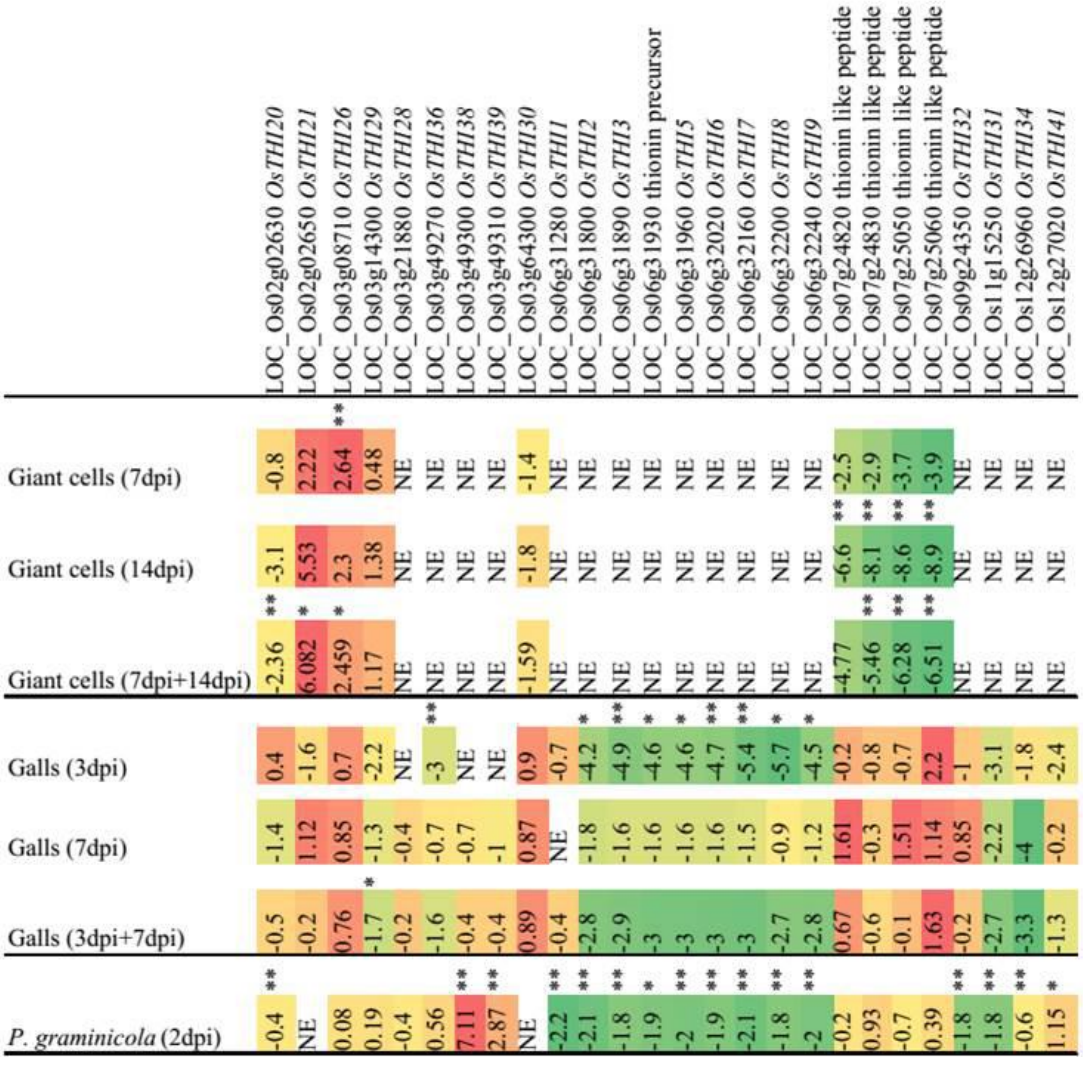
## 3.2 Results

### 3.2.1 The response of thionin genes upon *Meloidogyne graminicola* and *Pythium graminicola* infection

The expression profile of thionin genes in RKN-infected galls (Kyndt *et al*., 2012a) and giant cells (GC, Ji *et al*., 2013, chapter 5) as well as the whole roots in the case of infection with the oomycete *Pythium* (De Vleesschauwer *et al*., unpublished data ) was compared with their corresponding uninfected control tissues. Table 3.1 shows the $Log_2FC$ value of thionin genes in these infected tissues compared to the control. In GCs, *OsTHI21* located on chromosome 2 and *OsTHI26* /*OsTHI29* on chromosome 3 are induced at both time points as well as in the combined dataset (7dpi and 14dpi) when compared with uninfected vascular cells. However, thionin like peptides, mainly on chromosome 7, are suppressed in GCs at 7dpi and 14dpi, although the suppression at 7dpi is not significant. In whole galls, nevertheless, the transcripts of thionins are rather suppressed at both 3dpi and 7dpi in comparison with uninfected root tips. The suppression is mainly seen for the thionin genes from chromosome 6, like for instance *OsTHI1, OsTHI2, Oshi3, OsTHI5, OsTHI6, OsTHI7, OsTHI8 and OsTHI9*. Correspondingly, those genes are also strongly suppressed in *Pythium graminicola* -infected root tissue.

As 15 out of 40 annotated thionin genes are located on chromosome 6, and most of them are s uppressed in galls as well as in roots infected by *Pythium.* We decided to focus on the thionin genes from chromosome 6. Due to the high similarity in coding sequences and untranslated re gions among those genes, it is very difficult to separate the expression of *OsTHI3, OsTHI5, O sTHI6, OsTHI7* and *OsTHI9* by primer design for qRT-PCR with DNA-binding dyes, therefor e, we only studied the expression of *OsTHI1* (LOC_Os06g31280), *OsTHI2* (LOC_Os06g318 00), and *OsTHI7+6* (combined, LOC_Os06g32160 + LOC_Os06g32020) in further investigat ions. Due to high homology, the primer pair designed for *OsTHI7* potentially also binds to *Os THI6*. Hence, we consider the gene expression data recorded by this primer pair as a combinat ion of the expression pattern of both genes *OsTHI7* and *OsTHI6.*

Table 3.1 Differential expression patterns ($log_2FC$) of rice thionin genes in *M. graminicola* infected giant cells (Ji *et al*., 2013; chapter 5) and galls (Kyndt *et al*., 2012a) at different time points after infection, as detected by mRNA-seq, and in *Pythium graminicola* infected roots at 2dpi as detected by microarray (De Vleesschauwer *et al*., unpublished data), in comparison with the uninfected control tissue. *:p≤0.1; **:p≤0.05. Down- regulated genes, green; up-regulated genes, red. NE: not expressed in this tissue




### 3.2.2 Rice thionin genes are suppressed at early time points in galls induced by *M. graminicola*

In order to analyze the expression of rice thionin genes in more detail during the compatible interaction between *M. graminicola* and *O. sativa*, the expression of *OsTHI1, OsTHI2* and *OsTHI7+6* in galls and corresponding uninfected root tips (as control) was investigated by qRT-PCR (Fig 3.1). The gene expression analysis was done at 4 time points which are representing the whole life cycle of *M. graminicola.* All thionin genes are strongly suppressed in galls compared to control root tips until 7dpi. Even at 14dpi, *OsTHI7+6* appears still sup-

pressed in galls, although it is not significant. However, *OsTHI1* and *OsTHI2* are strongly induced at 14dpi.

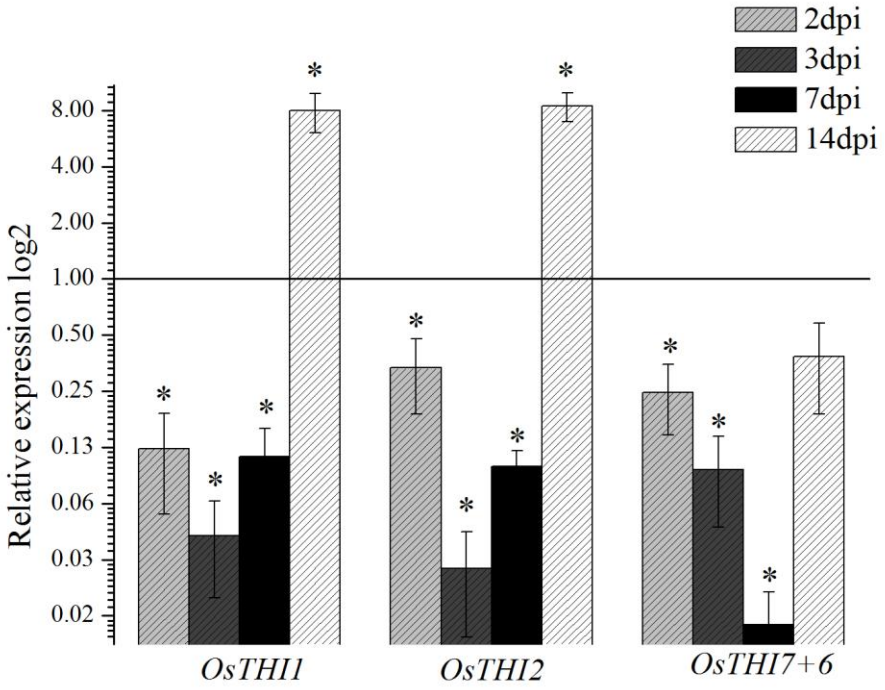



Fig 3.1 Relative expression levels of three rice thionin genes in *M. graminicola* induced galls compared to non-inoculated root tips at different time points after inoculation (dpi). Gene expression level was normalized using three internal reference genes, *OsEXP* and *OsUBQ5* and *OseIF5C*. Bars represent mean expression levels ±SE from 2 independent biological and three technical replicates each containing a pool of 8 plants. Data was obtained using REST2009 software. Significant differential expression ($P \leq 0.05$) in comparison with un-inoculated root tips (control expression level = 1) is indicated with asterisks. The black horizontal line shows the expression level in the untreated control plants (set at 1).

The qRT-PCR data (Fig 3.1) confirms and extends the transcriptome analysis of *M. graminicola* induced galls (Kyndt *et al*., 2012a), showing that *OsTHI7+6* is strongly and consistently suppressed in this compatible interaction. Hence, we selected *OsTHI7* as a candidate for further study. First, *OsTHI7*-promoter::*GUS* lines were generated and analyzed. In uninfected rice seedlings, GUS is only localized in the root tips meaning that OSTHI7 is expressed in the distal end of rice roots (Fig 3.2 E and F). The spatio-temporal localization of GUS in *M. graminicola* induced galls was also analyzed. GUS intensity is gradually lower during the development of galls (Fig 3.2 A-D). We further investigated the GUS expression in giant cells by cross sectioning of 7 dpi galls and corresponding root tips. As seen from Figure 3.3, there is nearly no GUS activity (pink staining, by dark field microscopy) was observed in 7dpi giant cells, but the pink stain was shown in control root tip sections.

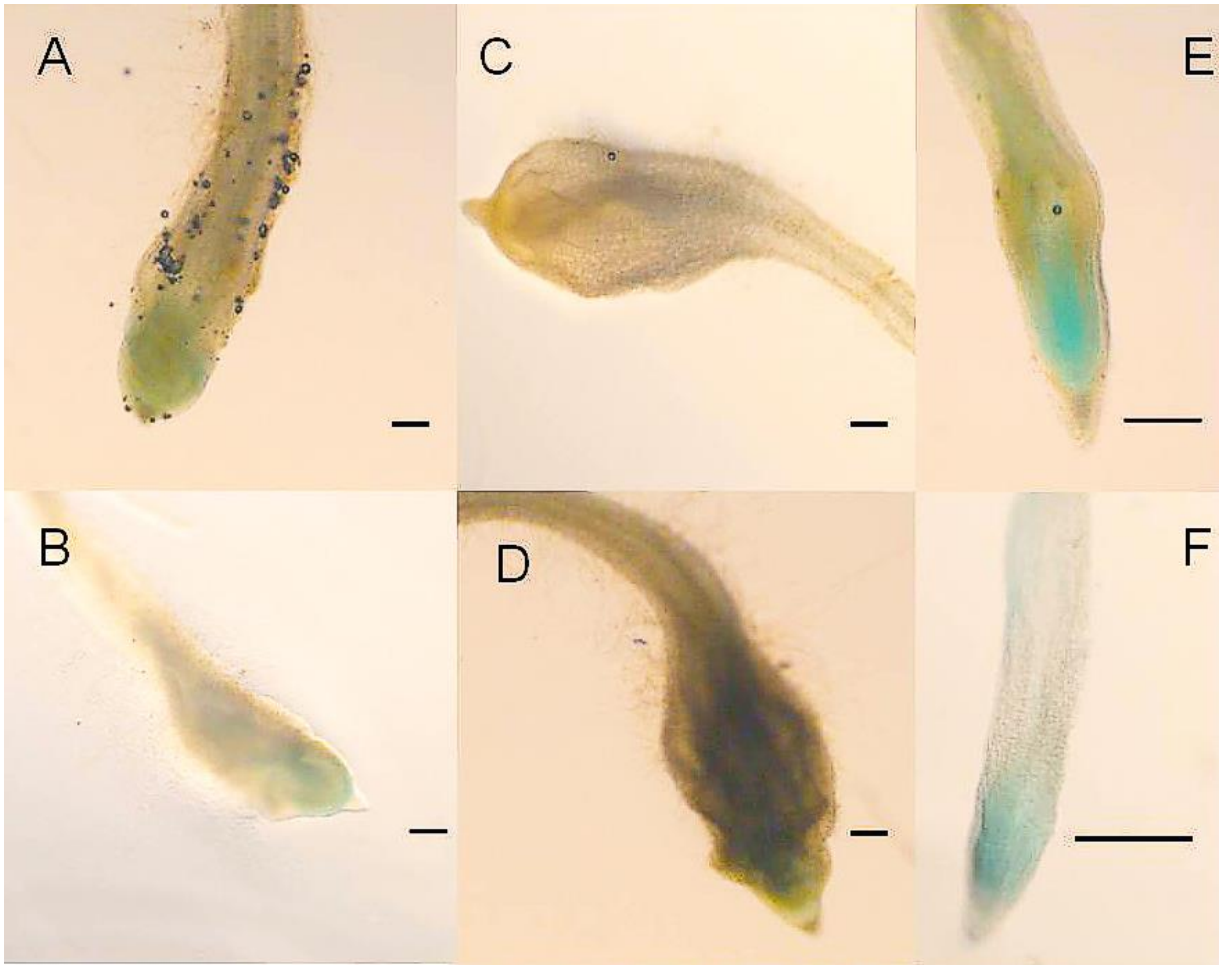



Fig 3.2 Spatio-temporal localization of GUS in *Meloidogyne graminicola* induced galls and in root tips of an uninfected *POsTHI7::GUS* line. **(A)** 2dpi **(B)** 3dpi **(C)** 7dpi **(D)** 14dpi **E** and **F**: un-inoculated root tips corresponding to the 7 and 14 dpi time points. Three independent lines were observed with similar results. GUS staining (blue) from one line was represented. Scale bar: 200 µm

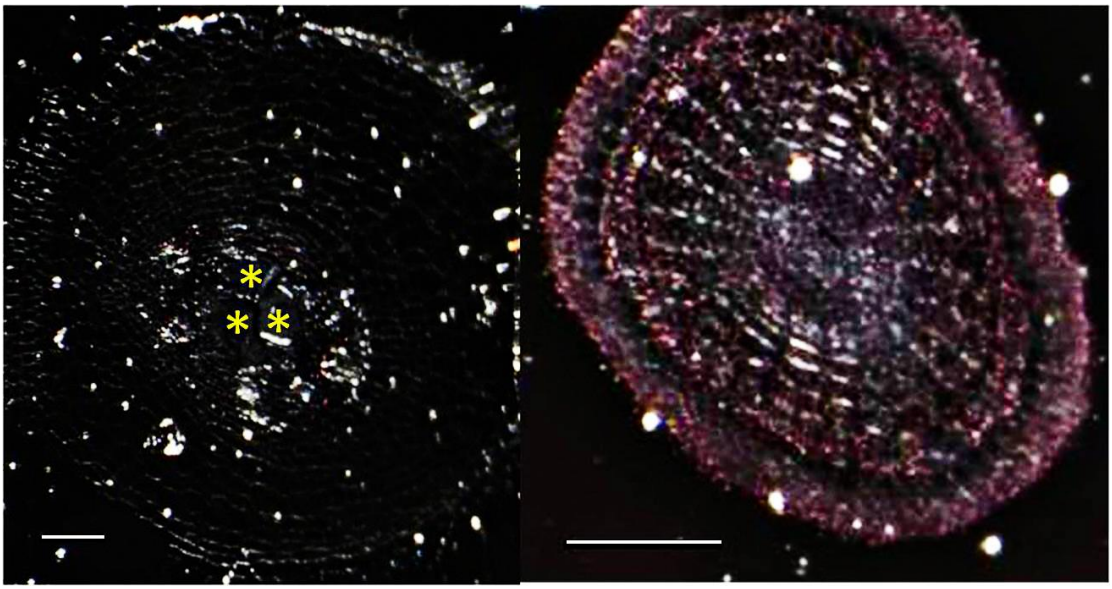


Fig 3.3 Thin section of 7dpi gall and corresponding root tip from the same line of *POsTHI7::GUS* as in Fig 3.2. GUS staining (pink color) was visualized by dark field microscopy. **(A)** 7dpi gall section **(B)** root tip section. *: giant cell, scale bar: 100 µm.

In conclusion, qRT-PCR data and histochemical analysis of GUS activity in *POsTHI7*::GUS lines showed that under normal growth conditions *OsTHI7* shows a root tip-specific expression pattern, and it is suppressed in giant cells and galls induced by *M. graminicola*.

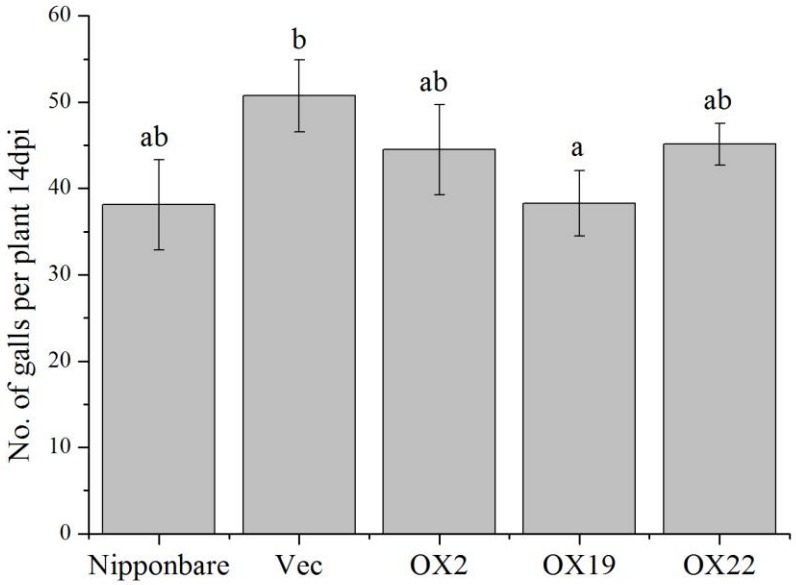
### 3.2.3 Overexpression of *OsTHI7* decreases plant susceptibility against *M. graminicola*

To find out whether *OsTHI7* can protect rice against *M. graminicola* infection, 30 independent T0 *OsTHI7* overexpression lines were generated, all the lines look phenotypically normal. The transformation was validated by PCR (data not shown) and semi-quantitative RT-PCR (Fig A5.A). The same vector, without insert, was also transformed to yield control plants.

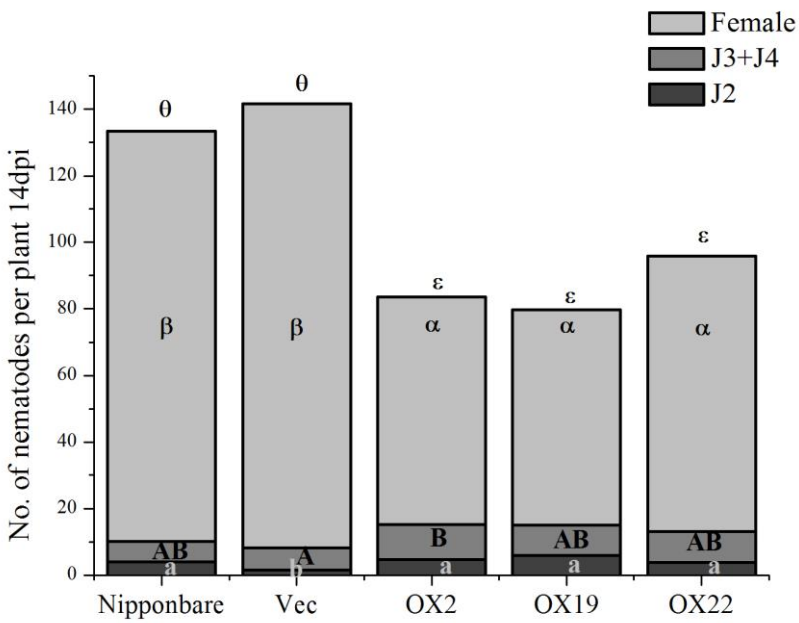
Three T1 hemizygous *OsTHI7* overexpression lines with single insertion (tested by segregation analysis) and good expression (Fig A5.B) were used in an infection experiment. As controls, one empty vector line and the wild type (cv. Nipponbare) were included. These three transgenic lines as well as the empty vector line look phenotypically normal (Fig A3). It has been demonstrated before that trans-gene or epigenetic effects of the transformation process can disrupt the homeostasis of a plant cell and lead to the expression of defence related proteins (Epple *et al*., 1997, Mittler *et al.*, 1995, Herbers *et al.*, 1996). However, in our case the expression of *PR1a* and *PR1b* is not changed in uninfected transformed plants compared to empty vector and Nipponbare control (Fig A5.B).

Two weeks after inoculation with *M. graminicola*, root and shoot length, number of galls, and different stages of nematodes were evaluated. At this time point, the root length of these five lines was not significantly different (Fig A4), however, the shoot length of the three overexpression lines was significant higher than the wild type plants Nipponbare (Fig A4). The number of galls was not significantly different in the *OsTHI7*-overexpression lines compared to control plants (Fig 3.4A). However, there was a significant reduction in the number of females and total nematodes per gall (average 39.2% reduction, Fig 3.4B) in the overexpression plants. Moreover, the galls in the control plants were relatively bigger than in the overexpression plants (Fig 3.4C).

A


No. of galls per plant 14dpi
60
50
40
30
20
10
0
ab
b
ab
a
ab
Nipponbare
Vec
OX2
OX19
OX22

B


Female
J3+J4
J2
No. of nematodes per plant 14dpi
140
120
100
80
60
40
20
0
θ
θ
ε
ε
ε
β
β
α
α
α
AB
A
B
AB
AB
a
a
a
a
a
Nipponbare
Vec
OX2
OX19
OX22

C

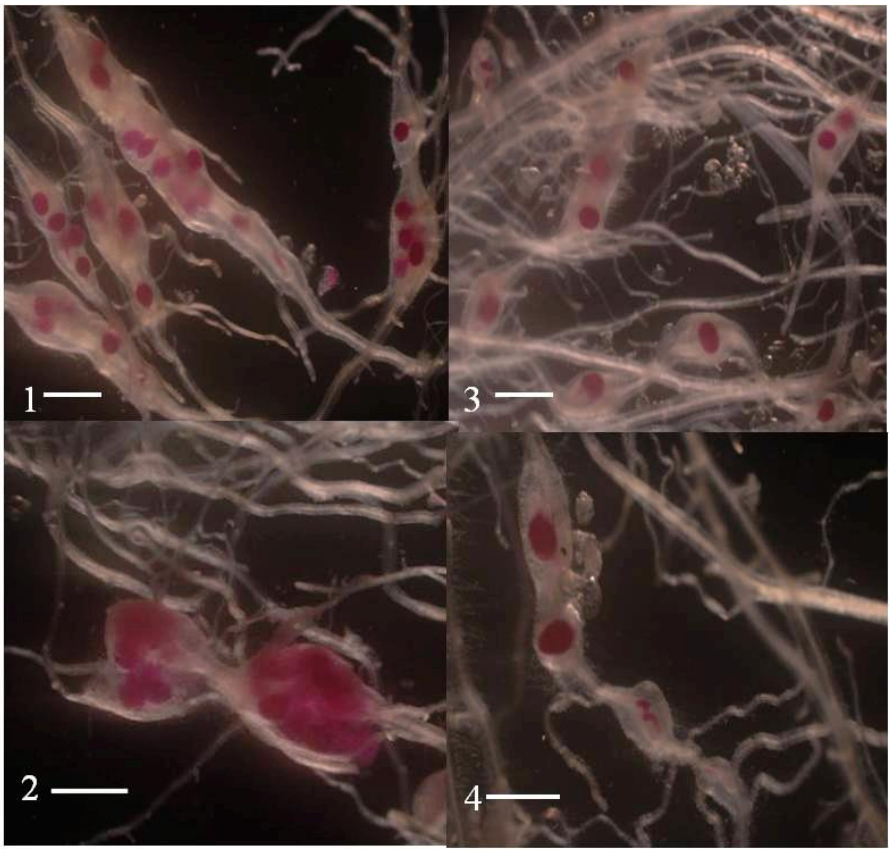


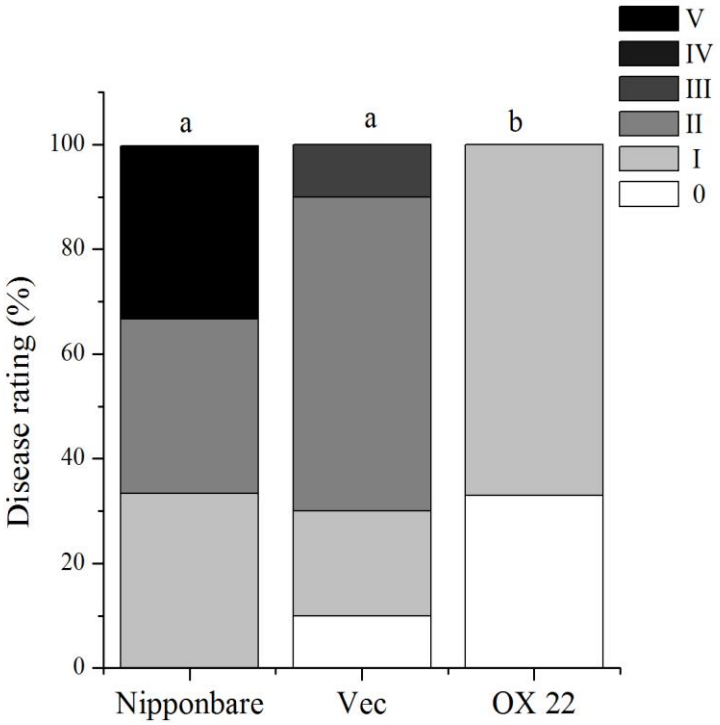

Fig 3.4 *OsTHI7* overexpressing plants exhibit reduced susceptibility to *M. graminicola*. Data obtained 14 days after inoculation (14dpi). (A) The number of galls in overexpression (OX2, OX19 and OX22) and control (Nipponbare and Empty Vector) plants. (B) The different developmental stages of *M. graminicola* within the galls in overexpression (OX2, OX19 and OX22) and control (Nipponbare and Empty Vector) plants. (C) Representative root system 14 days after infection with *M. graminicola* in different lines. 1. Empty Vector, 2. Nipponbare, 3. OX 19 and 4. OX2. Scale bar: 1mm. Bars represent means and ±SE of galls or nematodes per plant (n = 8). Different letters indicate statistically significant differences (Duncan's multiple range test with $P \leq 0.05$). a/b indicates the significant differences in J2 stage; A/B indicates the significant differences in J3+J4 stages. α/β indicates the significant differences in females, and θ/ε indicates the significant differences in the total of the infected nematodes. Data represent one of three independent experiments with similar results. The data obtained from the other two replicates are shown in addendum Fig A6. Vec: transgenic line containing an empty vector.

### 3.2.4 Overexpression of *OsTHI7* enhances plant tolerance to *Pythium graminicola*

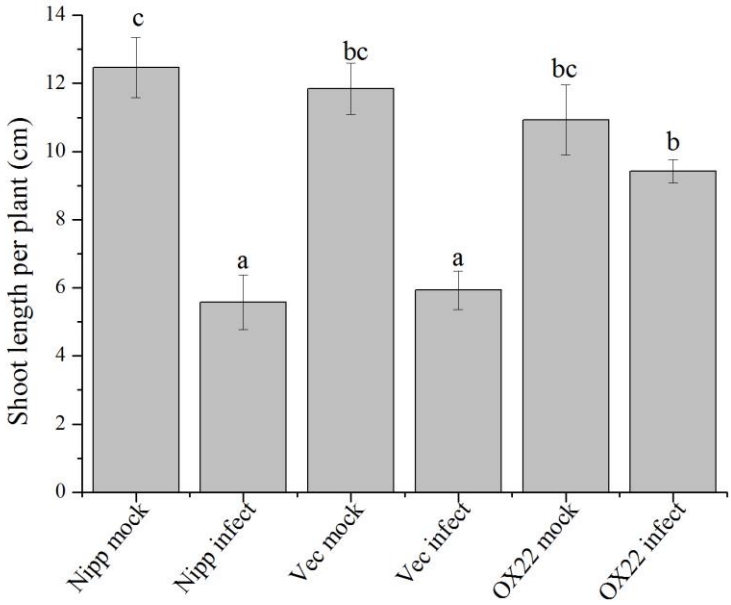
Since *OsTHI7* was also suppressed by the virulent oomycete *Pythium graminicola,* as shown by a microarray study (Table 3.1, De Vleesschauwer *et al*., unpublished data). We tested the effect of one overexpression line (OX22) after infection with *Pythium graminicola* to elucidate whether *OsTHI7* can also protect rice from other root pathogens. Seven days after *Pythium* infection, roots of wild type Nipponbare, empty vector and *OsTHI7*-overexpression plants all developed typical brown necrotic patches, and roots were stunted. There were no significant differences in disease index scores and disease rates of the root systems of controls and over-

expression plants (data not shown). However, shoots of the OX22-line appeared significantly healthier than the control lines, and with a higher shoot length and a lower disease index score (Fig 3.5).

A




**B**



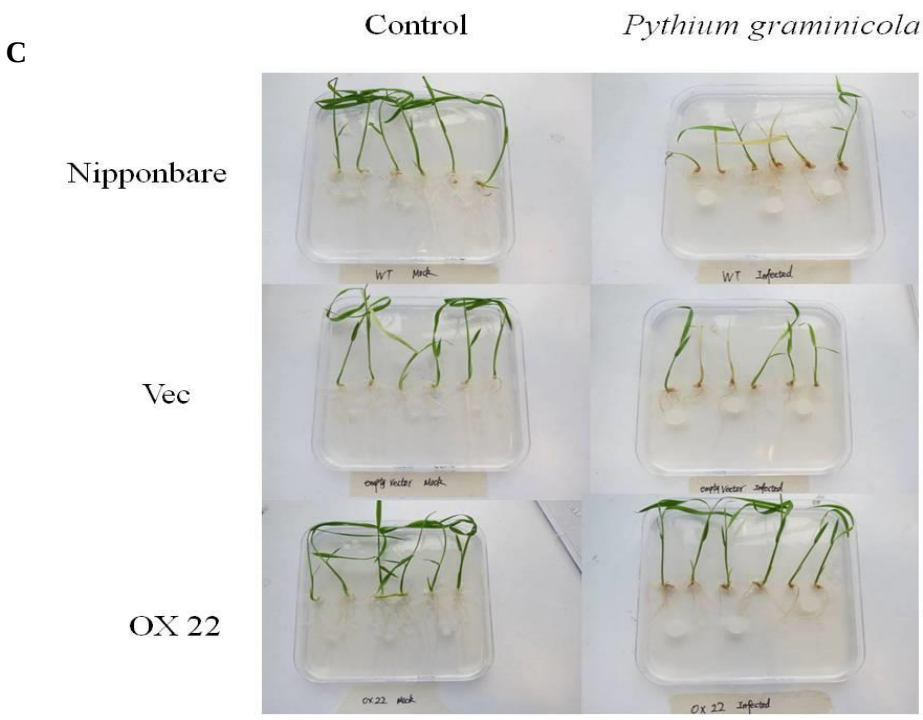



Fig 3.5 Susceptibility towards *Pythium graminicola* in *OsTHI7* overexpression line (OX22) in comparison with control (Nipponbare and Empty Vector) plants. OX22 enhanced rice shoot tolerance to root pathogen *Pythium graminicola* infection. Data obtained 7 days after infection. (A) The 0-to-V disease rating in control and overexpression plants. (B) The shoot length per plant. (C) Representative pictures were taken at the moment of disease scoring. Statistical analysis for disease rating was done using Mann Whitney test ($P \leq 0.05$). Statistical analysis for shoot length was done using Duncan's multiple range test $P \leq 0.05$. Bars represent means and ±SE from one biological replicates ($6 \leq n \leq 12$). Different letters indicate statistically significant differences. The data obtained from the other replicate are shown in addendum Fig A7. Vec: transgenic line containing an empty vector

### 3.2.5 OsTHI7 is localized in the secretory pathway in rice cells

As proteins within cells are spatially organized according to their roles, the study of protein subcellular localization is important to elucidate protein function. In order to further investigate the function of OsTHI7, a construct containing *OsTHI7* fused with *GFP* under the control of the maize ubiquitin promoter was transformed into rice plants and used to study the subcellular localization of OsTHI7. The leaves and roots of one week old *PUbi::OsTHI7-GFP* transgenic seedlings (T1) were viewed using a confocal microscope. As shown in Figure 3.6A, GFP (green color) was detected in punctuate structures in stomatal guard cells. These punctuate structures are likely Golgi bodies/or vesicles, and the bright spots (green color dots) are probably the result from aggregation of several Golgi bodies (Jung *et al.*, 2012). In roots, GFP was observed at the periphery of root cells (Fig 3.6B), however, the exact location is not clear.

Analysis of the OSTHI7 sequence by TargetP (Emanuelsson *et al*., 2000) and SignalP (Petersen *et al*., 2011) indicates that the peptide is likely to be secreted to the extracellular compartment (score 0.894), and sequence alignment with other known thionin peptides also indicates that OSTHI7 has a signal peptide (data not shown). Therefore, the GFP in the root is probably in the extracellular space. The protoplasts (big circle filled with green color and big circle in bright field) isolated from transgenic rice leaves showed that this peptide was located in the central vacuole since GFP expressed nearly in the whole protoplast (Fig 3.6C). The bright green dots in the protoplast are probably some cell orgenalles or the aggregations of this peptide, but this needs to be confirmed by markers.

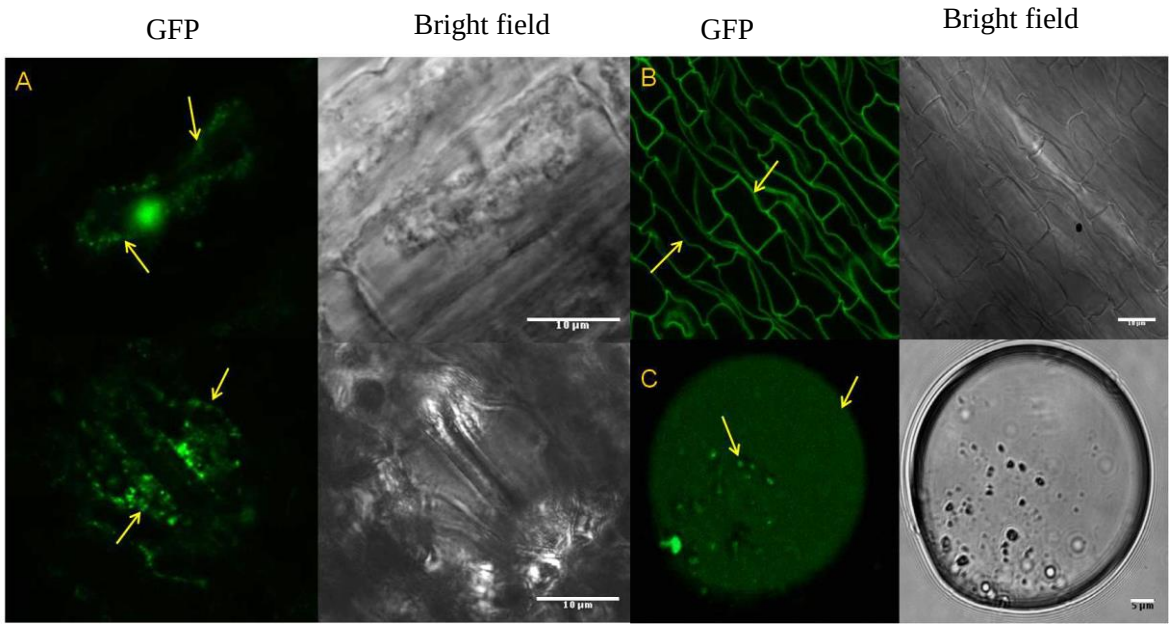



Fig 3.6 Confocal microscope images of different rice tissues expressing the *OsTHI7-GFP* under control of the ubiquitin promoter (Pubi::*OsTHI7-GFP*), similar observations from two different T1 lines. (A) In leaves of rice plants. Scale bar: 10 µm. (B) In roots of rice plants. Scale bar: 10 µm. (C) In leaf protoplasts of rice. Scale bar: 5 µm. A fluorescence pattern of GFP in the protoplast was targeted to a central vacuole. Yellow arrows indicate the observations described in the text.

Taken together, the results suggest that OsTHI7 is following in the secretory pathway, but whether it is secreted to the extracellular space or stays in the vacuole needs to be further investigation.

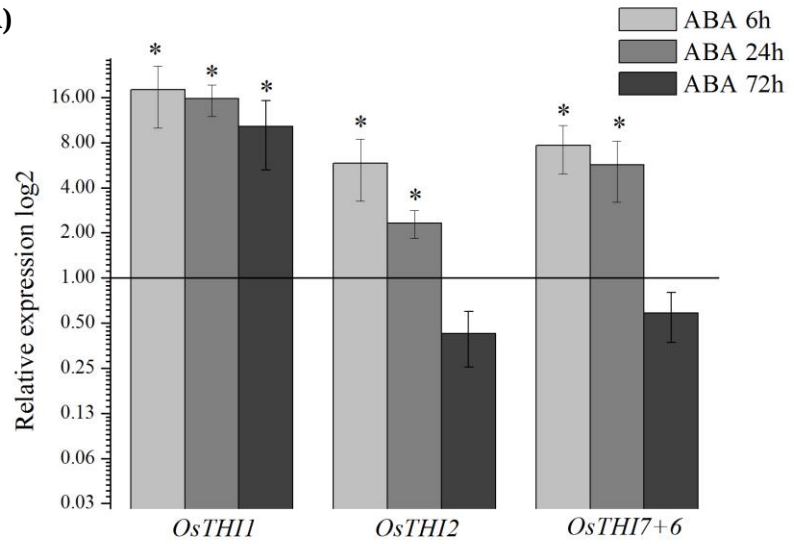
### 3.2.6 Rice thionin genes respond differently to plant hormones

Previous studies have shown that many PR-genes including plant thionin genes respond differentially to phytohormones (Bohlmann *et al*., 1998; Lee *et al*., 2000; Iwai *et al*., 2002; Kitanaga *et al*., 2006; Sels *et al*., 2008; Bari & Jones, 2009; Yazaki *et al*., 2003). Initial predictions using PLANT CARE indicated several hormone-related cis-elements in the promoter

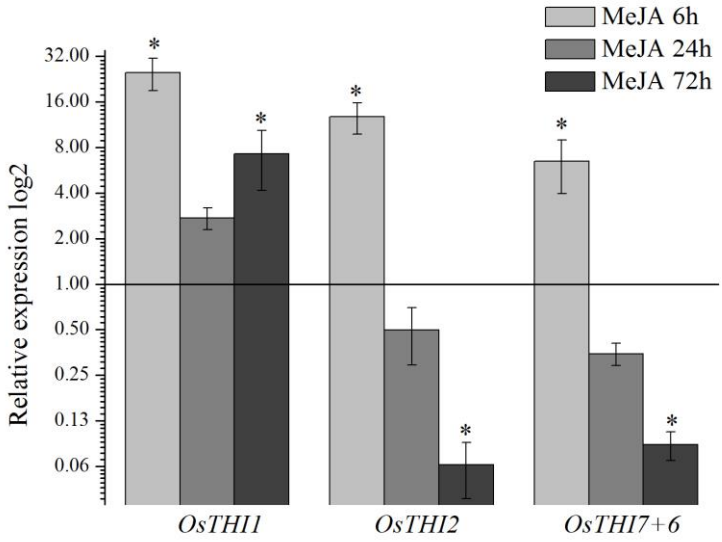
region of rice thionin genes (Table A1). Thefore, the expression pattern of these three thionin genes upon hormone treatments was investigated further by qRT-PCR.

Figure 3.7 shows that the root expression of the three thionin genes responds differently to plant hormone application. After 6 and 24 hours of ABA treatment, all thionin genes are significantly up-regulated; *OsTHI1* shows consistent induction at all the tested time points (Fig 3.7A). These results indicate that these thionin genes could be involved in the ABA mediated defence pathway. In the case of MeJA treatment, *OsTHI1* is responsive to MeJA at all-time points. *OsTHI2* and *OsTHI7+6* are up-regulated after 6h treatment but then attenuated gradually. After 72h treatment, mRNA levels of both genes were significantly down-regulated, which could be the result of feedback effects and/or modulation by crosstalk with other plant hormones. Upon BTH treatment, *OsTHI7+6* was persistently induced at all the investigated time points, while *OsTHI1* showed a very minor induction after 6h BTH treatment. *OsTHI2* was not significantly altered by this treatment (Fig 3.7C).

**(A)**


ABA 6h
ABA 24h
ABA 72h
Relative expression log2
16.00
8.00
4.00
2.00
1.00
0.50
0.25
0.13
0.06
0.03
OsTHI1
OsTHI2
OsTHI7+6

**(B)**


MeJA 6h
MeJA 24h
MeJA 72h
Relative expression log2
32.00
16.00
8.00
4.00
2.00
1.00
0.50
0.25
0.13
0.06
OsTHI1
OsTHI2
OsTHI7+6

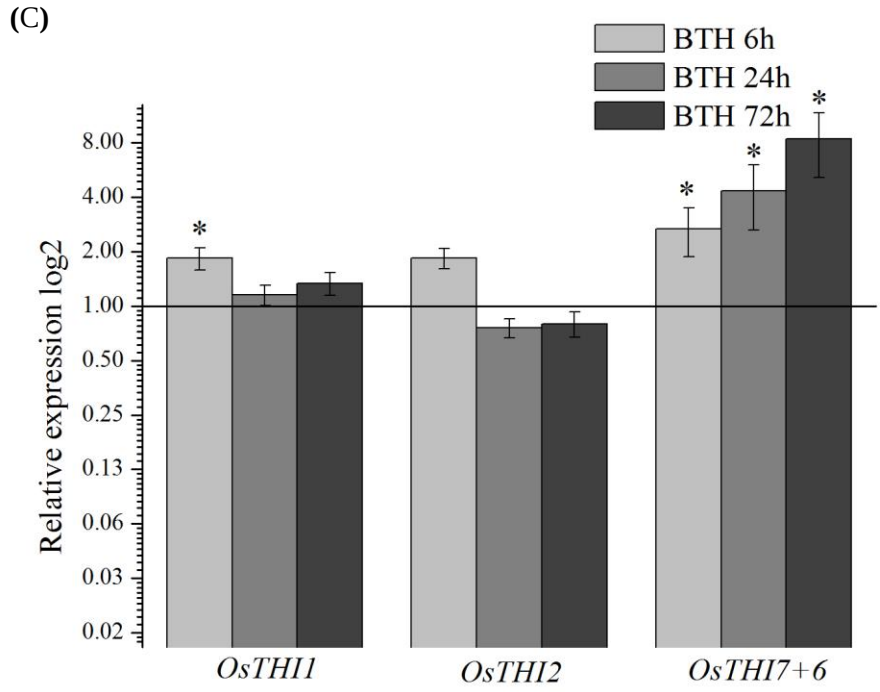



Fig 3.7 Analysis of the expression level of thionin genes in rice roots after root soaking in (A) 50 µm ABA, (B) 100 µm MeJA and (C) 250 µm BTH for 6h, 24h and 3days. Gene expression level was normalized using three internal reference genes, *OsEXP*, *OsUBQ5* and *OseIF5C*. Bars represent mean expression levels ±SE from 2 independent biological and three technical replicates, each containing a pool of 8 plant roots. Data was obtained using REST2009 software. Significant differential expression ($P \leq 0.05$) is indicated with asterisks in comparison with untreated control roots. The black horizontal line shows the expression level in the untreated control plants (set at 1).

For further validation of qRT-PCR data, GUS activity was studied in a *POsTHI7::GUS* line after 6h and 3 days phytohormone treatments. All the experimental conditions were the same as in the qRT-PCR assays. Our results in Figure 3.8G and H show that the untreated transgenic *OsTHI7*-Promoter::*GUS* line reveals consistent GUS staining only in the roots tips, which confirmed our previous observation that under normal growth conditions, OsTHI7 is confined to root tips. The *POsTHI7::GUS* line showed increased level of GUS-staining throughout the whole root system after 6h continuous ABA treatment. Apparently less GUS was detected in the root tips and proximal part of the roots after 3 days compared to 6h ABA treatment (Fig 3.8, A and B). In case of MeJA treatment, GUS induction was observed after 6h treatment but after 3days, there was nearly no GUS detected (Fig 3.8, C and D). Upon treatment with BTH, more GUS was localized throughout the root and not only in the root tip as in the control treatment (Fig 3.8, E and F). The results from our qRT-PCR and GUS-assays illustrate that rice thionin genes are hormone inducible.

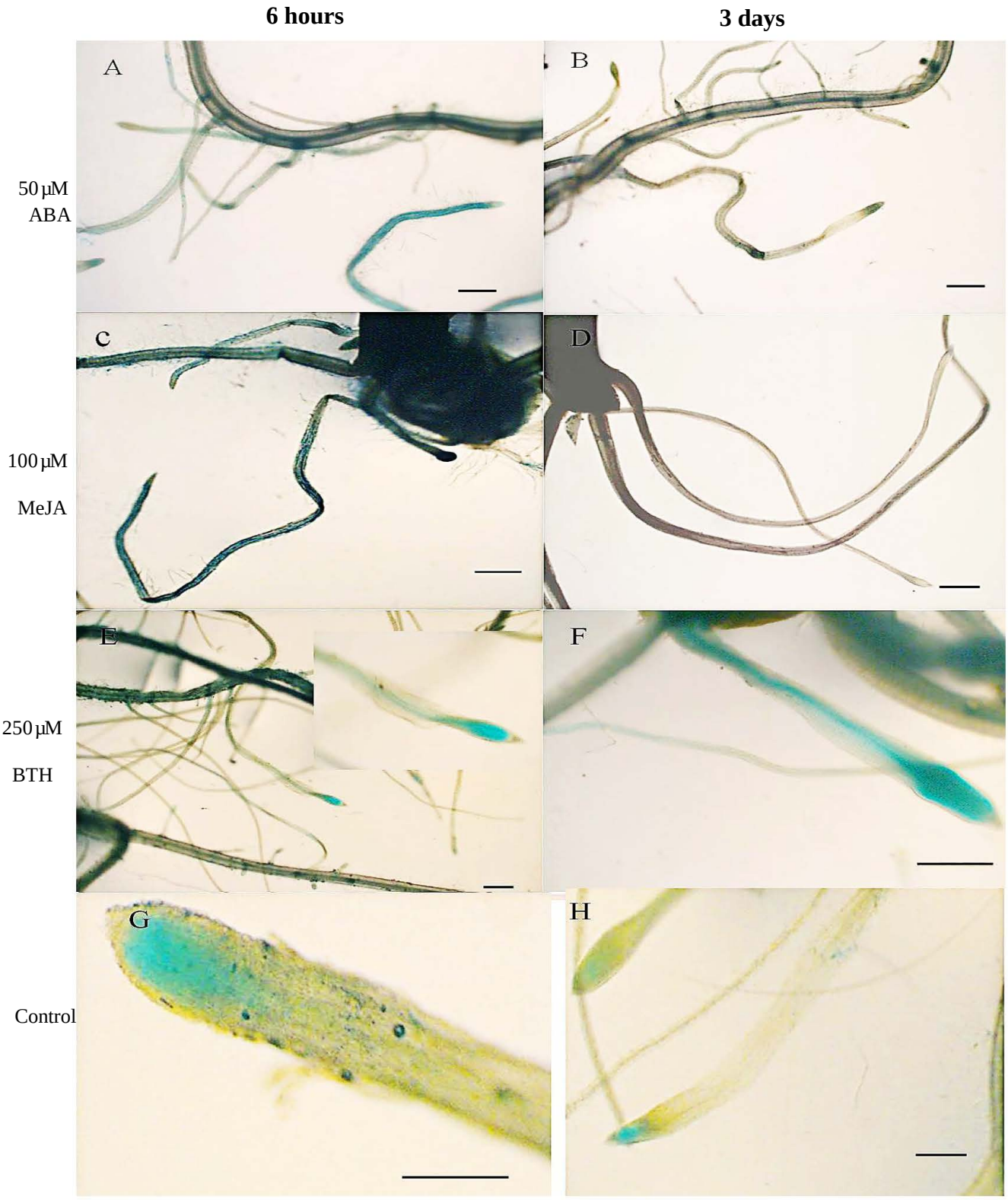



Fig 3.8 Analysis of GUS activity in one P*OsTHI7::GUS* line after phytohormone treatment for 6h and 3 days. Plant roots upon 50 µm ABA treatments (A) and (B), 100 µm MeJA treatments (C) and (D), and 250 µm BTH treatments (E) and (F). (G) and (H): control roots. Scale bar: 0.5mm.

## 3.3 Discussion

Thionins, identified as antimicrobial peptides and pathogenesis-related proteins, have been studied for decades (Edreva, 2005, Bohlmann & Apel, 1991), and many have shown that transgenic plants with enhanced thionin gene expression are more resistant to different fungal and bacterial pathogens (Bohlmann & Broekaert, 1994, Garcá Olmedo *et al*., 1998). Endosperm thionins of wheat are not only toxic to bacteria, yeast and fungi *in vitro*, but also show cytotoxic effects on insect larva (Garcá Olmedo *et al*., 1989). However, until now, there is no data on the effect of thionins towards nematodes. Furthermore, although there are a large number of thionin genes in the rice genome, there is no *in vivo* or *in vitro* research on the functional analysis of rice thionins.

In this study, the role of rice thionin genes in rice defence against root-knot nematodes was studied. Fifteen out of forty annotated thionin genes are on chromosome 6, and the coding sequences as well as amino acid sequences are highly similar. Probably the thionin genes on chromosome 6 have evolved through gene duplication, a process which has been shown to be very important for living organisms to get novel genes and to defend themselves against new pathogens (Martin & McInerney, 2009). To get more insight into the functions of rice thionins, we selected three rice thionin genes from rice chromosome 6 for further study, based on the available information from previous studies (Kyndt *et al*., 2012a). First, the expression of these thionin genes in root galls caused by *M. graminicola* was studied using qRT-PCR. Due to the high homology, it was impossible to design specific primer pairs for *OsTHI7 and OsTHI6* to be used in SYBR-Green qRT-PCR. Nevertheless, in the future, using fluorescent reporter probes might provide a solution for this problem. Four time points were chosen for this analysis. 2dpi and 3dpi are the time points when *M. graminicola* establishes a feeding site, 7dpi is a crucial time for nematode development, and 14dpi is almost the final time in the life cycle when the females start to produce eggs. Results show that *OsTHI7+6* is consistently down-regulated in galls at all the evaluated time points, although at 14dpi, the suppression is not significant. The observations are remarkably similar to the report of Kyndt *et al*. (2012a). *OsTHI1* and *OsTHI2* are both strongly down-regulated in 2, 3 and 7dpi galls, but considerably up-regulated at 14dpi. Most probably this induction is too late to provide effective plant defence. Down regulated thionin genes were also observed in *M. incognita* infected rice roots and after application other biotic stress which explored by Genevvestigator (Hruz et al., 2008). Suppression of thionin peptides was reported in the compatible interaction of barley with a virulent strain of *Erysiphe graminis* f. sp. *Hordei* compared to uninfected leaves, nevertheless, in an incompatible interaction, the level of thionins was not changed or slightly enhanced compared to control leaves (Ebrahim-Nesbat *et al*., 1993). These studies could indicate that the suppression of thionin genes is somehow contributing to a successful infestation. Since

*OsTHI7+6* is consistently down-regulated, we decided to study *OsTHI7* by genetic engineering for further elucidation of the role of *OsTHI7* in defence of rice against *M. graminicola*. The study of GUS activities in *POsTHI7::GUS* plants shows that OsTHI7 is weakly expressed at the root-tip. This peptide may have some functions to protect the fragile root tips from certain soil pathogens or insect infestation. However, as we know that the toxicity of PR-proteins is generally dose dependent (Stec, 2006) and cultivar Nipponbare is susceptible to *M. graminicola*, probably the endogenous *OsTHI7* level in the root tip is not strong enough to protect roots from *M. graminicola* infection. Alternatively, in our study, it has been shown that *M. graminicola* can strongly suppress thionin gene expression, which may avoid it to reach the toxic thionin dose for *M. graminicola*. Contrary to our observations, Iwai *et al*. (2002) detected no thionin gene expression at all in 2 weeks old rice roots, although they found the expression of endogenous thionin genes (several homologous genes) in coleoptiles of 4-day old plants using northern blot. This difference may be due to the different methodologies or materials. The qRT-PCR results were confirmed by the *PsOsTHI7::GUS* assays, where we additionally observed that there is nearly no GUS activity in 7dpi giant cells, whereas this gene is expressed in uninfected root sections. This result is also consistent with our study on giant cell transcripts by mRNA Seq ( Ji *et al*., 2013).

Next, we overexpressed *OsTHI7* in the susceptible cultivar Nipponbare. All of the tested three *OsTHI7*-overexpression lines showed less infection by *M. graminicola.* The enhanced resistance of the transgenic lines is probably due to a direct toxic effect of OSTHI7 to *M. graminicola*. Although we have not shown OSTHI7 has a toxic effect on nematodes, this peptide has all the characteristics which are important for toxicity (Bohlmann & Apel, 1991, Stec, 2006). It is basic and contains Tyr13 and Lys1, two residues that are determining toxicity by altering the permeability of cell membranes in various organisms, such as in cultured mammalian cells or fungal cells (Stec, 2006). When the roots were infected with *Pythium graminicola*, the shoots of OX22 also showed a significantly lower disease score and disease symptoms compared to control plants. In contrast, there were no significant differences on the length or disease symptoms in roots. These results indicate that this overexpression line is more tolerant to *Pythium graminicola* infection. However, the mechanism of the enhanced tolerance to *Pythium* infection is unclear.

Initial predictions by PSORT indicated that OSTHI7 has a large chance to be transported into the secretory pathway since it contains a predicted signal peptide. Our subcellular localization study shows that OSTHI7 is most probably translocated in the secretory pathway. However, the real localization still needs to be confirmed by co-localization with markers or by identification of the peptide in extracellular fluid. Previous studies have shown that the seed thionin and leaf thionin of barley were present in cell walls and vacuoles, respectively (Garcia-

Olmedo *et al.*, 1992, Bohlmann & Apel, 1987, Reimann-Philipp *et al.*, 1989, Iwai *et al.*, 2002). It has been shown that tobacco PR-1 protein and designated p14 protein of tomato, which is related to the PR-1 basic isoform of tobacco, were in two distinct locations: extracellular spaces or vacuoles (Vera *et al.*, 1989, Dixon *et al.*, 1991). The extracellular and vacuolar localization of OSTHI7 suggest that this peptide probably also has a dual localization. This localization may allow a direct toxic effect of OSTHI7 on *M. graminicola*, either by interacting with the external cuticle or by ingestion. Colgrave *et al.* (2010) reported that cyclotides, small disulfide-rich proteins having anti-microbial activity and insecticidal activity via disruption of cell membranes, had a toxic effect on the gastrointestinal nematode *Haemonchus contortus* of sheep, acting via its cuticle. Nevertheless, the exact mechanism of OSTHI7 decreased the susceptibility of rice plants against *M. graminicola* needs to be further studied.

Phytohormones not only coordinate all aspects of plant growth and development, but also modulate numerous stress or defence related responses in plants, such as the expression of PR proteins (Pieterse *et al.*, 2009, Pieterse *et al.*, 2012, Agrawal *et al.*, 2000). To understand the regulation of rice thionin genes by phytohormones, accumulation of the three thionin transcripts in rice roots was assessed after BTH, MeJA and ABA treatment to plant roots. The results revealed that all the transcripts were up-regulated by the three hormones at 6h, the earliest time-point in our test. The induction of the three rice thionin genes by treatment with BTH, MeJA and ABA at 6h indicates that those thionins genes may function as early defence genes. Our results demonstrate that *OsTHI1*, *OsTHI2* and *OsTHI7+6* are involved in ABA mediated defence response. ABA has been shown to play an important role in plant responses to abiotic and biotic stress (Wan & Li, 2006, Chinnusamy *et al.*, 2008, Cramer *et al.*, 2011). Yazaki *et al.* (2003) reported that *OsTHI2* was up-regulated in 30-day-old calli after a 3-day-treatment with either 50 µM ABA or 50 µM gibberellin acid. The presence of the ABRE motif in the promoter regions of these thionin genes suggests that they might be regulated directly by ABA through ABRE. It has been reported that exogenous application of MeJA to rice leaves significantly enhanced defence against *M. graminicola* in roots (Nahar *et al.*, 2011). The consistent induction of *OsTHI1* and the short-term effect on *OsTHI2* and *OsTHI7+6* upon MeJA treatment might contribute in part to the MeJA induced resistance against *M. graminicola*. When plants were treated with BTH, *OsTHI7+6* was induced dramatically. Nahar *et al.* (2011) reported that, although less strong than JA and ET, exogenous application of BTH induced a systemic resistance in rice roots to *M. graminicola*. Taken together with our infection experiments, these results indicate that *OsTHI7* may participate in the MeJA and SA mediated resistance against *M. graminicola*. Although in our qRT-PCR *OsTHI7* and *OsTHI6* could not be distinguished, the expression of *OsTHI7+6* in our qRT-PCR study upon hormone treatments was consistent with the expression of GUS in the *POsTHI7::GUS* line,

which indicates that *OsTHI7* and *OsTHI6* are regulated in a similar way or maybe that *OsTHI6* is not expressed. Moreover, there is a high similarity in the promoter sequences of *OsTHI7* and *OsTHI6* (98% identity).

Our results showed that some phytohormone responsive rice thionin genes do not have the known hormone related cis-acting elements in their promoter region, while some having specific hormone related cis-elements were not regulated by this specific phytohormone. This suggests that there are complex regulation networks between rice thionins and phytohormone signaling, and that they may for instance, be indirectly regulated by phytohormones (Liu *et al.*, 2009). The differential expression pattern of the four thionins upon hormone treatments indicates that, although there are high similarities in amino acid sequences among the thionin peptides, the response of the thionins to different stress may be different and diverse, as a consequence of evolution.

## 3.4 Conclusions

In this study, three thionins genes were shown to be locally down-regulated by rice root-knot nematode infection. Overexpression of *OsTHI7* lead to a lower number of nematodes in the transgenic plants demonstrating that *OsTHI7* may act as a defence gene *in vivo* against *M. graminicola*. However, more research needs to be done to study the mechanisms behind the induced resistance and the effect needs to be tested in the field. Moreover, the resistance to other plant parasitic nematodes or other pathogens still needs to be explored in the future. Furthermore, rice thionin genes, at least *OsTHI1*, *OsTHI2* and *OsTHI7+6* are regulated differently by plant defence- related hormones. This indicates that those thionin genes may be involved in different signal transduction pathways, and each of them has specific roles.

## 3.5 Materials and methods

### Plant material and growth conditions

Seeds of *O. sativa* cv. Nipponbare (GSOR-100; Genetic stocks oryza Collection, Washington DC, USA) were germinated on wet filter paper for 3 days at 30 ℃ and then transferred to SAP substrate (Reversat *et al.*, 1999) in PVC (polyvinyl-chloride) tubes and further grown at 26 ℃ under a 16h/8h light regime at a relative humidity of 70-75%.

### Nematode infection assay

The *M. graminicola* culture was provided by Prof. Dirk De Waele (Catholic University Leuven, Belgium) and was originally isolated in the Philippines. *M. graminicola* was maintained on *O. sativa* cv. Nipponbare in potting soil under the same temperature/light conditions as described above, and the nematodes were extracted using a modified Baermann funnel. Four-

teen-day-old rice plants were inoculated with about 250 second-stage juveniles of *M. graminicola* per plant or mock inoculated with water. To test thionin expression by qRT-PCR, 2dpi, 3dpi, 7dpi and 14dpi galls and corresponding root tips were collected and kept in -80 ℃ for RNA extraction. For the infection experiment on transgenic plants, the infection level of the plants was evaluated at 14dpi by counting the number of galls and nematodes per plant. To visualize the galls and nematode developmental stages, roots were stained with acid fuchsin as described by Nahar *et al*. (2011).

***Pythium graminicola* bioassay**

*Pythium graminicola* strain PB912 132 culture, inoculation and disease recording were according to Van Buyten and Höfte (2013). *Pythium graminicola* strain PB912 132 (Van Buyten and Höfte, 2013), isolated from a diseased aerobic rice field in Los Banos, The Philippines, and was cultivated on potato dextrose agar at 28 ℃ (PDA; Difco Laboratories). The husks of *Oryza sativa* cv. Nipponbare seeds were removed and seed surfaces were sterilized by 6% sodium hypochlorite for 20min, and subsequently rinsed three times with sterile water. Sterilized seeds were grown in square petri dishes (12x12cm) on standard strength Gamborg B5/1% plant agar medium at 28 ℃ (day)/26 ℃ (night) under 12-h photoperiod. Four days after germination, T1 seedlings were inoculated with PDA agar plugs containing *Pythium graminicola* strain PB912 132 in between each two plants. Control samples were mock-inoculated with the same size of PDA agar plugs. Disease symptom was only recorded on successful transformed T1 plants (checked by RT-PCR on leaves). Disease symptoms on rice shoots were rated on the basis of a disease severity scale: score 0, healthy shoots; I, shoot length more than 50% of the control, green culm and few yellow or brown spotted leaves; II, shoot length more than 34% of the control, slightly yellowing culm and yellow or brown spotted leaves; III, shoot length less than 34% of the control, slightly yellowing culm and yellow or brown spotted leaves; IV, shoot length less than 34% of the control, yellow culm and yellow or brown leaves; V, shoot length less than 34% of the control, brown, dried-out culm and leaves.

**Hormone treatment**

5-day old seedlings were grown hydroponically in 50% Hoagland solution. After 5 days, Hoagland was supplemented with methyl Jasmonate (MeJA, 100 µm, sigma-Aldrich NV/SA Bornem, Belgium), abscisic acid (ABA, 50 µm, Sigma-Aldrich NV/SA Bornem, Belgium) or Benzo-1, 2, 3-thiadiazole carbothioic acid S-methyl ester (BTH, 250 µm, Syngenta crop protection, Brussels, Belgium) solutions, which were prepared in 50% Hoagland solution and pure 50% Hoagland solution was used as control. The concentrations used have been opti-

mized before (Nahar *et al*., 2011, Nahar *et al*., 2012). After transfer to the hormone solution, root samples were collected at 6h, 24h and 72h for RNA extraction to study thionin gene expression. Two biological replicates, each composed of a pool of 8 individual plants, were taken.

**Semi-quantitative RT-PCR and quantitative real-time PCR**

RNA extraction and cDNA synthesis were performed and analyzed as described by Kyndt *et al*. (2012b). Semi-quantitative RT-PCR was used to detect the expression levels of *OsTHI7* in different overexpression lines on T0 generation. LOC_Os03g27010 (encoding an 'Expressed' protein) was used as internal control. qRT-PCR was performed and analyzed as described in Nahar *et al*. (2011) on T1 generation plants. The primer sequences used for RT-PCR and qRT-PCR are listed in Table 3.2. In all qRT-PCR experiments, expression of both *OsTHI7* and *OsTHI6* was detected simultaneously, as these two transcript sequences (including untranslated region) are highly similar and hence it was difficult to design gene specific primers in SYBR-Green qRT-PCR

Table 3.2 List of primers used in this study.

| Primers | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| **For qRT-PCR** | | |
| *OsTHI1* ( LOC_Os06g31280.1) | GCAGCATGAGCATATCCAAG | TTGCATTTCCCATCAACAAT |
| *OsTHI2* (LOC_Os06g31800.1) | ATGCAAGCATAAGGGCTTCA | GAGTGACAGAAATCAAGATAG |
| *OsTHI7+6* (LOC_Os06g32160 +LOC_Os06g32020) | TCGGGATGGATCCCTTTTGT | TTCATTGCTACCTCAGTTCCGA |
| *EXP* (LOC_Os03g27010) | TGTGAGCAGCTTCTCGTTTG | TGTTGTTGCCTGTGAGATCG |
| *UBQ5* (AK061988) | ACCACTTCGACCGCCACTACT | ACGCCTAAGCCTGCTGGTT |
| *EIF5C* (LOC_Os11g21990.1) | CACGTTACGGTGACACCTTTT | GACGCTCTCCTTCTTCCTCAG |
| **For cloning of genomic sequences** | | |
| *OsTHI7* DNA | GCTAGAAAGCATGGAAGGAGTG | CCTTATGCTTGCATGTTGCAC |
| *OsTHI7* DNA no stop | GCTAGAAAGCATGGAAGGAGTG | GGAAACAGCGGTGACAGTC |
| *OsTHI7* promoter | ACTCCCTTTCCCTCCCTGT | GCTTTCTAGCTTGTTTTCCAATG |
| **RT-PCR for testing the transformation on T0 generation** | | |
| For *OsTHIi7* OX and *GFP* fusion (no stop) | TCCAGGTGGAAGCAAAGAGT (part of *OsTHI7)* | GAACTTCAGGGTCAGCTTGC (part of e*GFP*) |
| For promoter-GUS and empty vector | | |
| *GUS* | CAACGAACTGAACTGGCAGA | GGCACAGCACATCAAGAGA |
| *eGFP* | ACGTAAACGGCCACAAGTTC | TGCTCAGGTAGTGGTTGTCG |
| **qRT-PCR and RT-PCR on T1 generation of OX and control plants** | | |
| *OsTHI7* cDNA | AACTGCGAGCCTCCTTACG | GGCAAAAAGAGTGCTCATGG |
| *OsPR1a* (AU163470) | GTATGCTATGCTACGTGTTTATGC | GCAAATACGGCTGACAGTACAG |
| *OsPR1b* ( AK107926) | ACGCCTTCACGGTCCATAC | AAACAGAAAGAAACAGAGGGAGTAC |

**Gene isolation, vector construction and rice transformation**

The *OsTHI7* genomic DNA sequence including two introns (737 bp), the *OsTHI7* genomic DNA lacking the stop codon (709 bp) and the DNA sequence upstream of the *OsTHI7* start codon (1994 bp) were amplified separately using PCR from *O. sativa* cv. Nipponbare. The primer sequences are provided in Table 3.2. The vector construction was performed by Gateway$^{R}$cloning (Life Tech). Vector PMBb7Fm21GW-UBIL was used for *OsTHI7* overexpression and *OsTHI7::GFP* fusion, and pBGWFS7,0 was used for promoter-GUS line construction. Both vectors were obtained from Plant Systems Biology (VIB, Belgium). All the inserted sequences were sequenced before introduction into *Agrobacterium*.

The binary vectors were introduced into *Agrobacterium tumefaciens* EHA105 cells using triparental mating. Transformed cells were selected on YEB agar plates (5g/L beef extract, 5g/L peptone, 1g/L yeast extract, 5g/L sucrose) containing spectinomycin (100 µg/ml). Rice transformation was done according to an in-house protocol based on the protocols of Zhang *et al.* (1997), Hiei *et al.* (1994), Paine *et al.* (2005). Transformed calli were selected using 50 µg/ml glufosinate (Sigma, Belgium), and the regenerated rice plants were grown in a growth chamber under 16/8 h of light/dark, 26 ℃ and 70-75% relative humidity. As a control, transgenic lines containing the PMBb7Fm21GW-UBIL vector without insert (empty vector) were generated.

Segregation analysis was done on seventy T1 seedlings by selection on 8mg/L Basta, and results were analyzed with the Chi Square test (P=0.05). Three overexpression lines and one promoter GUS lines with a 3:1 segregation were used for further experiments. One line transformed with the empty vector was used as control.

**Histological analysis of *OsTHI*7 promoter::GUS activity**

Histochemical GUS activity of whole seedlings was carried out as described by Delporte *et al.* (2011) with a few modifications. After reaction, the samples were washed several times using 70% ethanol and stored in 100% ethanol for a few days to wash out the excess dye and chlorophyll. Before transfer to microscopic slides, seedlings were incubated in lactic acid to clear the plant material, GUS staining was observed under a binocular microscope (Leica, S8APO, Germany).

**Embedding for transverse sections**

After GUS staining, galls and root tips were first rehydrated in 50% ethanol for 2h and 30% ethanol for 2h, and then incubated in water overnight. The rehydrated materials were fixed in 2% glutaraldehyde in 1x PIPES buffer and dehydrated in a series of ethanol solutions and in

absolute ethanol overnight. After dehydration, samples were infiltrated in Technovite 7100 (Heraeus Kulzer, Belgium) overnight. Afterwards, the samples were embedded in Technovite 7100 and solified at 37℃ for two days. After polymerization, samples were sectioned in 10μm in a Leica RM2265 motorized rotary microtome (Leica microsystems, Nussloch, Germany) and GUS staining was observed in dark field using a binocular microscope (Leica, S8APO, Germany).

**Subcellular Localization**

Leaves and roots were collected from one week old transgenic *PUbi::OsTHI7-GFP* plants, and cut into 1cm pieces, then directly mounted onto slides with a drop of glycerol. Protoplasts were extracted from 10 day old leaves cut into about 0.5mm strips using sharp razors. The strips were immediately transferred into enzyme solution (1.5% Cellulase RS, 0.75% Macerozyme R-10, 0.6 M mannitol, 10 mM MES at pH 5.7, 10 mM CaCl2 and 0.1% BSA) and incubated for 5 hours with 50 rpm shaking in the dark. By the end of the enzyme digestion, the solutions were shaken by hand for several seconds to release protoplasts to the enzyme solution. The digestion was terminated by adding an equal volume of W5 (154 mM NaCl, 125 mM CaCl2, 5 mM KCl and 2 mM MES at pH 5.7) solution. The protoplasts were collected by centrifugation at 1000 rpm for 3 min. After discarding the supernatant, the pellets were re-suspended in 50 μl W5 solution to be ready for microscopical analyses. The microscope pictures were taken using a Nikon A1R confocal system, mounted on a Nikon Ti microscope body.

The prediction of the subcellular localization of OSTHI7 was performed by using the software Signal P and Target P. The full length of the peptide (135 amino acids) was subjected to the search.

**Statistical analysis**

Data obtained from qRT-PCR was analyzed using the software Rest 2009. All other statistical analyses were performed using the software SPSS (version 21). The normality of data was checked by the Kolmogorov-Smirnov test of composite normality ($\alpha = 0.05$). Homogeneity of variance was checked by the Levene test ($P \leq 0.05$). Then analysis of variance (one-way ANOVA) and multiple comparisons of differences between treatments was performed (Duncan's multiple range test, $P \leq 0.05$). *Pythium* disease rate was analyzed by the non-parametric tests Mann Whitney ($P \leq 0.05$)

# CHAPTER 4

## β-aminobutyric acid–induced resistance against root-knot nematode in rice is based on increased basal defence

Hongli Ji, Tina Kyndt, Wen He, Bartel Vanholme and Godelieve Gheysen

Manuscript in preparation

## Abstract

The non-protein amino acid β-aminobutyric acid is known to protect plants against various pathogens. The mode of action is relatively diverse and specific in different plant-pathogen systems. To extend the analysis of the mode of action of BABA to plant parasitic nematodes in monocot plants, we evaluated the effect of BABA against the root-knot nematode (RKN) *M. graminicola* in rice. BABA-treatment of rice plants inhibits nematode penetration and resulted in delayed nematode and giant cell development. BABA-induced resistance (BABA-IR) was still functional in mutants or transgenics which are defective in salicylic acid (SA) biosynthesis and response, or abscisic acid (ABA) response. Pharmacological inhibition of jasmonic acid (JA) and ethylene (ET) biosynthesis showed that JA and ET are also not needed for BABA-induced resistance against rice RKN. However, histochemical and biochemical quantification and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) data indicate that BABA protects rice against RKN through the activation of basal defence mechanisms of the plant such as ROS accumulation, lignin formation and callose deposition.

## 4.1 Introduction

The non-protein amino acid β-aminobutyric acid (BABA) has been shown to induce resistance against a wide range of pathogens. It is not only effective against biotic stress factors, but also some types of abiotic stress (Cohen, 2002, Jakab *et al.*, 2001, Jakab *et al.*, 2005, Zimmerli *et al.*, 2008, Pastor *et al.*, 2013). The mechanisms of BABA-IR are diverse and they appear pathogen-system specific (Cohen, 2002, Jakab *et al*., 2001). In tobacco, BABA was no longer able to protect plants against Tobacco Mosaic Virus (TMV) in plants overexpressing the *NahG* gene, which decreases endogenous SA levels (Siegrist *et al.*, 2000). This indicates that BABA-IR against TMV in tobacco is SA-dependent. In addition, in *Arabidopsis*, priming by BABA against the bacterial pathogen *Pseudomonas syringae* pv. Tomato DC3000 and the fungal pathogen *Botrytis cinerea* depends on an intact SA signaling pathway, but is independent of a functional JA/ET pathway (Zimmerli *et al.*, 2000, Zimmerli *et al.*, 2001). However, *NahG* tobacco plants challenged with downy mildew showed no difference in protection by BABA compared to wild type plants (Cohen, 2001). BABA-IR against the oomycete pathogen *Hyaloperonospora parasitica* was still active in *Arabidopsis* genotypes impaired in either the SA, ET, or JA signaling pathway (Zimmerli *et al*., 2000). Moreover, BABA-IR against two necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina* were unaffected in *Arabidopsis* mutants impaired in camalexin synthesis, JA sensitivity, ethylene sensitivity and SA signaling (Ton & Mauch-Mani, 2004). Cytological investigations at sites of attempted penetration by those pathogens demonstrated the augmentation of callose-rich pa-

pillae (Zimmerli *et al*., 2000, Ton & Mauch-Mani, 2004), and the plant hormone ABA seems to play an important role in the BABA-primed callose deposition in *Arabidopsis* (Ton & Mauch-Mani, 2004). BABA-IR in lettuce against the oomycete *Bremia lactucae* also worked through a rapid encasement of the primary invading structures of the pathogen with callose and $H_2O_2$ accumulation (Cohen *et al*., 2010). In BABA-treated grapevine leaves, a strong ROS production was specifically observed in response to downy mildew *Plasmopara viticola* (Dubreuil-Maurizi *et al.*, 2010). However, BABA-treated sunflower inoculated with *Puccinia helianthi* (Amzalek & Cohen, 2007) showed no microscopic cellular responses.

A number of studies have demonstrated that BABA-IR can provide control against plant parasitic nematodes. The first study of BABA-IR against plant parasitic nematodes was carried out by Oka *et al* (1999). Application of BABA to tomato plants, either as a soil drench or a foliar spray, reduced root-galling or numbers of eggs produced by *M. javanica* (Oka *et al.*, 1999). Pre-treatment with BABA as a soil drench of 2mM significantly reduced the number of galls and egg masses induced by *M. javanica* in mung bean plants (Ahmed *et al.*, 2009). BABA-induced resistance was shown not only to be functional in dicots but also in monocots. On wheat, foliar sprays and soil drenches with BABA induced resistance against the cyst nematodes *H. avenae* and *H. latipons*, as well as RKN *Meloidogyne* spp. (Oka & Cohen, 2001). On pineapple, foliar sprays of BABA were effective against *M. javanica* (Chinnasri *et al.*, 2006). However, there is nearly no research on the molecular mechanisms of BABA-IR against plant parasitic nematodes. In the present study, we show that soil drenching with BABA is actively inducing resistance against RKN parasitism in rice roots. Studies with mutants or inhibitors point out that SA, JA and ET are not required for BABA-IR against RKN, while ABA might play a minor role. Instead, histochemical and cytological investigation of the defence reactions demonstrated that the basal defence of rice is enhanced with boosted hydrogen peroxide, lignin and callose accumulation in the BABA treated plants. Our data favor a model in which BABA induced resistance against *M. graminicola* acts through augmenting multiple facets of the plant basal defence independent of the SA, JA and ET pathways.

## 4.2 Results

### 4.2.1 BABA induces resistance against RKN in rice, by inhibiting nematode penetration and delaying nematode and giant cell development

*BABA induces resistance against M. graminicola in rice*

To determine if BABA can be used as an elicitor to induce rice resistance against the RKN, the effect of rice root susceptibility to *M. graminicola* was investigated after BABA treatment (Fig 4.1A). Compared to control plants pre-treatment with BABA resulted in a significantly

lower number of females per plant. This indicates that BABA is an excellent inducer of rice defence against *M. graminicola*.

*BABA-treated plants attract similar amounts of infective juveniles compared to control plants*

In order to obtain a more detailed understanding of BABA-induced resistance against *M. graminicola*, we continued to study at which infection stages the induced resistance is functional. Second stage juveniles (J2) in the soil are attracted to the root tips according to molecular signals coming from plant roots (Curtis *et al.*, 2009). We examined whether there is a difference in attraction of J2 in BABA versus control (water-treated) roots. Nine hours after initiation of the attraction assay, the number of nematodes within 1mm of the root tips was counted. The result in Figure 4.1B shows that there is no significant difference ($p > 0.05$) in number of J2 close to root tips of BABA-treated and control roots. From this data we can conclude that BABA does not influence nematode attraction to the roots.

*BABA-treated plants have lower M. graminicola penetration*

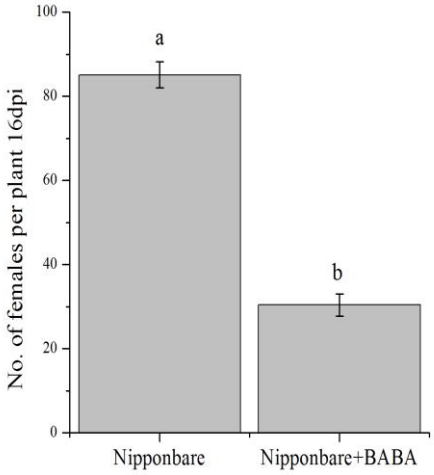
To further evaluate the active phase of BABA induced resistance against *M. graminicola*, the number of J2 inside roots was counted 50 hours after inoculation. As Figure 4.1C shows, BABA-treated plants exhibit significantly fewer number of J2 ($p<0.05$). This result illustrates that BABA induced resistance starts to be functional at the penetration stage. This effect could not be caused by toxicity effects of BABA on nematodes, since the nematode mobility is not impaired when J2 were incubated in BABA solution for 72 hours (data not shown).

*BABA-treatment of the plants delays M. graminicola development*

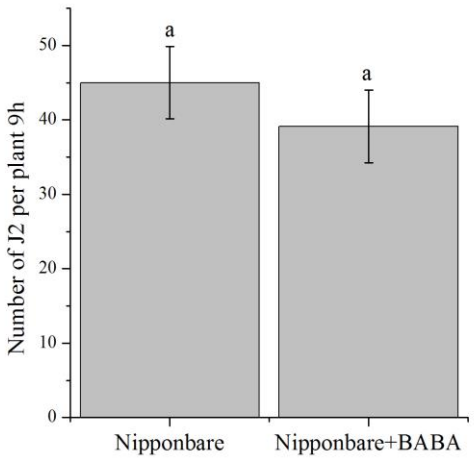
The developmental stages of *M. graminicola* in BABA treated plants were also recorded sixteen days after infection (dpi) and compared with control plants at this time point (Fig 4.2). The experiment was split in two parts: (i) pre-treatment: treatment with BABA (Fig 4.2A) for one day and then subsequently inoculated with RKN and (ii) post-treatment: pre-inoculation of RKN and two days later treated with BABA for two days (Fig 4.2B). At 16dpi, remarkably fewer and smaller females ($p<0.05$) were recorded in BABA treated roots in both experiments. The majority (87-94%) of nematodes in control plants had developed into females at 16dpi, however, much lower percentages of females were observed in BABA treated plants, with only about 52% and 9%, respectively, in pre-or post-treatment. Besides less females in BABA treated roots, the total number of nematodes in pre-treatment is significantly lower, which again confirms that fewer nematodes penetrate BABA-treated roots. The post-treatment has even a stronger effect on BABA induced resistance against RKN, as a large portion (91%) of nematodes remains in the juvenile stages. A slightly lower number of nematodes was also

observed in the post-treatment experiment, although one would expect that the nematodes had already entered the root system before the chemical was ap plied (at 2 dpi). Figure 4.2C shows representative galls at 16dpi in the control and BABA-treated plants. A delay of nematode development was clearly seen in BABA treated plants; the majority of the nematodes in the control plants were producing egg masses, while nematodes in BABA-treated plants were still in juvenile stages.

(A)




(B)



(C)

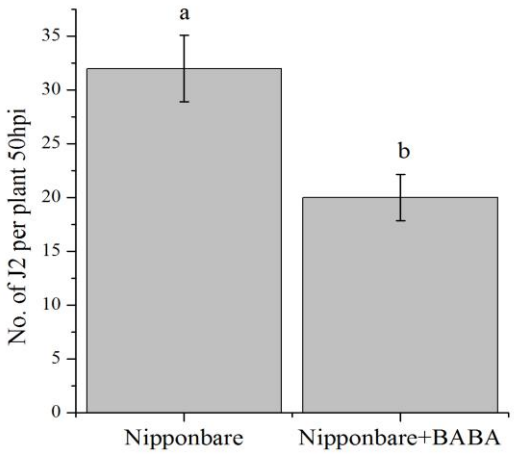
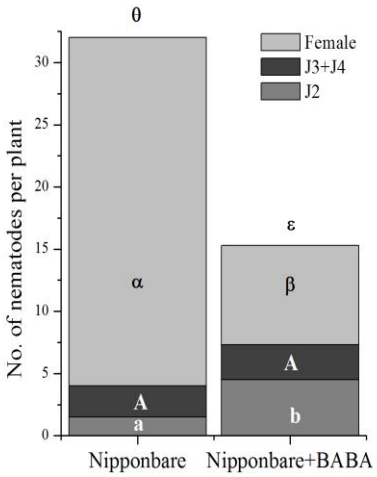
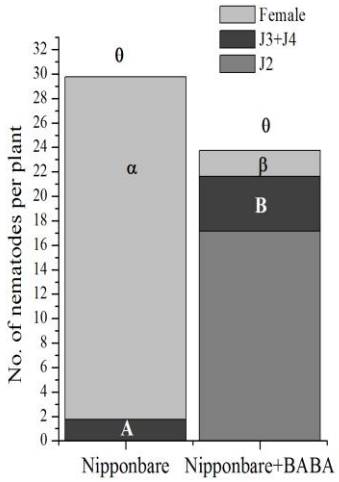



Fig 4.1 (A) Plants were soil drenched with 3.5mM BABA or water one day before RKN infection. Sixteen days after infection, females per plant were recorded. (B) Analysis of BABA-induced resistance against RKN on the stage of attraction. Plants were soil drenched with 3.5mM BABA or water one day before RKN infection. The number of J2 close to the root tips (about 1mm) were recorded at 9h after initiation of the experiment. (C) Effects of BABA on invasion of *M. graminicola* to rice plants. Plants were soil drenched with 3.5mM BABA or water one day before RKN infection. Number of nematodes was recorded 50 hours post infection. Bars represent means and ±SE of nematodes per plant (n = 8). Different letters indicate statistically significant differences ($P \leq 0.05$). Data represent one of two (B) or three (A and C) independent experiments with similar results. The data from other replicates are represented in addendum Fig A9.

(A)


θ
Female
J3+J4
J2
No. of nematodes per plant
ε
α
β
A
A
a
b
Nipponbare
Nipponbare+BABA

(B)


Female
J3+J4
J2
θ
θ
No. of nematodes per plant
α
β
B
A
Nipponbare
Nipponbare+BABA

**(C)**

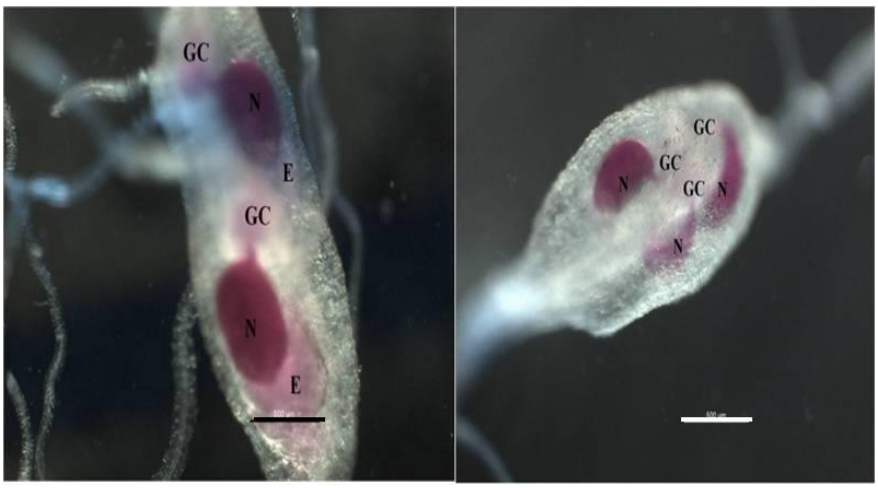

GC
N
E
GC
N
E
GC
GC
GC
N
N
N

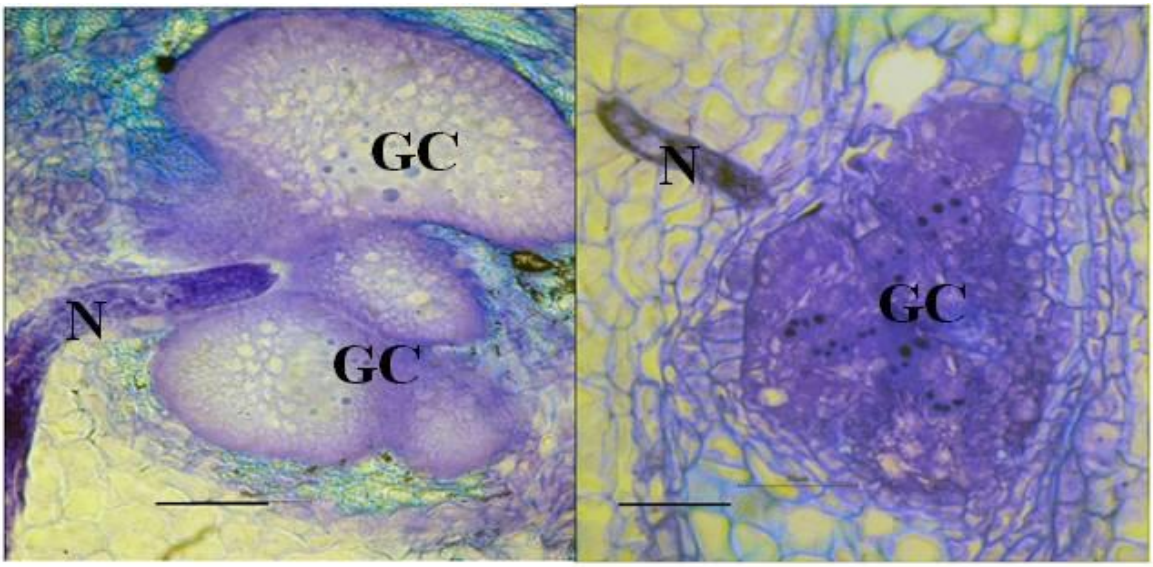
Fig 4. 2 Effects of DL-β-amino-n-butyric acid (BABA) on the development of *M. graminicola* in rice plants. (A) Pre-treatment of BABA: Plants were soil drenched with 3.5mM BABA or water one day before inoculation. Two days after inoculation, SAP was washed away from the roots and the plants were transferred to Hoagland solution for another 14 days. (B) Post-treatment of BABA: Two days after *M. graminicola* infection, roots were washed and transferred to 3.5 mM BABA solution or water for two days, subsequently, plants were transferred to pure Hoagland solution for another twelve days. Bars represent means and ±SE of nematodes per plant (n = 8). Different letters indicate statistically significant differences (P≤0.05). a/b indicates the significant differences in J2 stage; A/B indicates the differences in J3+J4 stages. α/β indicates the significant differences in females, and θ/ε indicates the significantly differences in all infected nematodes. Two independent experiments were conducted with similar results. The data obtained from the other replicates are shown in addendum Fig A10. (C) Developing RKN inside root galls at 16dpi, visualized with acid fuchsin, giant cells (GC), nematodes (N), and egg masses (E). Left: control; Right: roots treated with 3.5mM BABA. Scale bar = 500 µm.

The data presented in Figure 4.2 demonstrate that BABA is a strong inducer of plant defence against rice RKN. BABA treatment of plants not only impedes rice RKN penetration, but also inhibits their development.

*Histological response of giant cells in BABA-treated rice roots*

Both BABA-treated and control plants formed giant cells after 7 days of *M. graminicola* infection (Fig 4.3A). Although the giant cells are still enlarged cells with dense cytoplasm, multiple nuclei and thickened cell walls, they appear smaller and deformed in comparison with control giant cells. Probably these cells are not metabolically active enough to supply sufficient nutrients for nematode development. Correspondingly, nematode development at 7dpi was severely disrupted in the galls (Fig 4.3B).

(A)




(B) CONTROL BABA

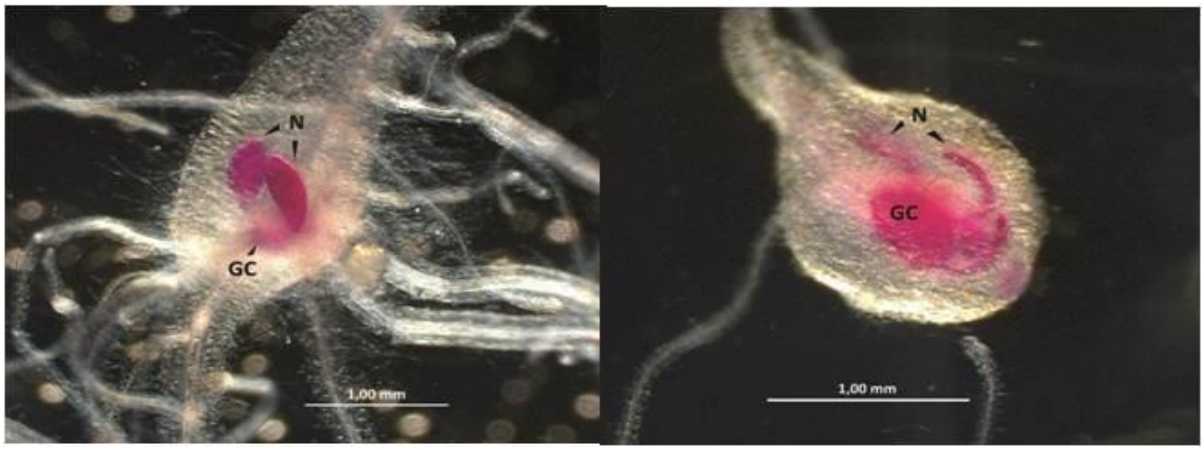



Fig 4.3 (A) Morphology of giant cells induced by RKN at 7dpi in water (control, left) and BABA-treated rice roots (right). Scale bar = 50μm. (B) Developing RKN inside 7dpi galls, visualized with acid fuchsin. Giant cells (GC); Nematodes (N). Scale bar = 1mm.

### 4.2.2 The expression of hormone-related genes in BABA-induced resistance against the rice RKN

To determine whether plant defence hormones are involved in BABA-induced resistance against *M. graminicola*, the expression of several hormone synthesis and response genes was investigated using qRT-PCR at different time points after BABA treatment, and different time points after inoculation with nematodes (Fig 4.4).

To check the importance of the SA pathway in BABA-induced resistance against *M. graminicola*, an SA biosynthesis gene (*OsICS1*) and a signaling gene (*OsWRKY45*) as well as the SA inducible gene *OsPR1b* were evaluated. OsICS1 is the rice isochorismate synthase, the first catalytic enzyme in the production of salicylic acid from chorismate during plant defence (Wildermuth *et al.*, 2001). *OsICS1* is significantly repressed in roots infected with *M. graminicola* at 1dpi and 3dpi, also when BABA is applied to the roots. However, the transcript levels of *OsICS1* do not significantly change in BABA- treated/uninoculated roots. OsWRKY45 is known to be a key regulator in the SA signaling pathway and involved in BTH induced resistance to *Magnaporthe grisea* and *Xanthomonas oryzae* pv. *oryzae* (Shimono *et al.*, 2007, Shimono *et al.*, 2012). Although the expression level of *OsWKRY45* is repressed in *M. graminicola* infected roots, its expression level is significantly up-regulated in BABA-treated inoculated and uninoculated roots at all the tested time points.

*OsPR1b* is generally seen as an SAR marker gene, it has been shown to be pathogen-inducible in rice after blast fungus and bacterial blight infection (Mitsuhara *et al.*, 2008). The transcript of *OsPR1b* is significantly up-regulated after *M. graminicola* infection at time 2 and time 3, but it does not respond to BABA treatment.

The JA biosynthesis gene *OsAOS2* (Mei *et al.*, 2006), which has been shown to be pathogen-inducible, and JA-inducible rice MYB transcription factor gene *OsJAMYB* both show a similar trend at all-time points. Both genes are significantly down-regulated in *M. graminicola* infected roots at 1 dpi. However, these two genes are up-regulated in all BABA-treated and inoculated roots in comparison to control roots.

The ET biosynthesis gene *OsACO7* and responsive gene *OsERF1* were used to evaluate the ET pathway. The qRT-PCR data indicate that *OsACO7* shows a significant down-regulation 1 day after BABA-treatment, and 1dpi in BABA treated and inoculated roots. *OsERF1* does not significantly respond to BABA treatment or to *M. graminicola* infection.

OsNCED3 is a key enzyme in the ABA biosynthesis pathway, and *OsLIP 9* is an ABA responsive gene. These genes do not strongly respond to inoculation at 1 and 3 dpi. However, BABA induces a strong up-regulation of *OsNECD3* at 2 days after treatment, both in unin-

oculated and inoculated roots. The ABA responsive gene shows a similar expression trend as *OsNCED3*, although it is a bit lower induced by BABA.

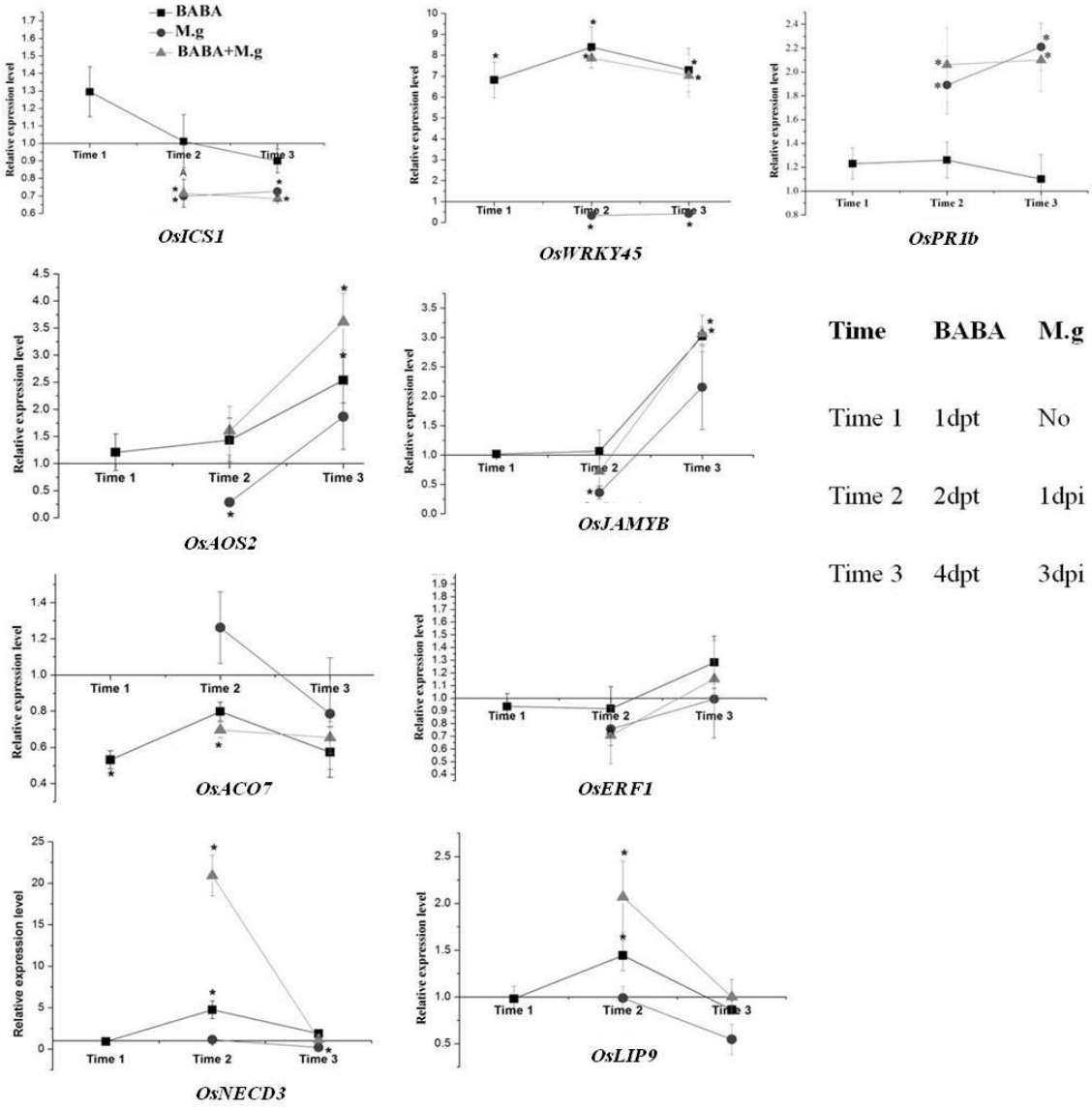
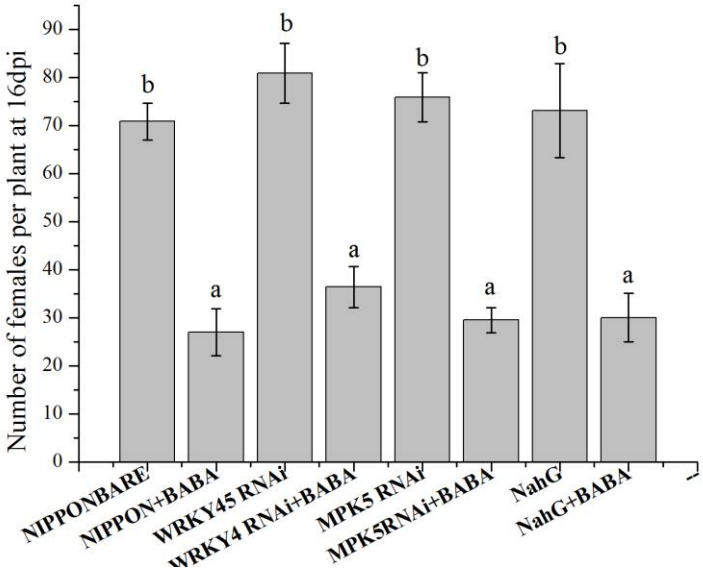



Fig 4.4 Analysis of the expression levels of defence hormone related genes in rice roots after soil application of BABA alone or with RKN infection at different times. Bars represent mean expression levels and ±SE from two biological replicates and three technical replicates, each containing a pool of eight plants. Asterisks indicate significant differential expression ($P \leq 0.05$). Data are shown as relative expression levels in comparison with the control roots (uninoculated and untreated). Gene expression levels were normalized using three internal reference genes, *OsEXP*, *OsEXPnarsai*, and *OsUBQ5*. M.g: *M. graminicola.*

### 4.2.3 Plants impaired, or less efficient in hormone-related pathways are still protected by BABA treatment

The qRT-PCR data described above showed that some genes involved in SA and ABA signal transduction pathways were strongly induced by BABA treatment (Fig 4.4). In order to obtain a more detailed understanding of the role of these defence signaling pathways in BABA-induced resistance against rice RKN, different rice transgenic plants were used to evaluate the efficiency of BABA-IR against *M. graminicola*.



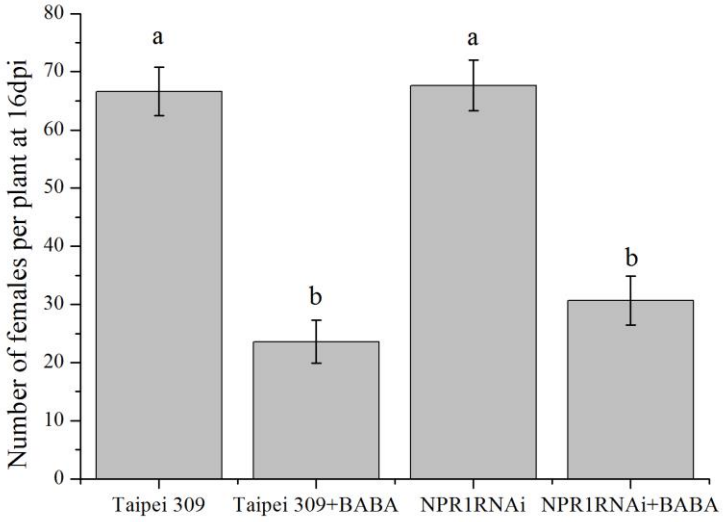



Fig 4.5 Effects of BABA against rice RKN in SA and ABA defective lines. 3.5mM BABA was soil drenched one day before nematode inoculation. The number of females per plant was counted at 16 dpi. Bars represent means and ±SE of females per plant (n = 8). Different letters indicate statistically significant differences (Duncan Multiple Range Test with $p \leq 0.05$). Data represent one of two independent experiments with similar results. The other replicate is shown in addendum Fig A11.

*NahG* plants express a bacterial salicylate hydroxylase that degrades SA to catechol (Gaffney *et al*., 1993) and this decreases the level of SA. *OsWRKY45* and *OsNPR1* are functioning in a signaling pathway downstream of SA, but are independent of each other (Shimono *et al.*, 2007). *NahG*, *WRKY45* RNAi and *NPR1* RNAi transgenic plants were used to check the importance of SA in BABA-IR. The data (Fig 4.5) show nematode reduction after application of BABA in all transgenic plants similar to the corresponding control lines.

The role of the ABA-inducible mitogen-activated protein kinase OsMPK5 in various biotic and abiotic stresses in rice is well documented (Xiong & Yang, 2003). Our experiments showed that there is no significant difference in nematode reduction by BABA-application in *OsMPK5* RNAi lines in comparison to the control.

### 4.2.4 Analysis of the mode of action of BABA using inhibitors of secondary metabolites

To further test whether the protection attributed to BABA depends on the accumulation of secondary metabolites, BABA was applied together with different chemical inhibitors of metabolite biosynthesis, 24h prior to nematode inoculation. Figure 4.6 illustrates the number of females developing on these plants at 16dpi. *In planta* inhibition of JA biosynthesis by DIECA and ET biosynthesis by AOA resulted in similar nematode reduction rates by BABA-application as in the control. Phenylalanine ammonia lyase (PAL) is the first enzyme in the phenylpropanoid pathway and is involved in the biosynthesis of the polyphenols (Hahlbrock & Grisebach, 1979). Blocking PAL biosynthesis by L-AOPP significantly decreased BABA-induced resistance against *M. graminicola*, with only about 40%-50% reduction rate compared to about 60%-70% reduction in the control. Similarly, blocking ABA biosynthesis by fluridone results in only 37.7%-40% nematode reduction by BABA-treatment (Fig 4.6). These results indicate that PAL and ABA are both partially involved in BABA-induced resistance against RKN, but not JA and ET.

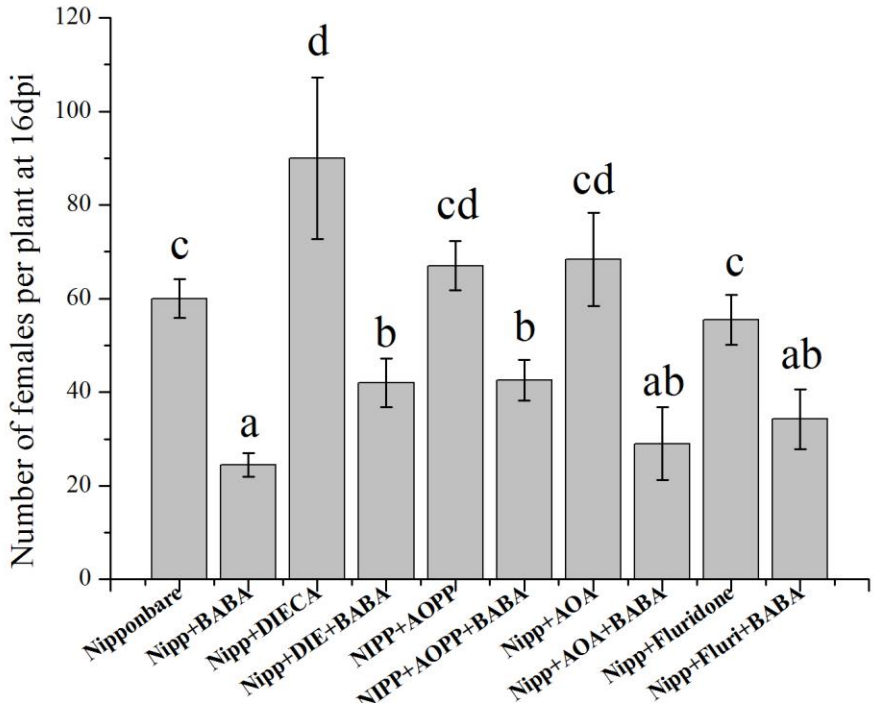


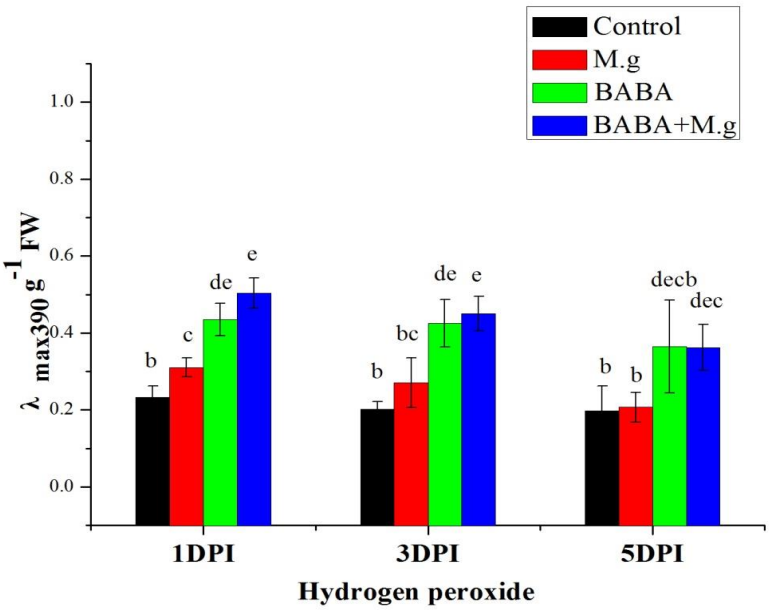

Fig 4.6 Effects of BABA in plants treated with inhibitors of secondary metabolites. 3.5mM BABA was soil drenched one day before nematode inoculation. The number of females per plant was recorded at 16dpi. Bars represent means and ±SE of females per plant (n = 8). Different letters indicate statistically significant differences (Duncan Multiple Range Test with $p \leq 0.05$). Data represent one of two independent experiments with similar results. The data from other replicate is in addendum Fig A12.

### 4.2.5 Reactive oxygen species generation

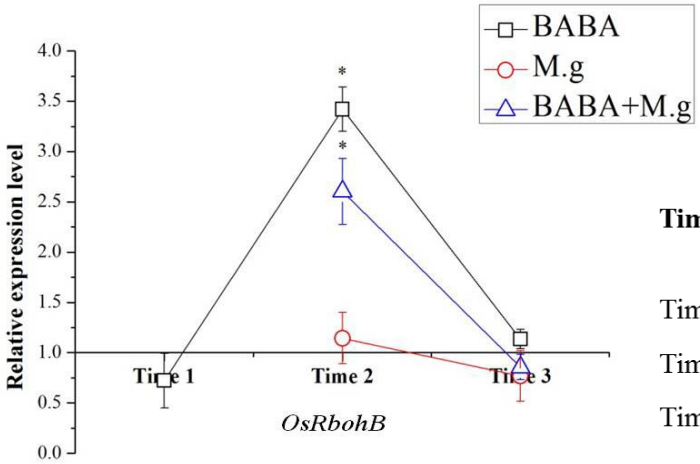
The production of reactive oxygen species during the oxidative burst is characteristic of the defence response in plants (Levine *et al.*, 1994). The hydrogen peroxide ($H_2O_2$) level was measured in rice roots upon BABA treatment. Increased levels of $H_2O_2$ were found in BABA treated plants, whether inoculated with *M. graminicola* or not (Fig. 4.7A). Nearly no changes in $H_2O_2$ generation over time were observed in untreated/uninoculated plants, but there is a small decrease in $H_2O_2$ production in roots infected with *M. graminicola*. This experiment was only conducted in one biological replicate and with a limited number of plants (4-5 plants), therefore, further study is needed.

The NADPH oxidases, which catalyze the reduction of oxygen to generate the superoxide anion, in plants also termed Respiratory Burst Oxidase Homolog (Rboh), were shown to be required for ROS accumulation in plant defence (Simon-Plas *et al.*, 2002, Torres *et al.*, 2006, Yoshioka *et al.*, 2003). Here we investigated the expression of one rice NADPH oxidase gene (*OsRbohB*) by qRT-PCR (Fig 4.7B). *OsRbohB* is strongly induced 2 days after BABA treatment in both uninoculated and inoculated plants.

(A)




(B)

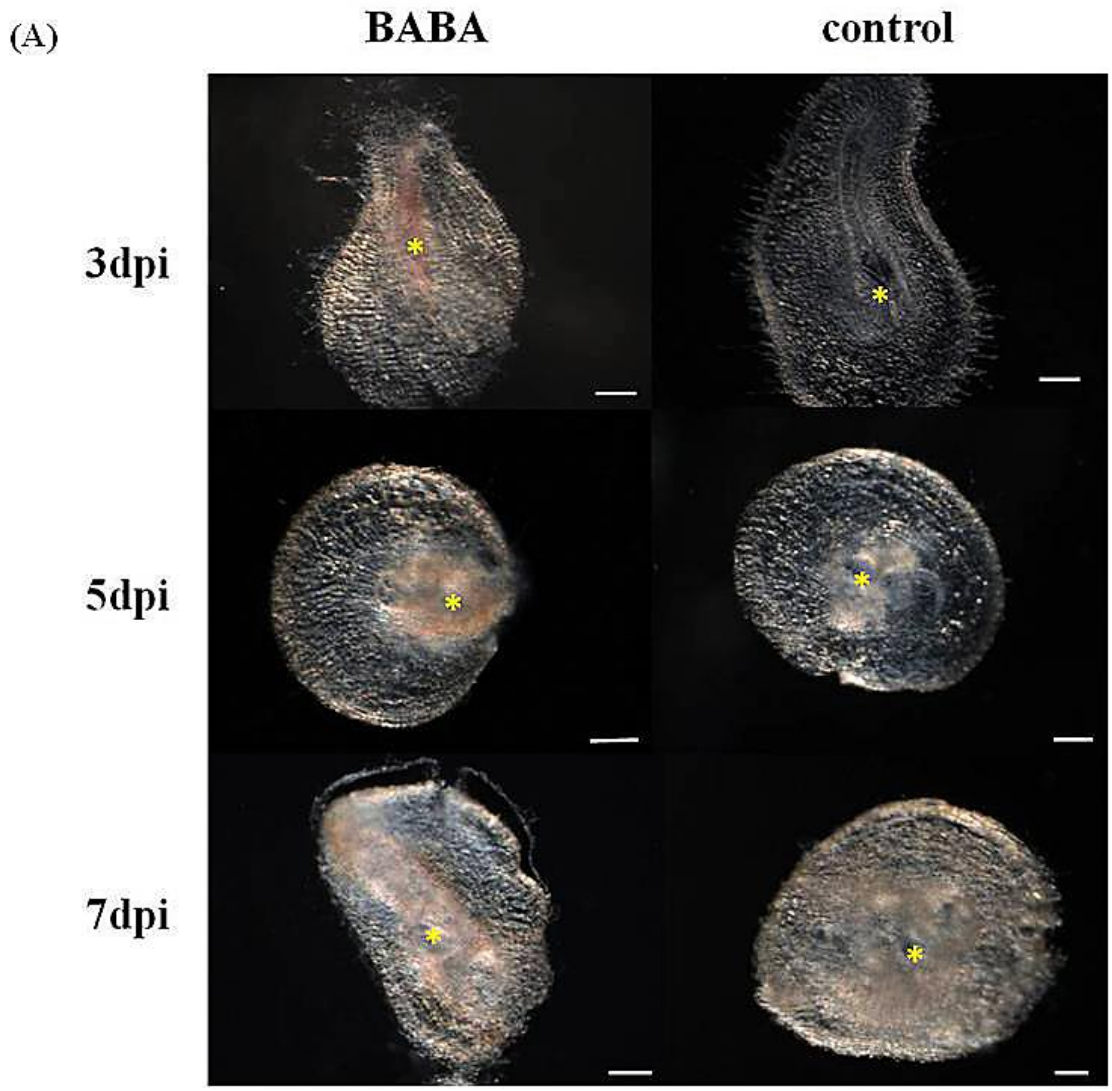



| Time | BABA | M.g |
|---|---|---|
| Time 1 | 1dpt | No |
| Time 2 | 2dpt | 1dpi |
| Time 3 | 4dpt | 3dpi |

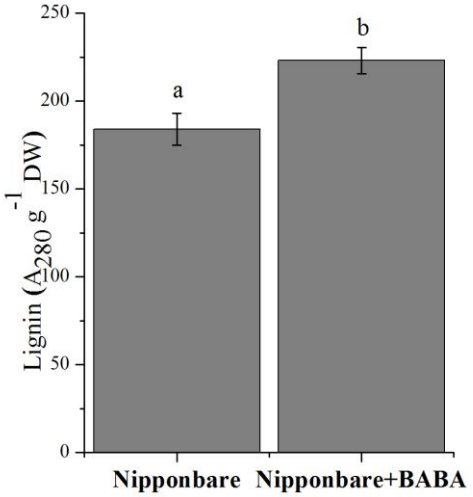
Fig 4.7 (A) The average value at λ max=390 per gram of fresh roots in BABA treated and water treated rice roots inoculated with *M. graminicola* or water. 3.5mM BABA or water was soil drenched 1 day prior to inoculation. Bars represent the mean and standard error of 4 to 5 plants. Different letters indicate statistically significant differences (Duncan Multiple Range Test with $p \leq 0.05$). (B) Analysis of *OsRbohB* expression in BABA treated and/or inoculated roots compared to untreated/uninoculated control at different time points. Bars represent mean expression levels and ±SE from two biological replicates and three technical replicates, each containing a pool of eight plants. Asterisks indicate significant differential expression ($P \leq 0.05$). Data are shown as relative expression levels in comparison with the control roots (uninoculated and untreated, set at 1). Gene expression levels were normalized using two internal reference genes, *OsEXPnarsai* and *OsUBQ5*. M.g: *M. graminicola*.

### 4.2.6 BABA-treated plants have increased lignin accumulation and related gene expression in roots

The observation in the inhibitor experiment (Fig 4.6) indicated that the gene *OsPAL* may play a role in BABA-induced resistance against RKN. PAL is an important enzyme in the phenylpropanoid pathway that catalyzes the deamination of phenylalanine to trans-cinnamic acid, a precursor for the lignin and flavonoid biosynthetic pathways (Dixon & Paiva, 1995). Lignin confers mechanical strength to plant secondary cell walls, which contributes to the basal defence against pathogens (Lewis & Yamamoto, 1990), including plant parasitic nematodes (Wuyts *et al.*, 2006b, Fogain & Gowen, 1995, Wuyts *et al.*, 2007). To better determine the mechanisms of BABA-IR against RKN, the phloroglucinol-HCL (Wiesner) reaction was used to detect lignin (syringyl and guaiacyl lignin) in gall sections at different time points (Fig 4.8A). A strong staining of lignin (red) was observed around feeding sites at all-time points in BABA-treated plants compared to untreated control (Fig 4.8A).



(B)




| Time | BABA | M.g |
|---|---|---|
| Time 1 | 1dpt | No |
| Time 2 | 2dpt | 1dpi |
| Time 3 | 4dpt | 3dpi |

(C)

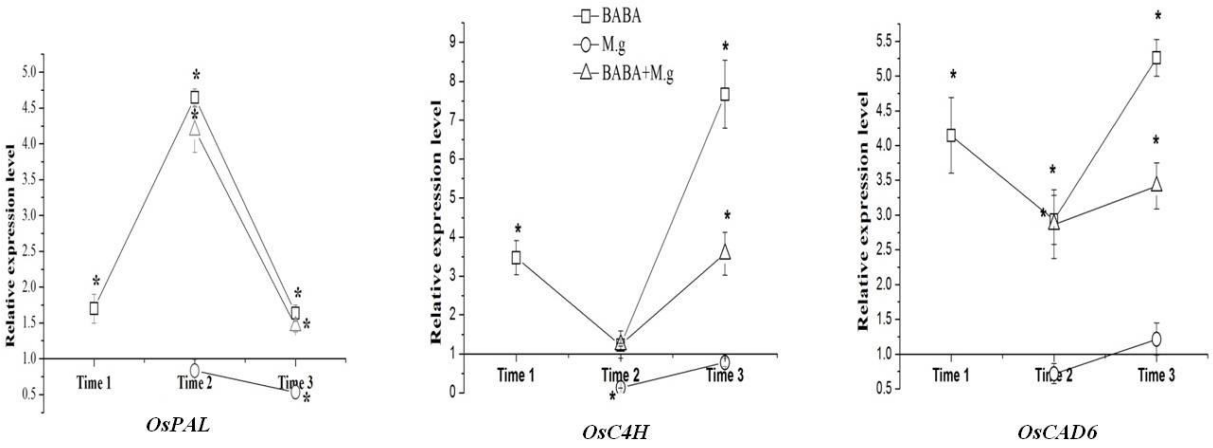



Fig 4.8 (A) Histochemical staining for lignin (pink-red color) in galls induced by *M. graminico*la at different time points. 100µm sections were stained with the Wiesner reagent to detect total lignin (guaiacyl and syringyl monomers). Scale bar = 100 µm. Yellow stars: giant cells. (B) Lignin content in the roots of rice seedlings. Measurements were made 1 day after BABA treatment or control (water) treatment. Bars represent the mean and ±SE of 20 plants. Different letters indicate statistically significant differences (Student T test with $p \leq 0.05$). (C) Analysis of the expression levels of three genes involved in ligni biosynthesis in rice roots after soil application of BABA alone or with RKN infection at different times compared to untreated and uninoculated control. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase. Bars represent mean expression levels and ±SE from two biological replicates and three technical replicates, each containing a pool of eight plants. Asterisks indicate significant differential expression ($P \leq 0.05$). Data are shown as

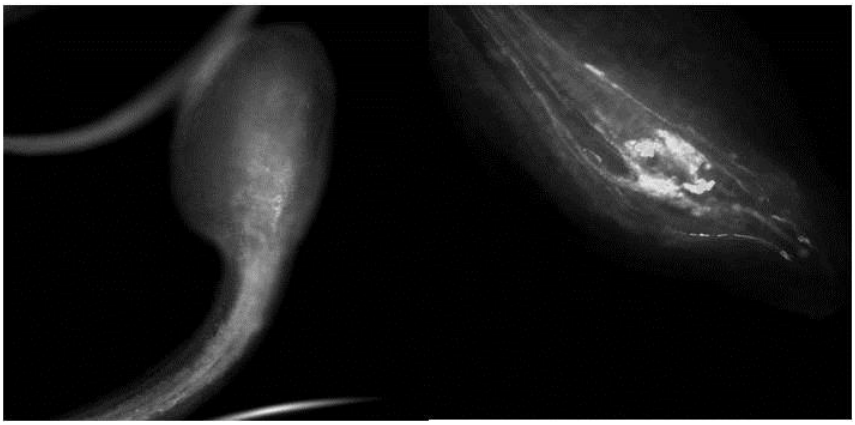
relative expression levels in comparison with the control roots (uninoculated and untreated, set as 1). Gene expression levels were normalized using two internal reference genes, *OsEXPnarsai* and *OsUBQ5*. M.g: *M. graminicola.*

Using the acetyl bromide method for quantification, a significant induction of lignin was also shown in rice roots 24 h after BABA treatment (Fig 4.8B). In line with these results, qRT-PCR on three important enzymes involved in lignin biosynthesis showed them to be significantly induced by BABA-treatment at different time points (Fig 4.8C). However, in non-BABA-treated but nematode-inoculated roots, a down-regulation of *OsC4H* (at 1 dpi) and *OsPAL* (at 3 dpi) was observed.

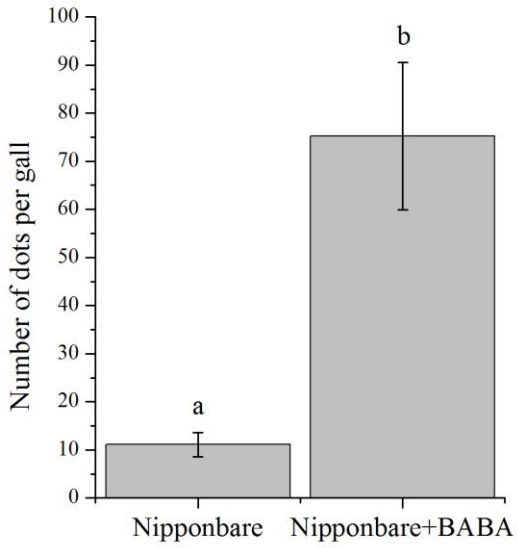
### 4.2.7 Callose deposition is enhanced in BABA-treated galls

Many researchers have reported that BABA induces callose deposition upon pathogen attack (Zimmerli *et al*., 2001, Ton & Mauch- Mani, 2004, Hamiduzzaman *et al.*, 2005). To explore further the mechanisms of BABA-induced resistance against *M. graminicola*, callose staining was performed on galls. Callose is a structural component of papillae and can be visualized after aniline blue staining with fluorescence microscopy (Adam & Somerville, 1996). Two days after *M. graminicola* infection, rice roots were washed up and transferred to 3.5mM BABA solution or water for another 2 days, and then roots including galls were used for callose staining. Representative pictures are shown in Figure 4.9A. Callose deposits were strongly induced by BABA in galls. The formed callose was more dense, bigger and prominent in BABA-treated galls than control galls. The big bright dots are probably the aggregation of many callose speckles, as reported by Ham *et al.*, (2007), they reported that 'big callose' was induced by *Pseudomonas syringae* pv. Phaseolicola NPS3121 with a diameter exceeding 20 µm. Moreover, the bright "dots" in each BABA-treated gall were significant more than in the control gall as measured by Image J (Fig 4.9B).

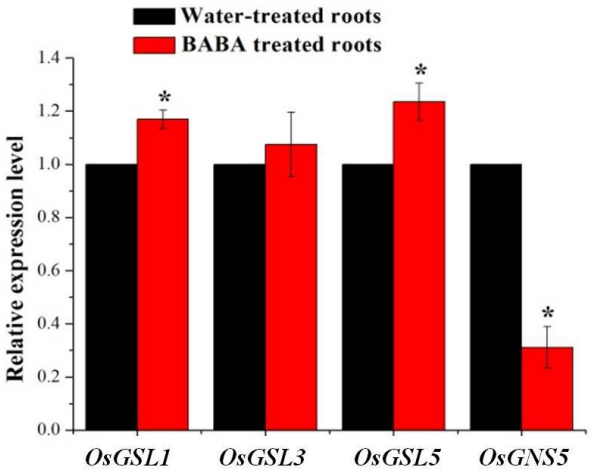
(A) Control BABA



(B)


Number of dots per gall
100
90
80
70
60
50
40
30
20
10
0
a
b
Nipponbare
Nipponbare+BABA

(C)


Water-treated roots
BABA treated roots
Relative expression level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
OsGSL1
OsGSL3
OsGSL5
OsGNS5

(D)

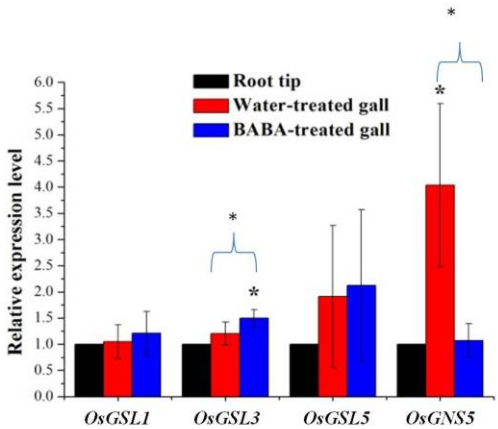

Root tip
Water-treated gall
BABA-treated gall
Relative expression level
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
OsGSL1
OsGSL3
OsGSL5
OsGNS5

Fig.4.9 **(**A) Callose detection in 4dpi water-treated and BABA-treated galls. (B) Quantification of callose deposition. The number of dots within the gall area was counted by ImageJ. Data are the mean and ±SE of dots from 8 galls. Different letters indicate significant differences (Student T test P≤0.05). (C) Analysis of the relative expression levels of callose synthesis and decomposition genes in BABA treated rice roots compared to control. (D) Analysis of the relative expression levels of callose synthesis and decomposition genes in BABA treated and water treated galls compared to non-treated/ non-inoculated root tips. Bars represent mean expression levels and SE from two biological replicates and three technical replicates, each containing a pool of eight plants. Asterisks indicate significant differential expression (P≤0.05). Gene expression levels were normalized using two internal reference genes, *OsEXPnarsai* and *OsUBQ5*.

Callose deposition is a dynamic process coordinated through the activities of callose synthase and the callose hydrolyzing enzyme β-1,3-glucanase (Hao *et al.*, 2008). The expression patterns of three callose synthase-encoding genes (*OsGSL1*, *OsGSL3* and *OsGSL5*) and one callose hydrolyzing gene (*OsGNS5*) were investigated by qRT-PCR (Fig 4.9C and D).

Upon BABA-treatment, *OsGSL1* and *OsGSL5* expression levels were clearly increased in roots, while *OsGNS5* showed a significant down-regulation (Fig 4.9C).

When looking at infected tissue, *OsGSL3* was significantly up-regulated in BABA-treated galls compared to control galls, as well as compared to un-treated/uninoculated root tips (Fig 4.9D). Although the other two callose synthase genes show a slightly higher expression level in BABA-treated galls, these differences were not significant. Interestingly, *OsGNS5* was clearly up-regulated in control galls; while in contrast, the expression level of this gene remained at the basal expression level in BABA treated galls. The results of the qRT-PCR confirm our microscopic observations that BABA induces the accumulation of callose by up-regulating callose biosynthesis and repressing its degradation, and *OsGNS5* is strongly induced in non-BABA-treated galls, exemplifying once again that this nematode strongly suppresses the plant innate immunity system.

## 4.3 Discussion

BABA has been reported for years to be a potent inducer of resistance in plants against a broad-spectrum of stress factors (Cohen, 2002, Jakab *et al*., 2001, Jakab *et al*., 2005, Zimmerli *et al*., 2008, Pastor *et al*., 2013). However, the molecular mechanisms underlying BABA-IR remain largely unknown, especially in the interaction of plants with plant parasitic nematodes. The research described here aims to provide a characterization of the role of BABA induced resistance against the rice root-knot nematode *M. graminicola* and the related defence mechanisms.

The data presented in this report show that BABA is a potent inducer of root defence against RKN attack in rice. Although this chemical does not influence nematode attraction towards the roots, treatment with BABA makes rice roots more difficult for RKN to invade as indicated by a lower number of nematodes in roots at 50 hpi. Furthermore, the development of juveniles into females was heavily inhibited, and giant cells in BABA-treated plants are much smaller than those in control plants. The action of BABA against RKN is not based on a direct toxic effect since 3.5mM BABA incubation did not exhibit any influence on nematode mobility *in vitro* (data not shown), which is in agreement with the other studies performed on many plant pathogens and nematodes (Oka & Cohen, 2001, Cohen, 2002, Jakab *et al*., 2001). Moreover, *in vivo* toxicity of BABA metabolites can also be ruled out as experiments using $C^{14}$-labeled BABA clearly demonstrated that the substance is not metabolized by the plant (Cohen & Gisi, 1994). Therefore, BABA seems to enhance rice defence against RKN attack.

To elucidate possible mechanisms contributing to BABA induced resistance against RKN, the involvement of hormone dependent defence pathways was examined. It has been reported that BABA-IR against TMV in tobacco, *Pseudomonas syringae* and *Botrytis cinerea* in *Arabidopsis* are based on an intact SA signaling pathway (Siegrist *et al*., 2000, Zimmerli *et al*., 2000). In this study, our data firstly showed that the transcripts of SAR marker *PR1b* and SA biosynthesis gene *ICS1* did not accumulate upon BABA-treatment. Secondly, BABA-IR was fully functional in the *NahG* line, and *NPR1* RNAi lines. Although the expression of *OsWRKY45* was significantly potentiated in BABA-treated roots, BABA succeeded to trigger defence against RKN in the *WKRY45* RNAi line, indicating that activation of *OsWRKY45* by BABA is not a prerequisite for defence against RKN. From these observations we conclude that the SA–dependent defence pathway does not contribute to BABA-IR against RKN.

Hydrogen peroxide accumulation has been implicated to perform several important functions in early defence responses of plants against pathogens (Lamb & Dixon, 1997, Apel & Hirt, 2004). It may cause direct pathogen destruction (Bestwick *et al.*, 1998), trigger hypersensitive cell death and activate defence related genes (Levine *et al*., 1994, Apel & Hirt, 2004), or serve as a secondary messenger in the systemic signaling network of plant cells (Shetty *et al.*, 2003). The accumulation of $H_2O_2$ in BABA treated plants has been repeatedly described. For instance, in lettuce, BABA treated plants induced accumulation of $H_2O_2$ in the mycelia of *Bremia lactucae* and adjacent mesophyll cells and altered the color of mycelia into red (Cohen *et al.*, 2011). In tomato, BABA induces an early oxidative burst and antioxidative defence mechanism, which leads to a suppressive effect on *Pseudomonas syringae* growth (Baysal *et al.*, 2007). In grapevine, a strong $H_2O_2$ production was observed in BABA-treated leaves after downy mildew *Plasmopara viticola* infection, and this process was correlated with an increased resistance (Dubreuil-Maurizi *et al*., 2010). Furthermore, BABA also in-

duced $H_2O_2$ production when infected by *M. javanica* in tomato (Sahebani & Hadavi, 2009) and cucumber (Sahebani *et al.*, 2011). In our experiments, treatment with BABA resulted in a stronger $H_2O_2$ generation in the rice roots, and this production of $H_2O_2$ lasts at least 5 days after nematode inoculation. Moreover, the transcript level of *OsRbohB* is strongly induced at 2 days after BABA-treatment in uninfected and infected plants. Therefore, the $H_2O_2$ production probably correlates with the induced resistance against RKN by BABA.

$H_2O_2$ could for instance also contribute to signaling events leading to enhanced lignin formation and callose deposition. Therefore, differences in lignin and callose content between BABA treated and water treated plants were analyzed. The lignin content was found to be significantly enhanced in one day BABA treated roots. Lignin, a major component of cell walls of vascular plants, is considered to be the first line of defence against the penetration of invading pathogens (Vance *et al.*, 1980, Lewis & Yamamoto, 1990, Nicholson & Hammerschmidt, 1992). Lignin can enhance mechanical strength of plant cell walls and make it less accessible to cell wall-degrading enzymes, which are secreted by plant parasitic nematodes for their penetration and migration in plant roots (Gheysen *et al.*, 2006). RNAi-mediated transient gene silencing of lignin biosynthesis genes in wheat led to a higher penetration efficiency of *Blumeria graminis f. sp. tritici* than control (Bhuiyan *et al.*, 2009). Resistant banana (*Musa* spp.) of burrowing nematode *Radopholus similis* had constitutively higher levels of lignin in the vascular bundle than susceptible cultivars (Fogain & Gowen, 1995, Wuyts *et al*., 2007). Hence, the high level of lignification in BABA-treated roots probably restricts *M. graminicola* invasion and therefore resulted in less nematode penetration in our experiment. Portillo *et al.* (2013) reported that genes such as phenylalanine ammonia lyase (PAL), cinnamate 4-hydoxylase (C4H), caffeate O-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD) involved in lignin monomer synthesis and lignin cross-linking pathways are strongly repressed in giant cells induced by *M. javanica* in tomato and *Arabidopsis* plants. Also in rice galls induced by *M. graminicola*, the phenylpropanoid pathway is known to be strongly repressed (Kyndt *et al.*, 2012a). PAL and C4H are two important enzymes in the phenylpropanoid pathway from where monolignol biosynthesis is derived (Howles *et al.*, 1996, Blount *et al.*, 2000), while, CAD and COMT are core enzymes leading to the biosynthesis of lignin in plants (Mee *et al.*, 2005). Therefore, suppressing the expression of those genes will affect lignin biosynthesis and result in lower lignin content. *Arabidopsis thaliana* mutants with impaired COMT function were more susceptible to various bacterial and fungal pathogens (Quentin *et al.*, 2009). Transgenic *Arabidopsis* containing elevated levels of syringyl lignin, significantly reduced the fecundity and development of *M. incognita* compared to the wild type control (Wuyts *et al*., 2006a). Accordingly, tobacco plants with reduced syringyl lignin content result in a faster life cycle of *M. incognita*. In our

study, BABA treated galls were characterized by a more pronounced lignin deposition than control galls, and genes involved in lignin biosynthesis are more strongly expressed in BABA-treated plants at different time points. PAL genes are significantly suppressed in giant cells induced by *M. graminicola* in rice (Ji *et al.*, 2013), and several researchers have reported that BABA treated plants have enhanced PAL activity which is correlated with disease resistance (Slaughter *et al.*, 2008, Baysal *et al.*, 2005). Application of 100 µM L-AOPP, an inhibitor of PAL, did not increase the susceptibility of Nipponbare to RKN, although there were a slightly higher number of females than the control. However, BABA induced resistance was partially compromised in L-AOPP treated plants with a 40%-50% reduction of females in comparison to nearly 60%-70% reduction in control plants. Taken together, we speculate that a higher *PAL* expression leads to increased levels of lignin, which contributes to less efficient penetration and/or delay of RKN development. However, the role of other phenylpropanoid compounds, like flavonoids and phytoalexins, in BABA-IR still needs to be investigated.

Callose plays important roles during the normal development of plants (Chen *et al.*, 2007), and it is known to be induced as a plant resistance response after exposure to a range of biotic and abiotic stresses (Jacobs *et al.*, 2003, Ueki & Citovsky, 2005, Luna *et al.*, 2011, Chen & Kim, 2009, Stone & Clarke, 1992). Callose deposition is one of the earliest plant responses against invading nematodes (Hussey *et al.*, 1992, Grundler *et al.*, 1998). It is generally deposited between the plasma membrane and the cell wall, and synthesized by callose synthase and degraded by β-1,3-glucanase (Kauss, 1996). It has been demonstrated that BABA induced callose deposition is correlated to the expression of resistance in several pathosystems (Zimmerli *et al*., 2000, Ton & Mauch-Mani, 2004, Hamiduzzaman *et al*., 2005, Cohen *et al*., 2011, Cohen *et al*., 2010, Walz & Simon, 2009, Barilli *et al.*, 2010). The authors proposed that callose deposition may (i) cause cell wall strengthening and restrict pathogen penetration; (ii) block the transport of nutrients and water to the feeding sites of nematodes, causing starvation and growth cessation of the nematodes. For example, overexpressing of *RAP2.6*, an ethylene response transcription factor gene in *Arabidopsis* plants enhanced callose deposition in syncytia and enhanced resistance against the beet cyst nematode *Heterodera schachtii* (Ali *et al.*, 2013). Similarly, callose deposition on the sieve plates of resistant rice is an important mechanism for reducing phloem translocation and phloem sap to the brown plant hopper pest (Hao *et al*., 2008). In our experiment, stronger fluorescence was found in BABA-treated galls, indicating abundant, compact callose deposits in those galls. In contrast, the callose signals were much fainter in water-treated galls. The callose synthase gene *OsGSL3* was significantly up-regulated in BABA treated galls, compared to uninfected root tips or water treated galls. Another two callose synthase genes, *OsGSL1* and *OsGSL5* were also induced in rice roots treated with BABA. *OsGNS5* on the other hand is strongly down-regulated in BABA treated

roots. *GNS5* plays critical roles in decomposition of the callose barriers, and it is hijacked by pathogens to weaken the physical barriers of the plant and hence promote infection (Akiyama *et al*., 1997). The down-regulation by BABA may allow callose to be maintained in plant roots. In contrast, our results show that RKN feeding induces the expression of this β-1, 3-glucanase, which is probably one of the mechanisms of RKN to overcome the physical barriers of the basal defence in susceptible rice plants. Similarly, the brown plant hopper can unplug sieve tube occlusions by activating β-1, 3-glucanase genes in susceptible rice plants (Hao *et al*., 2008). Taken together, we conclude that BABA activates genes for callose synthesis and suppresses callose degradation, leading to the accumulation of callose in BABA treated roots. This phenomenon may inhibit RKN penetration, giant cell expansion and female enlargement and ingestion of cytoplasm from giant cells. Several studies have been reported that BABA-induced callose deposition is based on the ABA signaling pathway (Ton & Mauch-Mani, 2004, Ton *et al.*, 2005, Flors *et al.*, 2005). There is an indication in our experiments that ABA is required for BABA-IR against RKN as BABA-IR was slightly impaired when the plants were co-treated with the ABA inhibitor fluridone. However, since fluridone inhibits ABA biosynthesis via the carotenoid pathway from which only a small fraction may be required for ABA biosynthesis (Taylor *et al*., 2000), fluridone probably provoked perturbations (i.e. inhibition of astaxanthin and lutein) other than the inhibition of ABA biosynthesis (Achuo 2005). Therefore, whether fluridone can successfully and specifically inhibit ABA production in our experimental conditions, and if callose deposition is dependent on ABA still needs to be further explored.

Our understanding of the mechanisms of BABA induced resistance against rice root-knot nematode RKN can be encapsulated in a model shown in Figure 4.10. BABA induces a multifaceted plant basal defence response in the rice roots, which inhibits nematode penetration and development. However, hormone-dependent signal pathways play no major roles in this induced resistance mechanism.

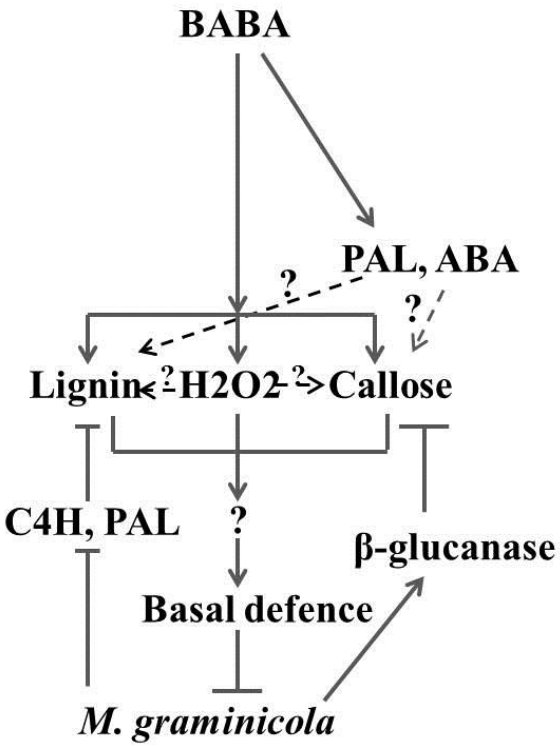



Fig.4.10 Model illustrating the mechanisms of BABA induced resistance against RKN in rice. Lines ending with arrows show activation. The line ending with a perpendicular short line indicates suppression. Dashed lines indicate possible effects, for which this paper does not provide evidence.

## 4.4 Conclusions

In summary, we have shown that BABA application to rice roots induced strong resistance against the RKN *M. graminicola* at different developmental stages. The induced resistance is independent of the defence signaling cascades SA, JA and ET, but coupled with enhanced multifaceted plant basal defence, including $H_2O_2$, lignin and callose accumulation. The cumulative results presented in this research highlight the possible actions of the non-protein amino acid BABA against RKN in rice.

## 4.5 Material and methods

### Plant materials and growth conditions

Rice (*Oryza sativa* L.) lines used in this work included of *O. sativa* cv. Nipponbare and cultivar Taipei 309 (NPR1 WT). The seeds of cv Nipponbare were provided by the US Department of Agriculture (GSOR-100) and corresponding SA-deficient *NahG* lines (Yang *et al*., 2004) and RNAi *OsMPK5* transgenic plants (Xiong & Yang, 2003) were kindly provided by YinongYang (Pennsylvania StateUniversity), *OsWRKY45* RNAi lines was a gift from Hiroshi Takatsuji (Plant Disease Resistance Research Unit, National Institute of Agrobiological Sciences, Ibaraki 305-8602, Japan); the *NPR1* mutant and its corresponding wild type Taipei 309

was kindly provided by Zuhua He (National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China). Seeds were germinated on wet filter paper for 3 days at 30°C and then transferred to SAP substrate (Reversat *et al*., 1999) in PVC (polyvinyl-chloride) tubes and further grown at 26 ℃ under a 16h/8h light regime at a relative humidity of 70-75%.

**Infection experiments**

The *M. graminicola* culture was provided by Prof. Dirk De Waele (Catholic University Leuven, Belgium) and was originally isolated in the Philippines. *M. graminicola* was maintained on *O. sativa* cv. Nipponbare in potting soil under the same temperature/light conditions as described above, and the nematodes were extracted using a modified Baermann funnel. Fourteen-day-old rice plants were soil drenched with 3.5mM BABA or water as control, and one day after they were inoculated with about 200 second-stage juveniles of *M. graminicola* per plant. In the case of a developmental assay, in pre-treatment of BABA, two days after inoculation, roots were washed and plants were put in Hoagland solution for another 14 days. In post-treatment of BABA, 2 days after *M. graminicola* infection, roots were washed and transferred to Hoagland+3.5mM BABA solution for 2 days, after that, plants were transferred to pure Hoagland. Infected roots were collected at 50 hours post infection (hpi) for penetration assay, 16 days post infection (16dpi) for development stage analysis , and then roots were boiled for 3 min in 0.8% acetic acid and 0.013% acid fuchsin. They were washed with running tap water and then destained in 5:100 ml acidified glycerol to visualize nematode developmental stages.

**Attraction assay**

14- day-old *O. sativa* cv. Nipponbare seedlings were soil drenched with 3.5mM BABA or water. One day after treatment, root tips were excised (about 5mm long) and each tip was put into a 1.5cm diameter well in a 24 well cell culture microplate. Each well contains about 150 J2 suspended in 500µl 0.8% water-agar. The microplates were incubated in a growth chamber (26℃) for 9 h after initiation of the assay, and then the number of nematodes touching the terminal 1mm of the root tip was counted. Eight replicates were included in each experiment and the experiment was done twice.

**Inhibitor experiment**

Chemical treatment was done with aminooxyacetic acid (AOA), Diethyldithiocarbamic acid (DIECA), L-2-aminooxy-3-phenylpropionic acid (L-AOPP) and fluridone, inhibiting eth-

ylene (ET) biosynthesis, jasmonic acid (JA) biosynthesis, phenylalanine ammonia-lyase (PAL) and abscisic acid (ABA) biosynthesis, respectively. All chemicals were dissolved in a few drops of ethanol before diluting in distilled water. The chemicals and concentrations used are as follows: AOA (50mM), DIECA (100 µM), L-AOPP (100 µm), and fluridone (0.1 µm). Intact 14-d-old seedlings were soil drenched with each inhibitor or each inhibitor plus 3.5mM BABA. All the experiments were independently repeated with similar results. All the chemicals were soil drenched 24 h before nematode inoculation.

**RNA extraction, cDNA synthesis, and qRT-PCR**

RNA extraction and cDNA synthesis were performed on total roots, galls or root tips and analyzed as described by Kyndt *et al*. (2012b). The primer sequences used for qRT-PCR are listed in Table 4.1

Table 4.1 Overview of the reference and target genes used in this study, showing their GenBank accession/locus numbers (MSU7.0) and the primer used for qRT-PCR

| Primers | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| *OsEXP* (LOC_Os03g27010) | TGTGAGCAGCTTCTCGTTTG | TGTTGTTGCCTGTGAGATCG |
| *OsUBQ5* (AK061988) | ACCACTTCGACCGCCACTACT | ACGCCTAAGCCTGCTGGTT |
| *OsEXPnarsai* (LOC_Os07g02340.1) | AGGAACATGGAGAA-GAACAAGG | CAGAGGTGGTGCAGATGAAA |
| *OsICS1* (NM_001069519 | TGTCCCCACAAAGGCATCCTG G | TGGCCCTCAACCTTTAAACATGCC |
| *OsdWRKY45* (Os05g0323900) | AATTCGGTGGTCGTCAAGAA | AAGTAGGCCTTTGGGTGCTT |
| *OsPR1b* (AK107926) | ACGCCTTCACGGTCCATAC | AAACAGAAAGAAACAGAGGGAGTAC |
| *OsAOS2* (Os03g12500) | CAATACGTGTACTGGTCGAAT GG | AAGGTGTCGTACCGGAGGAA |
| *OsJAMyb* (AY026332) | GAGGACCAGAGTGCAAAAGC | CATGGCATCCTTGAACCTCT |
| *OsACO7* (Os01g39860) | GGACTACTACCAGGGCACCA | GATTAGCGCACGCGATTTTA |
| *OsERF1* (EF061888.1) | GAGTCGTCCTTCTCCTCCTC | CCTCTCTTTCTCCGTTTCG |
| *OsNCED3* (Os03g40438) | GTTCAAGCTCCAGGAGATGC | AGAGGTGGAAGCAGAAGCAG |
| *OsLIP9* (AB011367) | CCGGCTACAGAGGAAGTGAG | TCTCCATGATCTTGCCCAGT |
| *OsRbohB* (NM001049555.1) | CTGGACAGGACCAAGAGCAG | ATCTTGAACGGAGCAGCACA |

| | | |
|---|---|---|
| *OsC4H* (NM_001061725) | CAGACTGGTGAGATCCGGTG | TTCCCCATTCGATCGACCAC |
| *OsCAD6* (NM_001058825 ) | TCGGTAAGAGGACGGTGAGT | TGTCGATGTCCCAGGTGATG |
| *OsPAL* (X16099.1) | TGTGCGTGCTTCTGCTGCTG | AGGGTGTTGATGCGCACGAG |
| *OsGSL1* (AP001389) | TGAGGACCTGCCACGATT | CACGCTGATTGCGAACAT |
| *OsGSL3* (AP003268) | TGGCAAGCGACCACATAG | AGACCTTAGCACGGACTG |
| *OsGSL5* (AP008212) | GTGGTGTCCCTGCTATGA | GTTGTTTGCTATT |
| *OsGNS5* (U72251) | TTGCGGCCATTCCTACAGT | TGGTGAGGGCGATGCTTG |

**Tissue processing for light microscopy**

Galls on the roots were excised from mock treated and BABA-treated plants 7 days after inoculation. Fixation of galls was done in 2% glutaraldehyde and 98% PIPES buffer overnight, then dehydration was done in a series of ethanol dilutions. Embedding of galls was conducted by using Technovit 7100 (Kulzer, Germany). 10µm thick sections were cut with a Leica RM2265 motorized rotary microtome (Leica microsystems, Nussloch, Germany) and stained with 0.05% toluidine blue O. Digital images were acquired with an Olympus BX51 microscope equipped with an Olympus ColorView III camera.

**Hydrogen peroxide content assay**

Hydrogen peroxide levels were determined according to Velikova *et al*. (2000). Root tissues (about 100 mg) were homogenized in an ice bath with 1 mL 0.1% (w/v) TCA. The homogenate was centrifuged at 14000 rpm for 15 min and 0.5 ml of the supernatant was added to 0.5 mL, 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1 M KI. The absorbance of supernatant was read at λ max=390 nm. One biological replicate was conducted with 4-5 plants.

**Lignin analyses and histochemical test**

Lignin quantification was performed according to Vanholme *et al.* (2013). Root tissues were collected one day after soil drench with 3.5mM BABA or water. Dry roots (5mg-7mg each) in 2ml vials were subjected to sequential extractions, each time for 30 min: at near-boiling temperatures in water (98 ℃), ethanol (76 ℃), chloroform (59 ℃), and acetone (54 ℃). The remaining cell wall residue was dried under vacuum and weighed again. Absorbance was measured at 280 nm using a Nano-Drop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Twenty samples were measured for each treatment.

Lignin histochemistry was examined using Wiesner reagent (Pomar *et al*., 2004). Galls harvested at different time points from BABA or mock treatments were embedded in 7% agarose, and were cut into 100µm sections with a Vibroslicer. Sections were incubated for 5 min in a phloroglucinol solution (1% phloroglucinol in 95% ethanol+HCL), and then the Wiesner reagent was changed by water. Sections were directly observed with a binocular microscope (Leica, S8APO).

**Callose staining**

The method of detection of callose deposition was adapted from Millet *et al*. (2010). Two days after nematode or water inoculation, plants were transferred to 3.5mM BABA or water for another 2 days. Whole roots were immediately fixed in a 3:1 ethanol: acetic acid solution overnight. The fixative was changed three times to ensure both thorough fixing and clearing of the tissues. Seedlings were rehydrated in 70% ethanol for 3 h, 50% ethanol for 3 h, 30% ethanol for an additional 2h and water overnight. After three water washes, roots were treated with 10% NaOH and placed at 37$^{o}$C for 2 h to make the tissues transparent. After three water washes, seedlings were incubated in 0.01% aniline blue (Sigma-Aldrich) in 150mM $K_2HPO_4$ solution, pH 9.5 for 25 min. The roots including galls were mounted on slides, and callose was observed immediately under UV (excitation, 390nm; emission, 460 nm). For each treatment 8 roots with several galls were observed. Callose quantification was done by using Image J.

# CHAPTER 5

## Transcriptional analysis through RNA sequencing of giant cells induced by *Meloidogyne graminicola* in rice roots

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## Abstract

One of the reasons for the progressive yield decline observed in aerobic rice production is the rapid build-up of populations of the rice root-knot nematode *Meloidogyne graminicola*. These nematodes induce specialized feeding cells inside root tissue, called giant cells. By injecting effectors in and sipping metabolites out of these cells, they reprogram the normal cell development, and deprive the plant of its nutrients. In this research, we have studied the transcriptome of giant cells in rice, after isolation of these cells by Laser Capture Microdissection. The expression profiles revealed a general induction of primary metabolism inside the giant cells. Although the roots were completely covered from light induction, we detected a remarkable induction of genes involved in chloroplast biogenesis and tetrapyrrole synthesis. The presence of chloroplast-like structures inside these dark-grown cells was confirmed by confocal microscopy. On the other hand, genes involved in secondary metabolism and more specifically the majority of defence-related genes were strongly suppressed in the giant cells. In addition, significant induction of transcripts involved in epigenetic processes was detected inside these cells at 7 days after infection.

## 5.1 Introduction

Biotrophic plant pathogens have evolved sophisticated strategies to manipulate their host. They derive all of their nutrients from living plant tissues, by making intimate contact with their host while avoiding a resistance response. Rice is one of the most important crop plants worldwide and an excellent model system for studying monocotyledonous plants. Estimates of annual yield losses due to plant-parasitic nematodes on this crop range from 10 to 25% worldwide (Bridge *et al*., 2005). One of the agronomically most important nematodes attacking rice is the rice root-knot nematode *Meloidogyne graminicola*. Attack of plant roots by sedentary plant parasitic nematodes, like the root-knot nematodes (RKN; *Meloidogyne* spp.) leads to the development of specialized feeding cells in the vascular tissue. The second stage juvenile of the RKN punctures selected vascular cells with its stylet, injects pharyngeal secretions, and this ultimately leads to the reorganisation of these cells into typical feeding structures called giant cells (GCs), from which the nematode feeds for the remainder of its sedentary life cycle (Gheysen and Mitchum, 2011). Morphological and physiological reprogramming of the initial feeding cell leads to nuclear enlargement, proliferation of organelles, metabolic activation, cell cycle alterations and cell wall changes (Gheysen and Mitchum, 2011). The hyperplasia and hypertrophy of the surrounding cells leads to the formation of a root gall, which is typically formed at the root tips in the case of the rice RKN *M. graminicola*. In comparison with other RKN, *M. graminicola* has a very fast life cycle. In well-drained soil at 22-29 ℃ the life cycle of *M. graminicola* is completed in 19 days. Swelling of the root tips is observed as early as 1 day post inoculation (dpi). At 3 dpi, terminal hook-like galls are clearly

visible (Bridge *et al*., 2005). After 3 moults the nematodes are mature, around 10-12 dpi. While most other RKN deposit egg masses at the gall surface, the *M. graminicola* females lay their eggs inside the galls, and hatched juveniles can re-infect the same or adjacent roots.

We have recently studied transcriptional reprogramming patterns in galls induced by the RKN *M. graminicola* in rice using deep RNA sequencing (Kyndt *et al*., 2012a). The there-reported gene expression differences reflect a combination of changes occurring in giant cells and surrounding gall tissues. Due to the technical difficulty of isolating giant cells from the root tissue, most transcriptome analyses have up till now focused on the whole gall tissue, in *Arabidopsis* and tomato (e.g. Bar-Or *et al*., 2005, Jammes *et al*., 2005). Nevertheless, though technically challenging, giant cell contents can be isolated using microaspiration (Wang *et al*. 2003) or laser capture microdissection (LCM; Portillo *et al*., 2009; Fosu-Nyarko *et al*., 2009, Barcala *et al*., 2010; Portillo *et al*., 2013). The research of Barcala *et al*. (2010) and Portillo *et al*. (2013) demonstrated the molecular distinctiveness between the giant cells and the surrounding gall tissue.

The goal of our research was to study the transcriptional changes in giant cells formed in rice roots upon RKN infection. LCM was combined with mRNA-Seq to study the giant cell transcriptome at two time points after infection. We have compared the data with reports from giant cells and complete galls induced by RKN in rice and other plant species. Some of the reported changes were independently validated by quantitative RT-PCR (qRT-PCR) and confocal microscopy. Our study highlights that key metabolic pathways, hormone homeostasis and epigenetic processes are affected during giant cell development.

## 5.2 Results

In this study, a c**o**mparative gene expression analysis was carried out to investigate the rice response to infection with a sedentary nematode species, *Meloidogyne graminicola*. This nematode induces the formation of specialized feeding sites called giant cells (GCs) in root tissue. For an in-depth analysis of the transcriptional reprogramming induced in these cells, they were isolated by laser capture microdissection (LCM) at 7 and 14 days post inoculation (dpi) and mRNA-Seq was carried out on the isolated RNA. Root cells from the vascular tissue of uninfected plants of the same age were used as control material. Transmitted light microscopical analysis revealed that at 7 dpi, the nematodes were at the juvenile 3 (J3) or J4 stage; at 14 dpi most of *M. graminicola* had matured and most females had laid eggs. The GCs contained a dense cytoplasm, the shape was oval or globular, and the cell wall was thick (Fig 5.1). Per biological replicate and time point 150-200 giant cell sections (Fig 5.1) or 300 control sections (from uninfected plants) were used for LCM of the giant and vascular cells, respec-

tively. After quality control, the cDNA was sequenced using the Illumina mRNA-Seq protocol.

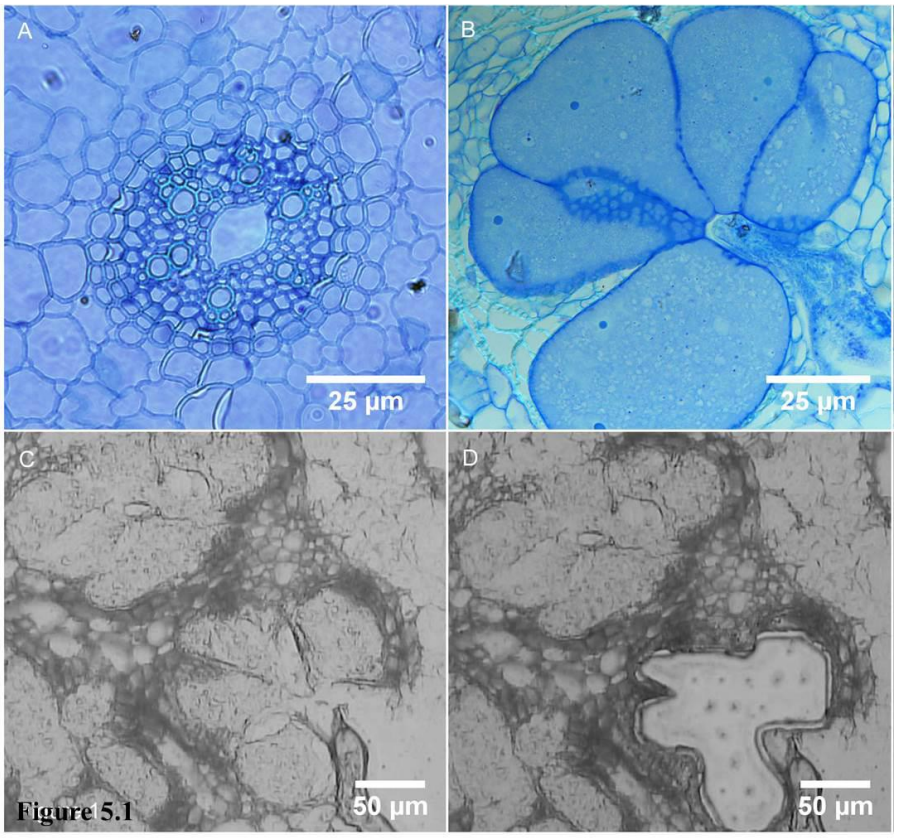


Fig 5.1 Transverse sections of giant cells (GC) formed by the root-knot nematode *Meloidogyne graminicola* in rice roots (*Oryza sativa* cv. Nipponbare). A: control vascular tissue B: 7 dpi GC. Scale bars in A and B: 25 µm. C: 7 dpi GCs before LCM; D: 7 dpi GCs after LCM. Scale bars in C and D: 50 µm.

In total 139,254,416 reads were acquired from infected and uninfected cells at the two time points. The data can be accessed through the GEO repository: GSE43577. The short reads were aligned against the whole reference genome sequence of rice cv. Nipponbare (MSU7.0) and 79.2 % of the sequenced fragments, 36 bp reads on each end of the fragments, could be mapped (Table 5.1). This mapping percentage is substantially higher than reported in our previous mRNA-Seq analysis on complete galls (Kyndt *et al*., 2012a); where on average only 49.20 % of the reads were mapped. The higher number of mapped reads in the current analysis can be explained by the fact that paired-end reads were used here, and because of the nature of the samples, specific isolated cells from within the root tissue have a lower chance of contaminants. The total length of mapped reads was over 10 billion bases, representing nearly 27-fold coverage of the rice genome and approximately 97-fold coverage of the annotated transcriptome. The expression of a total of 54,206 different rice transcripts was detected in the analyzed tissues. Correlation between the two biological replicates sequenced from each cell

type and at each time point was checked by means of Pearson correlation coefficient of the expression value of each gene after normalization. The average Pearson R between 2 biological replicates was 0.9959 (p-value <2.2 E-16).

Comparative gene expression profiling was performed by Gene Set Enrichment, pathway mapping and statistical analysis of differential gene expression levels between infected and uninfected cells at the different time points. In addition, a search was performed to detect novel transcriptionally active regions (nTARs) not annotated in the rice genome assembly MSU7.0, and alternative splicing patterns in the isolated rice cells.

### 5.2.1 Transcriptome changes in giant cells at 7 dpi

At 7 dpi, the nematodes feeding from the giant cells within the gall tissue were at the J3/J4 stage. A total of 42,756 different transcripts were found to be expressed in the collected cells at this time point. The expression level of all rice loci was compared between 7 dpi GCs and uninfected vascular cells in roots of the same age.

Gene Set Enrichment Analysis on relative expression levels ($log_2FC$) of all transcripts in the infected versus uninfected cells revealed that genes involved in 'biosynthetic process' (mainly translation), 'cell cycle', 'generation of precursor metabolites and energy', and 'cellular component organization', were strongly up-regulated at 7 dpi, while genes involved in 'tropism', 'signaling', 'response to stimulus' and 'secondary metabolic process' were generally down-regulated. Genes with a molecular function annotated as 'structural molecule activity' and 'nucleic acid binding' were generally higher expressed in 7 dpi GCs than in control cells (Fig 5.2).

Table 5.1 Overview of the obtained mRNA-Seq data from giant cells induced by nematode infection in rice and control cells from the vascular root tissue, and mapping of these sequences onto the rice genome.

| Sample | Total number of sequenced fragments | Total number of paired mappings | Number of mapped fragments | Number of unmapped fragments | % unique mapping |
|---|---|---|---|---|---|
| **Uninfected vascular cells at time point 7 dpi(1)** | 17031983 | 32558679 | 13967317 | 3064666 | 82,01 |
| **Uninfected vascular cells at time point 7 dpi (2)** | 16406474 | 30877769 | 12916523 | 3489951 | 78,73 |
| **Giant cells at time point 7 dpi(1)** | 18433445 | 36677998 | 14860282 | 3573163 | 80,62 |
| **Giant cells at time point 7 dpi (2)** | 18253140 | 34825239 | 14294260 | 3958880 | 78,31 |
| **Uninfected vascular cells at time point 14 dpi (1)** | 17135578 | 33563491 | 13585221 | 3550357 | 79,28 |
| **Uninfected vascular cells at time point 14 dpi (2)** | 17600487 | 33773375 | 13622898 | 3977589 | 77,40 |
| **Giant cells at time point 14 dpi (1)** | 16877180 | 37350477 | 13725022 | 3152158 | 81,32 |
| **Giant cells at time point 14 dpi (2)** | 17516129 | 37472359 | 13296867 | 4219262 | 75,91 |
| **Total** | 139254416 | 277099387 | 110268390 | 28986026 | 79,18 |
| **Reads (two/fragment)** | 278508832 | | | | |
| **Coverage of the rice genome** | 26,86 | | | | |
| **Coverage of the rice transcriptome** | 97,63 | | | | |

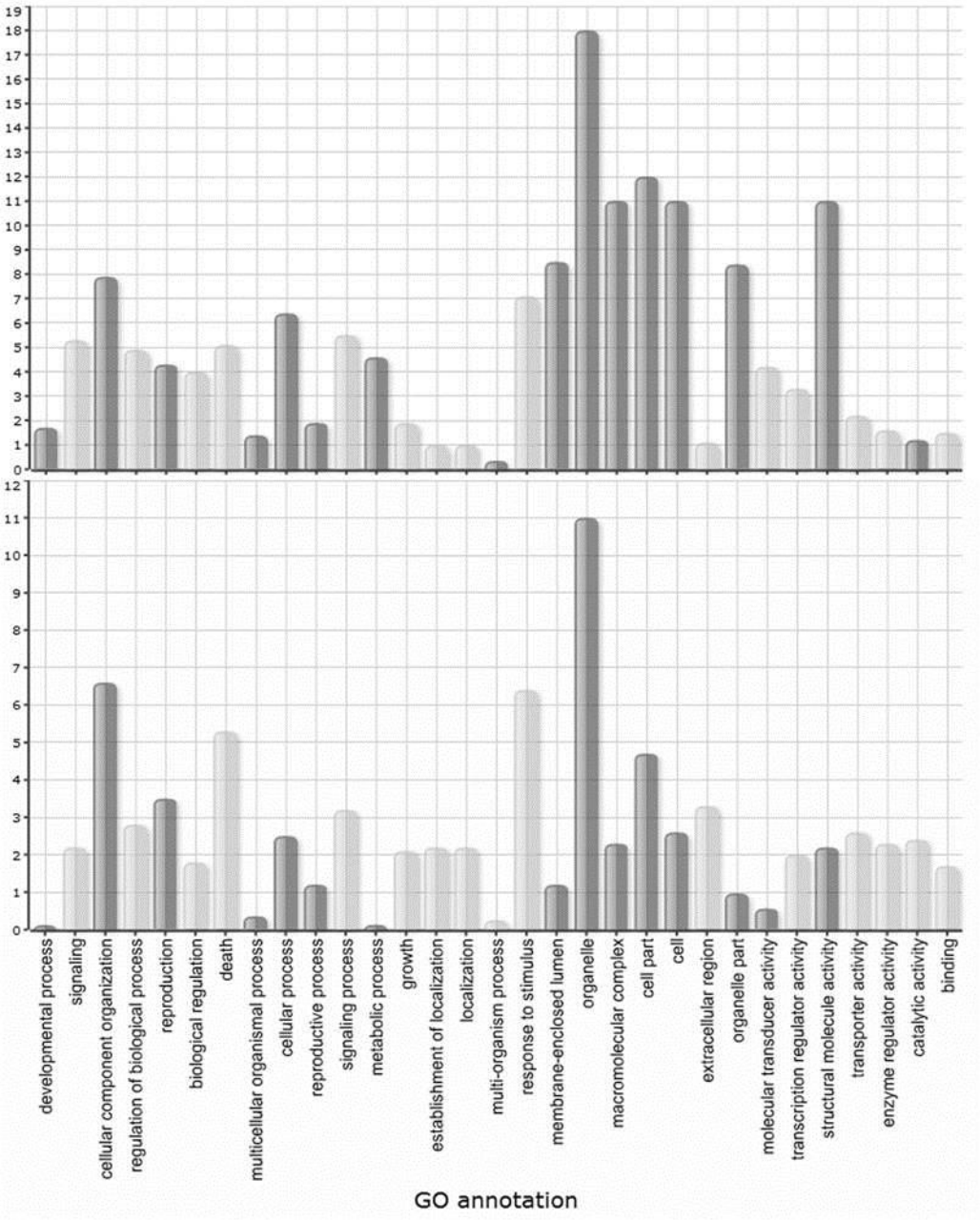


Fig 5.2 Parametric Analysis of Gene Set Enrichment of transcriptome data of giant cells induced by RKN in rice at 7 dpi (top) and 14 dpi (below). Z-scores (Y-axis) of all secondary level GO terms are shown. Bars in dark grey indicate GO terms that are up-regulated in the infected tissue versus the corresponding control, while light grey bars indicate GO-terms that are down-regulated in the infected tissue versus the corresponding control.

Pathway mapping with MapMan showed a significant modification of, for instance, glycolysis, starch metabolism, trehalose metabolism, homoserine biosynthesis, tetrapyrrole synthesis, phenylpropanoid pathway, flavonoid production, cell wall precursor synthesis and pectin esterases. Figure 5.3 shows the expression pattern of transcripts involved in tetrapyrrole biosynthesis, with a high frequency of transcripts induced in the GCs vs. control material. Higher

plants contain four classes of tetrapyrroles, namely chlorophyll, heme, siroheme, and phytochromobilin, and all of them play vital roles in various biological processes, including photosynthesis, respiration, nitrite and sulfite reduction, as well as various cellular processes including gene expression, protein import, and the assembly of essential proteins (Tanaka and Tanaka, 2007). They are predominantly synthesized in plastids. Figure 5.3 shows that almost all genes involved in the biosynthesis of siroheme, heme, phytochromobilin and chlorophyll *a*, but not chlorophyll *b*, are expressed at higher levels in giant cells than in control cells.

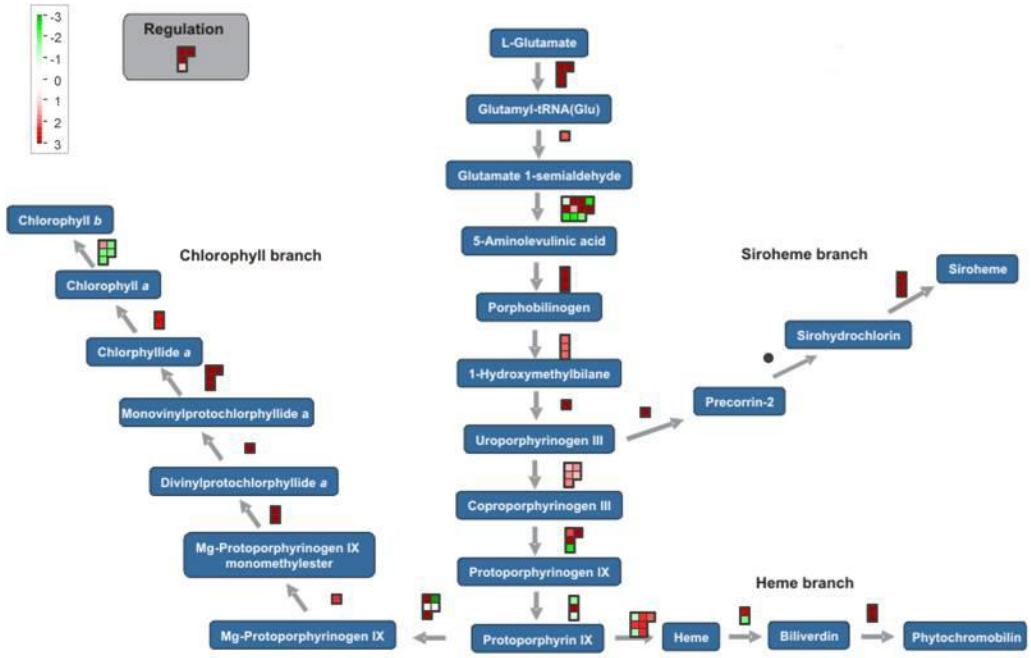


Fig 5.3 Mapman visualization of the expression profiles of genes involved in tetrapyrrole biosynthesis in 7 dpi giant cells. The visualization shows the observed differential expression patterns, based on the $\log_2$ fold changes of mRNA levels, in giant cells versus control cells. Dots show the different paralogous genes encoding the enzyme that catalyzes a certain step. Red dots indicate that the gene is up-regulated in infected tissue versus the corresponding healthy control, while green indicates down-regulation.

At 7 dpi 77,709 transcripts were found to be significantly differentially expressed (FDR<0.05), with 650 transcripts down-regulated, and 77,059 up-regulated in the giant cells (Table S5.2). Thirteen of them were chosen for independent validation based on their potentially interesting function, and their expression pattern in the giant cells was evaluated on an independent biological sample by qRT-PCR. The expression trend was confirmed in all but one case (Table S5.3). Differences in $Log_2$FC-values as obtained from mRNA-Seq and qRT-PCR are largely due to biological variation and/or to differences in the applied algorithms for estimating expression levels.

The genes with strongest down-regulation in GCs versus uninfected cells included transcripts encoding gibberellin 2-beta-dioxygenase, involved in gibberellin catabolism; chalcone synthase, involved in flavonoid production; protein disulfide isomerase, that catalyzes post-translational protein modifications through formation and breakage of disulfide bonds; and 2 NBS-LRR disease resistance proteins. Among the strongest up-regulated transcripts were those encoding 78 transporter proteins; OsBAK1, the BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1; ABIL2, known to be involved in regulation of actin and microtubule organization; 17 cell-cycle regulating cyclins; 10 tubulins; 6 members of the auxin-responsive OsIAA-gene family; 6 other auxin-response factors; and a remorin C-terminal domain containing protein.

### 5.2.2 Experimental validation of chloroplast and chlorophyll content in 7 dpi giant cells

Many reports on plant-nematode interactions have analyzed plants growing in Petri dishes in a day/night cycle (Sijmons *et al*., 1991). It is generally known that the developed nematode feeding sites contain chloroplasts under light influence (Orion and Wergin, 1982; Golinowski *et al*., 1996; Sijmons *et al*., 1991). Orion and Wergin studied this with electron microscopy and revealed the differentiation of chloroplasts from amyloplasts inside these light-induced giant cells in tomato. However, in the current study, roots were completely covered from light to preclude any artificial induction of chlorophyll and chloroplast formation. Interestingly, the results of the transcriptome data (Fig 5.3), reveal a strong induction of photosynthesis-related genes in dark-grown GCs, and hence we decided to confirm the presence of chlorophyll *a* inside the GCs using confocal microscopy. To this end, lambda stacks of fixed GC sections were acquired using spectral detection confocal microscopy and they were compared with images from fixed and fresh leaf sections. The confocal images demonstrated that fresh rice leaves contain dense arrays of strongly autofluorescent chloroplasts, about 5 µm in size and shaped as flattened disk (Fig 5.4A, D). After fixation, the fluorescent pattern in the leaf had changed into a more punctuate pattern, with chloroplast fluorescence limited to ~1 µm-sized foci. This punctuated effect is plausibly due to the fixation and (especially) the dehydration procedure (Fig 5.4B, C). Remarkably, similar fluorescent foci were observed inside the GCs, albeit to a lesser extent (Fig 5.4C, F) and some autofluorescence was also seen in the neighbouring cells of the GCs. However, the fluorescent foci and autofluorescence were never observed in healthy control roots (data not shown). To determine whether these foci in the GCs contained chlorophyll *a*, we compared their spectral profile with that of chloroplasts in fixed and fresh leaf sections (Fig 5.4G). Pedrós *et al*. (2008) reported that the chlorophyll *a* fluorescence emission spectrum is characterized by a major peak at 683 nm, which dominates the autofluorescence spectrum of chloroplasts in the deep red region. Indeed, inside chloroplasts from fresh leaf material a fluorescent peak with a maximum intensity at 683 nm was obtained

upon excitation at 488 nm. In chloroplasts from fixed leaf sections, this peak was slightly shifted towards 678 nm, possibly due to quenching effects. A very similar, although slightly shifted, peak was observed in the presumed chloroplasts from the GC sections, suggesting the presence of chlorophyll *a* in these structures. The slight shift of the peak and the deviation from the reference curves at the longer wavelengths may be due to the absence of other accessory pigments (e.g. carotenoids, xanthophyll's, and chlorophyll *b*) that contribute to the spectral profile.

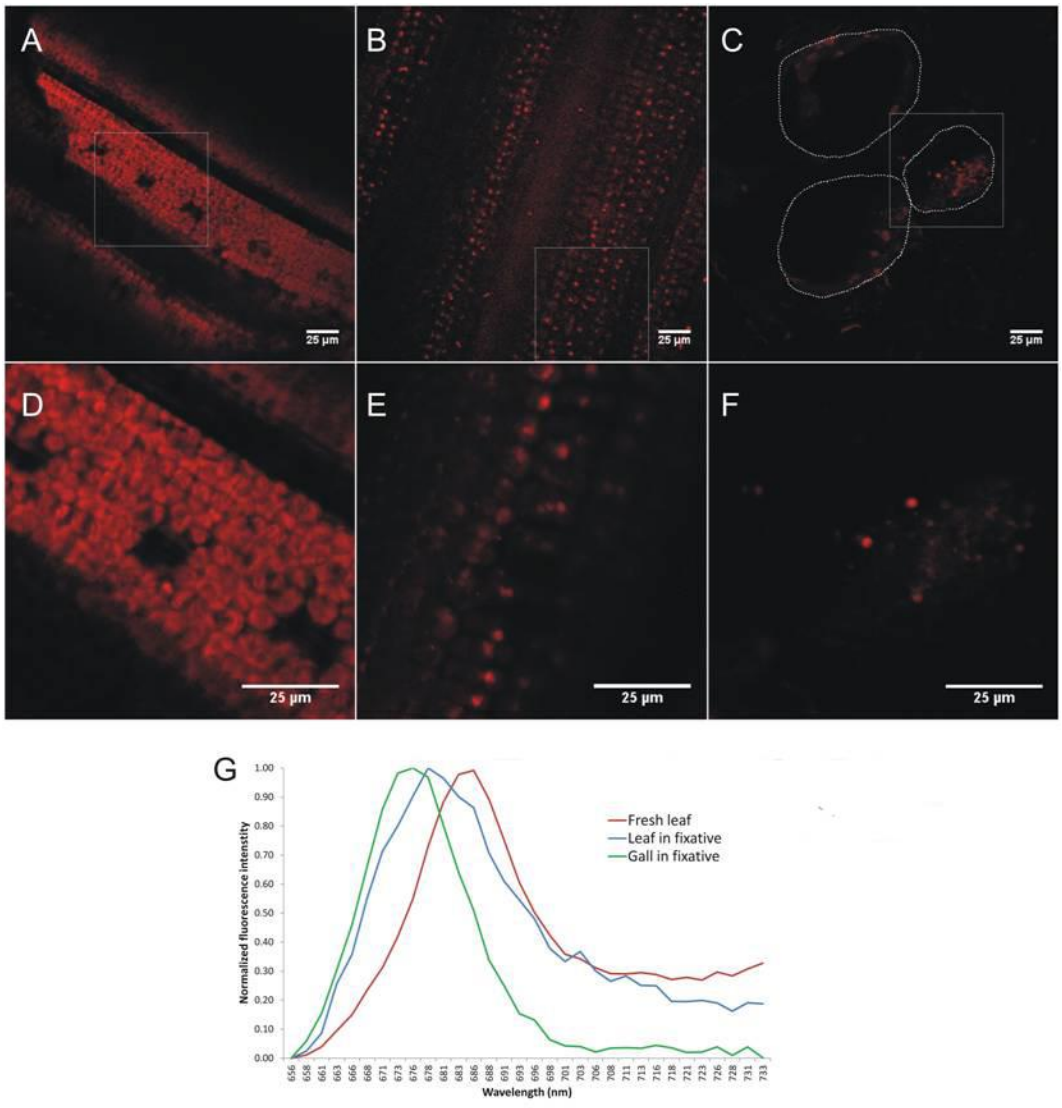


Fig 5.4 Confocal microscopy and spectral profiling of chloroplast (-like) autofluorescence. A-F: Confocal images were acquired from sections of fresh leaves (A, D), fixed leaves (B, E) and fixed giant cells (C, F) at two different zoom factors 1x (A, B, C) and 3x (D, E, F), using a spectral detector set to capture chlorophyll *a* autofluorescence (655.5nm-735.5nm). White squares in A, B and C, respectively, show the region that was magnified in D, E and F, respectively. The dotted lines in C delineate the boundaries of the giant cells. Note the difference in shape between chloroplasts of fresh and fixed leaf sections and the presence of chloroplast-like structures in the giant cell section. G: Average spectral profiles, measured as average intensity per wavelength interval across the lambda stack range for at least 4 (presumed) chloroplast regions.

### 5.2.3 Transcriptome changes in giant cells at 14 dpi

The expression level of all rice loci was compared between 14 dpi GCs and uninfected root vascular cells at the same time point. In total, 41,179 different transcripts were found to be expressed in the collected rice cells. While still ongoing at 7 dpi, the expansion of giant cells and cytoskeleton rearrangements end at 10–14 dpi in the case of *M. incognita* infection on *Arabidopsis* (de Almeida-Engler *et al*., 2004).

Gene Set Enrichment Analysis of relative expression levels ($log_2FC$) of all transcripts in the infected versus uninfected cells revealed that genes involved in 'photosynthesis', 'DNA metabolic process', 'cellular component organization', 'generation of precursor metabolites and energy' and 'cell cycle' were strongly up-regulated at 14 dpi, while genes involved in 'secondary metabolic process', 'response to stimulus' and 'cell death' were generally down-regulated. Transcripts coding for proteins with 'hydrolase' and 'nuclease' activity were generally up-regulated (Fig 5.2). When comparing Gene Set Enrichment of 7 and 14 dpi giant cells (Fig 5.2), similar trends are observed, although genes in the GO-categories 'metabolic processes', 'structural molecule activity', 'organelle part', 'membrane-enclosed lumen', and 'macromolecular complex' show less strong induction at 14 dpi than at 7 dpi. Pathway mapping with MapMan showed, similar to the 7 dpi data, a significant modification of starch and sucrose metabolism, trehalose metabolism, tetrapyrrole synthesis and the phenylpropanoid pathway. Additionally, significant modifications were detected in light reactions, flavonoid production and cell wall-related pathways in 14 dpi GCs.

2,884 transcripts were significantly differentially expressed at FDR<0.05. 238 of them were down-regulated, while 2,646 were up-regulated (Table S5.4). Among the strongest down-regulated genes, transcripts were found coding for nicotianamine synthase, involved in phytosiderophore biosynthesis; a glucan endo-1,3-beta-glucosidase precursor; alpha DOX2, involved in the synthesis of oxylipins; transcription factor WRKY71; 4 thionin-like peptides; flavonol synthase and phenylalanine ammonia lyase, both involved in the phenylpropanoid pathway. The strongest up-regulated transcripts included starch synthase; Cullin-1; AP2-like ethylene-responsive transcription factor AINTEGUMENTA, which regulates growth and cell numbers during organogenesis; roothairless-1; and genes involved in cell cycle control, such as those encoding cyclin-T1-1, cyclin-dependent kinase A-1 and cyclin-dependent kinase C-2.

### 5.2.4 Comparison between 7 & 14 dpi GCs and 7 dpi galls

A total of 942 genes was found to be differentially expressed (FDR<0.05) between control and giant cells at both 7 and 14 dpi. Correlation between the Log2FC-values (GC vs. respective control) of those 942 genes is Pearson R=0.85 (p<2.2E-16). This demonstrates that DEGs at 7 and 14 dpi show a similar transcriptional profile.

In the following paragraphs the transcriptional profiles of some specific pathways are explored in the GCs (7 and 14 dpi), in comparison with previously generated transcriptome data from 7 dpi whole-gall material of rice (Kyndt *et al*., 2012a).

*Transcripts involved in the metabolism of the phytohormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscissic acid (ABA) and gibberellic acid (GA)*

As already described by Barcala *et al*. (2010) and Portillo *et al*. (2013) for tomato and *Arabidopsis*, expression patterns in rice GCs were strongly different from the whole-gall transcriptional profile (Kyndt *et al*., 2012a). TableS5.5 shows the $Log_2FC$ of genes involved in biosynthesis of different plant hormones in 7 and 14 dpi GCs and 7 dpi whole gall material (data taken from Kyndt *et al*., 2012a), each in comparison with their corresponding uninfected control tissue. Results show that transcripts involved in SA-biosynthesis through the phenylpropanoid pathway are suppressed in GCs at both 7 dpi and 14 dpi, with for instance many paralogues encoding phenylalanine ammonia lyase much lower expressed in giant cells in comparison with uninfected vascular cells (Table S5.5). Some of these genes were found to be rather induced in 7 dpi whole galls. Many transcripts involved in jasmonate biosynthesis, e.g. encoding lipoxygenases, allene oxide synthase and 12-oxophytodienoate reductases, are suppressed in 7 and 14 dpi GCs versus uninfected vascular root cells. A lot of these transcripts are rather induced in 7 dpi whole galls (Table S5.3). The fact that GA-biosynthesis and signaling through DELLA proteins is generally induced in gall tissue was already reported by Kyndt *et al*. (2012a), and the data shown in the current study reveal that many of these enzymes are also expressed to a higher level inside GCs at both time points. The fact that genes involved in GA-degradation are also induced is potentially due to a feedback effect to control internal GA homeostasis.

A lot of transcripts involved in the production of abscisic acid are induced in 7 dpi galls, while many ABA-biosynthesis genes are repressed in 7 dpi GCs. In 14 dpi GCs both induced and repressed expression patterns are observed. Nevertheless, the transcript encoding 8-hydroxylase, involved in ABA degradation, is very strongly induced in the gall and GC tissue both at 7 and 14 dpi. In the ET-biosynthesis pathway, either strong induction or strong suppression was observed for different 1-aminocyclopropane-1-carboxylic acid (ACC)-synthase and ACC-oxidase paralogues, but no clear trend was seen, although slightly more transcripts encoding ACC-oxidase were found in 7 dpi galls.

*Transcriptional changes in genes involved in epigenetic modifications*

Different epigenetic processes, which are not necessarily independent of one another, have been described to affect gene expression (Berger, 2007). Many transcripts involved in (post)-

transcriptional gene silencing, like those encoding Dicer and ARGONAUTE proteins, are induced in the 7 dpi GCs and some also in whole 7 dpi gall material (Table 5.2), but this trend does not persist in the 14 dpi GC. Histone-modifying enzymes are also dramatically up-regulated in the 7 dpi GCs, while they show varying expression profiles in the 14 dpi GCs (Table 5.2). In complete gall tissue the transcriptional profile is diluted by the neighbouring cells, but also there one of these genes (LOC_Os01g59620) is significantly higher expressed in comparison with the uninfected root tips.

### 5.2.5 Novel transcripts and alternatively spliced transcripts

A total of 18,291 novel transcriptionally active regions (nTARs) were detected in the isolated cells (Table S5.6). A blast search was done against all ESTs from *Oryza sativa*, and all proteins predicted from the *O. sativa* genome project. 7,383 nTARs gave a significant hit (E <1E-4) against at least one EST from *O. sativa*. tBlastX against all proteins of *O. sativa* resulted in hits for 3,587 of the nTARs, indicating potential paralogy to a known rice transcript (Table S5.6).

To predict a potential function of these nTARs, a SwissProt/trembl search was done, and although this was successful for 4,719 transcripts, many of them were annotated as 'Putative uncharacterized protein, *O. sativa*'. Nevertheless, among the nTARS the following rice orthologues were detected: a Bowman-Birk type wound-induced proteinase inhibitor WIP1 (*Zea mays*), a receptor-like protein 12 (*A. thaliana*), disease resistance protein RGA2 (*Solanum bulbocastanum*), regulatory protein NPR3 (*A. thaliana*) and Gibberellin 20 oxidase 1 (*A. thaliana*) (Table S5.4). Among the nTARs, 12,185 nTARS showed a significant differential expression pattern in the GCs versus the uninfected vascular cells, with 2,214 nTARs significantly down-regulated in the GCs, and 9,971 significantly up-regulated (FDR<0.05). Among the down-regulated nTARs were for example transcripts showing homology to isoflavone reductase and carotenoid 9, 10(9', 10')-cleavage dioxygenase. Potential novel orthologues of callose synthase 3 and polygalacturonase are both significantly induced in the giant cells (Table S5.6). In addition to the nTARs, 16,063 alternatively spliced transcripts were detected (Table S5.6), among which 8,374 have significant differential expression patterns (2,465 down and 5,909 up-regulated).

Table 5.2 Transcriptional changes in genes involved in epigenetic mechanisms as obtained by mRNA-Seq on 7 dpi and 14 dpi giant cells (Vs. control vascular root cells) and 7 dpi galls (vs. control root tips; data extracted from Kyndt *et al*., 2012a). NE: Not expressed

| | **$Log_2$FC Gall 7 dpi vs. Control root tips** | **$Log_2$FC Giant Cell 7 dpi vs. control root cells** | **$Lo_{g2}$FC Giant Cell 14 dpi vs. control root cells** | **Annotation** |
|---|---|---|---|---|
| ***Post-transcriptional gene silencing*** | | | | |
| **LOC_Os01g16870** | 0,02 | 1,98 | 0,73 | argonaute |
| **LOC_Os01g16860** | 1,58 | 1,37 | NE | AGO4-2 |
| **LOC_Os10g34430** | 5,35 | 7,03 | 0,01 | Dicer |
| **LOC_Os04g43050** | 2,45 | 1,90 | 0,76 | Dicer |
| **LOC_Os04g39160** | 2,78 | 4,59 | 2,04 | RNA-dependent RNA polymerase |
| **LOC_Os01g34350** | 2,19 | 0,54 | -6,89 | RNA-dependent RNA polymerase |
| **LOC_Os02g58490** | -0,26 | 4,70 | 3,79 | PINHEAD |
| **LOC_Os04g47870** | 1,91 | 2,45 | 0,35 | PINHEAD |
| ***Histone modification*** | | | | |
| **LOC_Os01g56540** | 1,00 | 3,99 | NE | histone-lysine N-methyltransferase SUVR3 |
| **LOC_Os01g59620** | 2,16 | 4,25 | 4,25 | histone-lysine N-methyltransferase, H3 lysine-9 specific SUVH1 |
| **LOC_Os07g25450** | -0,09 | 5,87 | -0,15 | histone-lysine N-methyltransferase |
| **LOC_Os08g10470** | 0,03 | 7,56 | 4,91 | histone-lysine N-methyltransferase ASHR2 |
| **LOC_Os12g32374** | -0,88 | 4,89 | 2,20 | histone deacetylase 6 |
| **LOC_Os05g36920** | 0,88 | 5,53 | -3,11 | histone deacetylase |
| **LOC_Os10g28040** | -0,45 | 5,58 | -2,98 | histone acetyltransferase GCN5 |
| **LOC_Os06g38470** | 0,97 | 3,19 | 1,10 | histone deacetylase HDAC1 |

## 5.3 Discussion

During pathogen attack, a plant host modulates its gene expression to ward off the invader, while the pathogen produces effector proteins to manipulate the molecular machinery of the plant aiming at increased susceptibility. Characterizing gene expression in the cells that are specifically targeted by the pathogens provides insights into this complex molecular arms-race. To the best of our knowledge, this report represents the first successful combination of Laser Capture Microdissection with mRNA-Seq for expression profiling in the field of plant-nematode interactions. Recently, we have reported an mRNA-seq based transcriptome analysis of galls induced by *M. graminicola* in rice (Kyndt *et al*., 2012a). Within this gall, developing giant cells undergo repeated nuclear divisions without cytokinesis to form large multinucleate cells. As giant cells comprise only a small fraction of the gall material, the analysis of gene expression at the single-cell type level was required for a further in-depth study on these highly specialized nematode feeding cells. In the current study giant cells at 7 and 14 dpi and the corresponding control cells were isolated by LCM, and their transcriptomes were compared using mRNA-Seq.

In total 278 billion paired-end mRNA-Seq reads were generated, leading to estimated expression levels of more than 50,000 annotated and novel rice loci, with a coverage of approximately 100-fold the annotated transcriptome. Previous studies on complete gall tissue and microdissected giant cells from *Arabidopsis*, tomato and soybean (Bar-Or *et al*., 2005; Jammes *et al*., 2005; Barcala *et al*., 2010; Ibrahim *et al*., 2011) applied microarray analysis, and hence only transcripts represented on the arrays could be detected. The benefit of mRNA-Seq lies in the fact that next to annotated transcripts this technique allows the detection of novel splice junctions, novel transcripts, paralogues and rare transcripts (Sultan *et al*., 2008; Wilhelm *et al*., 2008; Zhang *et al*., 2010). Indeed, our analysis uncovered 18,291 putative novel transcriptionally active regions (nTARs) and 16,063 alternatively spliced transcripts expressed in the analyzed LCM-isolated rice cell types. Similar high levels of alternative splicing in rice have been reported by Lu *et al*. (2010) who estimated that ~48% of rice genes show alternative splicing patterns. Among the nTARs, 12,185 are differentially expressed, with 2,214 nTARs significantly down-regulated in the GCs, and 9,971 significantly up-regulated (FDR<0.05). These represent transcripts that might only be expressed at very low levels and/or under specific circumstances, making them undetected in previous studies.

In the following paragraphs, transcriptional changes observed in rice giant cells are being compared to expression results from *Arabidopsis* and tomato giant cells (Barcala *et al*. 2010; Portillo *et al*., 2013), complete galls from tomato, *Arabidopsis* and rice (Bar-Or *et al*., 2005; Barcala *et al*., 2010; Kyndt *et al*., 2012a, Portillo *et al*., 2013), and above-ground tissues of

nematode-infected rice plants (Kyndt *et al*., 2012b). Our results were also compared with data obtained from plants infected with cyst nematodes, another type of sedentary plant-parasitic nematodes that form feeding sites called syncytia (Ithal *et al*., 2007a, 2007b; Klink *et al*., 2007; Szakasits *et al*., 2009).

Metabolic changes in giant cells induced by RKN infection in rice

Feeding sites of sedentary endoparasitic nematodes, like Mel*oidogyne* spp. and cyst nematodes, are the only source of nutrients for these root parasites throughout their life (Jammes *et al*., 2005; Szakasits *et al*., 2009). This high demand for resources is reflected in the up-regulation of genes involved in the primary metabolism of the plant cell, with a prominent induction of starch production, something already observed by Barcala *et al*. (2010) in *Arabidopsis* giant cells. Also syncytia formed by cyst nematodes store carbohydrates by starch accumulation in the plastids (Hofmann *et al*., 2008), probably as a carbohydrate buffer and long-term storage to compensate for changing solute uptake by the nematode. In contrast with the induction of the primary metabolism, the secondary metabolism of the giant cells is strongly impaired, with for instance many down-regulated transcripts involved in phenylpropanoid production.

*The flavonoid side-chain of the phenylpropanoid pathway*

The first step in the phenylpropanoid pathway is catalysed by phenylalanine ammonia lyase (PAL). Strikingly, all PAL-homologues are suppressed in giant cells at both investigated time points. However, when looking at whole gall tissue many of them were repressed at 3 dpi (Kyndt *et al*., 2012a), but induced at 7 dpi (Table S5.5). After non-oxidative deamination of L-phenylalanine to trans-cinnamic acid by phenylalanine ammonia-lyase (PAL), the phenylpropanoid pathway branches into different side chains responsible for the biosynthesis of different metabolites, like lignin precursors, flavonoids, hydroxycinnamic acid esters and salicylic acid (Boudet, 2000).

Active suppression of the flavonoid pathway is an important feature of pathogenicity in many other plant pathogen-interactions (Oh and Collmer, 2005). In line with this view, we found with both mRNA-Seq and qRT-PCR that chalcone synthase, the key enzyme in the flavonoid side-branch of the phenylpropanoid pathway is significantly suppressed in rice giant cells (Table S5.3). Nevertheless, Hutangura *et al*. (1999) showed that chalcone synthase is induced around the invading nematode at 24 h after *M. javanica* infection in white clover (*Trifolium repens*) and that flavonoids were detectable in and around the feeding site within 48 h of the start of the infection process. Similarly, in other plant-nematode interactions, genes involved in flavonoid biosynthesis have been reported to be induced, e.g. upon migratory nematode

infection in rice (Kyndt *et al*., 2012a) and cyst nematode infection in soybean and *Arabidopsis* (Ithal *et al*., 2007a; Jones *et al*., 2007). Data from mutant lines impaired in flavonoid biosynthesis showed that they were either equally or more susceptible to *Heterodera schachtii* (Jones *et al*., 2007) supporting the view that flavonoids are produced by the plant as part of the defence response against nematodes. These metabolites have indeed been shown to have a direct negative effect on many nematode species (Wuyts *et al*., 2006b). Hence, local suppression of flavonoid biosynthesis in the giant cells might be an important strategy for the RKN to overcome host defence responses.

*Trehalose*

Prior to the current study, an activation of the trehalose metabolism was observed in whole galls induced by RKN in rice (Kyndt *et al*., 2012a) and systemic tissue of cyst nematode infected plants (Hofmann *et al*., 2010). Remarkably, in studies focusing on isolated nematode feeding sites several genes encoding trehalose-6-phosphate synthase, an enzyme needed to form trehalose-6-phosphate (T6P), were rather down-regulated (GCs: this study; syncytia: Szakasits *et al*., 2009). T6P has multiple functions in plants, not only in carbohydrate storage and metabolism, but also as a stress protectant, and as a metabolic signaling molecule involved in many plant pathogen-interactions (Fernandez *et al*., 2010) and cell wall modification (Bae *et al*., 2005). Furthermore, other transcripts that are known to be responsive to abiotic and biotic stimuli, like (receptor-like) protein kinases, stress and disease resistance-related proteins are generally strongly repressed in giant cells when compared to corresponding uninfected vascular root cells.

Dark-grown giant cells contain chloroplast-like organelles

Plant roots mainly develop non-photosynthetic plastids, such as starch-containing amyloplasts, but roots of several plant species have the potential to turn green when exposed to light (Flores *et al*., 1993). Upon light exposure, amyloplasts inside RKN-induced galls in tomato roots were reported to differentiate into chloroplasts (Orion and Wergin, 1982), and similar phenomena are generally seen in syncytia formed in nematode-infected plants under light influence (Sijmons *et al*., 1991; Golinowski *et al*., 1996). Also our previous transcriptome study of complete galls showed evidence of photosynthetic activity at 7 dpi, but again these results could have been biased because the tissue had not been covered from indirect light (Kyndt *et al*., 2012a). Up till now, this phenomenon had not been investigated in dark-grown giant cells. That is why, for the here-described experiments, the material was completely protected from light. To our surprise, a strong induction of photosynthesis-related transcripts and transcripts involved in the biogenesis of chloroplasts was consistently found in the GCs. Genes related to

the biosynthesis of tetrapyrroles, which mainly occurs inside chloroplasts, are induced in the giant cells (Fig 5.3). Both transcriptome data and microscopical analysis (Fig 5.4) confirmed that the 7 dpi giant cells even without light-stimulation hold chloroplast-like structures, which contain metabolites with a similar fluorescence emission spectrum as rice leaf tissue.

Sugar depletion due to nutrient sipping by the feeding nematode might be the trigger that activates photosynthetic gene expression (Oswald *et al*., 2001). Furthermore, recent findings suggest that phytohormones and environmental (stress) signals regulate the expression of genes that are related to tetrapyrrole metabolism. Whether there is a causal relationship between this disturbance of the hormone homeostasis and chlorophyll accumulation inside giant cells remains to be further studied.

Modulation of plant-hormone pathways in giant cells

Hormones act as signaling molecules in plants by mediating physiological responses, thereby coordinating growth and differentiation of cells as well as innate immunity.

Consistent with recent findings demonstrating a general induction of GA biosynthesis in galls on tomato and rice (Bar-Or *et al*. 2005, Kyndt *et al*., 2012a) and syncytia in soybean (Klink *et al*., 2007), giant cells accumulate high levels of transcripts coding for enzymes involved in GA production and response (such as catabolism and DELLA proteins) (Table S5.5). Gibberellins are important stimulators of cell division and elongation (Richards *et al*., 2001). These observations suggest that gibberellins are important players in the development, maintenance and maturation of giant cells. GA plays a critical role in controlling and coordinating cell division, cell expansion and, interestingly, also chloroplast biogenesis through influencing the DELLA protein family in leaf tissue of both dicot and monocot plant species (Jiang *et al*., 2012).

Transcripts necessary for jasmonate biosynthesis are suppressed in the 7 and 14 dpi giant cells, although this was not observed in whole gall tissue (Nahar *et al*., 2011; Kyndt *et al*., 2012a). Also Ithal *et al*. (2007b) detected a strong and consistent, but very local, suppression of the JA-pathway in isolated syncytia after cyst nematode infection in soybean. It is important to note that activation of the JA-pathway by external methyl jasmonate application is an effective way to protect potato, tomato and rice from RKN infection (Cooper *et al*., 2005; Nahar *et al*., 2011; Vieira dos Santos *et al*., 2013).

Although application of the SA-analogue BTH only resulted in slightly less gall information in rice (Nahar *et al*., 2011), it has recently been shown to have a strong negative effect on *M. chitwoodi* development in tomato and potato (Vieira dos Santos *et al*., 2013). SA is derived

from the phenylpropanoid pathway, and inn line with a general local and systemic repression of this pathway at 3 dpi in galls and systemic tissues in rice (Kyndt *et al*., 2012a and 2012b) and giant cells formed in *Arabidopsis* (Barcala *et al*., 2010), transcripts involved in the phenylpropanoid pathway are also strongly down-regulated in the isolated 7 and 14 dpi giant cells formed in rice roots. Ethylene (ET) is known to play a synergistic role with JA in plant innate immunity (Pieterse *et al*., 2009). Nahar *et al*. (2011) showed that ET-insensitive mutants and pharmacological inhibition of ET-biosynthesis in rice leads to significantly higher susceptibility for RKN, demonstrating that ET plays a role in defence against RKN in these plants. In addition, Fudali *et al*. (2013) showed that ET-overproducing *Arabidopsis* plants are less attractive to RKN. Nevertheless, ET has been suggested to be critical for syncytium formation during cyst-nematode infections in *Arabidopsis* (Goverse *et al*., 2000). Its ambiguous role in plant defence versus development of the feeding site, might explain why no clear trend concerning the ET-pathway was observed in the here-studied 7 dpi and 14 dpi giant cells (Table S5.5).

A role for epigenetic processes in transcriptional reprogramming of the root cells

Different epigenetic processes have been described to affect the transcriptome of plant cells: (1) cytosine methylation influences gene expression by altering transcription and chromatin structure, (2) histone modifications have an important impact on the structure of chromatin and can make DNA more or less accessible for transcription, (3) small RNAs, like miRNA and siRNAs influence gene expression through targeted degradation of mRNA (post-transcriptional gene silencing) or induction of methylation at complementary DNA sequences (transcriptional gene silencing).

Recent studies have shown the importance of small RNAs during cyst nematode feeding site (syncytium) formation. Sequences of known miRNAs as well as siRNAs were identified by sequencing small RNA libraries isolated from feeding sites induced by the cyst nematode *Heterodera schachtii* in *Arabidopsis* roots (Hewezi *et al*., 2008), and the data suggested a role for small RNAs mediating gene regulation processes during the plant-nematode interaction. Hewezi *et al*. (2012) reported a strong down-regulation of miR396 in early syncytial cells in comparison with the surrounding root tissue, when nematodes are at the J2 or early J3 stage. At later time points, when nematodes reached the J3/J4 stage, a specific miR396 up-regulation was observed in the developed feeding site, miR396 targets a set of Growth-regulation Factor genes (Hewezi *et al*., 2012).

In tomato giant cells, Portillo *et al*. (2013) reported that genes involved in epigenetic processes are induced from 3 dpi and increasing at 7 dpi. Histone acetylation/deacetylation and

methylation are important mechanisms regulating gene expression in plants (Zhou *et al*., 2010), and may be largely involved in responses to environmental stimuli (Chen and Tian, 2007; Servet *et al*., 2010). The here-reported transcriptional changes at 7 dpi in genes encoding histone modifying and small RNA processing enzymes (Table 5.2) confirm a role for epigenetic processes in transcriptional reprogramming of the root cells to form nematode feeding sites. Which transcriptional changes are specifically targeted by these enzymes, and what is their functional relevance, remains to be elucidated.

## 5.4 Materials and Methods

### Infection and LCM of giant cells

*Oryza sativa* cv. Nipponbare (GSOR-100, USDA) was germinated for 6 days at 30 ℃, transferred to SAP-substrate (Sand Absorbent Polymer; Reversat *et al*., 1999) and further grown at 26 ℃ under a 16h/8h light-regime. Extra care was taken to prevent any light influence on the roots of the plants. Nematodes were cultured and extracted as described before (Kyndt *et al*., 2012b). When 12 days old, plants were inoculated in SAP with 250 stage 2 juveniles of *Meloidogyne graminicola* per plant. Control plants were mock-inoculated with water. One day after inoculation the plants were transferred to a hydroponic culturing system with Hoagland solution (Reversat *et al.*, 1999) to synchronize the infection process. Infected and control roots were collected at 7 and 14 days post infection (dpi) and fixed in Farmer's fixative (3:1 Ethanol: Acetic Acid). The material was dehydrated in ethanol and then cryosectioned with a cryostat at -20 ℃. RKN form giant cells in the vascular tissue, but the type of cells specifically targeted is unknown and can even differ between individual nematodes in the same plant tissue (Endo, 1987), therefore a mixture of different types of cells from the vascular tissue of mature rice roots was used as control material in this study. Giant cells and control cells were captured using a Zeiss PALM Laser Microbeam according to the manufacturer's instructions (Fig 5.1). Captured cells were infiltrated in RNA extraction lysis buffer (Stratagene). Three independent biological replicates were taken at each time point, of which two were analyzed by mRNA-Seq. The third independent biological replicate was used for qRT-PCR validation. About 150-200 giant cell sections and 300 control sections (of uninfected plants) per biological replicate were used for giant cell and control cell isolation by LCM.

### RNA extraction, library preparation and Illumina GAIIx sequencing

RNA from LCM-isolated giant cells was extracted with the Absolutely RNA Nanoprep Kit (Agilent), followed by cDNA synthesis using the Ovation RNA-Seq System (NuGEN). This system is based on the Ribo-SPIA® technology (NuGEN) to generate high quality, linearly amplified cDNA from low amounts of RNA, and was specifically designed for next genera-

tion sequencing platforms. The obtained cDNA-concentrations varied between 4.8 and 6.6 µg per sample. cDNA integrity was confirmed using the Agilent BioAnalyzer 2100 (Agilent) and qRT-PCR with two reference genes (Table S5.1).

The full length cDNA was fragmented by sonication with a Covaris S2 ultrasonicator (Covaris). The mRNA-Seq library was constructed according to the NEB protocols E6040 (New England BioLabs). We used the multiplexing sequencing adapters provided in the Multiplexing Sample Preparation Oligo Kit (Illumina). Size selection of the library was performed on a 2% agarose gel (Low Range Ultra Agarose, Biorad 161-3107). The denatured library was diluted to a final concentration of 6 pM and loaded on a paired-end read flow cell (TruSeq v5 kit, Illumina). To minimize lane effects the samples were multiplexed. Each sample was sequenced in duplicate in 2 different lanes (4 lanes total with 8 MID tags per lane). After cluster generation, the multiplexed library was sequenced on an Illumina Genome Analyzer IIx (36 cycles, paired end).

**Mapping reads to genome data and annotated transcripts**

Reads were mapped to the *Oryza sativa* subsp. *Japonica* reference genome (build MSU7.0) in two phases using TopHat version 1.3.1 (Trapnell *et al*., 2009) and Cufflinks, version 1.0.3 (Trapnell *et al*., 2010). A detailed description of the workflow and settings used in the data analysis is given in Kyndt *et al*. (2012a).

**Identification of novel transcriptionally active regions (nTARs)**

The Cufflinks program generates a GTF file including all transcripts annotated in MSU7.0 and putative novel transcripts derived from the data. All putative nTARs marked as splice variants of known genes or located within intronic regions were disregarded and for the 18,291 remaining nTARs BLASTx searches were performed against Swiss-Prot and trEMBL and all predicted rice proteins (http://rice.plantbiology.msu.edu/). Homologues of the nTARs in rice ESTs were searched by tBLASTx (E<1e-4).

**Calculation, normalization and profiling of gene expression**

Expression was quantified per sample and per annotated or unannotated transcript as the sum of all reads that mapped to the respective gene exons with a 16 base pair tolerance on either side to compensate for potential errors in the gene annotation. Expression profiles were assessed using the R-package "baySeq", version 1.5.1. (Hardcastle and Kelly, 2010). To compensate for artificial differences in read distributions, the original library sizes were multiplied by additional normalisation factors calculated using the Trimmed Median of M-values method described in (Robinson and Oshlack, 2010) with standard settings as implemented in the edgeR package (version 2.0.3). A transcript was considered to be expressed if at least one

sequence read was mapped to it in one of the samples. For all further analyses the expression level of each transcript for each condition was estimated as the fold change (FC) of mapped reads relative to the controls. The FC was calculated as follows: reads were normalized as described earlier and averaged over the biological replicates. Before calculating the base 2 log of the ratio of these averages, the number of reads was increased by 1 in each group (to avoid 0-values).

**Gene Ontology and enrichment analyses**

Gene Ontology (GO) analysis and GO enrichment were performed using agriGO (Du *et al*., 2010). Parametric Analysis of Gene Set Enrichment (PAGE) (Kim and Volsky, 2005), based on differential gene expression levels ($log_2$FC), was executed. Benjamini and Hochberg false discovery rate analysis (FDR) was performed using the default parameters to adjust the PAGE P-values.

In addition, MapMan (Thimm *et al*., 2004) was used to visualize the expression of genes onto metabolic pathways and the WSR-test (with Benjamin and Hochberg correction) was used to test the statistical significance of differential expression of these pathways.

**Validation of mRNA-Seq by qRT-PCR**

Based on potential functional importance, 13 genes were selected for validation in an independent biological sample by qRT-PCR. Locus number of these transcripts, primer sequences and reaction efficiencies are presented in Supplementary Table S5.1. qRT-PCR was performed and analysed as described in Kyndt *et al*. (2012b), using 3 technical replicates.

**Confocal microscopy**

Galls were collected at 7 dpi and fixed in Farmer's fixative (3:1 Ethanol: Acetic Acid) overnight. They were dehydrated by a dilution series of 70-90-100% Ethanol (1h each) and stored at 4 ℃ for 7 days before microscopical analysis. To prepare samples for confocal microscopy, fixed galls were sliced in thin sections using a razor blade and mounted onto a slide in a drop of water. Images were acquired with a Nikon A1R confocal microscope, mounted on a Nikon Ti body and equipped with a 40 x (NA = 0.6) PLAN Fluor ELWD objective. Image acquisition was performed at a pinhole setting of 1 Airy Unit and a pixel size of 0.6 µm x 0.6 µm (zoom factor 1) or 0.2 µm x 0.2 µm (zoom factor 3). To capture the spectral fingerprint (lambda stack) of chloroplast pigment autofluorescence, samples were illuminated with a 488 nm Argon laser and emission was detected on a spectral 32-PMT detector, set at a resolution of 2.5 nm per detector within a range of 655.5 – 735.5 nm. As a reference, lambda stacks were also acquired from fresh and fixed leaf sections using identical settings. Spectral profiles of (presumed) chloroplasts were determined by measuring the average intensity per wave-

length interval (2.5nm) across the lambda stack range. Per condition, the average of the individual profiles of min. 4 equally sized chloroplast (-like) regions was calculated.

# CHAPTER 6

## General conclusions and perspectives

**Rice root-knot nematode (RKN) can be suppressed in rice by over-expressing one of its endogenous thionin genes**

RKNs migrate between the plant cells by enzymatic softening of the middle lamella of the root cells, causing hardly any necrosis during their invasion. In addition, RKNs obtain manipulative power to suppress plant defence for their feeding site establishment and development. For example, many genes involved in the phenylpropanoid pathways and defence-related hormone pathways are strongly attenuated in galls (Kyndt *et al.*, 2012a, Barcala *et al.*, 2010, Jammes *et al.*, 2005), as well as in giant cells (chapter 5). One of the rice thionin genes, *OsTHI7*, was observed in a transcriptome study to be down-regulated in 3dpi and 7dpi galls induced by *M. graminicola* (Kyndt *et al.*, 2012a). Corroborating and extending this knowledge, the results provided in chapter 3 show a clear attenuation of thionin transcripts in early developmental stages of galls, as observed by qRT-PCR and promoter-GUS assays. Thionins, known pathogen related proteins (PR protein), are a group of anti-microbial polypeptides that are thought to be involved in plant defence (Bohlmann & Apel, 1991). There are 40 putative paralogues of thionin in Japonica rice. This large number could be the result of evolution by the pressure of new variants of pathogens, and/or the fact that each gene might be uniquely responsive to specific external or internal stimuli, such as pathogens, hormones or expression in different tissues or developmental stages (Florack & Stiekema, 1994, Bohlmann & Apel, 1991). Indeed, rice thionin genes respond differently to plant defence related hormones, although the amino acid sequences of those thionin proteins are highly similar. In chapter 3, we studied the transcriptional response of three rice thionin genes, *OsTHI1*, *OsTHI2*, and *OsTHI7+6* upon BTH, MeJA and ABA application. *OsTHI7+6* was strongly and continuously induced by BTH, which indicates that *OsTHI7+6* is involved in SA mediated plant defence against rice RKN (chapter 3). *OsTHI1* expression in rice roots strongly responds to ABA treatment. Since ABA is a very important signaling molecule involved in abiotic stress, the potential correlation of *OsTHI1* with abiotic stress deserves to be further investigated. Our analysis was only conducted in root tissue, and since PR gene expression is associated with SAR, further analysis of the expression of thionin genes in shoot tissue will expand our knowledge on thionin and defence hormone interactions in rice. Moreover, in addition to the classical defence-related hormones SA, JA or ABA, other hormones such as brassinosteroid, cytokinins, auxins and gibberellins have been implicated as important players in plant defence (Bari & Jones, 2009, Pieterse *et al.*, 2012). It will be particularly interesting to test thionin gene expression in response to these hormones. In addition, these stimuli could also regulate other thionin genes, such as those located on other chromosomes. In this perspective, it is tempting to test the response of other rice thionin genes to certain stimuli. Due to the high similarity of coding sequences of thionin genes in rice, it was not easy to measure

the expression of a single gene. Nevertheless, in the future fluorescent probes might be used to solve this issue.

In chapter 3, we used the transgenic approach to study a single thionin gene in the interaction between thionins and rice RKN. Based on the observations in RNAseq analysis (Kyndt *et al*., 2012a) and qRT-PCR results (chapter 3), as well as the response to defence hormones (chapter 3), *OsTHI7* was thought to be a promising candidate for the transgenic study. Interestingly, our results show that overexpression of *OsTHI7* in the susceptible rice variety *Oryza sativa* cv Nipponbare not only decreases plants susceptibility to the RKN, resulting in a lower number of females and total number of nematodes per plants, but also enhances plant tolerance to *Pythium graminicola* infection. However, the exact mechanism by which OsTHI7 decreases susceptibility to pathogens is still unknown. The subcellular localization study shows that OsTHI7 is translocated in the secretory pathway and it has a dual localization in the extracellular spaces and vacuoles (chapter 3), which is in line with previous findings from other thionins (Vera *et al.*, 1989, Dixon *et al.*, 1991). It has been reported that thionins have a toxic effect on pathogenic fungi and bacteria by changing the permeability of their membranes (Bohlmann & Apel, 1991). The toxicity has been previously established *in vitro* (Bohlmann *et al.*, 1988, Florack & Stiekema, 1994), and emerging evidence also indicates that thionins are toxic to fungal pathogens *in vivo*. For instance, overexpression of the *Arabidopsis* homolog, *AtTHI2.1* decreased the susceptibility to *Fusarium oxysporum.* Fungi infecting cotyledons of transgenic lines had more abnormal hyphae, including hyperbranching via a direct toxic effect of the overexpressed thionin (Epple *et al.*, 1997). Cytotoxic effects on cultured mammalian cells have also been reported (Garc á Olmedo *et al.*, 1989). Endosperm thionins were toxic to mice, guinea pigs and rabbits or insect larva when the protein was injected intravenously or in the hemocoel, but not upon oral administration. Further deciphering the effect of OsTHI7 protein *in vitro* against nematodes may provide more insights on the effect of this protein effect against RKN. Moreover, in-depth detection of OsTHI7 distribution in feeding site by antibodies will be of particular value in supporting our hypothesis of a release of OsTHI7 from vacuoles upon feeding site formation. Besides the direct toxic effect, overexpression of *OsTHI7* may augment the expression of other defence-related genes. Although we have checked the expression of *PR1a* and *PR1b* in overexpressed plants and there are nearly no changes in overexpressed plants compared to the control, more defence-related genes should be checked to get a general overview of thionin induced resistance against RKN. The enhanced tolerance of *OsTHI7* overexpression plants against *Pythium graminicola* was revealed by a better shoot growth compared to the control plants, although the infected roots appeared to have a similar disease index as the control. The mechanism of this enhanced tolerance is unclear. Mechanisms of tolerance are quite diverse, including increased photosynthetic activi-

ty, reallocating resources, increase in nutrients uptake etc. (Rosenthal & Kotanen, 1994, Agrawal *et al.*, 1999, Tiffin & Inouye, 2000).

The resistance against other plant parasitic nematodes and pathogens or abiotic stress still needs to be explored and besides the number of nematodes or a disease index score to measure resistance, measuring the grain yield will be the most important parameter. Field trails may also offer a better idea about the future application of this transgenic plant.

**Rice RKN can be suppressed in rice by exogenous application of BABA**

Plants carry inducible mechanisms that protect plants in a time-dependent manner. Since the beginning of the 20$^{th}$ century, much research has been conducted on the use of induced resistance (IR) agents to reduce dependence on chemical pesticides. IR only uses endogenous genes to carry out efficient defence, which avoids introducing exogenous genes into plants and therefore it does not meet the public protest encountered by genetically modified plants (chapter 4). β-aminobutyric acid (BABA), a non-protein amino acid, is an IR agent that helps fending off a wide range of pathogens in dicotyledonous plants (Cohen, 2002, Jakab *et al.*, 2001, Jakab *et al.*, 2005, Zimmerli *et al.*, 2008, Pastor *et al.*, 2013). In line with the extensive work in dicots, this thesis revealed BABA can also induce resistance against RKN in monocots such as rice (chapter 4).

Results in chapter 4 show that BABA-IR against RKN in rice by inhibiting nematode penetration and delaying nematode and giant cell development. Hence, gaining insights into the ability of BABA-induced resistance against other nematodes or rice diseases will be helpful to consider whether BABA can be applied in the rice fields. Since not only RKN, but also other rice nematodes, such as white tip nematode (*Aphelenchoides besseyi*), rice root rot nematode (*Hirschmanniella oryzae*) and diseases caused by pathogenic bacteria and fungi are still among the major constraints on rice productivity. Intriguingly, the descendants of BABA primed *Arabidopsis* plants exhibit more resistance to oomycete pathogen *Hyaloperonospora arabidopsidis* compared to the descendants of unprimed plants (Slaughter *et al.*, 2012). This result is incentive to test whether it is also the case in rice. The fact that BABA does not have a direct toxic effect to pathogens, insects and nematodes (Oka & Cohen, 2001, Cohen, 2002, Jakab *et al*., 2001), is potentially another advantage to other methods for plant resistance, such as the application of nematicides or engineering plants with PR proteins (chapter 3). These methods need to be cautioned since they have ecological costs. Taken together, BABA seems to be a very valuable induced resistance elicitor with a broad spectrum pathogen protection; it is also durable and environmentally sound. However, there is still allocation costs associated with induced resistance. For instance, application of benzothiadiazole to wheat in the absence of pathogens reduces biomass, number of ears and grains (Heil *et al.*, 2000). High

concentrations of BABA induce a direct defence response in *Arabidopsis* against *Hyaloperonospora parasitica* and *Pseudomonas syringae* but reduce the relative growth rate up to 44% in the absence of pathogen infection (van Hulten *et al*., 2006). Hence, evaluation of yield losses caused by BABA -IR in rice will give another view to decide upon its application into the field. In our current study (chapter 4), a BABA concentration of 3.5mM was used, which is relative high compared to studies in dicotyledons. The lowest concentration of BABA inducing resistance against RKN is worthwhile to test, in order to evaluate the cost factors. Moreover, using a lower concentration of BABA will result in priming (chapter 1), which hardly causes any fitness cost since plants only activate the defence response upon pathogen challenge (van Hulten *et al*., 2006).

Results denoted in chapter 4 indicate that BABA induced resistance against rice RKN is different from Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR), which are SA and JA/ET dependent. However, this does not exclude the involvement of other types of hormones in addition to the pathways modulated by SA, JA. BABA induces resistance in some pathogen-plant systems through enhanced basal defence (chapter 1). In line with those findings, histochemical, biochemical and molecular analyses were performed. The results uncovered that BABA-IR against RKN is correlated with increasing levels of $H_2O_2$, lignin and callose (chapter 4). Employing inhibitor compounds or mutants to block $H_2O_2$, lignin or callose synthesis would provide further support for these conclusions. There are many reports showing BABA induced callose deposition is ABA dependent (Ton & Mauch-Mani, 2004, Ton *et al.*, 2005, Flors *et al.*, 2005). However, although we have found that BABA induced resistance against RKN was partially comprised in ABA inhibited plants, the link between ABA and callose deposition still needs to be further explored. In spite of the widespread interest of BABA-IR, there are still no data available on the transcriptome or proteome analysis in monocot such as rice treated with BABA. Dissecting the transcriptome changes in BABA treated rice plants will shed new light on the mode of BABA induced resistance in monocots. In the case of RKN, it will be interesting to focus on the transcriptome of giant cells in BABA treated plants (chapter 5). According to the transcriptome or proteome data, hopefully we can find the important genes which are responsible for BABA-IR in rice. Moreover, as the descendants of BABA pr**i**med *Arabidopsis* plants still exhibit resistance to oomycete challenge (Slaughter *et al*., 2010), studying the epigenetic modifications will be an interesting aspect for exploring the mechanism of BABA induced resistance. Another question is the identification of BABA modulators or receptors, which is essential to our understanding of BABA induced resistance.

**Transcriptional analysis reveals a general induction of primary metabolism and a strong suppression of defence-related genes inside the giant cells**

The J2 juveniles of *M. graminicola* are attracted to the roots and penetrate the roots just behind the root tips. When J2s settle down and start to feed, those feeding cells enlarge and are converted into multinucleate cells through synchronous nuclear divisions without cell division, forming so-called giant cells (GCs). Hyperplasia and hypertrophy of the surrounding cells lead to the formation of the typical galls at the root tips (chapter 1, Jones & Payne, 1978, Gheysen *et al.*, 2006). The gene expression in GCs is very distinct from the surrounding cells (Gheysen & Fenoll, 2002, Escobar *et al.*, 2011). To gain insight into GCs differentiation in rice upon RKN infection, we combined LCM with mRNA sequencing (mRNA-Seq) to study the giant cell transcriptome at two time points after infection (chapter 5). The expression profiles revealed a general induction of primary metabolism inside the giant cells. While genes involved in 'tropism', 'signaling', 'response to stimulus', and 'secondary metabolism were generally down-regulated. The majority of phenylalanine ammonia lyase homologues and defence related hormones such as JA and SA were suppressed in GCs, although this was not observed in whole-gall tissue (Nahar *et al*., 2011; Kyndt *et al*., 2012a). Hence, local suppression of those defence- related genes in the GCs might be an important strategy for the RKN to overcome host defence. Taking advantage of this obtained knowledge, further work should focus on deciphering the putative roles of the induced or suppressed genes in GCs, either by genetic engineering or other approaches. Interestingly, a remarkable induction was observed in genes involved in chloroplast biogenesis and tetrapyrrole synthesis in dark grown GCs. Whether it is due to sugar depletion by the feeding nematode or related to other signals such as phytohormones remains to be further studied. The transcriptional changes at 7 dpi in genes encoding histone-modifying and small-RNA-processing enzymes indicate a role for epigenetic processes in transcriptional reprogramming of the root cells to form nematode feeding sites. However, their functional relevance remains to be elucidated. Furthermore, it will be very interesting to explore the transcriptome changes in syncytia, the feeding sites of cyst nematodes (Gheysen *et al*., 2006), in rice and other plant species, such as wheat, to gain more in-depth insight of the distinct and conserved genes involved in the formation and maintenance of feeding sites.

# Summary

The dramatic growth of human global population requires an increase in sustainable agricultural production, particularly in the production of cereals, such as rice, wheat, and maize. However, plants are constantly exposed to a spectrum of diseases including viruses, fungi, bacteria, and nematodes, which cause yield loss. Presently, disease control is mainly based on the use of chemicals. However, due to the hazardous effect and non-specific toxicity of these chemicals or their degradation products on the environment and human health, the use of chemicals is often restricted. Therefore, producing crops with increased and durable resistance is a major focus in plant research. In the past, durable resistance to diseases has been sought through conventional breeding approaches. However, conventional breeding of resistance suffers from the serious limitation that it does not allow introduction of resistance genes into distant species. Genetic engineering has been promoted for three decades as a solution for this problem. The most straightforward approach is to add genes encoding proteins with antimicrobial properties, such as antimicrobial proteins, including the pathogenesis-related (PR) proteins. Those proteins have a direct a toxic effect on microbial pathogens, either demonstrated *in vitro* or *in vivo*. Moreover, those genes might increase plant preformed physical arriers and are involved in the defence signaling pathways. Enhanced disease resistance has been achieved not only using transgenic plants but can also be induced by a variety of abiotic and biotic inducers. Biotic inducers include infection by necrotizing pathogens which results in systemic acquired resistance (SAR), or by plant-growth-promoting rhizobacteria leading to induced systemic resistance (ISR). Abiotic inducers include chemicals which act at various facets in disease resistance as well as in abiotic stress tolerance. Among these abiotic inducers, β-aminobutyric acid (BABA), a non-protein amino acid exhibits a promising potential of induced resistance in plants. Although both engineering resistance and induced resistance hold great potential of disease control, little information is available on the efficacy of PR proteins against plant parasitic nematodes, and the knowledge of induced defence responses in the interaction between nematodes and monocots such as rice is still in its infancy. In this thesis, two approaches of improving rice resistance against RKN were studied, via genetic engineering resistance by overexpressing rice endogenous PR gene *OsTHI7* and through exogenous application of BABA. Moreover, to get more knowledge on the plant response to RKN infection, and in turn to supply more resources of effective genes for developing resistant plants, the transcriptome of giant cells in rice induced by RKN was analyzed.

In the first part of this thesis, a thorough molecular characterization of the rice thionin genes is reported (chapter 3). A clear attenuation of thionin transcripts in early developmental stages

of galls was observed by qRT-PCR and promoter-GUS assays. These results indicate that the RKNs have the power to suppress plant defence to enable a successful infection and establishment of the functional feeding site. Overexpression of *OsTHI7* decreases plant susceptibility against *M. graminicola*, as revealed by a lower number of females and lower total number of nematodes per plant. In addition, it enhances the plant tolerance to *Pythium graminicola* infection. The observed enhanced resistance might be explained by a toxic effect of plant thionins to the pathogens membranes. Moreover, although there are high similarities in coding sequences and amino acid sequences among the thionins, the three studied thionin genes respond differently upon phytohormone treatments, which may indicate that the functions of the gene family members are diverse and specific. All the data provided in this chapter point out that *OsTHI7* can act as a defence gene in vivo against RKN and enhances plant tolerance to *Pythium graminicola*. Moreover, rice thionin genes, which are responding to different signal transduction pathways, may serve to different functions, either in plant development or defence.

Aiming to further explore disease resistance in rice, attention was shifted to the induced resistance by BABA (chapter 4). The potential of BABA-induced resistance (IR) was investigated and the induced defence mechanisms underpinning BABA-IR in rice against RKN was deciphered. BABA application on rice plants inhibited nematode penetration and delayed nematode and giant cell development. Experiments with hormone biosynthesis inhibitors mtants and transgenic lines show that BABA-IR against RKN is SA, JA and ET-independent. In line with some research findings in other pathogen-plant systems, our data revealed that BABA-IR against RKN is correlated with increasing levels of $H_2O_2$, lignin and callose accumulation. The cumulative results presented in this research indicate that BABA induces a multifaceted plant basal defence response in rice roots, which in turn inhibits nematode penetration and development.

Transcriptional data of the plant response to nematode infection would offer another approach to improve plant resistance through analyzing differentially expressed genes. In chapter 5, we specifically isolated giant cells by laser capture microdissection (LCM). After RNA extraction from these cells, we applied mRNA sequencing (mRNA-Seq) to study the giant cell transcriptome at two time points after infection. The expression profiles revealed a general induction of primary metabolism inside the giant cells, but the majority of the defence-related genes were strongly suppressed in the giant cells. Moreover, transcripts involved in epigenetic processes were significantly induced in 7dpi giant cells. The results were also compared to expression results from *Arabidopsis* and tomato giant ells, as well as to complete galls and syncytia. This study identified several plant genes with differential expression in giant cells,

which may participate in feeding site formation and maintenance, or may be the plant response to nematode invasion. Such genes can be used to improve plant resistance in the future.

With this thesis, the understanding of rice-nematode interaction regarding thionin genes, BABA-IR and transcriptome in giant cells have significantly progressed. Such advances will not only progress our fundamental knowledge of plant response to nematodes attack, but also provide novel strategies to improve rice defence to the root-knot nematode *Meloidogyne graminicola*.

# Samenvatting

De dramatische toename van de wereldpopulatie vereist een verhoogde maar duurzame landbouwproductie vooral van granen zoals rijst, tarwe en maïs. Planten worden echter voortdurend bedreigd door ziekten en plagen zoals virussen, schimmels, bacteriën, insecten en nematoden. Deze biotische problemen worden veelal onder controle gehouden door chemische gewasbeschermingsmiddelen. Chemische middelen hebben echter vaak negatieve effecten op omgeving en menselijke gezondheid en daarom is het interessanter om gewassen te verkrijgen met een duurzame resistentie tegen ziektes en plagen. Aangezien veredeling van ziekteresistentie gelimiteerd is tot genen die in dezelfde of verwante soorten aanwezig zijn, kan genetische modificatie een alternatieve oplossing bieden voor bepaalde problemen. Het ligt voor de hand daarvoor genen uit te testen die coderen voor antimicrobiële eiwitten zoals de pathogenese- gerelateerde(PR)-eiwitten. Deze eiwitten kunnen een direct antimicrobieel effect hebben op pathogenen of pathways in de plant beïnvloeden. Naast het inbrengen van genetische resistentie kan een plant ook beschermd worden door het natuurlijk afweersysteem te activeren via abiotische of biotische inducers. Biotische inducers zijn bv. infectie door necrose-veroorzakende pathogenen wat dan resulteert in systemische verworven resistentie (SAR), of door plantengroeibevorderende rhizobacteria wat leidt tot geïnduceerde systemische resistentie (ISR). Bij de abiotische inducers kennen we bv. β-aminobutyric acid (BABA), een non-protein aminozuur dat een groot potentieel heeft voor geïnduceerde resistentie in planten. Alhoewel deze methoden zeer beloftevol zijn staat de kennis van geïnduceerde afweer in de interactie tussen nematoden en monocots zoals rijst nog in de kinderschoenen. In dit doctoraat bestuderen wij twee mogelijkheden om rijstresistentie tegen wortelknobbelnematoden (RKN) te verbeteren, via genetische modificatie van resistentie door overexpressie van het PR-gen *OsTHI7* en door exogene toediening van BABA. Bovendien proberen we nog meer informatie te verzamelen over de plantenrespons op RKN-infectie namelijk door transcriptoomanalyse van reuzencellen, om op die manier nog andere genen te identificeren die mogelijks kunnen gebruikt worden in nieuwe strategieën om planten resistent te maken tegen RKN.

In het eerste deel van dit doctoraat wordt een gedetailleerde moleculaire analyse uitgevoerd van de rijstthioninegenen, een groep van PR-eiwitten (hoofdstuk 3). Een duidelijke attenuatie van thioninetranscripten in vroege ontwikkelingsstadia van gallen werd aangetoond via qRT-PCR en promoter-GUS assays. Deze resultaten wijzen erop dat RKNs de plantenafweer kunnen onderdrukken omzo een succesvolle infectie te kunnen uitvoeren. Overexpressie van *OsTHI7* vermindert de gevoeligheid van de plant tegen *M. graminicola*, te zien aan een lager totaal aantal nematoden in de plant en een lager aantal mature vrouwtjes. Bovendien zijn deze

overexpressieplanten meer tolerant tegen *Pythium graminicola* infectie. De verhoogde resistentie zou kunnen verklaard worden door een toxisch effect van plant thionines op pathogeenmembranen. Bovendien is aangetoond dat de expressie van de verschillende thionines op een andere manier gereguleerd wordt door hormonen, wat er op wijst dat deze thionines mogelijks andere functies hebben in plantenontwikkeling en afweer. Het potentieel van BABA-ge ïnduceerde resistentie (IR) en de onderliggende mechanismen die de resistentie van rijst tegen RKN verhogen werden bestudeerd in hoofdstuk 4. Toediening van BABA aan rijstplanten inhibeerde nematodepenetratie en vertraagde de ontwikkeling van nematoden en reuzencellen. Experimenten met inhibitoren van hormoonbiosynthese, mutanten en transgene lijnen toonden aan dat BABA-IR tegen RKN onafhankelijk is van SA, JA of ET. Zoals in de literatuur beschreven voor andere plant-pathogeeninteracties werd ook hier gevonden dat BABA-IR gecorreleerd is met hogere concentraties aan $H_2O_2$, lignine en callose. Dit toont aan dat BABA een veelzijdige-plantenafweer induceert in rijstwortels die zowel de penetratie als de ontwikkeling van nematoden afremt.

Transcriptionele gegevens van de plantenrespons op nematodeninfectie kan de basis vormen voor nieuwe strategie ën om de plant te beschermen. In hoofdstuk 5 hebben we reuzencellen geïsoleerd via "laser capture microdissection" (LCM). Na RNA-extractie uit deze cellen werd mRNA sequencing (mRNA-Seq) uitgevoerd om de expressie-patronen te analyseren op twee tijdstippen na infectie. De expressieprofielen wezen op een algemene inductie van het primaire metabolisme in reuzencellen en een algemene repressie van genen betrokken bij de afweer. Transcripten betrokken bij epigenetische processen zijn significant opgereguleerd op 7 dagen na inoculatie. De resultaten werden ook vergeleken met gegevens van tomaat en *Arabidopsis*. Deze studie identificeerde verschillende plantengenen met een differentieel expressiepatroon in reuzencellen. Dergelijke genen kunnen betrokken zijn bij reuzencelontwikkeling of bij de afweer van de plant tegen infectie en kunnen dus mogelijks gebruikt worden in strategie ën om de plantenafweer te verbeteren.

In deze doctoraatsthesis is er significante vooruitgang geboekt in het begrijpen van de rijst-nematode interactie meer bepaald in relatie tot thioninegenen, BABA-IR en het transcriptoom in reuzencellen. Deze gegevens bieden ni leen meer fundamenteel inzicht in de interactie maar kunnen ook nuttig zijn om de resistentie van rijst te verhogen tegen de nematode *Meloidogyne graminicola*.

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Jingjusi road 20, 610066 Chengdu, China

**2000-2004**: Southwest China University

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Professional career

-UGent-BOF PhD Scholarship (2009-2013): The Ghent University open competitive scholarship for doing PhD at the Ghent University of Belgium

-VLIR-UOS (2007-2009): The Flemish competitive scholarship for doing International Masters in Nematology at the Ghent University of Belgium

**III. Publications with peer reviewing**

**Hongli Ji**, Godelieve Gheysen, Simon Denil, Keith Lindsey, Jennifer F. Topping, Kamrun Nahar, Annelies Haegeman, Winnok H. De Vos, Geert Trooskens, Wim Van Criekinge, Tim De Meyer and Tina Kyndt (2013). Transcriptional analysis through RNA sequencing of giant cells induced by *Meloidogyne graminicola* in rice roots. Journal of Experimental Botany*64 (12): 3885-3898*
Tina Kyndt, **Hongli Ji**, Bartel Vanholme and Godelieve Gheysen (2013). Transcriptional silencing of RNAi constructs against nematode genes in *Arabidopsis*. Nematology 15 (5):519-528

**IV. Conference and symposia contributions**

-Ji HL., Kyndt T., Gheysen.G. The morphology of giant cells induced by root-knot nematode *M. graminicola.* Proceedings of the fourth COST 872 Annual Meeting, Lisbon, Portugal, 24-27th May, 2010. **Poster presentation**

- Ji HL., Kyndt T., He W., Gheysen.G. The effects of BABA on rice defence against the root-knot nematode *Meloidogyne graminicola.* Plant Diseases and Resistance Mechnisms, Vienna, Austria. 20-22nd Feb. 2013. **Poster presentation**
- Ji HL., Kyndt T., He W., Gheysen.G. The effects of BABA on rice defence against the root-knot nematode *Meloidogyne graminicola.* 65th International Symposium on Crop Protection, Gent, Belgium, 21st May, 2013. **Oral presentation**
- Ji HL.,Kyndt T.,Ullah C., Gheysen G. Investigating the role of thionins in the interaction between root -knot nematodes and rice. 10th International Congress of Plant Pathology, Beijing, China, 25-30th August, 2013. **Oral presentation**

**V. Training**

Workshop Model Organisms: Nature gift to translational Research. Flemish Training Network in Life Sciences. 29-31st August 2012, Hasselt

Workshop Next Generation Sequencing. Flemish Training Network in Life Sciences. 17th-21st September 2012, Leuven

Introduction to Linux for bioinformatics. VIB Bioinformatics Training & Service Facility (BITS).12th and 19th October 2012.Gent

Basic bioinformatics concepts, databases and tools. VIB Bioinformatics Training & Service Facility (BITS). 22nd and 26th October 2012, Leuven

Analysis of public microarray data using Genevestigator. VIB Bioinformatics Training & Service Facility (BITS). 6th and 9th November 2012, Gent

**VI. Master students and theses supervision**

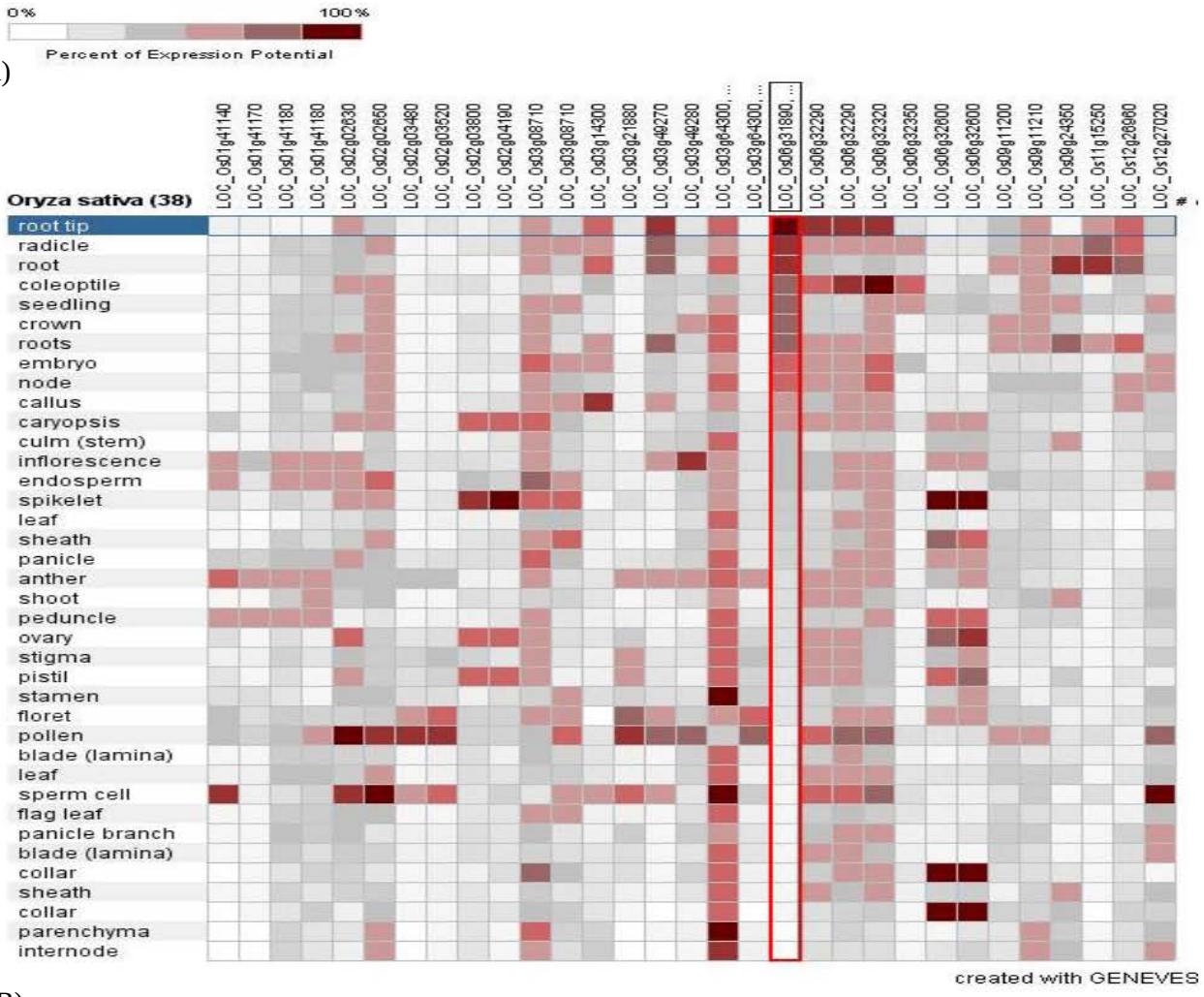
GONZALEZ-SANTIN L. LORENA (2010-2011). Characterisation of rice genes up-regulated after *Meloidogyne graminicola* infection. Ghent University, Belgium. Thesis submitted in partial fulfillment to obtain the degree of European Master of Science in Nematology.

WEN HE (2011-2012). The effects of DL-Beta-Amino-n-Butyric acid (BABA) on rice defence against the root-knot nematode, *Meloidogyne graminicola.* Thesis submitted in partial fulfillment to obtain the degree of Master of Science in Nematology.

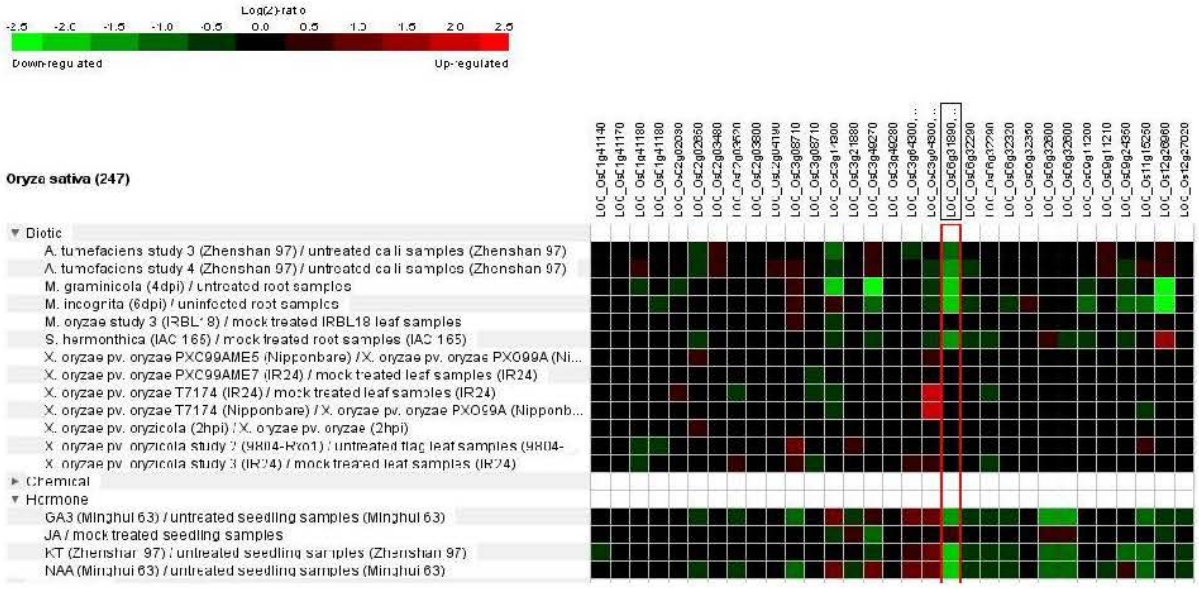
CHHANA ULLAH (2012-2013). The role of thionins in the interaction between *Meloidogyne graminicola* and rice (Oryza sativa L.) Thesis submitted in partial fulfillment to obtain the degree of Master of Science in Nematology.

# Addendum

(A)


0%
100%
Percent of Expression Potential
Oryza sativa (38)
root tip
radicle
root
coleoptile
seedling
crown
roots
embryo
node
callus
caryopsis
culm (stem)
inflorescence
endosperm
spikelet
leaf
sheath
panicle
anther
shoot
peduncle
ovary
stigma
pistil
stamen
floret
pollen
blade (lamina)
leaf
sperm cell
flag leaf
panicle branch
blade (lamina)
collar
sheath
collar
parenchyma
internode
created with GENEVES

(B)


Log(2)-ratio
-2.5 -2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5
Down-regulated
Up-regulated
Oryza sativa (247)
Biotic
A. tumefaciens study 3 (Zhenshan 97) / untreated calli samples (Zhenshan 97)
A. tumefaciens study 4 (Zhenshan 97) / untreated calli samples (Zhenshan 97)
M. graminicola (4dpi) / untreated root samples
M. incognita (6dpi) / uninfected root samples
M. oryzae study 3 (IRBL 8) / mock treated IRBL18 leaf samples
S. hermonthica (IAC 165) / mock treated root samples (IAC 165)
X. oryzae pv. oryzae PXO99AME5 (Nipponbare) / X. oryzae pv. oryzae PXO99A (Ni...
X. oryzae pv. oryzae PXO99AME7 (IR24) / mock treated leaf samples (IR24)
X. oryzae pv. oryzae T7174 (IR24) / mock treated leaf samples (IR24)
X. oryzae pv. oryzae T7174 (Nipponbare) / X. oryzae pv. oryzae PXO99A (Nipponb...
X. oryzae pv. oryzicola (2hpi) / X. oryzae pv. oryzae (2hpi)
X. oryzae pv. oryzicola study 2 (9804-Rep1) / untreated flag leaf samples (9804-
X. oryzae pv. oryzicola study 3 (IR24) / mock treated leaf samples (IR24)
Chemical
Hormone
GA3 (Minghui 63) / untreated seedling samples (Minghui 63)
JA / mock treated seedling samples
KT (Zhenshan 97) / untreated seedling samples (Zhenshan 97)
NAA (Minghui 63) / untreated seedling samples (Minghui 63)

| Locus Number | Putative function | Locus Number | Putative function | Locus Number | Putative function |
|---|---|---|---|---|---|
| **LOC_Os01g41140** | *OsTHI*18 | **LOC_Os02g04190** | *OsTHI*23 | **LOC_Os06g32350** | *OsTHI*12 |
| **LOC_Os01g41170** | *OsTHI*27 | **LOC_Os03g08710** | *OsTHI*26 | **LOC_Os06g32600** | *OsTHI*15 |
| **LOC_Os01g41180** | *OsTHI*19 | **LOC_Os03g14300** | *OsTHI*29 | **LOC_Os09g11200** | *OsTHI*33 |
| **LOC_Os02g02630** | *OsTHI*20 | **LOC_Os03g21880** | *OsTHI*28 | **LOC_Os09g11210** | *OsTHI*35 |
| **LOC_Os02g02650** | *OsTHI*21 | **LOC_Os03g49270** | *OsTHI*36 | **LOC_Os09g24350** | *OsTHI*32 |
| **LOC_Os02g03480** | *OsTHI*24 | **LOC_Os03g49280** | *OsTHI*37 | **LOC_Os11g15250** | *OsTHI*31 |
| **LOC_Os02g03520** | *OsTHI*25 | **LOC_Os06g32290** | *OsTHI*10 | **LOC_Os12g26960** | *OsTHI*34 |
| **LOC_Os02g03800** | *OsTHI*22 | **LOC_Os06g32320** | *OsTHI*11 | **LOC_Os12g27020** | *OsTHI*41 |
| **LOC_Os03g64300** | *OsTHI*30+*OsTHI*39 | | | | |
| **LOC_Os06g31890** | *OsTHI3+OsTHI5+OsTHI6+OsTHI9+OsTHI7+OsTHI2* | | | | |

Fig A.1 (A) Rice thionin genes expression in different tissues. (B) Differential expression patterns ($Log_2FC \geq 1.5$, $P \geq 0.05$) of rice thionin genes under different biotic stress or hormone treatments. Data were generated by Genevestigator, and shows an overview of all public available micro-array studies on these genes (Hruz *et al.*, 2008 ). LOC_Os06g31890 is highlighted because this probe binds to the here-studied thionin genes.

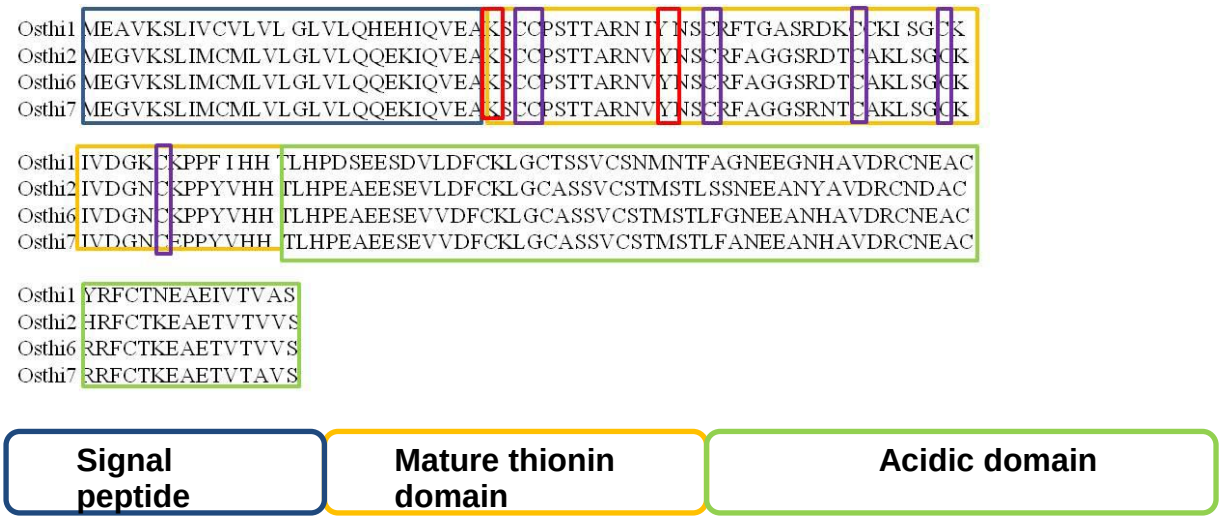



Fig A2 Upper: Multiple alignments of four rice thionin genes. Amino acid sequences deduced from rice annotation (http://rice.plantbiology.msu.edu/cgi-bin/putative_function_search.pl). Sequences were aligned using MUSEL multiple alignment software (http://www.ebi.ac.uk/Tools/msa/muscle/), and manually modified. The conserved six cysteine residues are indicated by a purple box; the toxicity defining residues (Tyr13 and Lys1) are indicated in red. Lower: Typical thionins contain a signal peptide, a mature thionin domain and an acidic domain.

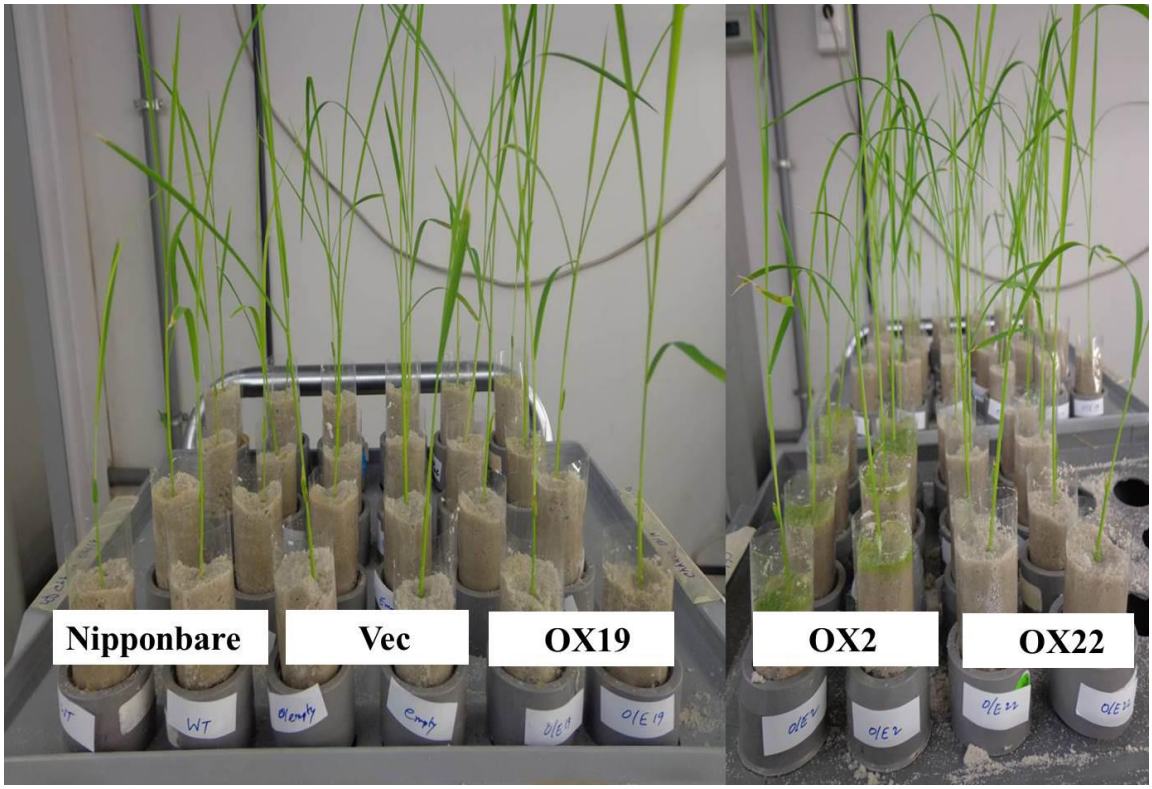



Fig A3 The phenotype of *OsTHI7* overexpression lines (OX19, OX2 and OX22) and control plants (Nipponbare and Vec) 14-day-old before *M. graminicola* inoculation. Vec: transgenic line containing an empty vector

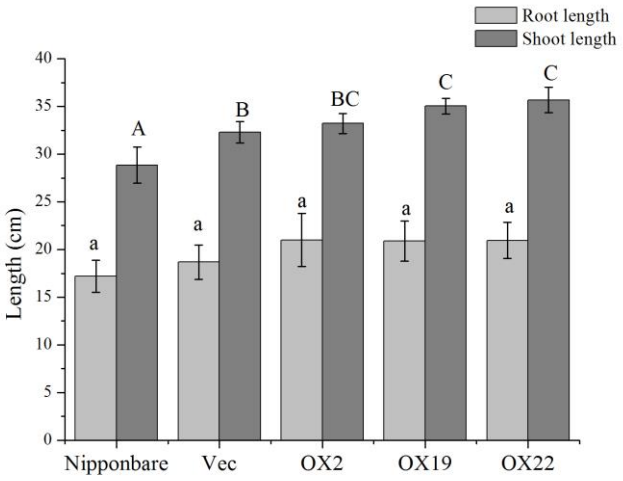



Fig A4 Shoot and root length of control and *OsTHI7* over-expression lines at 14days after infection with *M. graminicola* (28- day-old seedlings). Bars represent means and ±SE of nematodes per plant (n=24). Different letters indicate statistically significant differences (Duncan's multiple range test with $P \leq 0.05$). Small letters indicate the significant differences in root length; big letters indicate the differences in shoot length. Vec: transgenic line containing an empty vector

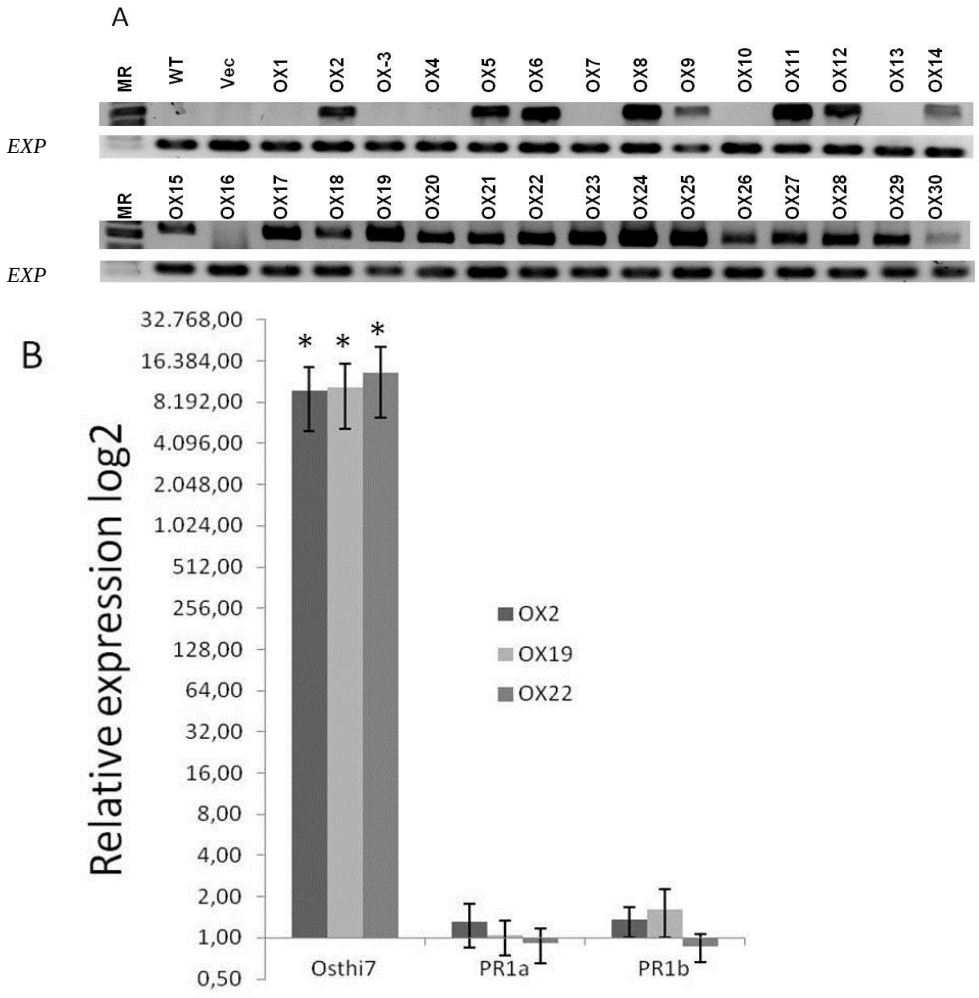
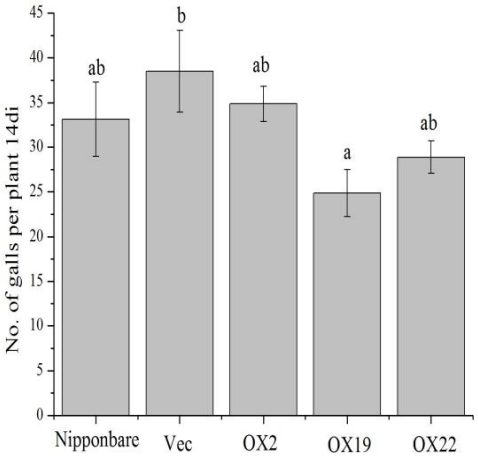
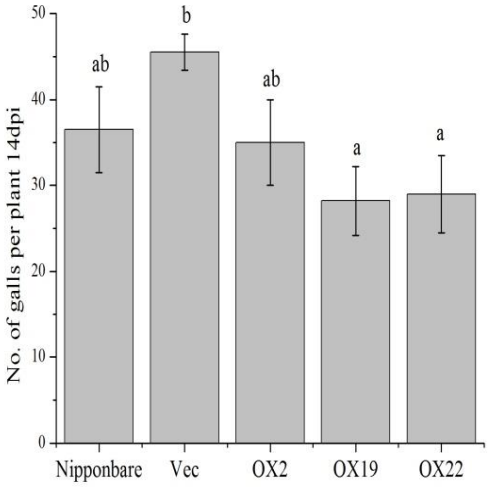


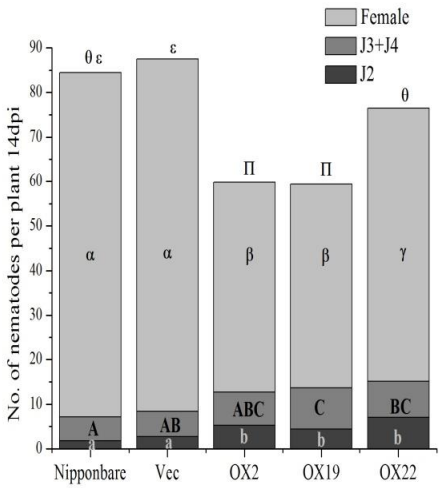

Fig A5 Analysis of transgenic plants overexpressing *OsTHI7*. (A) Evaluation of the construct expression in leaves in the T0 generation of *OsTHI7* overexpression plants by semi-quantatitive reverse transcriptase PCR. **(**B) Evaluation of the *OsTHI7*, *PR1a* and *PR1b* expression level in roots of the T1 generation of *OsTHI7* overexpression plants, by qRT-PCR. Gene expression level was normalized using two internal reference genes, *OsEXP*, *OsUBQ5*. Bars represent mean expression levels ±SE from two independent biological and three technical replicates, each containing a pool of 8 plant roots. Data was obtained using REST2009 software. Significant differential expression ($P \leq 0.05$) is indicated with asterisks in comparison with empty vector roots (set as 1).

(A)


No. of galls per plant 14di
45
40
35
30
25
20
15
10
5
0
ab
b
ab
a
ab
Nipponbare
Vec
OX2
OX19
OX22


No. of galls per plant 14dpi
50
40
30
20
10
0
ab
b
ab
a
a
Nipponbare
Vec
OX2
OX19
OX22

(B)


No. of nematodes per plant 14dpi
Female
J3+J4
J2
90
80
70
60
50
40
30
20
10
0
θ ε
ε
Π
Π
θ
α
α
β
β
γ
A
AB
ABC
C
BC
a
a
b
b
b
Nipponbare
Vec
OX2
OX19
OX22

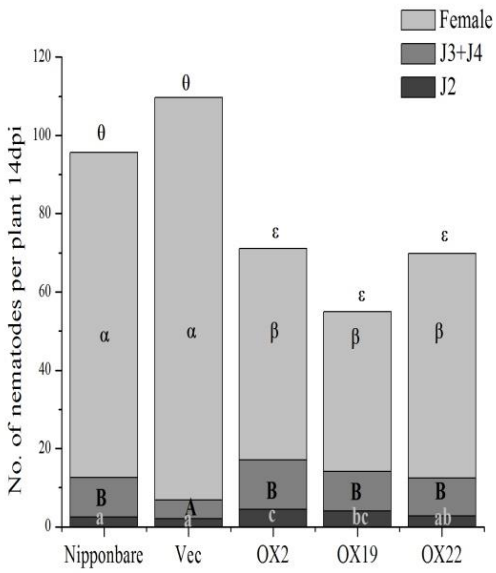
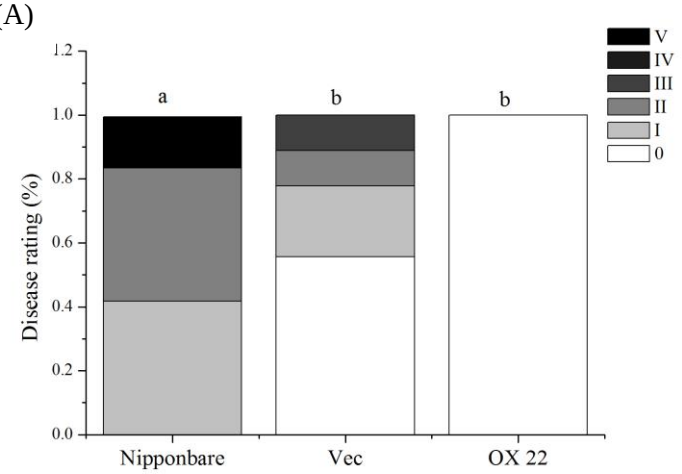



Fig A6 *OsTHI7* overexpressing plants exhibit reduced susceptibility to *M. graminicola*. Data obtained 14 days after inoculation (14dpi). (A) The number of galls in overexpression (OX2, OX19 and OX22) and control (Nipponbare and Empty Vector) plants (B) The different developmental stages of *M. graminicola* within the galls in overexpression (OX2, OX19 and OX22) and control (Nipponbare and Empty Vector) plants. Bars represent means and ±SE of nematodes per plant (n = 8). Different letters indicate statistically significant differences (Duncan's multiple range test with P≤ 0.05). a/b indicates the significant differences in J2 stage; A/B indicates the significant differences in J3+J4 stages. α/β indicates the significant differences in females, and θ/ε indicates the significant differences in the total of the infected nematodes. Data represent the other two independent experiments than the one in Fig 3.4. Vec: transgenic line containing an empty vector.

(A)



(B)

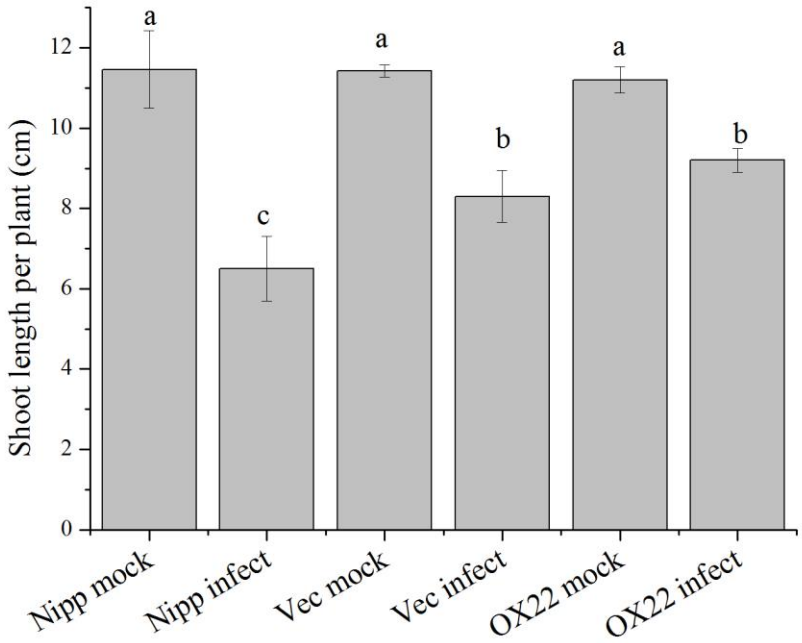
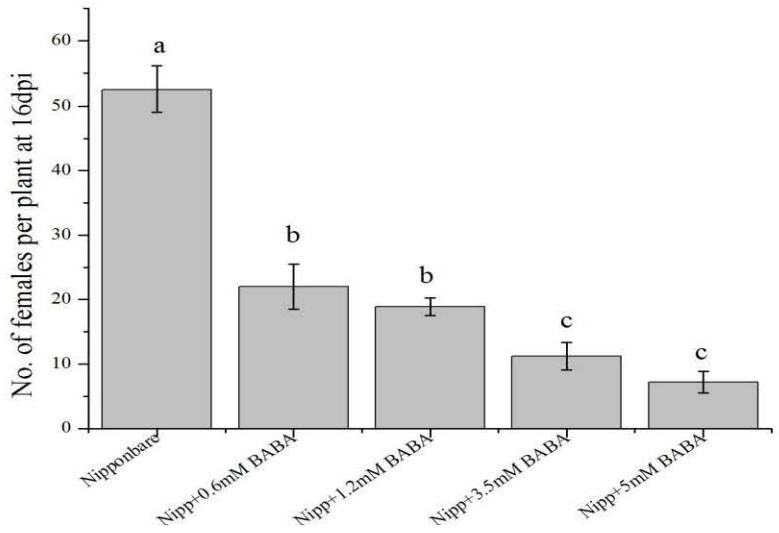
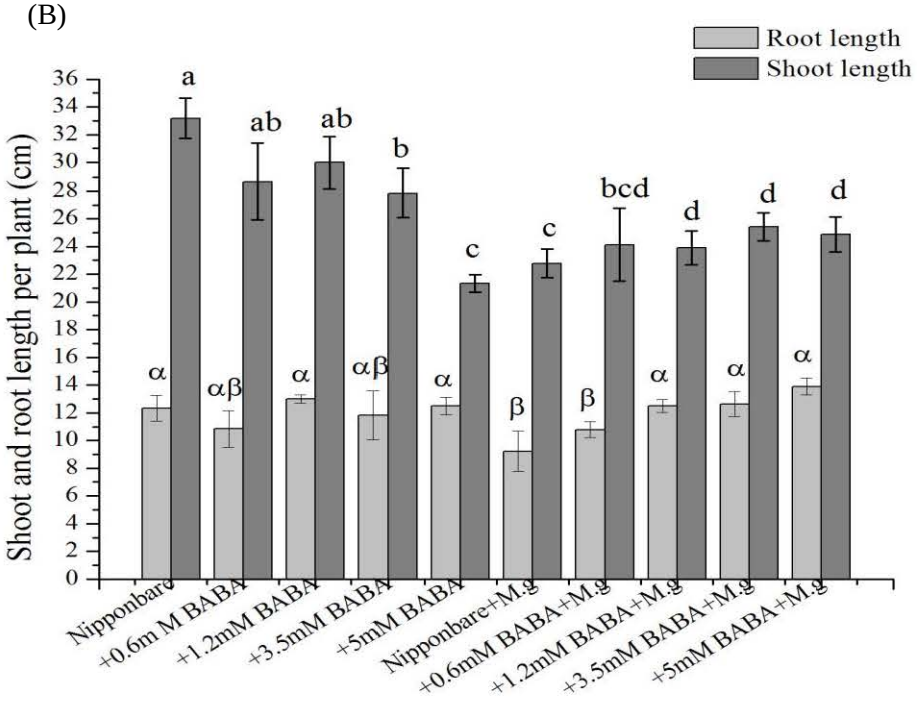



Fig A7 Susceptibility towards *Pythium graminicola* in *OsTHI7*overexpression line (OX22) in comparison with control (Nipponbare and Empty Vector) plants. OX22 enhanced rice shoot tolerance to root pathogen *Pythium graminicola* infection. Data obtained 7 days after infection. (A) The 0-to-V disease rating in control and overexpression plants. (B) The shoot length per plant. Statistical analysis for disease rating was done using Mann Whitney test ($P \leq 0.05 = \alpha$). Statistical analysis for shoot length was done using Duncan's multiple range test $P \leq 0.05$. Bars represent means and ±SE from the other biological replicate than the one in Fig 3.5 ($6 \leq n \leq 12$). Different letters indicate statistically significant differences. Vec: transgenic line containing an empty vector.

(A)


No. of females per plant at 16dpi
0
10
20
30
40
50
60
a
b
b
c
c
Nipponbare
Nipp+0.6mM BABA
Nipp+1.2mM BABA
Nipp+3.5mM BABA
Nipp+5mM BABA

(B)


Root length
Shoot length
Shoot and root length per plant (cm)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
a
ab
ab
b
c
c
bcd
d
d
d
α
αβ
α
αβ
α
β
β
α
α
α
Nipponbare
+0.6m M BABA
+1.2mM BABA
+3.5mM BABA
+5mM BABA
Nipponbare+M.g
+0.6mM BABA+M.g
+1.2mM BABA+M.g
+3.5mM BABA+M.g
+5mM BABA+M.g

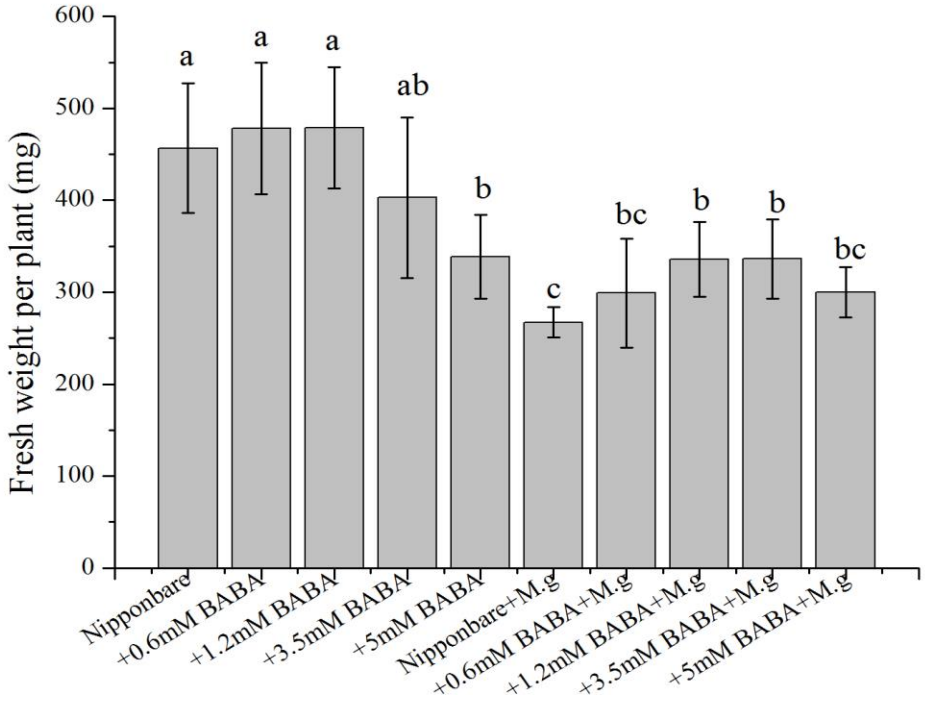
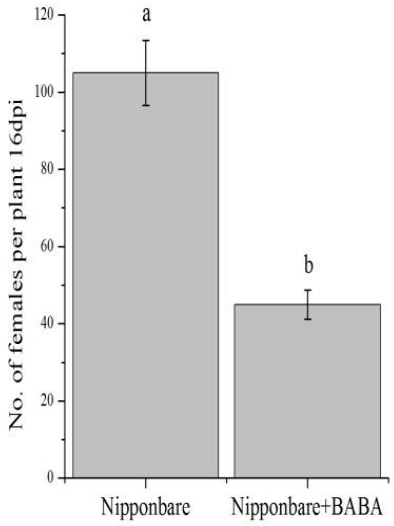
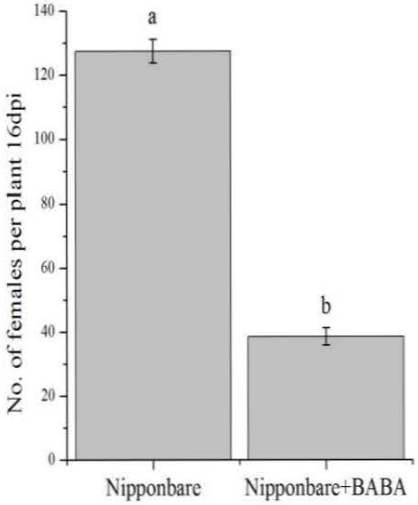


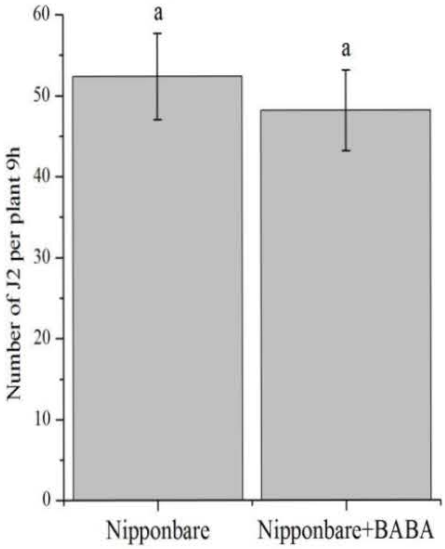

Fig A8 (A) The effect of different concentrations of BABA on the number of females (B) The effect of different concentrations of BABA on the fitness (Above: on shoot and root length; Below: on fresh weight) of rice plants (30-day-old) at 16dpi, with (+Mg) and without nematode infection. Different concentrations of BABA were applied as soil drench one day before nematode inoculation. Bars represent the mean and ±SE per plant (n=8). Different letters indicate statistically significant differences (Duncan Multiple Range Test with $p \leq 0.05$).

(A)




No. of females per plant 16dpi
140
120
100
80
60
40
20
0
a
b
Nipponbare
Nipponbare+BABA

(B)


Number of J2 per plant 9h
60
50
40
30
20
10
0
a
a
Nipponbare
Nipponbare+BABA

(C)

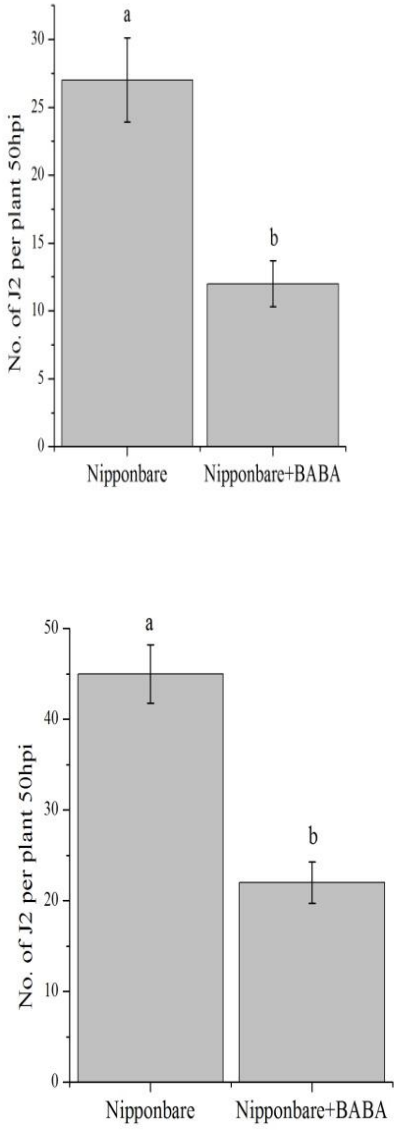
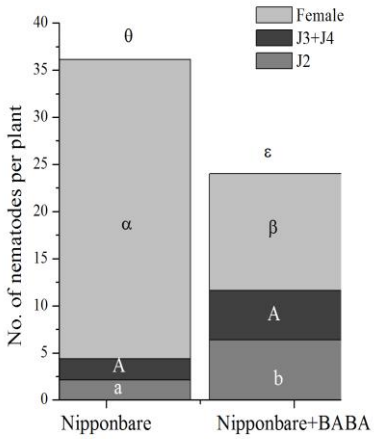



Fig A9 (A) Plants were soil drenched with 3.5mM BABA one day before RKN infection. Sixteen days after infection, females per plant were recorded. Data represent the other two independent experiments than the one in Fig 4.19(A). Bars represent means and ±SE of females per plant (n = 8). (B) Analysis of BABA-induced resistance against RKN on the stage of attraction. 3.5mM BABA was soil drenched one day before nematode inoculation. The number of J2 close to the root tips (about 1mm) were recorded at 9h after initiation of the experiment. Bars represent means and ±SE of nematodes per plant (n = 8). Data represent the other replicate than the one in Fig 4.1 (B). (C) Effects of BABA on invasion of *M. graminicola* to rice plants. 3.5mM BABA was soil drenched one day before nematode inoculation. Number of nematodes was recorded 50 hours after infection. Bars represent means and ±SE of nematodes per plant (n = 8). Data represent the other two independent experiments than the one in Fig 4.1 (C). Different letters indicate statistically significant differences (Duncan Multiple Range Test with $p \leq 0.05$).

(A)




(B)

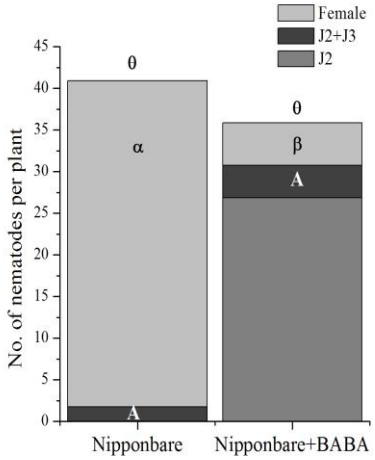



Fig A10 Effects of DL-β-amino-n-butyric acid (BABA) on the development of *M. graminicola* in rice plants. (A) Pre-treatment of BABA: Plants were soil drenched with 3.5mM BABA or water one day before inoculation. Two days after inoculation, SAP was washed away from the roots and plants were transferred to Hoagland solution for another 14 days. (B) Post-treatment of BABA: Two days after *M. graminicola* infection, roots were washed and transferred to 3.5 mM BABA solution or water for two days, subsequently; plants were transferred to pure Hoagland solution for another twelve days. Bars represent means and ±SE of nematodes per plant (n = 8). Different letters indicate statistically significant differences ($P \leq 0.05$). a/b indicates the significant differences in J2 stage; A/B indicates the differences in J3+J4 stages. α/β indicates the significant differences in females, and θ/ε indicates the significant differences in all infected nematodes. Data represent another replicate than the one in Fig 4.2 (A) and (B), respectively.

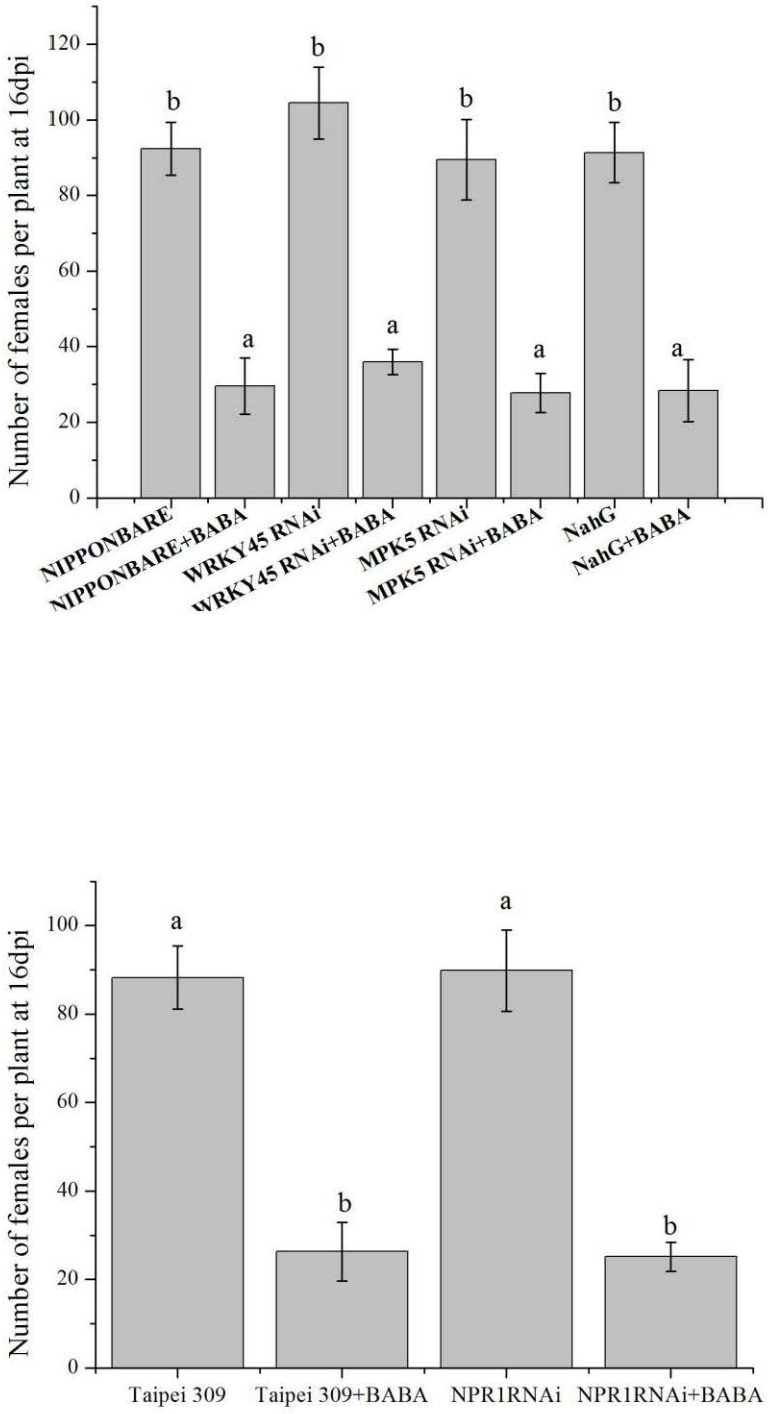



Fig A11 Effects of BABA against RKN in SA and ABA defective lines. 3.5mM BABA was soil drenched one day before nematode inoculation. The number of females per plant was counted at 16 dpi. Bars represent means and ±SE of females per plant (n = 8). Different letters indicate statistically significant differences (Duncan Multiple Range Test with $p \leq 0.05$). Data represent one of another replicate than the one in Fig 4.5.

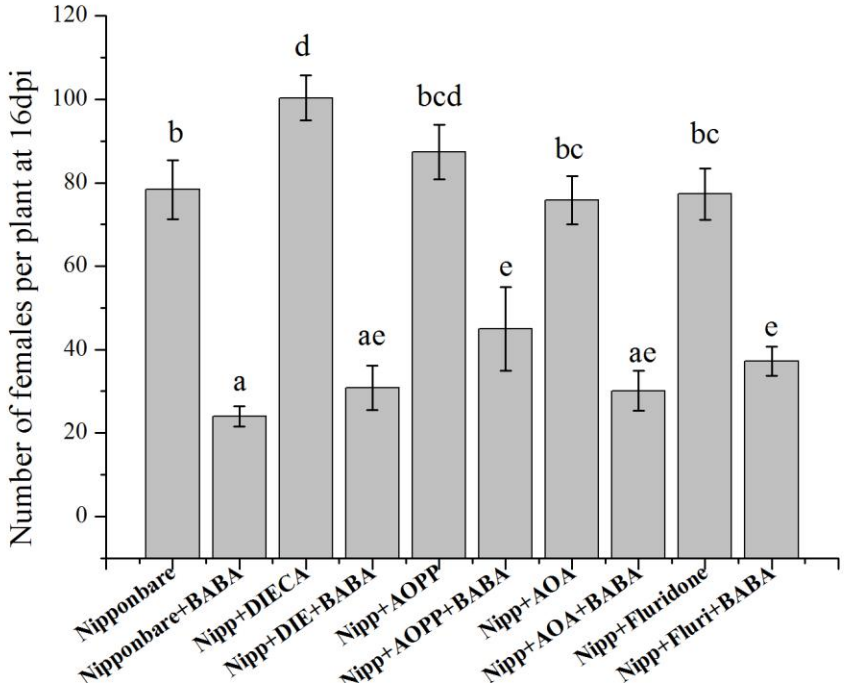



Fig A12 Effects of BABA in plants treated with inhibitors of secondary metabolites. 3.5mM BABA was soil drenched one day before nematode inoculation. The number of females per plant was recorded at 16dpi. Bars represent means and ±SE of females per plant (n= 8). Different letters indicate statistically significant differences (Duncan Multiple Range Test with $p \leq 0.05$). Data represent one of another independent experiment than the one in Fig 4.6.

Table A1 Analysis of putative hormone cis-elements present in the 1.5kb promoter regions of four rice thionin genes by use of the PLANT CARE database.

| Motif / position | *OsTHI1* | *OsTHI2* | *OsTHI6* | *OsTHI7* | **Putative functions** |
|---|---|---|---|---|---|
| ABRE | 329,707 | 811 | none | none | Involved in the abscisic acid responsiveness |
| GARE | 822 | 1153 | none | none | Involved in gibberellin responsiveness |
| TCA | 732 | none | none | none | Involved in salicylic acid responsiveness |
| CGTCA | none | 164 | 160 | 86,160 | Involved in methyl jasmonate responsiveness |

The supplementary data of chapter 5 is available at JXB online:

http://jxb.oxfordjournals.org/content/64/12/3885/suppl/DC1