Susceptibility genes, a novel strategy to improve resistance against the root-knot nematode *Meloidogyne incognita*

Sonja Warmerdam

Propositions

1. Nematode resistance based on stacking less conducive alleles of susceptibility factors will be more durable than the use of major resistance genes.
   (this thesis)

2. DSC1 and WRKY19 operate as TIR-NLR receptor pair in basal innate immunity to root-knot nematodes.
   (this thesis)

3. Too much valuable biological meaning of data is overlooked due to strict application of statistical rules.

4. Genetic studies with twins will provide insight in inherited traits specific to cancer development.

5. The estimation of jump height during a volleyball game based on flight time does not asses the body posture and is therefore not specific enough to measure jump height.

6. Having kids improves your ability to solve problems.

7. Playing a team sport makes you a better researcher.

Propositions belonging to the thesis entitled:
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Thesis committee

Promotor
Prof. Dr Jaap Bakker
Professor of Nematology
Wageningen University & Research

Co-promotor
Dr Geert Smant
Associate professor, Laboratory of Nematology
Wageningen University & Research

Dr Aska Goverse
Associate professor, Laboratory of Nematology
Wageningen University & Research

Other members
Prof. Dr Joop J.A. van Loon, Wageningen University & Research
Prof. Dr Yuling Bai, Wageningen University & Research
Prof. Dr Tina Kyndt, Ghent University, Belgium
Dr Petra M. Bleeker, University of Amsterdam

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Sonja Warmerdam
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Chapter 1

General introduction

Biotic stress is a major constraint in global food security

Agriculture currently faces the enormous challenge of producing sufficient food for a rapidly growing world population. It is expected that by the end of the twenty-first century the world population will consist of around 11 billion people (United Nations, 2015). To be able to feed everyone, food production needs to double by the end of this century (United Nations, 2009). There are several possibilities to increase global food production, most of which involve productivity gains (Jaggard *et al.*, 2010; Foley *et al.*, 2011; Crist *et al.*, 2017). For instance, crop yield can be increased by more efficient usage of both freshwater supplies and proper application of fertilizers (Oerke, 2006; Foley *et al.*, 2011). However, a significant part of the global losses in agricultural productivity is caused by pests and diseases, resulting in a yield gap of 20-40% (Oerke, 2006; Jaggard *et al.*, 2010). To increase the global food production it is beneficial to reduce the crop losses due to pest and disease.

Impact of root-knot nematodes in agriculture

Crop plants are continuously attacked by a wide range of above- and belowground plant microbes and parasites. Plant-parasitic nematodes are thought to be the main cause of biotic stress in food crops, resulting in around $157 billion annual losses each year (Abad *et al.*, 2008; Fuller *et al.*, 2008; Jones *et al.*, 2013; Bebber *et al.*, 2014). Plant parasitic nematodes belong to the phylum Nematoda, which currently includes approximately 40.000 described species divided over 12 major clades (Holterman *et al.*, 2006). Plant parasitism as life strategy arose independently multiple times in at least four of these clades. The most primitive plant parasites are migratory ectoparasites, whereas the most advanced plant parasites are sedentary endoparasites (Smant *et al.*, 2018). Two groups of sedentary endoparasites are cause for major concern in agriculture, these two groups are commonly referred to as cyst nematodes and root-knot nematodes.

Root-knot nematodes are formally classified as the genus *Meloidogyne*, which currently includes of more than 95 species (Elling, 2013; Jones *et al.*, 2013). Three *Meloidogyne* species of particular agronomic importance in the tropics and subtropics are sometimes collectively referred to as the tropical root-knot nematodes (i.e., *M. incognita*, *M. arenaria*, and *M. javanica*). These three *Meloidogyne* species often occur in so-called mixed populations. They are extremely polyphagous and therefore have a very broad host range, including hundreds of different monocotyledons and dicotyledons. *M. incognita* and *M. arenaria* have recently been marked as the most invasive of all currently known plant pathogens and parasites with a presence in more than 190 countries (Bebber *et al.*, 2014).

Biology of *Meloidogyne incognita*

M. incognita is an obligate biotroph that only feeds on living cells located within the vascular cylinder of the root of a host plant (Gheysen & Mitchum, 2011). The life cycle of *M. incognita* starts when freshly hatched second stage juveniles (J2s) in the soil are attracted to the root tip of a host plant. They invade host roots close to the root elongation zone by physical and enzymatic breakdown of cell walls of the root epidermis (Figure 1). The infective J2s then migrate further intercellularly through the root cortex and meristem to enter the vascular cylinder from below. Inside the vascular cylinder, the J2s establish a permanent feeding site (Wyss & Grundler, 1992; Caillaud *et al.*, 2008). To this end, infective J2s carefully perforate the cell wall of a small number of host cells with their stylet and inject pharyngeal gland secretions into the apoplast and cytoplasm of these cells. These secretions are thought to induce major cellular changes in recipient host cells (Gheysen & Mitchum, 2011; Escobar *et al.*, 2015). The most distinctive of these changes is enormous cellular expansion, which is why these host cells are referred to as giant cells. The formation of giant cells involves several rounds of acytokinetic mitosis and endoreduplication leading to multinucleate cytoplasm and polytene chromosomes (Kyndt *et al.*, 2013; Engler *et al.*, 2016). The tissue around the permanent feeding sites of root-knot nematodes typically shows signs of hyperplasia, which gives rise to the hallmark tumor-like malformations in nematode-infected roots.

Infective J2s also use their stylet to selectively withdraw large amounts of plant assimilates from giant cells, which triggers the formation of sink-like features in host cells inside the permanent feeding site. For instance, the development of secondary cell wall ingrowths is thought to sustain the flow of assimilates from the phloem into the giant cells. The uptake assimilates from the giant cells enables J2s to undergo three more moults to reach the adult stage within a couple of weeks after the establishment of the permanent feeding site. Adult females of *M. incognita* reproduce asexually by obligate mitotic parthenogenesis. The females produce offspring as eggs which are held together in a gelatinous matrix that remains attached to the females. Embryonic development of first stage juveniles (J1s) and subsequent moulting into J2s occurs inside the eggs (Kyndt *et al.*, 2013). Whilst remaining inside the eggs, these pre-parasitic J2s can survive relative harsh environmental conditions for a considerable amount of time.

Feeding site formation is a crucial step in the biology of root-knot nematodes but it is also the element in the interaction between root-knot nematodes and plants that is least understood. It most likely involves the manipulation of various plant developmental processes. Several studies point at modifications of plant cell walls, changes in mitotic cell cycle, and local alterations in hormone homeostasis as key phenomena in the ontogeny of giant cells (Gheysen & Mitchum, 2011; Kyndt *et al.*, 2013; Engler *et al.*, 2016). Furthermore, transcriptome analyses of giant cell-enriched root tissue from *Arabidopsis thaliana* infected with root knot nematode*s* show that more than one thousand genes are differentially

regulated in association with nematode infections (Jammes *et al.*, 2005; Fuller *et al.*, 2007; Barcala *et al.*, 2010). However, it should be noted that many of these genes may be regulated in response to nematode feeding, but are not necessarily required for the ontogeny of giant

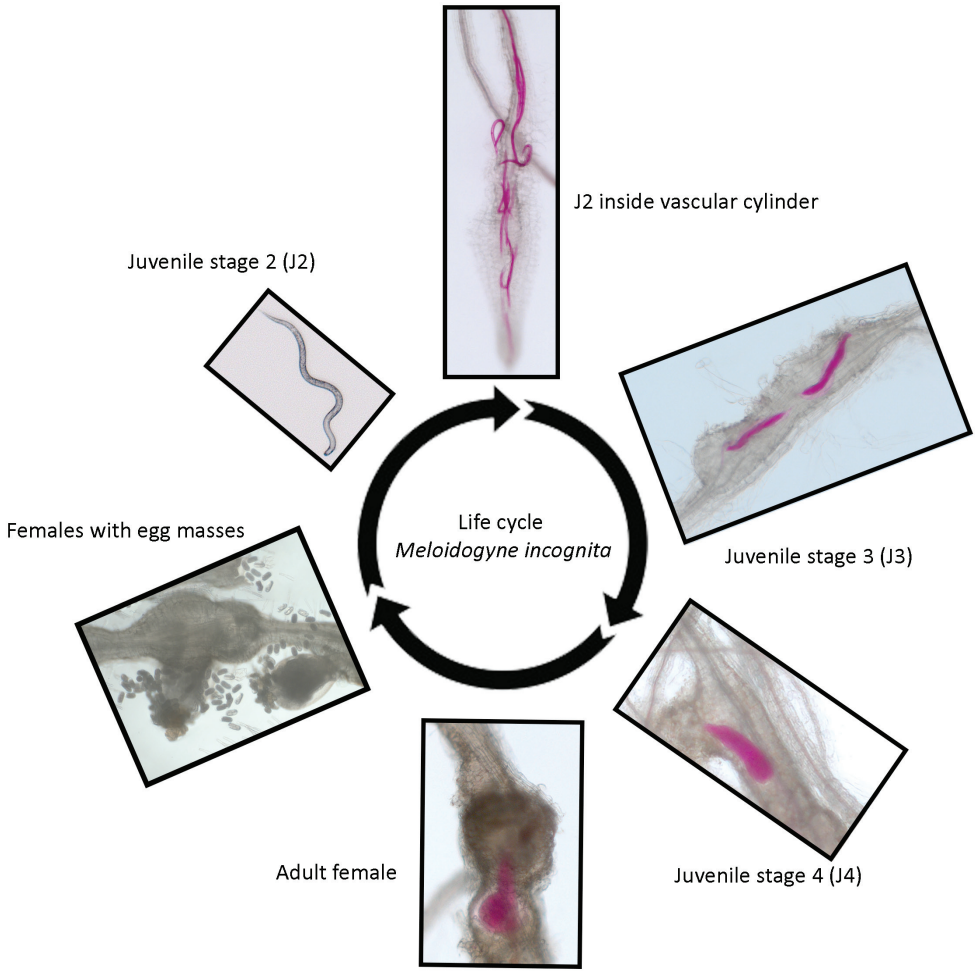

Fig. 1. Life cycle of the root-knot nematode *Meloidogyne incognita*. A pre-parasitic second stage juvenile (J2) present in the soil will migrate towards the host and enters the root. The parasitic J2 migrates in between the cells into the vascular cylinder, where it will initiate a feeding site for the uptake of nutrients. It moults into a third juvenile stage (J3) upon successful feeding site initiation and continuous feeding will lead to another moult into a fourth juvenile stage (J4). A J4 will become an adult female which produces egg masses as progeny. Each egg contains a first stage juvenile (J1), which will moult in the egg into a pre-parasitic J2 to start a new life cycle. Images were obtained from Arabidopsis roots infected with *M. incognita* using a dissection microscope and nematodes were stained with acid fuchsin red for visibility.

Major resistance to *M. incognita*

The three main strategies to manage root-knot nematodes in food crops at the moment are chemical control, crop rotation, and use of nematode resistant cultivars. For decades, root-knot nematodes have been controlled chemically by periodic application of pesticides. Nowadays, most of these pesticides are under strict regulatory bans because of their high toxicity for humans and environment (Fuller *et al.*, 2008). Crop rotation with host and non-host plant species can also manage population densities of the mostly polyphagous root-knot nematodes in experimental situations. For instance, cover crops such as marigolds and bermuda grass can limit disease severity by root-knot nematodes, but they are not able to completely eradicate nematode infestations in the field (Netscher & Taylor, 1979; Hooks *et al.*, 2010; Xie *et al.*, 2016). This is largely because of the ability of nematode eggs to survive for many years in the soil in the absence of host plants. Therefore, the use of resistant cultivars is currently the most important pest management strategy in areas heavily infested with root-knot nematodes (Williamson & Kumar, 2006; Fuller *et al.*, 2008; Wesemael *et al.*, 2011).

Resistance to root-knot nematodes in commercial cultivars is based on the introgression of major resistance (*R*) genes from wild relatives of crop species. Major resistance genes to root-knot nematodes have been identified in tomato (*Mi* genes) (Kaloshian *et al.*, 1995; Kaloshian *et al.*, 1998; Rossi *et al.*, 1998), pepper (*Me* genes) (Djian-Caporalino *et al.*, 2007), prunes (*Ma* genes) (Lu *et al.*, 1999; Claverie *et al.*, 2004), potato (*Mc* genes) (Janssen *et al.*, 1996) and carrot (*Mj* genes)(Ali *et al.*, 2013). For instance, the resistance gene *Mi-1.2* from *Solanum peruvianum* has been successfully used for decades to breed tomato cultivars with high level of resistance to *M. incognita*, *M. javanica* and *M. arenaria* (Rossi *et al.*, 1998). Most *R*-genes belong to the nucleotide-binding site leucine-rich repeat (NB-LRR) superfamily (Boller & Felix, 2009; Zipfel, 2014; Mantelin *et al.*, 2015). The *R*-gene *Mi-1.2* encodes an NB-LRR protein that carries a coiled-coil domain and a Solanaceous-specific domain at its amino terminus. Remarkably, *Mi-1.2* does not only confer resistance to three tropical root-knot nematode species, but also to whiteflies, psyllids and aphids (Kaloshian *et al.*, 1995; Vos *et al.*, 1998; Nombela *et al.*, 2003; Casteel *et al.*, 2006). The exact mechanism underlying the ability of *Mi-1.2* to confer resistance to multiple pathogens is unknown.

Two natural phenomena are currently threatening the use of *Mi-1.2*. The first is the rising soil temperature due to global warming. Nematode resistance based on *Mi-1.2* is temperature sensitive. Soil temperatures above 28°C at the time of host invasion render *Mi-1.2*-resistance ineffective towards root-knot nematodes (Williamson, 1998). Secondly, the wide spread use of *Mi-1.2* over the years has also resulted in selection of virulent field populations across most major tomato producing areas, which further limits its use (Kaloshian *et al.*, 1996; Semblat *et al.*, 2000; Devran & Söğüt, 2010).

Basal defense against *M. incognita*

While nematode resistance based on major *R*-genes has been studied for a long time, basal defence to root-knot nematodes in plants is largely an unchartered area of research. Root-knot nematodes use their stylet to puncture through plant cell walls potentially liberating small cell wall fragments that can be recognized as elicitors of basal defense responses (Gheysen & Mitchum, 2011; Goverse & Smant, 2014; Mantelin *et al.*, 2015). It is thought that these damage-associated molecular patterns (DAMPs) as well nematode-associated molecular patterns (NAMPs) might activate basal plant defenses via surface-localized immune receptors. Recognition of DAMPs and NAMPs could then lead to DAMP- or NAMP-triggered immunity for nematodes, which generally involves a broad repertoire of chemical responses in plant cells. These chemical responses may include the production of reactive oxygen species, various hydrolytic enzymes, and protease inhibitors (Jones & Dangl, 2006; Zipfel, 2014). However, not so much is known about DAMPs uniquely associated with nematode infections in plants, and the importance of damage-triggered immunity to root-knot nematodes is not well understood. By contrast, nematodes commonly release a group of small glycolipid molecules named ascarosides that are able to function as NAMPs and induce basal defense responses in plant (Manosalva *et al.*, 2015). This suggests that basal defense responses triggered by NAMPs may contribute to the overall susceptibility of plants to infections by root-knot nematodes. Various recent studies focusing on the early changes in the transcriptome in nematode-infected plant lend support to this hypothesis (Ji *et al.*, 2015; Zhou *et al.*, 2015; Teixeira *et al.*, 2016). To what extent basal defense responses restrict the virulence of *M. incognita* is hard to judge. Particularly, because this polyphagous root-knot nematode species seems to have adapted its effector repertoire to suppress basal defense responses during the onset of parasitism (Jaouannet *et al.*, 2012).

Nematode resistance by loss-of-susceptibility

An alternative approach to make plants more resistant to nematode infections independent of major *R*-genes intents to make use of allelic variation in so-called plant susceptibility genes (*S*-genes) (Pavan *et al.*, 2010; Schie & Takken, 2014). As a theoretical concept, *S*-genes are defined as being required for compatible plant-pathogen interactions. By extension, loss-of-function alleles of *S*-genes may limit the ability of a pathogen to cause disease (Panstruga, 2003; de Almeida Engler *et al.*, 2005). *S*-genes can be categorized into two types. Products of type 1 *S*-genes facilitate growth and development of the pathogen. By contrast, type 2 *S*-genes code for negatively regulators of plant defense. Besides the two types specified, *S*-genes can be involved in different processes during plant-pathogen interaction. For plant nematode interactions it is likely that *S*-genes of type 1 are involved in the attraction of nematodes to the roots. During the invasion of the nematode is it more likely that *S*-genes involved in defense, type 2, are more important. While during establishment of the feeding

site a *S*-genes can either be type 1 or type 2. During feedings site expansion and maintenance it is more likely that *S*-genes are of type 1.

When an *S*-gene belongs to type 1, loss of function could alternate growth and development of the plant resulting in adverse pleotropic effects (de Almeida Engler *et al.*, 2005; Schie & Takken, 2014). In this case, the *S*-gene is unsuitable to enhance resistance, as the gain by making the plant resistant is lost due to growth problems. When the S-gene belongs to the type 2 group, there is less risk of developing adverse pleiotropic effects during loss of function, however, defense against other pathogens could be altered (de Almeida Engler *et al.*, 2005; Schie & Takken, 2014). To overcome the possible negative side effects of loss of function of *S*-genes it is possible to use allelic variants that are present in other genetic backgrounds. Allelic variants can have different expression levels which could still induce resistance but reduces the adverse pleiotropic effects.

Strategies to identify *S*-genes for *M. incognita* in plants

Various strategies have been employed to identify plant genes specifically involved in susceptibility to infections by nematodes. Most of these strategies use expression of a gene in association with nematode infections as a key criterion to prioritize it for further research. For instance, genes specifically regulated in permanent feeding sites of sedentary nematodes have been identified in a promoter-tagging strategy. This method makes use of a library of transgenic Arabidopsis lines with random insertions of a promoter-less beta-glucuronidase (GUS) construct (Goddijn *et al.*, 1993). Arabidopsis lines harboring inserts in regulatory sequences up- or down- stream of nematode *S*-genes are expected to show specific GUS expression upon nematodes infections. However, with the development of techniques for transcription studies, like cDNA AFLP, cDNA libraries, gene expression microarray and RNAseq largely replaced the promoter-tagging approach. These transcription studies identified a whole list of S-genes for different plant-pathogen interactions (Schie & Takken, 2014). Nowadays, mainly gene expression microarrays and RNAseq are used to investigate plant-nematode interactions. A key development changing the application of whole transcriptome analyses has been the minimal amount of nematode-infected plant tissue that is needed for these experiments (e.g., whole nematode-infected roots, excised galls, and individual giant cells). An evident weakness of these methods is their inherent focus on differential gene expression which makes them unsuitable to identify *S*-genes that are not significantly regulated in association with nematode infections in plants.

An alternative to identify *S*-genes is the use of forward genetics like genome wide association (GWA) mapping. GWA mapping is based on studying associations between a large number of SNPs across a genome and complex traits within a sample of genetically diverse individuals from a natural population (Zhu *et al.*, 2008). The use of natural accessions can identify *S*-genes based on available allelic variation (Alonso-Blanco & Koornneef, 2000; Koornneef *et al.*, 2004).

Scope of this thesis

The overall objective of this thesis was to identify novel *S*-genes for *M. incognita* in *Arabidopsis thaliana* using a genome-wide association mapping strategy. We selected Arabidopsis as a model host of *M. incognita* for this study for several reasons. First, Arabidopsis responds to *M. incognita* in a similar manner as cultivated plant species (Engler *et al.*, 2016). Second, we could not find any prior information in the literature pointing at the presence of segregating major *R*-genes to *M. incognita* in natural Arabidopsis inbred lines (Niebel *et al.*, 1994). Segregating major *R*-genes can tremendously complicate studies focusing on the genetic architecture of disease susceptibility in plants. And, third, for genetic studies in plants Arabidopsis is still the organism of choice. Over the years the Arabidopsis research community has developed an unparalleled set of tools to study plant biology in depth.

Furthermore, we used GWA mapping to resolve the architecture of susceptibility of Arabidopsis to *M. incognita*, because this method does not result in a strong bias towards genes differentially regulated in association with nematode infections. As GWA mapping relies on linkage between reproductive rate of *M. incognita* and natural alleles of Arabidopsis, the translation of our findings can also be relatively efficient by searching for similar polymorphisms in homologous genes in crop species.

In **Chapter 2,** we first challenged a natural collection of 340 Arabidopsis inbred lines with *M. incognita* to quantify the number of egg masses per plant at six weeks after inoculation. We used GWA mapping to map quantitative variation of this metric on the genome of Arabidopsis. This revealed four novel QTLs associated with reproductive rate of *M. incognita* in Arabidopsis, two of which we studied in more detail. To avoid pursuing false positive loci we applied stringent selection criteria. However, by doing so we could only resolve 50 percent of the heritable variation in this trait.

To resolve all of the heritable variation in the reproductive rate of *M. incognita* in Arabidopsis, we lowered the threshold for significant associations in the GWA mapping in **Chapter 3.** This led to the identification of 15 additional QTLs associated with the reproductive rate of *M. incognita* in Arabidopsis. To challenge the higher risk of false discovery, we further investigated the QTLs with both the smallest effect size and lowest statistical support (QTL12). QTL12 is located on chromosome 4 and harbors *Ethylene Responsive Factor 6 (ERF6)*. We demonstrate that *ERF6* functions as a co-regulator of susceptibility to *M. incognita* in Arabidopsis.

In **Chapter 4** we investigate QTL13 on chromosome 4 where we identify two genes that are members of the NB-LRR gene family. As there is no evidence for major *R*-gene resistance against *M. incognita* in Arabidopsis we here further characterize QTL13. This led to the identification of the genes *DOMINANT SUPPRESSOR OF CAMTA 3 RESISTANCE 1 (DSC1)* and *TIR-NB-LRR-WRKY19 (WRKY19)* that do not confer major resistance but play a role in susceptibility to *M. incognita*.

In **Chapter 5,** we investigate QTL2 on chromosome 5 harbouring three genes all of which contribute to the quantitative variation in susceptibility of Arabidopsis to *M. incognita*. The three genes encode a RING-variant domain-containing protein RU-BOX1 and two novel F-box proteins, FFBD1 and FRNI1. Further investigation showed that the mechanisms underlying the effects of RU-BOX1 and FRNI1 on susceptibility of Arabidopsis to *M. incognita* may involve gibberellic acid and auxin-mediated signalling and responses.

Overall, we showed that with GWA mapping it is possible to resolve the genetic architecture underlying the reproductive rate of *M. incognita* in Arabidopsis. In **Chapter 6**, I discuss the in this thesis used strategy to identify *S*-genes which can be used to create durable resistance against nematodes.

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Chapter 2

Genome wide association mapping of the architecture of susceptibility to the root-knot nematode *Meloidogyne incognita* in *Arabidopsis thaliana*

Sonja Warmerdam
Mark G. Sterken
Casper C. van Schaik
Marian E.P. Oortwijn
Octavina C. A. Sukarta
Jose L. Lozano-Torres
Marcel Dicke
Johannes Helder
Jan E. Kammenga
Aska Goverse
Jaap Bakker
Geert Smant

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Abstract

Susceptibility to the root-knot nematode *Meloidogyne incognita* in plants is thought to be a complex trait based on multiple genes involved in cell differentiation, growth, and defence. Previous genetic analyses of susceptibility to *M. incognita* have mainly focussed on segregating dominant resistance genes in crops. It is not known if plants harbour significant genetic variation in susceptibility to *M. incognita* independent of dominant resistance. To study the genetic architecture of susceptibility to *M. incognita,* we analysed nematode reproduction on a highly diverse set of 340 natural inbred lines of *Arabidopsis thaliana* with genome-wide association mapping. We observed a surprisingly large variation in nematode reproduction among these lines. Genome-wide association mapping revealed four quantitative trait loci (QTLs) located on chromosomes 1 and 5 of *A. thaliana* significantly associated with reproductive success of M. incognita, none of which harbours typical resistance gene homologs. Mutant analysis of three genes located in two QTLs showed that the transcription factor BRASSINAZOLE RESISTANT1 and a F-box family protein may function as (co-)regulators of susceptibility to *M. incognita* in Arabidopsis. Our data suggests that breeding for loss-of-susceptibility, based on allelic variants critically involved in nematode feeding, could be used to make crops more resilient to root-knot nematodes.

Introduction

Polyphagous root-knot nematodes significantly undermine agricultural productivity in major food crops all over the world (Jones *et al.*, 2013). In a recent study on biotic risk factors of global food security, the tropical root-knot nematode *Meloidogyne incognita* was ranked as the most invasive plant disease-causing agent (Bebber *et al.*, 2014). For decades, root-knot nematode infestations have been controlled by applications of chemical pesticides. However, most pesticides against root-knot nematodes currently face regulatory bans for their high human and environmental toxicity. The phasing-out of chemical pesticides to root-knot nematodes has significantly increased the global demand for nematode-resistant crops. However, for only a few crops, such as tomato, prune, carrot, and pepper, highly specific dominant resistance genes against root-knot nematodes are available (Williamson & Kumar, 2006; Davies & Elling, 2015).

Two natural phenomena currently threaten the use of dominant resistances to root-knot nematodes, the first of which is genetic selection for resistance-breaking nematode populations. For instance, most of the commercial cultivars of tomato (*Solanum lycopersicum*) carry introgressions of the dominant *Mi-1.2* gene from the wild tomato species *S. peruvianum*, which in many areas is no longer able to confer high levels of resistance to several tropical root-knot nematodes species (e.g., *M. incognita*, *M. javanica*, and *M. arenaria;* (Castagnone-Sereno *et al.*, 2013)). Findings of virulent field populations of *M. incognita* in tomato with the *Mi-1.2* gene are not a particularly recent development (Kaloshian *et al.*, 1996; Semblat *et al.*, 2000). However, their widespread dispersal across major tomato-producing regions has lately turned them into a major concern for growers. Secondly, many known dominant resistances to tropical root-knot nematodes are temperature sensitive and rising soil temperatures by global warming may render them ineffective (Jacquet *et al.*, 2005).

Root-knot nematodes are obligate biotrophs that feed for weeks on living cells within the vascular cylinder of the root of a host plant. Soil-born second stage juveniles (J2s) of *M. incognita* invade the roots at the transition zone close to the root tip. The J2s then migrate intercellularly through the root cortex towards the root meristem, where they enter the vascular cylinder from below. Inside the vascular cylinder the J2s establish a permanent feeding structure consisting of several giant nurse cells (Caillaud *et al.*, 2008). For the initiation of these giant cells, the J2s redirect the differentiation and growth of vascular cells into large transfer cell-like units. The exact molecular mechanisms underlying the cellular transformation of vascular parenchyma into giant cells are not well understood. However, it is clear that giant cell formation involves alterations in a wide range of fundamental molecular and cellular processes, including epigenetic control of gene expression, cell cycle regulation, plant cell wall modifications, and cytoskeletal rearrangements (Kyndt *et al.*, 2013). Prolonged feeding on giant cells enables the J2s to moult three times into the adult female stage. After a couple of weeks, adult female root-knot nematodes produce offspring as an aggregate of eggs held together by a gelatinous matrix (Kyndt *et al.*, 2013).

Giant cells are a polygenic trait of nematode-infected plants, involving hundreds of different plant genes. Studies on giant cell-enriched root tissue from *Arabidopsis thaliana* infected with *M. incognita* revealed more than three thousand differentially regulated genes in a comparison with uninfected root tissue (Jammes *et al.*, 2005). Similarly, around one thousand genes appeared to be differentially regulated in giant cell-enriched tissue of *M. incognita* at 21 days post inoculation in Arabidopsis compared to uninfected tissue (Fuller *et al.*, 2007). A similar number of differentially expressed genes were identified in a comparison of microdissected giant cells and neighbouring vascular cells in Arabidopsis at three days post inoculation with the tropical root knot nematode *M. javanica* (Barcala *et al.*, 2010). Although not all genes regulated in association with giant cell formation will be causally linked to this process, allelic variation in specific subsets of these genes may quantitatively affect the susceptibility of a host plant to root-knot nematodes.

Quantitative traits can be mapped onto specific genome loci by exploiting linkage disequilibrium (LD) between allelic variants (i.e., single nucleotide polymorphisms [SNPs]) and a particular trait in a set of individuals. Genome-wide association (GWA) mapping expands on this principle by studying associations between a large number of SNPs across a genome and complex traits within a sample of genetically diverse individuals from a natural population (Zhu *et al.*, 2008). At present, the richest resources for GWA mapping between SNPs and complex traits in plants centre on large collections of natural inbred lines of Arabidopsis (Atwell *et al.*, 2010; Cao *et al.*, 2011; Weigel, 2012). Arabidopsis serves as a model organism to study plant responses to all kinds of abiotic and biotic stresses, including infections by root-knot nematodes (Sijmons *et al.*, 1991). Genome-wide associations between allelic variants and responses to a variety of biotic and abiotic stresses have recently been mapped onto the genome of Arabidopsis (Kloth *et al.*, 2012; Bac-Molenaar *et al.*, 2015; Kloth *et al.*, 2016; Davila Olivas *et al.*, 2017). Moreover, multi-trait genome-wide association mapping has been used to reveal cross-correlations between SNPs and resistances to different biotic and abiotic stresses in Arabidopsis, including for instance parallels in responses to osmotic stress and root-knot nematodes (Thoen *et al.*, 2017).

In theory, plants could be made more resistant to nematode infections by selecting for less conducive allelic variants of genes that critically determine susceptibility (i.e., *S*-genes; (de Almeida Engler *et al.*, 2005; van Schie & Takken, 2014)). Given the current problems with dominant resistance genes in food crops, we asked ourselves whether plants harbour significant natural variation in susceptibility to root-knot nematodes, which is not related to dominant resistance. Here, we present the results of a GWA study of quantitative variation in susceptibility to the root-knot nematode *M. incognita* in Arabidopsis. Our primary interest was to analyse allelic variation in genes that do not resemble major resistance gene homologs. To this purpose, we chose to work with Arabidopsis, because earlier research suggested that dominant resistance to *M. incognita* may be absent in this species (Niebel *et al.*, 1994). In fact, it is not so likely that the resistance gene repertoire of Arabidopsis,

with its native range in more temperate regions of Europe and Asia (Beck *et al.*, 2008), has undergone extensive adaptations to tropical root-knot nematodes (e.g., *M. incognita*). Natural Arabidopsis inbred lines are also particularly well suited for GWA mapping of disease susceptibility, because they allow for repeatedly phenotyping of genetically identical individuals in notoriously variable *in vitro* bioassays with nematodes. In total, we found eight SNPs in our GWA study to be significantly associated with the reproductive rate of *M. incognita* in 340 Arabidopsis lines. By using the predicted LD decay for the Arabidopsis genome, we aggregated the SNPs into four genomic regions, two of which we examined in more detail in this paper. Our data on the candidate genes in these loci demonstrate that the transcription factor BRASSINAZOLE RESISTANT-1 and a F-box family protein in Arabidopsis most likely (co-)regulate susceptibility to *M. incognita*.

Materials and Methods

Plant material

For genome-wide association mapping, we used a population consisting of 340 natural inbred lines selected from a global HapMap collection of *Arabidopsis thaliana (L.)* Heynh (http://bergelson.uchicago.edu/wp-content/uploads/2015/04/Justins-360-lines.xls). The homozygous T-DNA insertion mutant lines Salk_052305C (hereafter referred to as *gsp1-1*) and Salk_050274C (hereafter referred to as *frni1-1*), the ethyl methanesulfonate (EMS)-induced mutant line *bzr1-1D*, and the *BZR1:CFP* gene fusion reporter line were obtained from the Nottingham *Arabidopsis* Stock Centre (Alonso *et al.*, 2003). The *bzr1-1D* and *BZR1:CFP* lines were originally described in Wang et al. (2002). The *bzr1-1D*, *BZR1:CFP*, *gsp1-1* and *frni1-1* lines were all generated in the background of *A. thaliana* Col-0.

The homozygosity of T-DNA inserts was checked with PCR on genomic DNA isolated from leaf material (Holterman *et al.*, 2006) of twelve seedlings using primer combinations as indicated in supplemental table S1. The following conditions were used for PCR: 10 min at 94°C, 35 cycles of 30 s at 94°C, 1.5 min at 60°C, and 1 min at 72°C, and a final incubation of 10 min at 72°C. The PCR amplification products were analysed by agarose gel electrophoresis.

Nematode infection assays

Eggs of *Meloidogyne incognita* were obtained by treating tomato roots infected with *M. incognita* (strain 'Morelos' from INRA, Sophia Antipolis, France) with 0.05% v/v NaOCl for three minutes. Roots were rinsed with tap water and the eggs were collected on a 25µm sieve. Next, the eggs were incubated in a solution of 2.4 mM NaN_3 for 20 minutes while shaking. Thereafter, the eggs were rinsed with tap water and incubated on a 25µm sieve in a solution of 1.5 mg/ml gentamycin and 0.05 mg/ml nystatin in the dark at room temperature. Hatched juveniles were collected after four days and surface sterilized (0.16 mM $HgCl_2$, 0.49 mM NaN_3, 0.002% v/v Triton X-100) for 10 min. After surface sterilization, the juveniles were rinsed three times with sterile tap water and transferred to 0.7% Gelrite solution (Duchefa biochemie).

To generate cultures of Arabidopsis seedlings in vitro, seeds were vapour-sterilized (in 0.7 M NaOCl and 1% HCl in tap water) for 5 h and transferred to a 6-well cell culture plate containing Murashige and Skoog (MS) medium with vitamins 4.7 g/L (Duchefa Biochemie), 58.5 mM sucrose and 5 g/L Gelrite (Duchefa biochemie). The 6-well plates with seeds were incubated in the dark at 4°C for 3 days. Next, the seeds were allowed to germinate at 21°C under 16h light / 8h dark conditions. To determine the susceptibility of the 340 natural Arabidopsis inbred lines and the *bzr1-1D*, *gsp1-1*, and *frni1-1* mutant lines, one-week-old seedlings were manually transferred to wells in a new 6-well plate containing MS medium and incubated for 7 more days at 21°C under a 16h light and 8h dark regime. Each well

contained only one seedling. Next, the seedlings were inoculated with 180 infective J2s of *M. incognita* per plant and incubated at 24°C in the dark.

To be able to count the number of infective juveniles in the *bzr1-1D*, *gsp1-1* and *frni1-1* mutant lines at 7 days post inoculation whole roots were stained with acid fuchsin. To this end, clean roots were first incubated in 16.8 mM NaOCl for 5 minutes, and thereafter in tap water for 10 minutes. Next, the roots were transferred into an acid fuchsin solution (0.2 M acid fuchsin and 0.8 % glacial acetic acid in tap water) and heated in a microwave oven for 30s. After cooling down, roots were transferred to 40% glycerol and the number of juveniles were counted by visually inspecting the roots with a dissection microscope.

The number of egg masses per plant were counted six weeks after inoculation by visually inspecting the roots with a dissection microscope. The natural inbred lines were screened in batches of twenty accessions, including Col-0 as reference in each batch. Each inbred line was tested in at least four technical replicates. The average number of egg masses per plant, the standard error of mean, the number of technical replicates (n) of each inbred line are summarized in supplemental table S2. The data was analysed for narrow sense heritability and genome wide associations as described below.

To determine the susceptibility of the *bzr1-1D*, *gsp1-1*, and *frni1-1* mutant lines, the number of juveniles and egg masses per plant was statistically analysed using two-way ANOVA and post-hoc Tukey HSD test in R (version 3.0.2, www.r-project.org). Each line was tested in at least three independent experiments and 18 replicates per experiment. Both genotype and experiment number were used as factors to test for significance in the ANOVA.

To collect nematode-infected roots for gene expression analysis by quantitative reverse transcription PCR (qRT-PCR), freshly germinated seven days old seedlings were transferred to 12 cm square plates containing MS medium and placed vertically for seven more days at 21°C under a 16h light and 8h dark regime. Each plate contained four seedlings. Next, the seedlings were inoculated with 180 infective J2s of *M. incognita* per plant and incubated horizontally at 24°C in the dark. In parallel, seedlings in plates without juveniles were also incubated horizontally at 24°C in the dark to serve as uninfected controls. Furthermore, whole root systems of a subset of the seedlings were collected just prior to the inoculation with juveniles. Similarly, at seven days post inoculation whole root systems were collected of inoculated and non-inoculated seedlings. Root systems of 12 seedlings were aggregated to make one sample, which was snap frozen in liquid nitrogen and then stored at -80 °C until further use. Three biological replicates were performed for each experiment.

Quantitative reverse transcription PCR

Expression analysis for gene of interest was performed on the stored root samples produced during the nematode infection study. Whole root systems were cut from aerial parts of

the seedlings and snap frozen in liquid nitrogen. Total RNA was isolated from whole roots of twelve 14-days-old plants of *gsp1-1, bzr1-1D, frni1-1* and Col-0 wildtype. The frozen root systems were homogenized using TissueLyser (Qiagen) two times for 30 seconds. Total RNA was extracted from 100 mg of the homogenate with the Maxwell Plant RNA kit (Promega Corporation) using the Maxwell 16 Robot (Promega Corporation) according to the manufacturer's protocol. The amount of total RNA per sample was determined by spectrophotometer ND-1000 (Isogen Life Science). First strand cDNA was synthesized from total RNA using Superscript III First-Strand synthesis system (Invitrogen) according to manufacturer's protocol. Samples were analysed by quantitative PCR using Absolute SYBR Green Fluorescein mix (Thermo Fisher Scientific). cDNA matching *Arabidopsis thaliana* elongation factor 1 alpha was amplified as a reference for constitutive expression using primers as indicated in table S1 (Czechowski *et al.*, 2004). To quantify the expression level for the gene of interest specific gene primers were used (Table S1). For the qRT-PCR 5 ng cDNA was used with the following conditions: 15 min at 95°C, forty cycles of 30 s at 95°C, 30 s at 62 °C, and 30 s at 72°C, and a final incubation of 5 min at 72°C. Relative expression ratio between the gene of interest and the reference gene was calculated as described elsewhere (Pfaffl, 2001). Relative expression ratio was statistically analysed for significance with a two-way ANOVA and post-hoc Tukey HSD test in R (P-value<0.05).

Root phenotypes

Arabidopsis seedlings were allowed to germinate and grow for 14 days on MS medium as described above. To determine the number of root tips and root length of the seedlings, the complete plants were transferred from the media onto a plastic tray with water. Next, the leaves of the seedlings were removed and the roots were spread out over the surface of the tray. A scan of the roots was made with a photo scanner (EPSON perfection V800). The scan was analysed to measure root length using WinRHIZO package for Arabidopsis (WinRHIZO pro2015, Regent Instruments Inc.). Number of root tips was counted by visually inspecting the scan. Differences in the number of root tips and the root length per plant were statistically analysed for significance with a two-way ANOVA and post-hoc Tukey HSD test in *R* (P-value<0.05).

Confocal microscopy of BZR1-CFP

Seeds of the Arabidopsis *BZR1:CFP* reporter line and Col-0 were vapour sterilized and incubated for three days at 4°C in the dark as described above. Next, twenty seeds were transferred to 12 cm square plates with MS media and placed vertically in a growth chamber at 21°C with 16h light and 8 h dark regime. After five days, the seedlings were inoculated with 25 surface-sterilized juveniles of *M. incognita* per plant and placed vertically at 24°C in

the dark. Three days post inoculation seedlings were transferred to a microscope slide and analysed with the Zeiss LSM 710 confocal microscope. Seedlings were incubated in 0.5 µg ml^{-1} propidium iodide in phosphate-buffered saline to stain the plant cell walls. The emission spectra were set to 463-538 nm and 586-719 nm for CFP and propidium iodide, respectively. Non-adjusted images were analysed with ImageJ, wherein pixel intensity of the root area was compared to the background. Data of two independent experiments, including the analysis of 10 seedlings per experiment, was analysed with a two-way ANOVA and post-hoc Tukey HSD test in R. Images were enhanced in brightness for publication in print.

Narrow sense heritability

To estimate the amount of variation that can be explained by genome-wide association mapping, we calculated the narrow-sense heritability. To this end, we employed a mixed model approach using Efficient Mixed-Model Association (EMMA) based on restricted maximum likelihood (REML) to estimate the variance components as described (Kang *et al.*, 2008; Rockman *et al.*, 2010). The kinship matrix was calculated using all 214,051 SNPs (Horton *et al.*, 2012) and narrow sense heritability was calculated as

$$h^2 = \frac{V_G}{V_G + V_E}$$

where V_G is the genetic variance and V_E is the residual variance, as estimated by REML (excluding SNPs with a frequency < 0.05 from the estimation).

Genome-wide association mapping

Genotypic means of the egg mass data was used as input for the genome wide association mapping using 214,051 SNPs (Horton et al 2012) using rrBLUP and the TAIR10 database (Yu *et al.*, 2006; Endelman, 2011). First, a kinship matrix based on all SNPs was constructed to correct for population structure. Second, association mapping was done, excluding SNPs with a frequency <0.05 from analysis. SNPs with a –log10(p) > 5.0 were considered significantly associated with phenotypic variance. To determine the false discovery rate at this threshold for significance, an empirical multiple testing threshold was calculated by permutation. The trait levels were randomly assigned to the genotypes, where after the association mapping was performed as described above. This procedure was repeated 1000 times resulting in a false discovery rate of 0.2 at –log10(p) > 5.0. To calculate how much of the total narrow sense heritability can be explained by significantly associating SNPs we used an additive linear model incorporating all SNPs in order to avoid a bias in SNPs capturing the same variation.

The linkage disequilibrium between SNPs was calculated using correlation analysis. First, the SNPs were converted to binary traits (either 0 or 1), which was possible since the HapMap genetic map was constructed with SNPs with only two variants per site. Per two locations the correlation between SNPs was calculated by Pearson correlation (as provided by R). The squared correlations were reported since the direction of the correlation does not confer

real information (as the conversion to binary was arbitrary).

Results

Quantitative variation in susceptibility to *M. incognita*

Arabidopsis has not been systematically analysed for intraspecific variation in susceptibility to *M. incognita* before. To investigate whether Arabidopsis harbours any significant quantitative variation in susceptibility to *M. incognita*, we tested seedlings of 340 natural inbred lines of the Arabidopsis HapMap population with nematode bioassays *in vitro*. These natural inbred lines were phenotypically screened for reproductive success of *M. incognita* in batches of twenty accessions, including Columbia-0 (Col-0) as reference in each batch. Approximately sixty accessions were tested multiple times in different batches to monitor consistency across different batches. Six weeks after inoculation, the average number of egg masses of *M. incognita* on the 340 accessions ranged from 5 to 45 per plant (Fig. 1; Table S2). Inoculations with *M. incognita* on Col-0 resulted on average in 12 egg masses per plant. Based on our extensive phenotype screening we concluded that Arabidopsis harbours large quantitative variation in susceptibility to *M. incognita*.

To estimate how much of the variance in reproductive success of *M. incognita* was caused by underlying genetic variation in the Arabidopsis lines, we calculated the narrow-sense heritability. Using 214,051 SNPs as basis for the genetic similarity, we estimated that 52 percent of the variation in susceptibility to *M. incognita* was attributable to additive genetic variation between the different Arabidopsis lines. We therefore decided to use our data set for identifying genome-wide associations between SNPs in Arabidopsis and susceptibility to

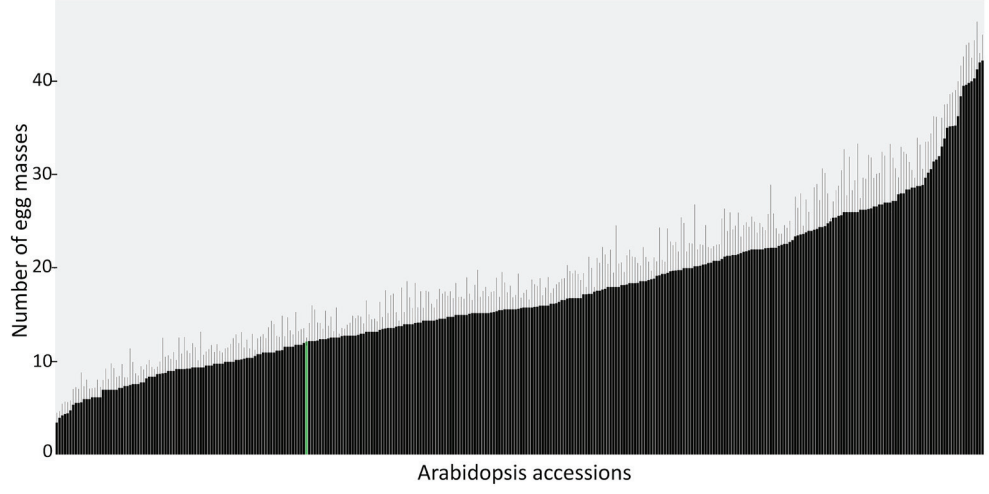

Figure 1. Quantitative variation in susceptibility of *Arabidopsis thaliana* to the root-knot nematode *M. incognita*. Average number of egg masses per plant including standard error of the mean on 340 natural inbred lines of Arabidopsis (accessions) at 6 weeks after inoculation with 2nd stage juveniles of *M. incognita.* The green bar indicates the number of egg masses per plant for Col-0, which was used as a reference throughout this study.

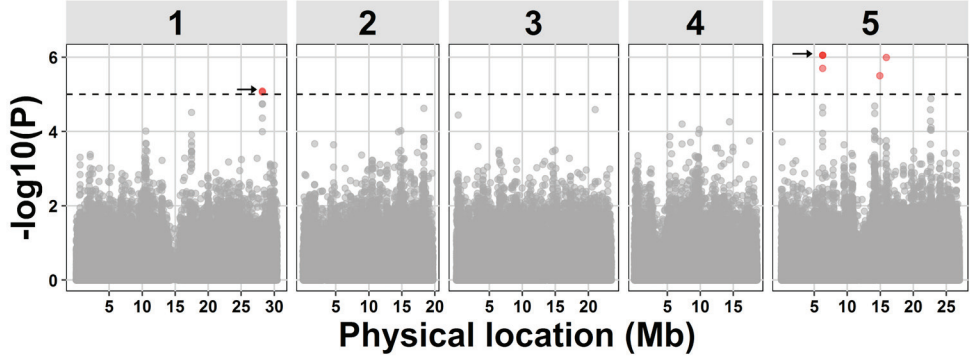

Figure 2. Manhattan plot of associations between 199,252 SNPs and the number of egg masses per plant of *M. incognita* in Arabidopsis. Dashed horizontal line indicates threshold for significance in GWA mapping set at –log10(p) = 5. Red dots indicate the positions of eight significantly associated SNPs, of which five are overlapping and are indicated by the arrows, where on chromosome 5 three SNPs are overlapping. Numbers one to five in grey rectangles mark the five chromosomes of Arabidopsis.

M. incognita.

Four QTLs for susceptibility to *M. incognita* in Arabidopsis

To link allelic variation in Arabidopsis to reproductive success of *M. incognita*, we mapped genome-wide associations underlying the number of egg masses per plant using linear mixed models (Yu *et al.*, 2006; Endelman, 2011). Only SNPs with a minor allele frequency above 0.05 (199,252 SNPs) were included in the analysis. We identified significant associations between eight SNPs and the number of egg masses per plant six weeks after inoculation with *M. incognita* in Arabidopsis (threshold for significance –log10(p)>5; Fig. 2). Furthermore, by using an additive linear model incorporating all SNPs again, we calculated that 22 percent of the total variation in susceptibility of Arabidopsis can be linked to these eight SNPs.

Linkage disequilibrium (LD) in populations of natural inbred lines of Arabidopsis decays on average within 10 Kb (Kim *et al.*, 2007). Based on this predicted LD decay, we aggregated the eight SNPs into four QTLs located on two chromosomes (Table 1). We also analysed the specific LD between the eight significantly associated SNPs (Fig. S1). As expected, only low LD was observed between SNPs located in different QTLs (r^2 < 0.11). However, moderate LD was observed for the SNPs in QTL1 on chromosome 1 (r^2 = 0.55), while strong LD was observed for the four SNPs in QTL2 on chromosome 5 (r^2 > 0.99). The two SNPs marking QTLs 3 and 4 segregate independently (r^2 = 0.01). In conclusion, allelic variation in at least four genome locations is linked to quantitative variation in susceptibility to *M*. *incognita* in

our population of Arabidopsis natural inbred lines.

To further investigate the genetic architecture underlying the reproductive success of *M. incognita* in Arabidopsis, we focused on QTL1 and QTL2 located on chromosomes 1 and 5, respectively. QTL1 is marked by two significantly associated SNPs with moderate LD (markers Chr1.28187392 and Chr1.28188151). These two SNPs were located within 1 Kb distance from each other (Table 1 and Fig. 3a). SNP marker Chr1.28187392 is located in *BRASSINAZOLE-RESISTANT 1* (*BZR1*; AT1G75080) (Fig. 3A). The neighbouring SNP marker Chr1.28188151 is located in a predicted gene in complementary orientation encoding a putative DNA glycosylase superfamily protein (AT1G75090; hereafter named *GSP1*). Marker Chr1.28188151 was in strong LD (r^2 > 0.94) with three other markers at this locus (i.e., Chr1.28187959, Chr1.28187978, and Chr1.28188103), which were just below our threshold for significance in the GWA. Marker Chr1.28188103 was located in *GSP1*, while Chr1.28187959 and Chr1.28187978 were located in the regions where transcripts of *BRZ1* and *GSP1* overlap.

We used SNPs markers Chr1.28187392 and Chr1.28188151 to determine the most susceptible and the least susceptible haplotype for QTL1. Arabidopsis lines harbouring a C at Chr1.28187392 (n=278) were less susceptible to *M. incognita* than those harbouring a G (n=71). Similarly, lines harbouring a G at Chr1.28188151 (n=247) were also less susceptible than those harbouring a C (n=102). These polymorphisms occurred in four haplotype combinations: CC (n=32); GC (n=62); CG (n=232); and GG (n=2). Interestingly, lines with the most prevalent CG haplotype (e.g., Col-0) were also the least susceptible to *M. incognita*,

Table 1. Eight SNPs significantly associated with reproductive success of *M. incognita* aggregate into four quantitative trait loci (QTLs) located on two chromosomes of Arabidopsis. With possible alleles for each SNP position, frequency of lines harbouring the SNP and level of significance of the association of an individual SNP.

QTL	Chromosome	Position (bp)	SNP	SNP frequency	-log10(p)	Effect size	SNP located in gene
1	1	28187392	C:G	278:71	5.1	5.62	At1G75080
		28188151	C:G	102:247	5.1	5.10	At1G75090
2	5	6263591	A:T	139:210	6.1	5.31	At5G18780
		6263577	A:T	139:210	6.1	5.31	At5G18780
		6263644	A:T	140:209	5.7	5.18	At5G18780
		6263678	C:G	139:210	6.1	5.31	At5G18780
3	5	14913458	A:T	288:66	5.5	5.35	At5G37540
4	5	15904331	C:T	290:59	4.47	4.47	At5G39740

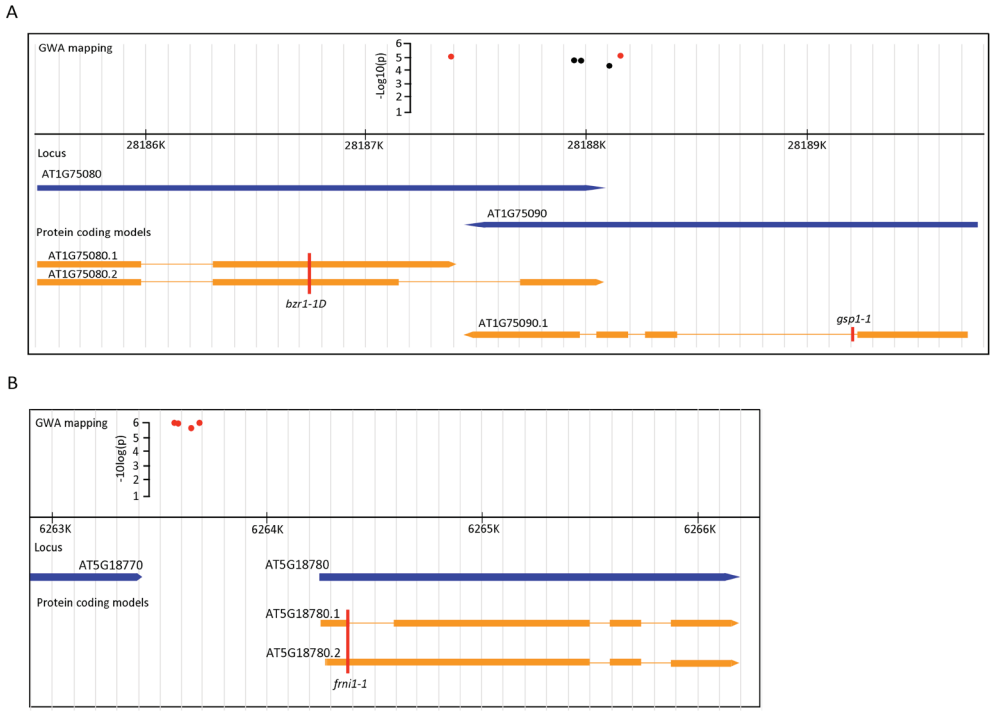

Figure 3. Overview of the genomic region harboring QTL1 and QTL2 located on chromosome 1 and 5, respectively. (A) genomic region of QTL1. The red dots represent the significantly associated SNPs. The black dots represent three SNPs in strong linkage disequilibrium, but with –log10(p) scores below 5. The blue arrows represent two predicted genes (At1G75080 and At1G75090) in complementary orientation. Transcripts deriving from these genes are indicated in orange, with rectangles marking the protein coding exons. The red vertical line marked as *bzr1-1D* indicates the position of the dominant EMS mutation in the *BZR1* gene. The red vertical line marked as *gsp1-1* indicates the position of the T-DNA insert in a homozygous knock-out mutant of *GSP1.* (B) genomic region harboring QTL2. The red dots represent the significantly associated SNPs. The blue arrows represent two predicted genes (At5G18770 and At5G18780) in similar orientation. Transcripts deriving from At5G18780 are indicated in orange, with rectangles marking the protein coding exons. The red vertical line marked as *frni1-1* indicates the position of the T-DNA insert in a homozygous knock-out mutant of *FRNI1*.

which could point to a selective advantage of this haplotype (Fig. S2).

Next, we focused on four significantly associated SNP markers with strong LD (i.e., Chr5.6263591, Chr5.6263577, Chr5.6263644, and Chr5.6263678) that mark QTL2 on chromosome 5. The SNPs are all located in an intergenic region approximately 600 base pairs upstream of predicted gene At5G18780 (Fig. 3b). Two splice variants have been observed for At5G18780, both with unknown function. The protein encoded by At5G18780 is annotated as F-box/Ribonuclease inhibitor-like superfamily protein of 441 amino acids (hereafter *FRNI1*). The predicted topology of *FRNI1* includes an amino terminal F-box of 50 amino acids long (pfam 00646), seven leucine-rich repeats with similarity to ribonuclease inhibitor 1 (RNI), and a carboxy terminal FBD domain (pfam08384) that is found in F-box

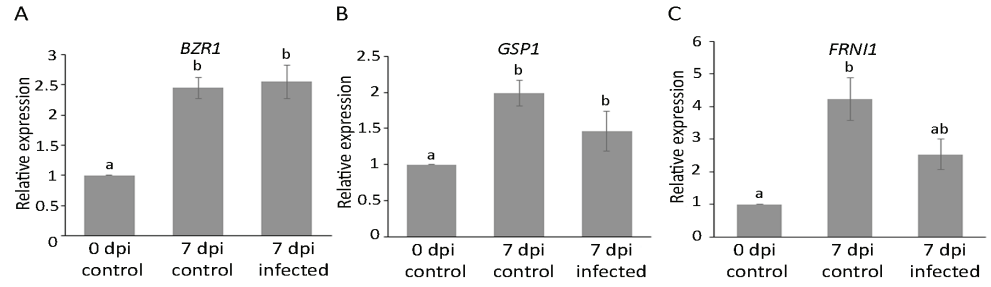

Figure 4. Relative expression of *BZR1*, *GSP1* and *FRNI1* in infected and non-infected roots of wildtype Arabidopsis Col-0 plants at 7 days post inoculation (dpi) with *M. incognita*. The expression levels of *BZR1* (A), *GSP1* (B) and *FRNI1* (C) are given as ratios relative to the expression levels of these genes at the time of inoculation. Data reflect gene expression levels in whole roots collected at the time of inoculation with *M. incognita* (0 dpi control), in whole roots collected at 7 days after mock-inoculation (7 dpi control) and 7 days after inoculation with *M. incognita* (7 dpi infected). The bars represent average values based on three independent biological samples with three technical replicates per biological sample. Error bars represent standard error of the mean. Different characters indicate statistical difference determined by ANOVA with post-hoc Tukey HSD (P-value<0.05).

domain containing plant proteins.

BZR1 and FRNI1 (co-)regulate reproductive success of *M. incognita*

To find further support for a role of *BZR1*, *GSP1*, and *FRNI1* in susceptibility of Arabidopsis to *M. incognita*, we first assessed their expression levels in roots of infected and non-infected seedlings using quantitative reverse transcription PCR (qRT-PCR). The expression of the genes was determined in whole root systems collected at the time of inoculation and at seven days after inoculation in infected and non-infected plants. This set up allowed us to study the developmental regulation of the genes in young Arabidopsis seedlings, as well as their regulation in response to infection by *M. incognita*. *BZR1*, *GSP1*, and *FRNI1* were all upregulated in non-infected roots of Arabidopsis seedlings, as they developed in the seven days after the time of inoculation (Fig. 4). The expression of both *GSP1* and *FRNI1* was significantly down-regulated in nematode-infected roots at same time point post inoculation (P-value<0.05). By contrast, infection by *M. incognita* did not alter the developmentally regulated expression of *BZR1*. As we used whole root systems, dilution effects could keep local changes in expression of *BZR1* at the infection site below the detection limits of the qRT-PCR. To address this concern, we also investigated the expression of *BZR1* with confocal microscopy of the Arabidopsis BZR1:CFP reporter line at 3 days after inoculation with *M. incognita* (Fig. S3). Based on image analysis, we concluded that infections with *M. incognita* do not lead to significant changes in *BZR1* expression at the infection site of the nematodes.

To test if *BZR1*, *GSP1*, and *FRNI1* are required for reproductive success of *M. incognita*, we challenged several Arabidopsis mutant lines with infective juveniles in a bioassay.

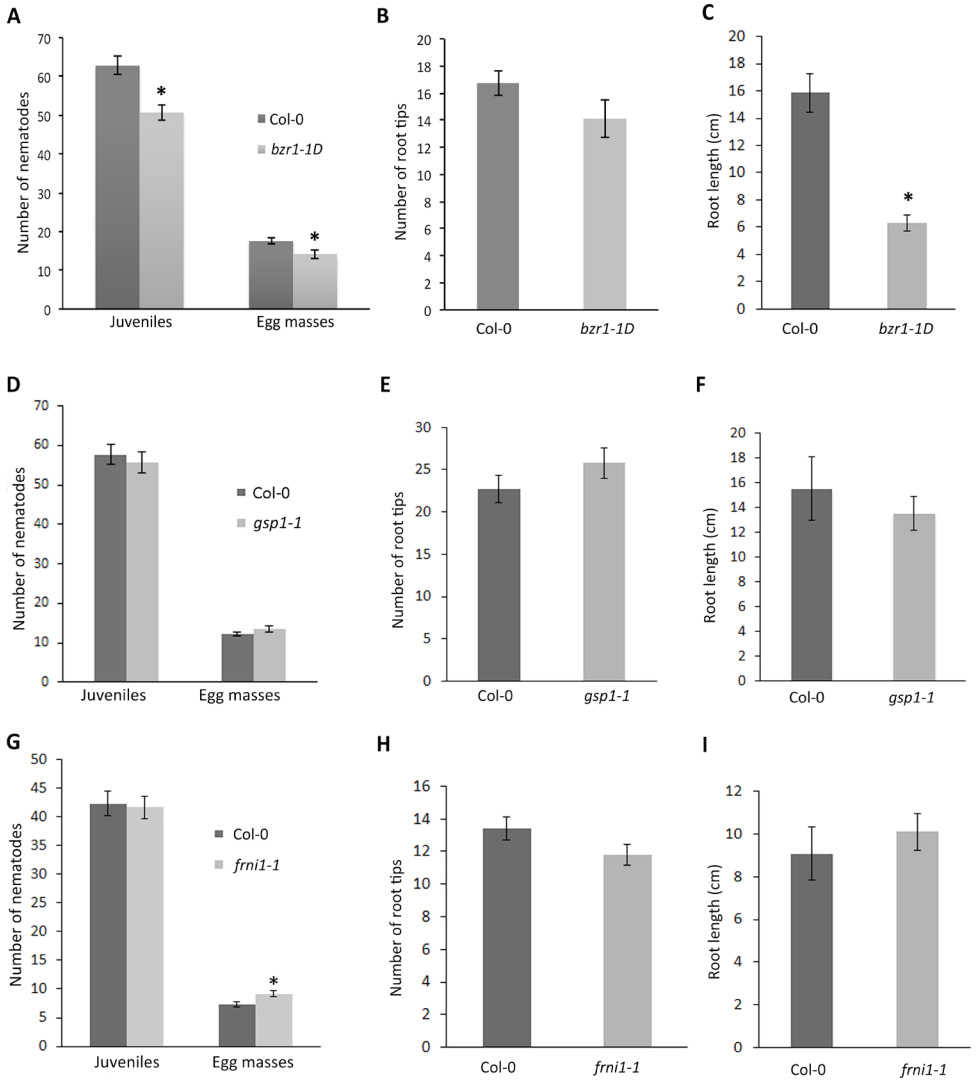

Figure 5. Susceptibility of a dominant positive EMS mutant *bzr1-1D* and homozygous T-DNA insert mutants *gsp1-1* and *frni1-1* of Arabidopsis to *M. incognita*. (A) Number of juveniles at 7 days post inoculation and egg masses per plant at 6 weeks post inoculation on *bzr1-1D* and wildtype Arabidopsis plants. (B) Number of root tips and (C) the total root length of seedlings of *bzr1-1D* at the age of inoculation. (D) Number of juveniles at 7 days post inoculation and egg masses per plant at 6 weeks post inoculation on *gsp1-1* and wildtype Arabidopsis plants. (E) Number of root tips and (F) the total root length of seedlings of *gsp1-1* at the age of inoculation. (G) Number of juveniles at 7 days post inoculation and egg masses per plant at 6 weeks post inoculation on *frni1-1* and wildtype Arabidopsis plants. (H) Number of root tips and (I) the total root length of seedlings of *frni1-1* at the age of inoculation. (A,D,G) Bars reflect the averages and standard error of the mean of three independent experiments (n >50). (B,C,E,F,H,I) Bars represent the mean and the standard error of mean of three independent experiments (n>12). Data was statistically tested for significance with ANOVA with post-hoc Tukey HSD (*P-value<0.05).

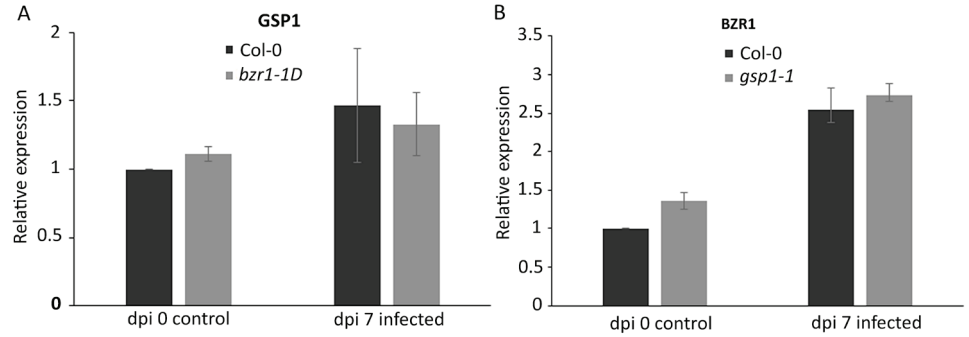

Figure 6. Mutation in the Arabidopsis *bzr1-1D* line does not affect expression of neighbouring *GSP1*, and vice versa. (A) Relative expression of *GSP1* in *bzr1-1D* and wildtype Arabidopsis plants at 7 days after inoculation with *M. incognita*. (B) Relative expression of *BZR1* in *gsp1-1* and wildtype Arabidopsis plants at 7 days after inoculation with *M. incognita*. Gene expression levels are determined with quantitative reverse transcription PCR and presented here as a ratio relative to the expression level in wildtype Arabidopsis plants at the time of inoculation. The bars represent average values based on three independent biological samples with three technical replicates per biological sample. Error bars represent standard error of the mean.

Homozygous Arabidopsis T-DNA knockout mutants of *BZR1* have a lethal phenotype and could not be used to test the involvement of this gene in the reproductive success of *M. incognita*. Instead, we analysed the susceptibility of the dominant positive EMS mutant Arabidopsis line *bzr1-1D* in our bioassays with *M. incognita* (Wang *et al.*, 2002). Both the number of J2s of *M. incognita* per plant at 7 days post inoculation and the number of egg masses per plant at 6 weeks post inoculation were significantly reduced on the *bzr1-1D* mutant line compared to the wildtype Arabidopsis plants (Fig. 5a). The *bzr1-1D* mutant harbours a functional mutant allele of the *BZR1* transcription factor that makes it insensitive to the brassinosteroid (BR) biosynthetic inhibitor brassinazole (Wang *et al.*, 2002). Seedlings of the *bzr1-1D* mutant typically show anomalous root architecture under specific light conditions (Wang *et al.*, 2002), and this could affect susceptibility to nematode infections. Indeed, in our experimental set-up the average total root length at the time of inoculation was significantly smaller in *bzr1-1D* mutants as compared to wildtype Col-0 plants (Fig. 5c). More importantly, susceptibility of plants to root-knot nematodes is known to depend on the number of available root tips at the time of inoculation. This parameter of root architecture was not significantly different between the *bzr1-1D* mutant and the wildtype Arabidopsis plants (Fig. 5b).

A homozygous Arabidopsis T-DNA mutant line was available for the *GSP1* gene, which harbours an insert in the predicted first intron of the coding sequence of *GSP1* (Fig. 3a). The expression of *GSP1* was strongly reduced in roots of the *gsp1-1* mutant line, but not completely knocked-out (Fig. S4a,b). Despite this reduction in gene expression the number of egg masses per plant at 6 weeks post inoculation was not significantly different in the *gsp1-1* mutant line, when compared to wildtype plants (Fig. 5d). Similarly, the number of infective juveniles per plant at 7 days post inoculation was also not significantly different

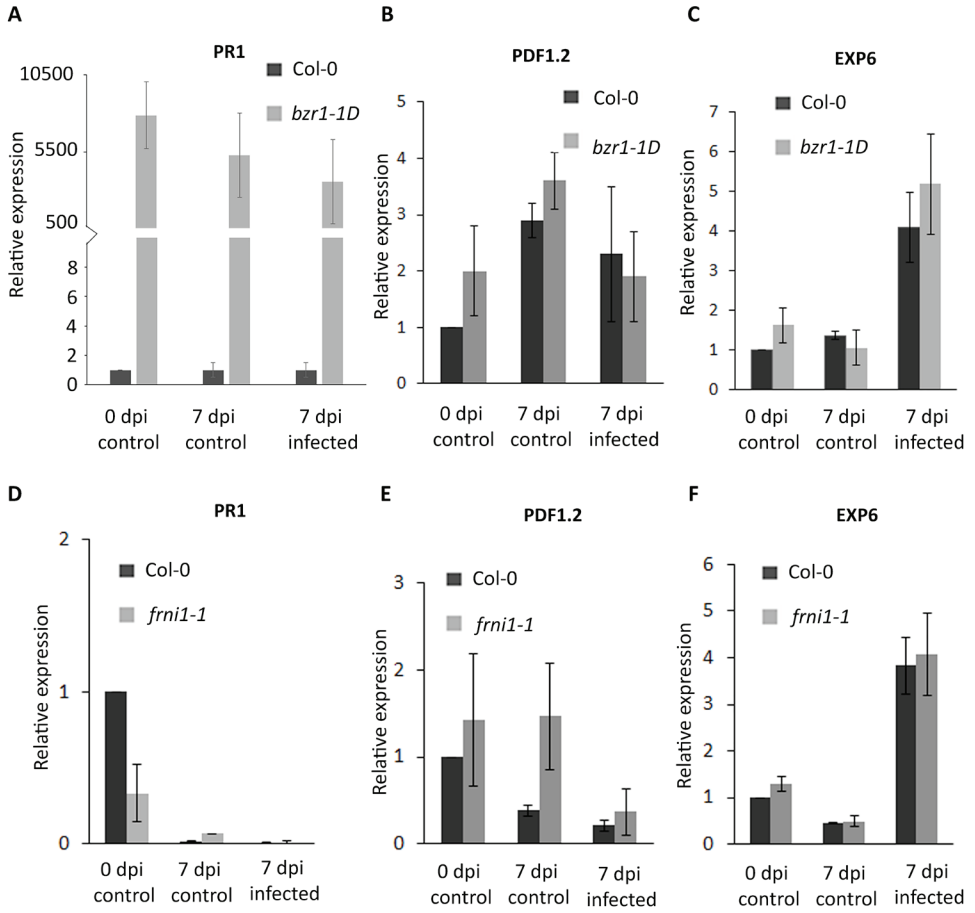

Figure 7. Differential expression of marker genes for salicylic acid (*PR1*) and jasmonic acid (*PDF1.2*) dependent defense responses and plant cell expansion (*EXP6*) in the *bzr1-1D* and *frni1-1* mutant and wildtype Arabidopsis plants. Relative expression levels of *PR1* (A), *PDF1.2* (B) and *EXP6* (C) in *bzr1-1D* and wildtype Arabidopsis plants. Relative expression levels of *PR1* (D), *PDF1.2* (E) and *EXP6* (F) in *gsp1-1* and wildtype Arabidopsis plants. Gene expression levels are determined with quantitative reverse transcription PCR and presented here as a ratio relative to the expression level in wildtype Arabidopsis plants at the time of inoculation. Data reflects expression in whole roots collected at the time of inoculation with *M. incognita* (0 dpi(days post inoculation) control), in whole roots collected at 7 days after mock-inoculation (7 dpi control) and 7 days after inoculation with *M. incognita* (7 dpi infected). The bars represent average values based on three independent biological samples with three technical replicates per biological sample. Error bars indicate standard error of the mean.

between the *gsp1-1* mutant and wildtype plants. Furthermore, the root architecture of this mutant was not significantly different from wildtype Arabidopsis plants (Fig. 5e,f).

BZR1 and *GSP1* are located in antisense direction and their coding sequences partially overlap. *BZR1* and *GSP1* could therefore act as cis-natural antisense pairs, which could lead to the formation of siRNA and thus transcript breakdown. To test this, we analysed the expression of *GSP1* in *bzr1-1D* and the expression of *BZR1* in *gsp1-1.* In a comparison with wildtype Arabidopsis seedlings at 7 days post inoculation, the expression of *GSP1* was not altered by the EMS mutation in *bzr1-1d*, and vice versa the expression of *BZR1* was not altered by the T-DNA insert in *gsp1-1* (Fig. 6). Our data therefore showed that it is unlikely that the phenotype of *bzr1-1D* mutation arises through it actions on transcript levels of *GSP1*.

A homozygous Arabidopsis knock-out line was also available for the *FRNI1* gene, which harbours a T-DNA insert in the first predicted exon of *FRNI1* (Fig. 3b). Quantitative RT-PCR showed that the expression of *FRNI1* was completely knocked-out in roots of the *frni1-1* mutant line (Fig. S4c,d). The number of egg masses on roots of the *frni1-1* mutant line at six weeks post inoculation was significantly higher as compared the wildtype Arabidopsis plants (Fig. 5g). By contrast, we observed no significant difference in the number of juveniles inside roots at seven days post inoculation between *frni1-1* and wildtype Arabidopsis plants. The root architecture of the *frni1-1* mutant was also not significantly different from wildtype Arabidopsis plants (Fig. 5h,i). Altogether, we concluded that *BZR1* and *FRNI* most likely function as (co-)regulators of reproductive success of *M. incognita* in Arabidopsis. Allelic variation in these genes may therefore contribute to quantitative variation in susceptibility to *M. incognita* in our collection of Arabidopsis lines. By contrast, despite its down-regulation in association with nematode infections, allelic variation in *GSP1* is less likely to be causal for quantitative variation in susceptibility of Arabidopsis to *M. incognita*.

BZR1 is a master regulator of both cell proliferation, differentiation, and defence. As such it regulates cell elongation, which is evident from the reduced root growth phenotype of the *bzr1-1D* mutant. Reduced cell growth may affect the expansion of nematode-induced giant cells, but we could not exclude that the *bzr1-1D* mutant is also affected in its ability to mount a defence response to *M. incognita*. We therefore analysed the expression of markers for salicylic acid- and jasmonic acid-related defence responses (i.e., At2G14610 [*PR1*] and A5G44120 [*PDF1.2*]) and a marker for cellular expansion (i.e, At2G28950 [*EXP6*]) in nematode-infected roots of the *bzr1-1D* mutant line and wildtype Arabidopsis. Surprisingly, the expression of *PR1* was constitutively and highly upregulated at the time of inoculation and at 7 days post inoculation in both infected and non-infected *bzr1-1D* mutants when compared to wildtype Arabidopsis plants (Fig. 7a). By contrast, the marker genes for jasmonic acid-dependent defences and cellular expansion were not differentially regulated in *bzr1-1D* and wildtype Arabidopsis (Fig. 7b,c). The function of the FRNI1 is not known, and

we therefore conducted a similar marker gene experiment on nematode-infected roots of the *frni1-1* mutant line. However, none of the marker genes was differentially regulated between *frni1-1* and wildtype Arabidopsis plants (Fig. 7d-f).

Discussion

Most genetic analyses of wild relatives of crop plant species to date have focussed on identifying new sources of dominant resistance to nematodes, while largely disregarding natural variation in susceptibility. Here, we used genome-wide association (GWA) mapping to assess the genetic underpinnings of a large variation in susceptibility to the root-knot nematode *M. incognita* in a population of natural inbred lines of Arabidopsis. By applying a –log10(p) score of 5 as threshold for significance (corresponding to a false discovery rate of 0.2), we identified four QTLs in Arabidopsis associated with the number of egg masses of *M. incognita* at six weeks post inoculation (Fig. 2). These four QTLs most likely harbour allelic variants that are causally related to reproductive success of *M. incognita* on Arabidopsis.

The ability of Arabidopsis to support reproduction of root-knot nematodes is thought to be a complex trait involving many different plant genes (Jammes *et al.*, 2005; Dubreuil *et al.*, 2007; Barcala *et al.*, 2010; Portillo *et al.*, 2013; Cabrera *et al.*, 2014). Given the anticipated complexity of this trait, the number of QTLs significantly associated with reproduction of *M. incognita* in our GWA study was relatively small. It is possible that the genetic architecture underlying susceptibility of Arabidopsis to *M. incognita* is much simpler than previously thought. However, it should be noted that only 22 percent of the observed phenotypic variance can be linked to the four QTLs identified our GWA study. This could indicate the presence of an abundance of rare alleles (with minor allele frequencies below 0.05), which are capturing most of the variation but were excluded from GWA mapping. Alternatively, many QTLs for susceptibility to *M. incognita* in the Arabidopsis genome may have small effect sizes that cannot be detected with the resolution of our bioassays. In either case, our GWA study most likely underestimates the complexity of the genetic architecture of susceptibility to *M. incognita* in Arabidopsis.

Others have investigated the genetic architecture of responses to biotic stresses in Arabidopsis by accepting a less stringent threshold for significant associations in GWA mapping ((e.g., -log10(p)> 4); (El-Soda *et al.*, 2015; Kloth *et al.*, 2016; Kooke *et al.*, 2016; Davila Olivas *et al.*, 2017). Similarly relaxing the stringency in our analysis would result in significant associations between 36 SNPs (located within 19 QTLs) and reproduction of *M. incognita* on Arabidopsis. Lowering the threshold for significance in the GWA mapping may thus reveal more common alleles with smaller effect sizes in our population of Arabidopsis lines (Korte & Farlow, 2013; Kooke *et al.*, 2016). However, this would also raise the false discovery rate to sixty percent, which would reduce the chances of identifying causal genes in follow up studies.

To assess whether our GWA study (using stringent criteria) can help to identify genes involved in susceptibility of Arabidopsis to *M. incognita*, we focused on two SNPs located in QTL1 on chromosome 1 (Fig. 3). Chr1.28187392 showed moderate LD (r^2=0.55) with Ch1.28188151, while LD seems to rapidly decay with SNPs directly flanking Chr1.28187392 and Chr1.28188151. Based on the locations of the two significant SNPs and the predicted LD decay in this region, we concluded that *BZR1* and *GSP1* were the only two candidates in this region that could contribute to the variance in susceptibility of Arabidopsis to *M. incognita*.

Our infection assays with the dominant positive *bzr1-1D* mutant line showed that BZR1 most likely acts as a rate-limiting factor in the reproductive success of *M. incognita* in Arabidopsis (Fig. 5). The number of juveniles inside seedlings during the early stages of parasitism and the number of egg masses at 6 weeks post inoculation was consistently smaller on the *bzr1-1D* mutant when compared to wildtype Col-0. BZR1 is constitutively active in the *bzr1-1D* mutant line, which simulates the accumulation of BR (Wang *et al.*, 2002). Under specific light conditions the dominant *bzr1-1D* mutation results in an anomalous root architecture. In our nematode infection assays the number of root tips at the time of inoculation was not significantly different between *bzr1-1D* mutant line and wildtype Col-0. This is important because the invasion of Arabidopsis by *M. incognita* only occurs in the transition zone close to root tips (Sijmons *et al.*, 1991). However, the reduced total root length of the *bzr1-1D* mutant could point at defects in cell growth, which may affect the expansion of nematode-induced giant cells.

The transcription factor BZR1 is at the end of a signalling cascade which is activated by BRI1/BAK1 co-receptor complex upon detection of brassinolide (Jaillais & Vert, 2016). The activation of BR-signalling in Arabidopsis results in the dephosphorylation and translocation to the nucleus of BZR1, where it binds to DNA and specifically activates or represses the expression of almost one thousand genes (Sun *et al.*, 2010; Di Rubbo *et al.*, 2011). BR-signalling plays a crucial role in determining cell growth by promoting elongation of differentiated cells, but also by regulating the transition between cell cycle progression and cell differentiation (Jaillais & Vert, 2016). Aberrant progression through the mitotic cell cycle, extensive cell elongation and expansion are all considered essential steps in the ontogeny of giant cells in nematode-infected roots of *A. thaliana* (de Almeida Engler & Gheysen, 2013; Kyndt *et al.*, 2013; Vieira *et al.*, 2013). The outcome of brassinosteroid (BR)-signalling in roots is cell type and position specific, but generally antagonises the effect of auxin (Chaiwanon & Wang, 2015). BZR1 regulates the expression of several genes related to auxin biosynthesis and signalling (Sun *et al.*, 2010). For instance, BZR1 directly represses the expression of PIN auxin efflux carriers involved in directing polar auxin transport towards root tips (i.e., *PIN3* and *PIN4*; (Feraru & Friml, 2008; Sun *et al.*, 2010; Vragović *et al.*, 2015). Recently, it was shown that development of *M. incognita* is hampered on Arabidopsis knock-out mutants of *PIN3* and *PIN4* (Kyndt *et al.*, 2016). PIN3 and PIN4 are thought to be involved in redirecting the flow of auxin during giant cell formation. Similarly, BZR1 regulates the expression of genes

involved in plant cell wall plasticity, which is a fundamental requirement for cell growth but also for the expansion of giant cells (e.g., *EXPA1*; (Jammes *et al.*, 2005; Sun *et al.*, 2010)).

Furthermore, BZR1 also regulates the trade-off between growth and immunity, which may explain the constitutive upregulation of *PR1* that we observed in the *bzr1-1D* mutant (Lozano-Duran *et al.*, 2013; Lozano-Durán & Zipfel, 2015). The loss of susceptibility to *M. incognita* in the *bzr1-1D* mutant could therefore also reflect alterations in basal immunity of Arabidopsis seedlings. This latter scenario would be in agreement with the recently observed enhanced resistance of transgenic *Lotus japonicus* plants ectopically expressing the Arabidopsis *bzr1-1D* allele to feeding by onion thrips (Miyaji *et al.*, 2014). Altogether, we conclude that BZR1 most likely (co-)regulates susceptibility to *M. incognita* in *A. thaliana* through its role in plant cell growth, basal defence, or both. Allelic variation in *BZR1* could therefore be casual for some of the observed variance in reproductive success of *M. incognita* in our population of Arabidopsis lines.

The second SNP marker significantly associated with the number of egg masses of *M. incognita* per plant in QTL1 was located in the first exon of *GSP1*, a putative DNA glycosylase superfamily protein. The function of *GSP1* has not been studied before, but based on sequence homology it is predicted to be involved in base-excision repair of DNA (Manova & Gruszka, 2015). Despite the fact that *GSP1* is strongly down-regulated upon infection by *M. incognita* at 7 days post inoculation, the homozygous knock-down mutant Arabidopsis line of *GSP1* in the Col-0 background showed no altered susceptibility to *M. incognita*. This finding suggests that *GSP1* is regulated in association with, but not required for, reproduction of *M. incognita* in Arabidopsis. However, it should be noted that the Arabidopsis Col-0 line carries the non-susceptible haplotype (i.e., CG) for this locus, and that a knock-down by the T-DNA insert in *gsp1-1* may therefore not lead to further reduction in susceptibility. In conclusion, we have found no evidence suggesting that allelic variation in *GSP1* significantly contributes to the variance in susceptibility of Arabidopsis to *M. incognita.*

Four co-segregating SNPs marking QTL2 on chromosome 5 pointed at FRNI1 as co-regulator of susceptibility of Arabidopsis to *M. incognita*. The SNP markers are located in the putative regulatory region upstream of the predicted coding sequence of *FRNI1*, where they might affect expression levels of this gene. Unlike *BZR1*, *FRNI1* is strongly downregulated in nematode-infected roots of wildtype Arabidopsis Col-0 plants. Alterations in *FRNI1* expression are therefore likely to affect susceptibility of Arabidopsis to *M. incognita*. In fact, our data showed that the compete loss of *FRNI1* expression in the *frni1-1* knock-out mutant resulted in a small but significant increase in the number of egg masses per plant. So far, no function has been ascribed to FRNI1, but its architecture as an F-box-like and RNI-like protein suggests that it might be involved in protein-protein interactions. More specifically, the F-box is defined as a component of the E3 ubiquitin ligase complex SCF (Skp1-cullin-F-box protein ligase), which targets proteins to the 26S proteasome for degradation (Lechner *et al.*, 2006). F-box-containing proteins are involved in a many cellular process in plants, including hormone signalling and defence responses. The lack of differential expression of *PR1*, *PDF1.2*, and *EXP6* in nematode-infected roots of *frni1-1* mutant and wildtype Arabidopsis plants offered no clue as to whether *FRNI1* co-regulates susceptibility by affecting defence, development, or both. Further investigations are therefore needed to shed light on the function of FRNI1 in Arabidopsis.

The main objective of this study was to explore the natural variation in susceptibility to *M. incognita* of Arabidopsis, which is thought to lack dominant resistance genes to this nematode species. In our phenotype screening of the Arabidopsis lines we observed an unexpected large variation in reproductive success of *M. incognita*. Extensive variation in susceptibility was also observed within a smaller set of forty-five Arabidopsis inbred lines challenged with the northern root-knot nematode *Meloidogyne hapla* (Boiteux *et al.*, 1999). As the natural distribution of *M. hapla* and *A. thaliana* in temperate regions may have overlapped, this variation could be partly based on segregating major resistance genes. We found no evidence by GWA mapping that the large phenotypic variance in susceptibility to *M. incognita* is based on the presence of segregating dominant resistance genes linked to any of the QTLs. By contrast, GWA mapping of susceptibility to *Meloidogyne graminicola* in rice cultivars identified eleven genomic loci, at least one of which harbours major resistance gene homologs (Dimkpa *et al.*, 2016). Our data thus indicate that plants could be made more resistant to infections of root-knot nematodes by selecting unfavourable alleles of *S*-genes that are essential for giant cell initiation, expansion, and maintenance. However, it remains to be investigated if these loss-of-susceptibility alleles can be exploited by plant breeders to improve the resilience of crops without experiencing undesirable pleiotropic effects on other agronomically important traits.

Acknowledgements

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Supplemental information

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Chapter 3

Mediater of tolerance to abiotic stress ERF6 co-regulates susceptibility of Arabidopsis to *Meloidogyne incognita*

Sonja Warmerdam
Mark G. Sterken
Casper C. van Schaik
Marian E. P. Oortwijn
Jose L. Lozano-torres
Jaap Bakker
Aska Goverse
Geert Smant

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Abstract

Root-knot nematodes transform vascular host cells into permanent feeding structures to selectively withdraw their nutrients from host plants during the course of several weeks. The susceptibility of host plants to root-knot nematode infections is thought to be a complex trait involving many genetic loci. However, genome-wide association (GWA) analysis has so far revealed only four quantitative trait loci (QTLs) linked to the reproductive success of the root-knot nematode *Meloidogyne incognita* in *Arabidopsis thaliana*, which suggests that the genetic architecture underlying host susceptibility could be much simpler than previously thought. Here, we report that, by using a relaxed stringency approach in a GWA analysis, we could identify 15 additional loci linked to quantitative variation in the reproductive success of *M. incognita* in Arabidopsis. To test the robustness of our analysis, we functionally characterized six genes located in a QTL with the lowest acceptable statistical support and smallest effect size. This led us to identify *ETHYLENE RESPONSE FACTOR 6* (*ERF6*) as a novel susceptibility gene for *M. incognita* in Arabidopsis. ERF6 functions as a transcriptional activator and suppressor of genes in response to various abiotic stresses independent of ethylene signalling. However, whole-transcriptome analysis of nematode-infected roots of the Arabidopsis *erf6-1* knockout mutant line showed that allelic variation at this locus may regulate the conversion of aminocyclopropane-1-carboxylate (ACC) into ethylene by altering the expression of 1-aminocyclopropane-1-carboxylate oxidase 3 (ACO3). Our data further suggest that tolerance to abiotic stress mediated by ERF6 forms a novel layer of control in the susceptibility of Arabidopsis to *M. incognita*.

Introduction

Below-ground attacks of crops by plant-parasitic nematodes are a major constraint in global food production (Fuller *et al*., 2008). Outbreaks of plant-parasitic nematodes can lead to substantial annual economic losses, amounting to $157 billion per year (Abad *et al*., 2008). The tropical root-knot nematode *Meloidogyne incognita* is ranked as one of the most rapidly spreading biological threats of agricultural productivity, occurring in more than 143 countries worldwide (Bebber *et al*., 2014). The extensive dispersal of *M. incognita* can be attributed to its wide host range, encompassing more than 1000 plant species from 200 different genera (Perry *et al*., 2009; Trudgill, 1997). For decades, infestations of root-knot nematodes in crops have been controlled by the application of chemical pesticides. However, because of regulatory bans on these compounds, the control of root-knot nematodes has become more reliant on major resistance genes in recent years (Wesemael *et al*., 2011; Williamson and Kumar, 2006). Unfortunately, the panel of major genes currently available for the breeding of novel resistances to root-knot nematodes into crops is extremely small. As a consequence of strong selection pressure by only a few widely used major resistance genes, the emergence of resistance-breaking populations of *M. incognita* has recently turned into a major concern for growers across different continents (Davies and Elling, 2015; Kaloshian *et al.*, 1996; Semblat *et al*., 2000).

Root-knot nematodes need to establish a permanent feeding site inside a host plant to develop into the reproductive stage and to produce offspring. To this end, freshly hatched second-stage juveniles (J2s) of *M. incognita* are attracted to the root tip of a nearby host plant. They invade the root directly above the elongation zone in the root tip. Next, invasive J2s migrate intercellularly through the cortex towards the root meristem, where they enter the vascular cylinder from below. Inside the vascular cylinder, the J2s establish a permanent feeding structure consisting of several giant cells. Giant cells are vascular cells transformed into large transfer cell-like units. Feeding on giant cells enables J2s to moult three times into the adult female stage, whilst remaining attached to the permanent feeding structure. After a couple of weeks of feeding, adult female root-knot nematodes produce offspring as an aggregate of eggs held together by a gelatinous matrix (Caillaud *et al*., 2008; Gheysen and Mitchum, 2011; Kyndt *et al*., 2013).

Whole-transcriptome analyses of giant cell-enriched tissue of roots infected with root-knot nematodes show that feeding site formation is accompanied by the differential regulation of more than 1000 genes (Barcala *et al*., 2010; Fuller *et al*., 2007; Jammes *et al*., 2005). The large number of genes regulated in association with feeding site formation suggests that the reproductive success of *M. incognita* might be a highly polygenic trait in plants. However, it should be noted that many genes may be regulated in response to the massive cellular changes induced by feeding nematodes, but may not necessarily be required for the susceptibility of plants to nematode infections. Nonetheless, allelic variation in genes

that do enable initiation, expansion and maintenance of giant cells may translate into quantitative variation in susceptibility to nematode infections. If this holds true, gain- and loss-of-function alleles of these so-called susceptibility genes may be used in the future to make crops more resilient to nematode infections (de Almeida Engler *et al*., 2005; van Schie and Takken, 2014).

Previously, we have used a genome-wide association (GWA) mapping approach to assess whether quantitative variation in susceptibility to *M. incognita* can be linked to allelic variation in specific loci (quantitative trait loci, QTLs) in Arabidopsis (Warmerdam *et al*., 2018). In that study, we found four QTLs significantly associated with the number of egg masses of *M. incognita* per plant at 6 weeks post-inoculation. We noted that, by using a threshold for significant associations of –log10(*P*) = 5, the accumulated effect sizes of the alleles in these four loci could account for only 50% of the heritable variation in susceptibility of Arabidopsis to *M. incognita*. We also noted that reducing this threshold to the level of –log10(*P*) = 4 would have explained all of the heritable variation in susceptibility of Arabidopsis to *M. incognita*. However, relaxing the stringency of our GWA approach would also have increased the risk of pursuing false positives. Here, we describe how we challenged this higher risk of false discovery under relaxed criteria by functionally characterizing six genes located at QTL13, which, in terms of statistical support and effect size, had a relatively high chance of being a false positive. This led us to identify *ETHYLENE RESPONSE FACTOR 6* (*ERF6*) as a regulator of susceptibility to *M. incognita* in Arabidopsis. The transcription factor ERF6 has not been associated previously with susceptibility to *M. incognita*, but its role as an activator and repressor of abiotic stress response genes may shed new light onto the role of tolerance to abiotic stress in feeding site formation.

Materials and methods

Plant material

The following homozygous Arabidopsis T-DNA insertion mutant lines were obtained from the Nottingham *Arabidopsis* Stock Centre (Alonso *et al.*, 2003): SALK_087356C with T-DNA insert in At4G17490 (*erf6-1*); SALK_059419C with T-DNA in At4G174505 (*duf239-1*); SALK_087258C with T-DNA in At4G147510 (*uch3-1*), SALK_129052C with T-DNA insert in At4G17520 (*hln-1*), and SALK_104999C with T-DNA insert in At4G17530 (*rab1c-1*). The T-DNA insert in all mutant lines interrupted the (predicted) open reading frames in the coding sequences of the genes. The T-DNA insert lines were all generated in the background of Col-0 (N60000), which was used as wildtype Arabidopsis throughout this study.

The presence and homozygosity of the T-DNA insert in the mutant lines was checked with PCR on genomic DNA isolated from leaf material of twelve seedlings (Warmerdam *et al.*, 2018). To determine if the wild type allele or the T-DNA insert was present in the mutants, we used different combinations of gene specific forward and reverse primers and combinations of gene specific primer and T-DNA-insert specific Lbl3.1 primer (Supplemental table S2; (Alonso *et al.*, 2003)). For the PCR, we used the following conditions: 10 min at 94°C, 35 cycles of 30 s at 94°C, 1.5 min at 60°C, and 1 min at 72°C, and a final incubation of 10 min at 72°C. The PCR amplification products were analyzed by agarose gel electrophoresis.

We also checked the expression of the affected gene by the T-DNA insertion line with reverse transcription quantitative PCR (RT-qPCR). Total RNA was isolated from whole roots of twelve 14 days-old plants of the specific T-DNA insertion mutant as described below for the microarray analysis. First strand cDNA was synthesized from total RNA using Superscript III First-Strand synthesis system according the manufacturers standard protocol (Invitrogen). cDNA samples were used as template in quantitative PCR with the Absolute SYBR Green Fluorescein mix (Thermo Fisher Scientific). cDNA matching *Arabidopsis thaliana* elongation factor 1-alpha (At5G60390) was amplified as a reference for gene constitutive expression (Czechowski *et al.*, 2004). To quantify the expression level for the gene of interest, we used a gene specific forward and reverse primers (Table S2). For the PCR, we used the following conditions: 15 min at 95°C, forty cycles of 30 s at 95°C, 30 s at 62 °C, and 30 s at 72°C, and a final incubation of 5 min at 72°C. Relative expression ratio between the gene of interest and the reference gene was calculated as described elsewhere (Pfaffl, 2001).

Nematode bioassays

Eggs of *Meloidogyne incognita* were obtained by treating tomato roots infected with *M. incognita* (strain 'Morelos' from INRA, Sophia Antipolis, France) with 0.05% v/v NaOCl for

three minutes. Roots were rinsed with tap water and the eggs were collected on a 25 µm sieve. Next, the eggs were incubated in a solution of 2.4 mM NaN_3 for 20 min while shaking. Thereafter, the eggs were rinsed with sterile tap water and incubated on a 25 µm sieve in a solution of 1.5 mg/ml gentamycin and 0.05 mg/ml nystatin in the dark at room temperature. Hatched juveniles were collected after 4 days and surface-sterilized using 0.16 mM $HgCl_2$, 0.49 mM NaN_3, 0.002% v/v Triton X-100 for 10 min. After surface sterilization, the juveniles were rinsed three times with sterile tap water and transferred to 0.7% Gelrite solution (Duchefa biochemie).

To test the susceptibility of Arabidopsis seedlings, seeds were vapour sterilized (with 7.1 M NaOCl and 1% HCl in tap water) for 5 h and transferred to a 6-well cell culture plate containing Murashige and Skoog (MS) medium (MS with vitamins 4.7 g/L (Duchefa biochemie), 58.5 mM sucrose and 5 g/L Gelrite). The 6-well plates were first incubated in the dark at 4°C for 3 days. Thereafter, the seeds were allowed to germinate at 21°C under 16h light / 8h dark conditions in a growth cabinet for seven more days. Individual one-week-old seedlings were subsequently transferred to separate wells in a new 6-well plate containing MS medium. The seedlings were incubated for 7 more days at 21°C under a 16h light and 8h dark regime. Next, individual seedlings were inoculated with 180 infective J2s of *M. incognita* per plant and incubated at 24°C in the dark. The number of egg masses per plant were counted six weeks after inoculation by visually inspecting the roots with a dissection microscope. Unless indicated otherwise, the differences in counts per plants were statistically analysed using two-way ANOVA and post-hoc Tukey's HSD test in R (version 3.0.2, www.r-project.org). Each Arabidopsis genotype was tested in at least three independent experiments and 10 replicates per experiment. Both genotype and experiment were used as factors to test for significance.

To collect roots of infected and non-infected Arabidopsis seedlings for gene expression analyses with microarrays and RT-qPCR, seeds were vapour sterilized (with 7.1 M NaOCl and 1% HCl in tap water) for 5 h and transferred to a 6-well cell culture plate containing Murashige and Skoog (MS) medium (MS with vitamins 4.7 g/L (Duchefa biochemie), 58.5 mM sucrose and 5 g/L Gelrite). The 6-well plates were first incubated in the dark at 4°C for 3 days. Thereafter, the seeds were allowed to germinate at 21°C under 16h light / 8h dark conditions in a growth cabinet for seven days. Next, four seedlings were transferred to 12 cm square plates containing MS medium, which were placed vertically in a growth cabinet for 7 days at 21°C under a 16h light and 8h dark regime. Next, individual seedlings were inoculated with 180 infective J2s of *M. incognita* per plant and incubated horizontally at 24°C in the dark. Samples of twelve whole root systems were collected at the time of inoculation, at 7 days after inoculation with *M. incognita*, and at 7 days after being mock-inoculated. The samples were stored at -80 degrees Celsius until further use.

Root phenotypes

Arabidopsis seedlings were allowed to germinate and grow for 14 days on MS medium as described above for the nematode bioassays. To determine the total root length and number of root tips of the seedlings, the complete plants were transferred from the media onto a plastic tray with water. Next, the leaves of the seedlings were removed and the roots were spread out over the surface of the tray. The total root length was measured using WinRHIZO package for Arabidopsis (EPSON perfection V800, WinRHIZO pro2015, Regent Instruments Inc.). The number of root tips was counted manually based on the scan that was made using WinRHIZO. Differences in the total root length per seedling in cm and number of root tips were statistically analysed with a two-way ANOVA and post-hoc Tukey's HSD test in R (P-value<0.05).

Genome wide association mapping

The GWA mapping was performed as described previously (Warmerdam *et al.*, 2018). In short, the GWAS function from the "R"-package rrBLUP was used for applying the EMMAX method for kinship-based GWAS using REML (Kang *et al.*, 2010; Yu and Buckler, 2006). The kinship matrix was calculated using the A.mat function (Endelman, 2011), using the 214051 SNPs of the panel as input (Thoen *et al.*, 2017). Linkage was calculated as the R^2 (from the Pearson correlation) between marker pairs in R (version 3.3.3, x64).

Heritability estimations

The narrow-sense heritability was estimated using EMMA (Kang *et al.*, 2008; Rockman *et al.*, 2010). We used the kinship matrix (minimum allele frequency >5%) of the strains to estimate the genotypic variation (V_G) and the residual variance (V_E) using REML. These were used to calculate the narrow-sense heritability by

$$h^2 = \frac{V_G}{V_G + V_E}$$

Where h^2 is the narrow-sense heritability. In order to determine the amount of heritable variation explained by the set of SNPs detected, the inclusion threshold was lowered from –log10(p) = 6.1 to 4.0 in steps of 0.1. For each set of SNPs, an additive ANOVA model was constructed to determine the phenotypic variation captured by the set of SNPs. As measure of model complexity versus explanatory power, the Bayesian information criterion (BIC) was calculated using the BIC function in R.

RNA isolation

Expression analysis for gene of interest was performed on the stored root samples produced during the nematode infection study. Whole root systems were cut from aerial parts of the seedlings and snap frozen in liquid nitrogen. Total RNA was isolated from whole roots of twelve 14-days-old plants of *erf6-1*, *duf239-1*, *uch3-1*, *rab1c-1* and Col-0 wildtype. The frozen root systems were homogenized using TissueLyser (Qiagen) two times for 30 seconds. Total RNA was extracted from 100 mg of the homogenate with the Maxwell Plant RNA kit (Promega Corporation) using the Maxwell 16 Robot (Promega Corporation) according to the manufacturer's protocol. A DNAse treatment was included in the Maxwell Robot processing. The amount of total RNA per sample was determined by spectrophotometer ND-1000 (Isogen Life Science).

Reverse transcription quantitative PCR

For reverse transcription quantitative PCR (RT-qPCR) first strand cDNA was synthesized from total RNA using Superscript III first strand synthesis system (Invitrogen) according to manufacturer's protocol. Samples were analysed by quantitative PCR using Absolute SYBR Green Fluorescein mix (Thermo Fisher Scientific). cDNA matching *Arabidopsis thaliana* elongation factor 1 alpha was amplified as a reference for constitutive expression using primers as indicated in table S2 (Czechowski *et al.*, 2004). To quantify the expression level for the gene of interest specific gene primers were used which were all checked for amplification efficiency (Table S2). For the RT-qPCR 5 ng cDNA was used with the following conditions: 15 min at 95°C, forty cycles of 30 s at 95°C, 30 s at 62 °C, and 30 s at 72°C, and a final incubation of 5 min at 72°C. Relative expression ratio between the gene of interest and the reference gene was calculated as described elsewhere (Pfaffl, 2001). Relative expression ratio was statistically analysed for significance with a two-way ANOVA and post-hoc Tukey's HSD test in R (P-value<0.05).

Microarray analysis

For microarray analysis, approximately 825 ng of total RNA of each sample was used for gene expression analysis on an *Arabidopsis* V4 Gene Expression Microarray (4x44K, Agilent Technologies). The probes were re-blasted against TAIR11 using the BLASTN function of the command line blast tool (version 2.6.0, win64). The default settings were used and the top-hit was used as probe annotation. Probes with multiple hits were censored (Camacho *et al.*, 2009). The total RNA was fluorescently labelled for two-colour microarray analysis and subsequently used for hybridization to the probes on the slides according to the manufacturer's protocols (QuickAmp Protocol, Agilent Technologies). Binding of fluorescent RNA to the probes on the microarray was measured with a High-Resolution C Microarray

Scanner and Feature Extraction Software (Agilent). Four independent replicates were made of roots of Col-0 at the time of inoculation (0 dpi), Col-0 at 7 days after inoculation, and *erf6-1* at 7 days after inoculation. Three independent replicates were made of roots of Col-0 at 7 days after inoculation, *erf6-1* at the time of inoculation (0 dpi), and *erf6-1* at 7 days after inoculation. All microarray data has been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6711.

After scanning the microarrays, the spot intensities were not background corrected prior to analysis (Zahurak *et al.*, 2007). Gene expression profiles were normalized using the Loess (within array normalization) and the quantile method (between array normalization) (Smyth and Speed, 2003). The normalized intensities were log2-transformed for further analysis.

A linear model was used to identify differentially expressed genes in a side-by-side comparison. The following treatments were compared: Uninfected roots of Col-0 versus *erf6-1* plants at the time of inoculation, uninfected roots of Col-0 versus *erf6-1* 7 days after mock inoculation, infected roots of Col-0 versus *erf6-1* plants at 7 days after inoculation and uninfected roots versus infected roots of Col-0 plant at 7 days after the time of inoculation.

We used the linear model

$$E_i = T_i + \text{error}$$

wherein the log2-normalized expression (E) of spot i (i in 1, 2, ..., 45220) was explained over treatment (T). Afterwards, the obtained significances were corrected for multiple testing using the false discovery rate (FDR) procedure in p.adjust obtaining q-values (Benjamini and Hochberg, 1995).

Enrichments were calculated using a hypergeometric test, as provided in "R". The following databases used were mined from TAIR11 (Berardini *et al.*, 2015; Lamesch *et al.*, 2012). Gene ontology, Gene ontology slim, gene classes, phenotypes. Furthermore, the MapMan gene ontology database (based on TAIR10) was used (Thimm *et al.*, 2004). Enrichments were done on gene annotations, not on spots (*e.g.* a gene covered by multiple spots is counted as one). Gene groups were filtered as follows: the group needed to consist of at least 3 genes and the overlap should be 2 at a minimum.

Results

Stepwise lowering of the threshold for significant associations in our original analysis from −log10(*P*) = 5 to −log10(*P*) = 4 revealed 28 additional single nucleotide polymorphisms (SNPs), resulting in a total number of 36 SNPs significantly associated with the number of egg masses of *M. incognita* per plant (Fig. S1; Table S1, see Supporting Information). Of this set of 36 SNPs, 12 showed strong linkage ($R^2 > 0.8$): four SNPs around position Chr1:28.18Mb, six SNPs around position Chr5:6.26Mb and two SNPs around position Chr5:22.68Mb (Fig. S2, see Supporting Information). By using the predicted average decline in linkage disequilibrium (LD) in Arabidopsis within 10 kb (Kim et al., 2007), we aggregated the remaining SNP markers into 19 QTLs (Table 1), two of which have already been described in more detail in Warmerdam et al. (2018). It should be noted that multiple SNPs aggregated into the same QTL when their LD regions overlapped, resulting in four QTLs spanning more than 20 kb. Altogether, by accepting −log10(*P*) = 4 as a threshold for significance, we could map all of the allelic variation (in 19 QTLs) underlying the heritable phenotypic variation in the reproductive rate of *M. incognita* in our population of Arabidopsis lines.

The cost associated with the lowering of the threshold for significance is an increase in the false discovery rate to 0.55. To challenge this high risk of false positive SNPs, we investigated QTL13 marked by only one SNP (Chr4.145290) with significance on the threshold value [−log10(*P*) = 4] and within the subset with the smallest effect size (3.83 egg masses). SNP marker Chr4.145290 is located at genomic position 9765013 of chromosome 4 inside the fifth predicted intron of gene model At4G17505 (Fig. 1). At4G17505 is annotated as a gene encoding a putative protein domain with unknown function (*DUF239*).

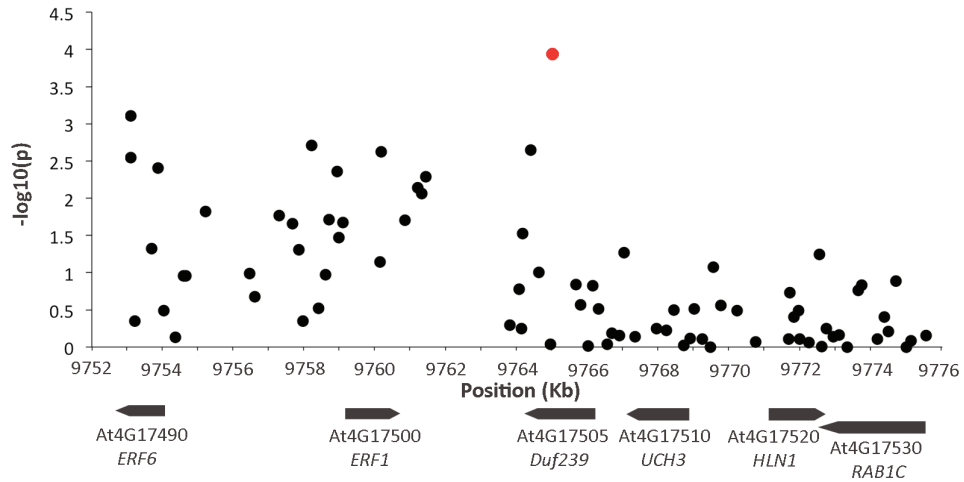

Fig. 1. Genome region surrounding SNP marker Chr4.145290 (red dot) on chromosome 4 of Arabidopsis. The black dots represent the SNPs located in the 10 Kb region up- and downstream of Chr4.145290. The vector arrows represent the predicted genes in this region with accessions numbers and abbreviations of the corresponding names.

Table 1. Details of 36 SNPs significantly associated with reproductive success of *M. incognita* aggregated into 19 QTLS. QTL1 to 4 were previously identified (Warmerdam *et al.*, 2018). [+]SNP ratio is the ratio of alleles within the population of 349 Arabidopsis natural inbred lines. -Log10(P) si the level of significance of the association of individual SNP and number of egg masses of *M. incognita* per plant.

QTL	Chromosome	Position (bp)	Alleles	SNP ratio	-Log10(P)	Effect size	SNP located in gene	Genes in 20 Kb LD
5	1	10540036	A:G	34:315	4	5.95	At1G30050	At1G30030, At1G30040, At1G30050, At1G30060, At1G30070, At1G30080
6	1	17480468	C:T	149:200	4.5	3.40	At1G47570	At1G47560, At1G47565, At1G47570, At1G47578, At1G47580, At1G47590, At1G47595
1	1	28186622	G:T	268:81	4	4.86	At1G75080	At1G75030, At1G75040, At1G75050, At1G75060, At1G75070 At1G75080, At1G75090, At1G75100, At1G75110, At1G75120, At1G75130
		28187392	C:G	278:71	5.1	5.62	At1G75080	
		28187959	C:T	247:102	4.7	4.96	At1G75080/ At1G17090	
		28187978	A:T	247:102	4.7	4.96	At1G75080/ At1G17090	
		28188103	C:T	104:245	4.4	4.73	At1G75090	
		28188151	C:G	102:247	5.1	5.10	At1G75090	
7	2	14465496	A:C	107:242	4	4.39	NA	At2G34230, At2G34238, At2G34240, At2G34250, At2G34260, At2G34270, At4G34280, At2G34290, At2G34300
8	2	14848612	G:T	150:199	4	4.28	At2G35250	At2G35215, At2G35220, At2G35230, At2G35240, At2G35250. At2G35260, At2G35270
9	2	18338888	A:G	211:138	4.6	4.56	At2G44440	At2G44400, At2G44410, At2G44420, At2G44430, At2G44440, At2G44450, At2G44460
10	3	287272	C:T	131:218	4.4	4.19	At3G01800	At3G01770, At3G01780, At3G01790, At3G01800, At3G01810, At3G01820, At3G01830. At3G01840

Table 1. continued

QTL	Chromosome	Position (bp)	Alleles	SNP ratio	-Log10(P)	Effect size	SNP located in gene	Genes in 20 Kb LD
11	3	21020421	C:G	130:219	4.6	3.48	At3G56750	At3G56720, At3G56740, At3G56750, At3G56760, At3G770
12	4	7211220	C:T	104:245	4.2	4.15	At4G12030	At4G12010, At4G12020, At4G12030, At4G12040, At4G12050
13	4	9765013	C:T	133:216	4	3.83	At4G17505	At4G17490, At4G17500, At4G17505, At4G17510, At4G17520, At4G17530
14	4	9843096	C:T	96:253	4.1	4.30	At4G17680	At4G17660, At4G17670, At4G17670, At4G17680, At4G17690, At4G17695
15	4	14391100	A:G	108:241	4.3	3.36	NA	At4G29160, At4G29170, At4G29180, At4G29190, At4G29200, At4G29210
2	5	6250487	A:G	139:210	4	4.62	At5G18740	At5G18700, At5G18710, At5G18720, At5G18730, At5G18740, At5G18748, At5G18750, At5G18755, At5G18760, At5G18770, At5G18780, At5G18790, At5G187800, At5G18810, At5G18820
		6253982	C:T	202:147	4.7	4.84	NA	
		6261603	A:T	109:240	4.5	4.88	At5G18770	
		6263591	A:T	139:210	6.1	5.31	At5G18780	
		6263577	A:T	139:210	6.1	5.31	At5G18780	
		6263644	A:T	140:209	5.7	5.18	At5G18780	
		6263678	C:G	139:210	6.1	5.31	At5G18780	
16	5	14116311	A:T	253:96	4	5.05	At5G35965	At5G35650, At5G35960, At5G35965, At5G35970
17	5	14139771	C:G	218:131	4	4.54	NA	At5G35995, At5G36001, At5G36002, At5G36005, At5G36010, At5G36015. At5G36020, At5G36030
		14152182	C:T	179:170	4.7	4.65	NA	
		14156930	G:T	210:139	4.5	4.31	At5G36015	
3	5	14913458	A:T	288:61	5.5	5.35	At5G37540	At5G37520, At5G37530, At5G37540, At5G37550, At5G37560
4	5	15904331	C:T	290:59	6	4.47	At5G39740	At5G39700, At5G39710, At5G39720, At5G39730, At5G39740, At5G39750, At5G39760
18	5	22583411	C:G	19:330	4.6	8.30	At5G55800	At5G55770, At5G55780, At5G55790, At5G55800, At5G55810, At5G55820, At5G55830, At5G55835, At5G55840, At5G55850, At5G55855, At5G55856, At5G55860
		22601633	A:C	17:332	4	7.95	At5G55840	
		22602265	G:T	17:332	4.3	8.07	At5G55840	
19	5	22668012	A:G	63:286	4.9	5.24	At5G55970	At5G55940, At5G55950, At5G55960, At5G55970, At5G55980, At5G55990, At5G56000, At5G56010, At5G56020, At5G56030 At5G56040
		22683923	C:G	31:318	4	5.72	At5G56010	
		22684613	C:T	29:320	4.1	5.89	At5G56020	

With the average LD decline in Arabidopsis (±10 kb) in mind, we identified in total six predicted genes that could be linked to SNP marker Chr4.145290, and which could therefore contribute to the observed variation in the number of egg masses of *M. incognita* per plant at 6 weeks post-inoculation.

ERF6 regulates the susceptibility of Arabidopsis to M. incognita

To determine which of the genes predicted for QTL13 could play a role in the reproductive success of *M. incognita* in Arabidopsis, we investigated several homozygous T-DNA insertion lines available for this locus. First, we tested an Arabidopsis line harbouring a T-DNA insert in *DUF239*, which harbours SNP marker Chr4.145290 (*DUF239*), but which was not significantly altered in susceptibility to *M. incognita* compared with wild-type Arabidopsis plants (Fig. 2B). The T-DNA insert in this line is located at the predicted 5'-end of the coding sequence of At4G17505, but this does not seem to affect the amplification of reverse transcribed mRNA matching the sequence further to the 3'-end (Fig. S2B). It should be noted that the gene model At4G17505 harbouring SNP marker Chr4.145290 is based on predictions only and has not been experimentally confirmed with cDNA (TAIR11).

An Arabidopsis line harbouring a T-DNA insert in At4G17490, encoding *ERF6*, contained an average of 28% more egg masses per plant than the corresponding wild-type Arabidopsis

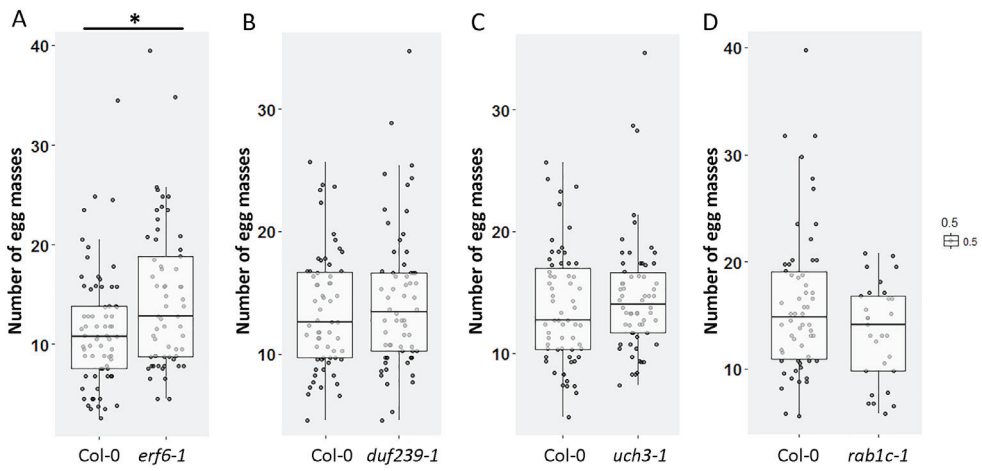

Figure 2. The number of egg masses of *M. incognita* per plant on homozygous T-DNA insertion mutants *erf6-1 (*ERF6*), duf239-1 (*DUF239*), uch3-1 (*UCH3*) and rab1c-1 (*RAB1C*)* of Arabidopsis at six weeks after inoculation. (A) Number of egg masses on *erf6-1* and wildtype Arabidopsis plants. (B) Number of egg masses on *duf239-1* and wildtype Arabidopsis plants. (C) Number of egg masses on *uch3-1* and wildtype Arabidopsis plants. (D) Number of egg masses per plant on *rab1c-1* and wildtype Arabidopsis plants. Data points are from three independent experiments with a total of n>30. Data is statistically analysed using ANOVA and post hoc Tukey's HSD test (* P<0.05).

plants (Figs 2A and S3C, see Supporting Information). The mRNA level of the *ERF6* transcript in this mutant line (*erf6-1*) was significantly reduced, suggesting that the increase in susceptibility is indeed caused by *ERF6* (Fig S2C). Unfortunately, for the neighbouring locus At4G17500, encoding *ETHYLENE RESPONSE FACTOR 1a* (*ERF1a*), no homozygous T-DNA insertion line was available. It should be noted that *ERF1a* is sometimes also referred to as *AtERF1* and is distinct from *ERF1* (At3G23240), which is located on chromosome 3 (Nakano et al., 2006).

Two T-DNA insertion lines for genes located to the right of marker Chr4.145290 (i.e. At4G17510 and At4G17530; Fig. 1) did not show a significantly different number of egg masses per plant when compared with wild-type Arabidopsis plants (Fig. 2C,D). The homozygous T-DNA insert at the 3'-end of the predicted coding sequence of At4G17510, encoding *UBIQUITIN C-TERMINAL HYDROLASE 3* (*UCH3*), interrupted the open reading frame, but did not significantly alter the amplification of mRNA upstream of the insert (Fig. S3D). The T-DNA insert in locus At4G17530, encoding *RAB GTPASE HOMOLOG 1C* (*RAB1C*), reduced the transcript level of this gene (Fig. S3E). Seeds of Arabidopsis lines harbouring a T-DNA insert in At4G17520, encoding *HYALURONAN/mRNA BINDING FAMILY*

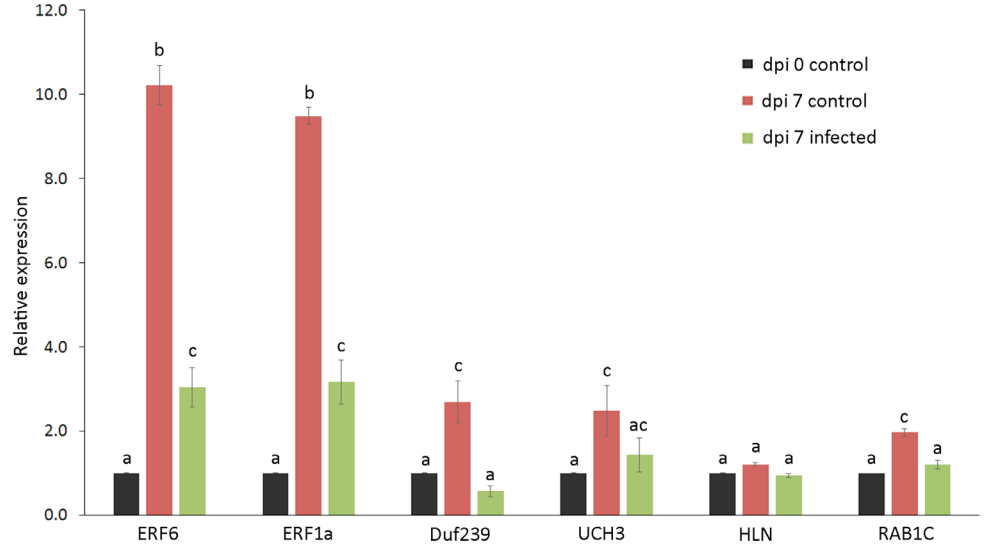

Figure 3. Relative expression of *ERF6*, *ERF1a*, *Duf239*, *UCH3*, *HLN* and *RAB1C* in roots of wildtype Arabidopsis Col-0 plants upon infection with *M. incognita*. Data reflect gene expression levels determined with reverse transcription quantitative PCR in whole roots collected at the time of inoculation with *M. incognita* (dpi 0 control), in whole roots collected at 7 days after mock-inoculation (dpi 7 control), and 7 days after inoculation with *M. incognita* (dpi 7 infected). The bars represent average values relative to the expression at 0 dpi. The data was based on three independent biological samples with three technical replicates per sample. Error bars represent standard error of the mean. Different characters indicate statistical differences for each gene of interest separately as determined by ANOVA with post-hoc Tukey's HSD (P-value<0.05).

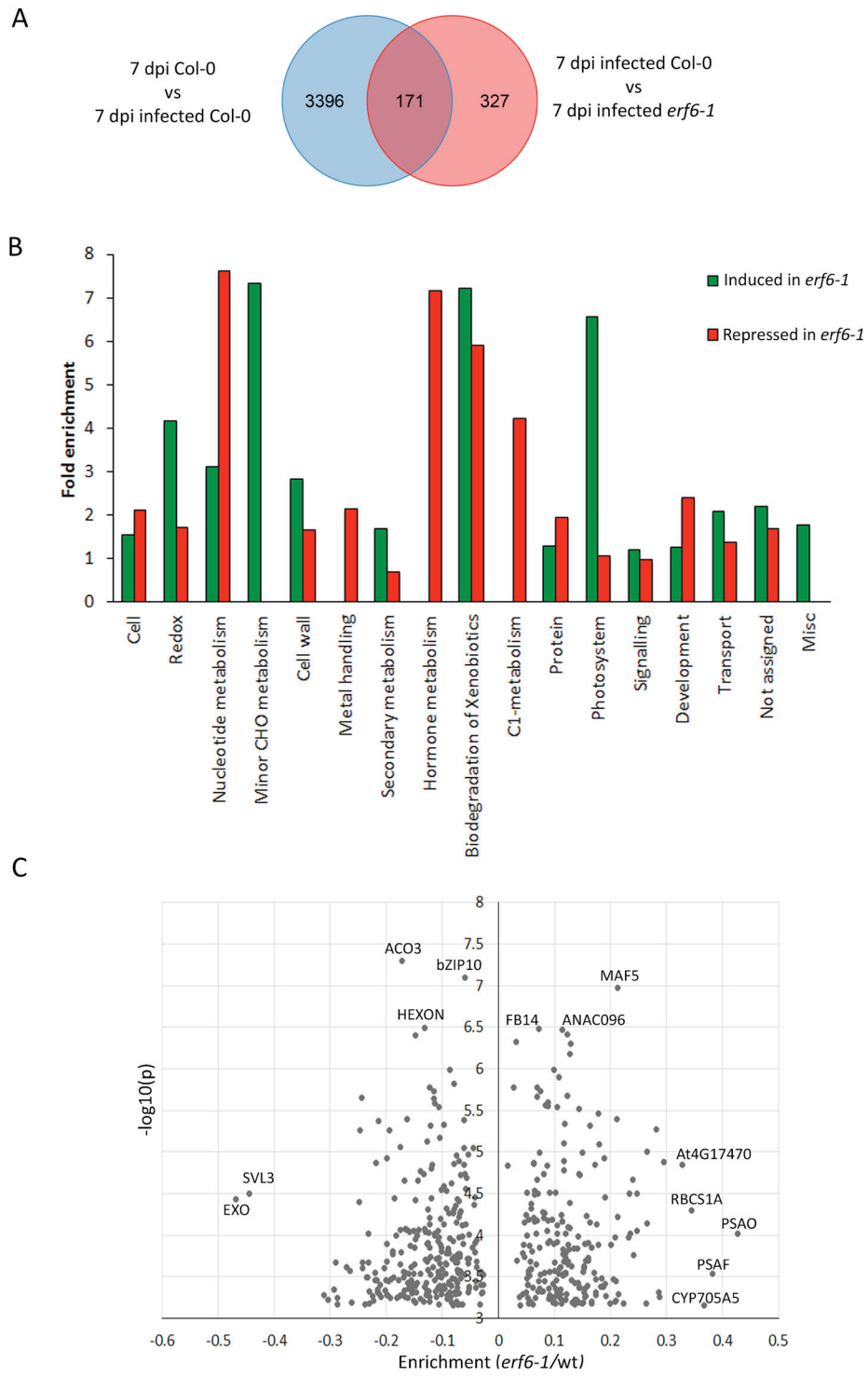

Figure 4. Differential expression of genes in wildtype Arabidopsis Col-0 and *erf6-1* mutant at 7 days after inoculation (dpi) with *M. incognita*. (A) Venn diagram of the number of differently expressed genes upon inoculation with *M. incognita* in wildtype Arabidopsis plants (7 dpi mock Col-0 vs 7 dpi infected Col-0) and *erf6-1* mutant plants (7 dpi infected Col-0 vs 7 dpi infected *erf6-1*). (B) Functional classification of 498 differently expressed genes between *erf6-1* and wildtype Arabidopsis plants at 7 dpi of *M. incognita* into MapMan bins. On the x-axis are the main BINs to which the differently expressed genes belong. The Y-axis represents the fold enrichment of the number of genes within a bin. (C) Volcano plot of the 498 differently expressed genes in nematode-infected roots of *erf6-1* mutant plants and wildtype Arabidopsis Col-0 plants. The x-axis reflects the enrichment in mean-normalized expression levels and the Y-axis indicates the level of significance (–log10(P)). Genes strongly regulated in *erf6-1* mutant plants (mean-normalized enrichment <-0.30 or >0.30) and with high statistically support (-log10(P)>6.5) are labelled with their abbreviated gene names. As expected, *ERF6* is by far the most down-regulated gene in *erf6-1* mutant plants (mean-normalized enrichment factor of -1.97) but is not shown in the plot.

PROTEIN (*HLN*), showed an aberrant germination phenotype and could therefore not be used in nematode bioassays. We therefore concluded that, for QTL13, only *ERF6* is likely to be involved in the reproductive success of *M. incognita* in Arabidopsis. Importantly, the number of root tips and the total root length of seedlings of the *erf6-1* T-DNA mutant line were not significantly different from those of wild-type Arabidopsis plants (Fig. S4, see Supporting Information). The increase in reproductive success of *M. incognita* in this mutant can therefore not be attributed to significant changes in root morphology.

To find further support for the involvement of QTL13 in nematode infections, we analysed the expression of the six genes by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in whole Arabidopsis roots at 7 days post-inoculation (dpi) with *M. incognita*. We also included samples of wild-type Arabidopsis seedlings at the time of inoculation to assess whether these genes undergo a significant developmental regulation in roots in the absence of root-knot nematodes. Both *ERF6* and *ERF1a* were strongly up-regulated in non-infected plants during the first 7 days after mock inoculation, but were significantly repressed in plants infected with *M. incognita* (Fig. 3). The relative expression of *DUF239* followed largely the same pattern over the three samples. The relative expression of *UCH3* and *HLN* was not significantly different between infected and non-infected Arabidopsis roots. The relative expression of *RAB1C* showed a small, but significant, difference between infected and non-infected Arabidopsis roots at 7 dpi.

ERF6 affects the expression of abiotic stress response genes

Our data showed that the transcription factor ERF6 most probably regulates the susceptibility of Arabidopsis to *M. incognita* infection. To gain more insights into the genes regulated by ERF6, we studied changes in the transcriptome of whole roots of the *erf6-1*mutant and the corresponding wild-type Arabidopsis at the time of inoculation (0 dpi) and at 7 dpi with *M. incognita* using gene expression microarrays. We also included mock-inoculated seedlings to monitor developmentally regulated genes in both the *erf6-1* mutant and

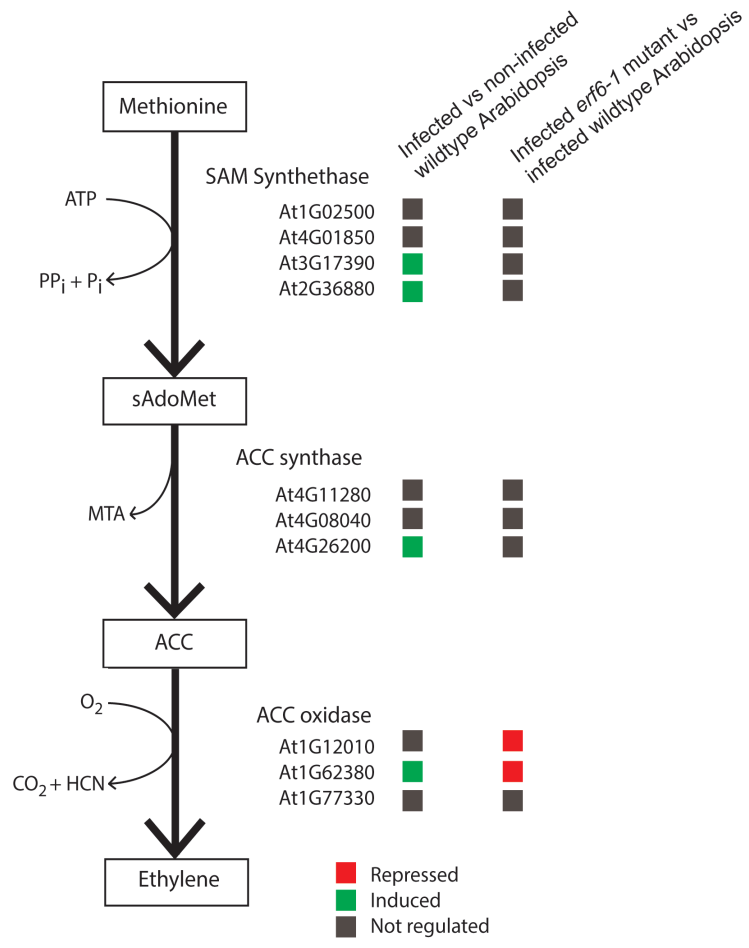

Figure 5. Differential regulation of genes encoding enzymes involved in ethylene biosynthesis pathway in upon infection in whole roots of *erf6-1* mutant and wildtype Arabidopsis plants at seven days after inoculation with *M. incognita*. The coloured boxes indicate induction (green) or repression (red) of gene expression in a comparison between nematode-infected versus non-infected roots of wildtype Arabidopsis plants (left column). The boxes in the right column indicate induction or repression of gene expression in nematode-infected roots of *erf6-1* versus wildtype Arabidopsis plants.

wild-type Arabidopsis plants. We found no differentially expressed genes between the *erf6-1* mutant and the wild-type plants at the time of inoculation and 7 days later in non-infected plants ($q < 0.05$). This showed that *ERF6* does not have a significant impact on global gene expression in developing roots of non-infected Arabidopsis seedlings in the first 7 days after inoculation.

As expected, *M. incognita* had a profound effect on the transcriptome in roots of wild-type Arabidopsis at 7 dpi. We found 3567 differentially expressed genes in the comparison between infected and non-infected roots of wild-type Arabidopsis. Of this set, 171 genes were differentially expressed between nematode-infected roots of the *erf6-1* mutant and wild-type Arabidopsis. Lastly, we identified 327 genes that were differentially expressed in nematode-infected roots of the *erf6-1* mutant, but not in infected roots of wild-type Arabidopsis plants. In conclusion, ERF6 has a significant impact on the expression of 498 genes in nematode-infected roots of Arabidopsis seedlings at 7 dpi (Fig. 4A; Table S4, see Supporting Information).

To determine which cellular processes are most probably affected by ERF6 in nematode-infected roots of Arabidopsis, we first performed an enrichment analysis using MapMan bins (Thimm et al., 2004). The set of 498 genes differentially regulated by ERF6 in nematode-infected roots of Arabidopsis was enriched for processes referred to as redox, nucleotide metabolism, minor carbohydrate metabolism, biodegradation of xenobiotics, C1 metabolism, photosystem and hormone metabolism (fold enrichment > 2.5) (Fig. 4B). MapMan enrichment analysis uses genome annotation terms, but does not include the actual fold changes or statistical significances of the differences in transcripts per gene. We therefore focused further on genes that stood out because of an exceptionally large change in expression level (i.e. mean normalized change in probe intensity of <−0.3 or >0.3), because of high statistical support of the changes (−log10(P)

> 6.5), or both (Fig. 4C). The application of these criteria resulted in a list of 15 genes with an overrepresentation of transcription factors, photosystem components and mediators of abiotic stress responses (Table 2).

ERF6 is thought to be involved in tolerance to abiotic stress regulated by reactive oxygen species (ROS) and 1-aminocyclopropane-1-carboxylic acid (ACC; Sewelam et al., 2013). Strikingly, the gene with highest statistical support for being down-regulated in a comparison between nematode-infected roots of the *erf6-1* mutant line and wild-type Arabidopsis encodes 1-aminocyclopropane-1-carboxylate oxidase 3 (At2G12010; ACC oxidase 3 or ACO3), which regulates the accumulation of ACC in plant cells by its conversion into ethylene. ACC is derived from methionine and *S*-adenosyl-methionine (SAM) in a series of reactions catalysed by SAM synthetases and ACC synthases. In a comparison between infected and non-infected roots of wild-type Arabidopsis plants, we observed that nematode infection is associated with an up-regulation of SAM synthetase 3 and 4, ACC synthase 7 (ACS7) and ACC oxidase 2 (ACO2), but not ACO3 (Fig. 5; Table S3, see Supporting Information). However, in the comparison between nematode-infected roots of the *erf6-1*mutant and wild-type Arabidopsis, we observed a down-regulation of both ACO2 and ACO3 at 7 dpi with *M. incognita* in our gene expression array data (Fig. 5).

Table 2. The fifteen genes either most strongly or significantly regulated in roots of *erf6-1* and wildtype Col-0 plants seven days after inoculation with *M. incognita*. Genes are indicated with their locus identifier (in TAIR). Relative expression to the mean (Log_2-value). Level of significance before (-log10(P)). Involvement in response to different types of stress.

Gene ID	Relative expression	-Log10 (P)	Bon-ferroni	Description	Stress response‡
AT1G12010	-0.17259	7.29	0.0009	1-Aminocyclopropane-1-carboxylate oxidase 3 domain (ACO3)	Oxidative stress (Sewelam *et al.*, 2013; Wang *et al.*, 2013); Osmotic stress (Dubois *et al.*, 2015).
AT4G02640	-0.05964	7.10	0.0009	Basic leucine zipper transcription factor (bZIP10)	Oxidative stress (Kaminaka *et al.*, 2006)
AT5G65080	0.21294	6.97	0.0010	MADS-box transcription factor (MAF5)	Unknown
AT5G54850	-0.13181	6.49	0.0016	Hexon like	Unknown
AT1G20800	0.07244	6.48	0.0016	F-box family protein (FB14)	Unknown
AT5G46590	0.11422	6.47	0.0016	NAC transcription factor ANAC096	Dehydration and osmotic stress (Xu *et al.*, 2013)
AT4G08950	-0.46799	4.43	0.0433	Phosphate-responsive 1 family protein (EXO)	Salt stress (Renault *et al.*, 2013) Oxidative stress (Charron *et al.*, 2008)
AT3G20520	-0.44503	4.49	0.0114	Glycerophosphodiester phosphodiesterase-like family protein (SVL3)	Salt stress (Ma *et al.*, 2006)
AT2G29470	-0.31112	3.28	0.0432	Glutathione S-transferase tau 3 (GSTU3)	Toxic catabolic process; Oxidative stress (Loeffler *et al.*, 2005)
AT1G70260	-0.30337	3.22	0.0465	Nodulin MtN21 /EamA-like transporter family protein (RTP1)	Oxidative stress (Pan *et al.*, 2016)
AT4G17470	0.32912	4.85	0.0076	Alpha/beta-Hydrolases superfamily protein	Unknown.
AT1G67090	0.34508	4.29	0.0148	Ribulose bisphosphate carboxylase small chain 1A (RBCS1A)	Unknown (chloroplast)
AT5G47990	0.36753	3.16	0.0497	Cytochrome P450, family 705, subfamily A, polypeptide 5 (CYP705A5)	Unknown (chloroplast)
AT1G31330	0.38302	3.54	0.0331	Photosystem I subunit F (PSAF)	Unknown (chloroplast)
AT1G08380	0.42666	4.01	0.0196	Photosystem I subunit O (PSAO)	Unknown (chloroplast)

Discussion

Recently, we have used GWA mapping to resolve the architecture of susceptibility to *M. incognita* in a population of 340 Arabidopsis natural inbred lines (Warmerdam et al., 2018). We linked the susceptibility to *M. incognita* to allelic variation in only four loci on two chromosomes of Arabidopsis, which was below our expectations. We therefore reasoned that, by using a relatively high threshold for significance (i.e. –log10(*P*) > 5) in our original analyses (Warmerdam *et al*., 2018), we might have disregarded valuable signals. Moreover, the SNPs identified in our original analysis could only explain 50% of the additive heritable phenotypic variation, providing further support for the idea that, by relaxing the stringency of our analysis, we might be able to resolve more components of the architecture of susceptibility. Here, we showed that, by accepting $-\log_{10}(P) \geq 4$ as the threshold for significant associations between SNPs in Arabidopsis and the reproductive success of *M. incognita*, we could resolve all of the heritable phenotypic variation in our population of Arabidopsis lines (Table S1).

As the false discovery rate increases by lowering the threshold of significance for association mapping, we challenged the risk of pursuing false positives by further investigating QTL13. The association of susceptibility with QTL13 was based on a single SNP with a significance on the threshold value [−log10(*P*) = 4] and with the smallest effect size of all SNPs meeting this criterion. SNP Chr4.145290, marking QTL13, is located within the predicted coding sequence AtG17505 (*DUF239*). However, we found no further evidence to conclude that allelic variation in *DUF239* contributes to the variation in susceptibility of Arabidopsis to *M. incognita*.

Five other genes are located within the 20-kb region around SNP Chr4.145290 and could therefore also be causally linked to the susceptibility to *M. incognita* (Kim *et al*., 2007*)*. However, we noted that Chr4.145290 marks a sharp boundary between a region harbouring SNPs with relatively high −log10(*P*) values at one side and a region with relatively low −log10(*P*) values at the other (Fig. 1). This suggests that the decay in LD may not be evenly distributed around Chr4.145290, and that the three genes (i.e. *UCH13*, *HLN1* and *RAB1C*) located in the flanking region with lower statistical support are less likely to be causally linked to susceptibility (Fig. 2). Indeed, our analyses with T-DNA mutant lines and the expression of the genes provide no further evidence that *UCH13*, *HLN1* and *RAB1C* are involved in the susceptibility of Arabidopsis to *M. incognita*.

The two remaining genes in QTL13, *ERF1a* and *ERF6*, belong to the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) superfamily of transcription factors. Members of the AP2/ERF superfamily are divided into four families based on the number of AP2/ERF domain repeats. Together with 120 other relatives, ERF1a and ERF6 form the largest class of AP2/ERF transcription factors in Arabidopsis. ERF family members typically harbour only one AP2/ERF domain of about 70 amino acids, which binds DNA at an AGCCGCC motif (GCC-box)

in promoter regions of the target genes. The ERF family is further divided into 12 subgroups based on conserved motifs outside the AP2/ERF domain, some of which are involved in the activation and deactivation of the transcription factors. ERF1a belongs to subgroup XIa, whereas ERF6 is assigned to subgroup IXb. Our data showed that both ERF1a and ERF6 are strongly down-regulated in association with nematode infections in roots of Arabidopsis seedlings at 7 dpi (Fig. 3). Unfortunately, no homozygous T-DNA insertion mutant line was available for the *ERF1a* gene in the Arabidopsis stock centres. Therefore, we cannot exclude the possibility that allelic variation in *ERF1a* contributes to the variation in reproductive success of *M. incognita* in our population of Arabidopsis inbred lines.

The significantly higher number of egg masses of *M. incognita* in the knockout mutant line *erf6-1*, when compared with wild-type Arabidopsis plants, showed that ERF6 most probably functions as a susceptibility gene for *M. incognita* in Arabidopsis (Fig. 2). *ERF6* is thought to be functionally redundant with *ERF5*, which is the closest relative of *ERF6* within ERF subgroup IXb (Moffat *et al*., 2012; Son *et al*., 2012). However, our data suggest that *ERF5* and *ERF6* are not redundant in co-regulating the susceptibility to *M. incognita* in Arabidopsis. The single *erf6-1* mutant line, harbouring an intact *ERF5* gene, was more susceptible to nematodes. Previously, the *erf6-1* mutant has been shown to be more susceptible to the virulent *Pseudomonas syringae* DC3000 strain when compared with wild-type Arabidopsis plants, which also points to the functional diversification of both genes (Son *et al*., 2012). Furthermore, if *ERF5* and *ERF6* function as redundant susceptibility genes for *M. incognita*infections in Arabidopsis, we would probably have found significant associations linked to *ERF5* in our GWA analyses. We observed no such association between the number of egg masses of *M. incognita* per plant and SNPs in LD with *ERF5* on chromosome 5. We therefore conclude that allelic variation in *ERF6*, but not in *ERF5*, may contribute to the quantitative variation in reproductive success of *M. incognita* in our population of Arabidopsis inbred lines.

The transcriptional activity of ERF6 in Arabidopsis is regulated by MITOGEN-ACTIVATED PROTEIN KINASE3 (MPK3)/MPK6 in response to biotic and abiotic stress. MPK3/MPK6-mediated phosphorylation stabilizes ERF6 and enables its targeting to the nucleus, where it binds to the GCC-box in promoters of target genes. The GCC-box is found in promoters of pathogenesis-related genes typically associated with ethylene/jasmonic acid-dependent responses, and is a common target of multiple ERF transcription factors belonging to different subgroups (Fujimoto *et al*., 2000; Song *et al*., 2005). It is therefore possible that the enhanced susceptibility of the *erf6-1* mutant line is caused by weakened plant defence responses. However, our MapMan enrichment analysis of genes differentially expressed in nematode-infected roots of the *erf6-1* mutant vs. wild-type Arabidopsis does not point to alterations in responses to biotic stresses (Fig. 4B). Furthermore, the expression of specific pathogenesis-related genes known to be either repressed or activated by ERF transcription factors (e.g. PR1 [At2G14160], PR2 [At3G57260], PR3 [At3G12520], PR4 [At3G04720],

PDF1.1 [At1G75830] and PDF1.2 [At5G44420]) was not significantly altered in nematode-infected *erf6-1* mutant plants. We therefore have no evidence to conclude that the increased susceptibility to *M. incognita* of the *erf6-1* mutant line is related to suppressed plant defence. Nonetheless, it should be noted that we only assessed gene expression at 7 dpi and we cannot exclude the possibility that ERF6 regulates the expression of defence-related genes at earlier and later time points.

However, many of the genes differentially regulated in the nematode-infected roots of *erf6-1* plants at 7 dpi are involved in responses to abiotic stresses (Fig. 4C; Table 1). This aligns well with earlier observations of ERF6 as a transcriptional regulator of genes involved in tolerance to osmotic stress (Dubois *et al*., 2013, 2015), ROS (Sewelam *et al*., 2013; Wang *et al*., 2013), high light treatment (Moore *et al*., 2014; Wang *et al*., 2013) and cold (Wang *et al*., 2013). Mild osmotic stress is thought to induce elevated cellular levels of ACC, which could, in a similar fashion to ROS, activate MPK3/MPK6 and, further downstream, ERF6 (Dubois *et al*., 2015; Wang *et al*., 2013). In addition, elevated levels of ACC are also known to induce *ERF6* expression in Arabidopsis seedlings (Son *et al.*, 2012), but it is not known whether this requires the conversion of ACC into ethylene and whether it is ethylene that regulates the expression of ERF6. Recently, it has been shown that ACC itself can function as a signalling molecule and is able to regulate growth and development in plants independent from ethylene (reviewed in Van de Poel and Van Der Straeten, 2014). Our data suggest that ERF6 may affect the conversion of ACC into ethylene in nematode-infected roots by regulating the expression of ACO2 and ACO3 (Fig. 5; Table 1), which could point to a key role for ethylene in ERF6-mediated susceptibility to *M. incognita*. However, unlike several other ERF transcription factors, ERF6-mediated expression of target genes has been shown to be independent of ethylene signalling (Meng et al., 2013). Alternative signalling pathways are therefore likely to contribute to the ERF6-regulated susceptibility of Arabidopsis to *M. incognita*, possibly involving ACC as a signalling molecule (Dubois *et al*., 2015).

Recently, we have shown in a multi-trait analysis, using the same panel of natural inbred lines of Arabidopsis, a strong positive correlation (R = 0.8) between SNPs significantly associated with the response to osmotic stress [i.e. increase in biomass under polyethylene glycol 8000 (PEG8000) treatment] and the reproductive success of *M. incognita* (Thoen *et al*., 2017). Interestingly, no such correlation was found between the susceptibility to *M. incognita*and responses to salt, drought and heat. This indicates that alleles specifically regulating tolerance to osmotic stress in Arabidopsis may also regulate the susceptibility to *M. incognita*. Permanent feeding cells induced by *M. incognita* display enormous cellular hypertrophy, which could be a response to the strongly elevated osmotic pressure in host cells caused by phloem unloading of carbohydrates and amino acids (Smant *et al.*, 2018). Allelic variation in ERF6 may therefore affect the ability of Arabidopsis to accommodate high osmotic pressures, which could affect giant cell expansion and thereby the reproductive success of *M. incognita*.

In addition to alterations in stress response genes, we also noted a strongly enhanced expression of various components of photosystem I in nematode-infected roots of the *erf6-1*mutant line (e.g. PSAO, PSAF, RBCS1A; Fig. 4C). This observation is puzzling as, during the *in vitro* bioassays with *M. incognita*, the Arabidopsis roots were not exposed to light. Differential regulation of components involved in photosynthesis is often observed in association with nematode infections in plants, and our findings are unlikely to be an artefact caused by specific environmental factors in our experimental set-up. Even in soil-grown plants, nematode-induced permanent feeding cells can harbour large numbers of chloroplast-like organelles, but the role of these structures is not well understood (Golinowski *et al*., 1996; Kyndt *et al*., 2012).

In conclusion, in this study, we have shown that, by accepting a lower threshold for significance and higher false discovery rate in GWA mapping, we were able to resolve all of the heritable variation in susceptibility to *M. incognita* in our population of Arabidopsis lines. In total, we identified 19 QTLs significantly associated with the reproductive rate of *M. incognita* on Arabidopsis, some of which may still be false positives. However, by focusing on an SNP marker Chr4.145290 that we initially considered to be a probable false positive, we gained insight into the role of ERF6 as a regulator of abiotic stress responses in plant–nematode interactions.

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Supplemental information

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Chapter 4

The TIR-NLR pair *DSC1* and *WRKY19* contributes to basal immunity of Arabidopsis to the root-knot nematode *Meloidogyne incognita*

Sonja Warmerdam
Mark G. Sterken
Octavine C. A. Sukarta
Casper C. van Schaik
Marian E. P. Oortwijn
Jose L. Lozano-Torres
Jaap Bakker
Geert Smant
Aska Goverse

Submitted to BMC plant biology

Abstract

Ecotypes of *Arabidopsis thaliana* can display large quantitative variation in susceptibility to the root-knot nematode *Meloidogyne incognita*, which is thought to be independent of dominant major resistance genes. However, in an earlier genome-wide association study of the interaction between Arabidopsis and *M. incognita* we identified a quantitative trait locus harboring homologs of dominant resistance genes but with minor effect on susceptibility. Here we report on the characterization of two of these genes encoding the TIR-NB-LRR immune receptor DSC1 (DOMINANT SUPPRESSOR OF Camta 3 NUMBER 1) and the TIR-NB-LRR-WRKY-MAPx protein WRKY19 in nematode-infected Arabidopsis roots. Nematode infection studies and whole transcriptome analyses using the Arabidopsis mutants showed that *DSC1* and *WRKY19* co-regulate susceptibility of Arabidopsis to *M. incognita*. Given the head-to-head orientation of *DSC1* and *WRKY19* in the Arabidopsis genome our data further suggests that both may function as a TIR-NLR immune receptor pair. Unlike other TIR-NLR pairs involved in dominant disease resistance in plants, DSC1 and WRKY19 most likely regulate basal levels of immunity to root-knot nematodes.

Introduction

The root-knot nematode *Meloidogyne incognita* is currently ranked as one of the most invasive plant disease-causing agents having major impact on global agricultural productivity (Bebber *et al.*, 2014). Infective second stage juveniles (J2) of *M. incognita* penetrate a host at the root elongation zone. Thereafter, they migrate through the cortex to the root tip and enter the vascular cylinder via the columella and quiescence center. Inside the differentiating vascular cylinder, the J2 carefully puncture the cell walls of several host cells with their stylet to initiate the formation of a permanent feeding site (Caillaud *et al.*, 2008; Gheysen & Mitchum, 2011; Goverse & Smant, 2014; Mantelin *et al.*, 2015). This permanent feeding site includes several giant cells, which are formed by major structural and metabolic changes in host cells most likely in response to stylet secretions of *M. incognita* (Gheysen & Mitchum, 2011; Escobar *et al.*, 2015). Juveniles of *M. incognita* take up their nutrients from these giant cells during the course of several weeks while undergoing three molts to enter the adult stage. Adult females produce eggs, which are held together in a gelatinous matrix at the surface of the roots (Gheysen & Mitchum, 2011; Goverse & Smant, 2014; Mantelin *et al.*, 2015).

Plants have developed several lines of defense to protect themselves against attacks by parasitic nematodes (Goverse and Smant, 2014). The first line of defense is thought to be structural where plants make use of rigid cell walls to prevent host invasion (i.e., by migratory ectoparasites). Next, plant cells carry surface-localized receptors to detect molecular patterns in the apoplast that are uniquely associated with host invasion by endoparasitic nematodes (Jones & Dangl, 2006; Cook *et al.*, 2015; Mantelin *et al.*, 2015). For example, root-knot nematodes release small glycolipids commonly referred to as ascarosides that are recognized as invasion-associated molecular patterns (Manosalva *et al.*, 2015). The exposure of Arabidopsis seedlings to these ascarosides activates basal plant defenses to a broad range of pathogens. Furthermore, Arabidopsis mutant analyses including *BAK1* have shown that receptor-mediated basal immunity plays a significant role in the susceptibility of plants to root-knot nematodes (Teixeira *et al.*, 2016). Interestingly, root-knot nematodes seem to have adapted to some extent to this line of defense with effectors capable of selectively suppressing responses activated by surface-localized immune receptors (Jaouannet *et al.*, 2012; Chen *et al.*, 2013; Lozano-Torres *et al.*, 2014; Chen *et al.*, 2015).

At a later stage in the infection process, nematode resistant plants can counteract the establishment of a permanent feeding site with effector-triggered immunity, which is predominantly based on sensing nematode effectors by intracellular immune receptors (Williamson & Kumar, 2006; Goverse & Smant, 2014). Effector-triggered immunity to root-knot nematodes involves a hypersensitive response-type of programmed cell death in and around giant cells, which interrupts the flows of assimilates towards the feeding nematodes. As a consequence of insufficient supply of nutrients this type of major resistance induces a developmental arrest in juveniles.

The largest group of intracellular plant immune receptors belong to the nucleotide-binding site leucine-rich repeat (NLR) superfamily of immune receptors. These NLRs consist of central nucleotide-binding domain attached to a C-terminal leucine-rich repeat (LRR) domain and a variable N-terminal domain that can either be a coiled-coil (CC) or a Toll-interleukin-1 receptor (TIR)-like domain (Boller & Felix, 2009; Takken & Goverse, 2012; Zipfel, 2014; Mantelin *et al.*, 2015). Three intracellular receptors capable of conferring resistance to *M. incognita* have been cloned to date, one of which is a CC-NLR receptor (*Mi-1.2* from *Solanum peruvianum*) and two are classified as TIR-NLR proteins (*Ma* from *Prunus cerasifera* and *PsoRPM2* from *Prunus sogdiana*) (Williamson, 1998; Claverie *et al.*, 2011; Zhu *et al.*, 2017). Most of the commercially grown tomato varieties (*Solanum lycopersicum*) carry introgressions of the *Mi-1.2* gene, conferring high levels of resistance to several tropical root-knot nematode species (e.g., *M. incognita, M. javanica* and *M. arenaria*)(Rossi *et al.*, 1998; Williamson, 1999). Resistance based on *Mi-1.2* is currently losing efficacy in the field due to its temperature sensitivity and because of natural selection of virulent nematode populations (Kaloshian *et al.*, 1996; Williamson, 1998; Devran & Söğüt, 2010). The breakdown of *Mi-1.2* resistance and the small number of major resistances genes currently available for root-knot nematodes has prompted a search for alternative strategies to develop durable nematode resistant crops.

Previously, we used genome-wide association mapping to identify less conducive allelic variants of genes that critically determine susceptibility of Arabidopsis to *M. incognita* (i.e. *S*-genes; Warmerdam *et al*., 2018; Warmerdam *et al*., 2019). In total, we identified 19 QTLs in the genome of Arabidopsis contributing to quantitative variation in reproductive success of *M. incognita*. We selected Arabidopsis as a model hosts for our studies because it is thought to lack major resistance to *M. incognita*. However, one of the QTLs with minor effect on susceptibility of Arabidopsis to *M. incognita* harbors homologs of the TIR-NLR class of resistance genes. Here, we report on the functional characterization of these TIR-NLR genes that were previously annotated as *DSC1* and *WRKY19*. *DSC1* encodes a typical TIR-NLR immune receptor (Lolle *et al.*, 2017), whereas *WRKY19* also includes a WRKY domain and MAPx domain at the C-terminus (Rushton *et al.*, 2010). Our data from mutant analyses and whole transcriptome analyses suggest that the coordinated activity of DSC1 and WRKY19 as a receptor pair may be involved in regulating basal defense of Arabidopsis to *M. incognita*.

Material and Methods

Plant material

The following homozygous Arabidopsis T-DNA insertion mutant lines were obtained from the Nottingham *Arabidopsis* Stock Centre (Alonso *et al.*, 2003): SALK_145043 with T-DNA insert in At4G12010 (*dsc1-1*); SALK_014300 with T-DNA in At4G12020 (*wrky19-1*); SALK_201408 with T-DNA in At4G12030 (*bat5-2*). The T-DNA insert lines were all generated in the background of Col-0 (N60000), which was used as wildtype Arabidopsis throughout this study.

The presence and homozygosity of the T-DNA insert in the mutant lines was checked with PCR on genomic DNA isolated from leaf material of twelve seedlings (Warmerdam *et al.*, 2018). To determine if the wild type allele was present in the T-DNA mutant we used a different combination of primers for each line (Table S1). To determine if the T-DNA insert was present we used the gene specific primer and T-DNA-insert specific Lbl3.1 primer (Alonso *et al.*, 2003). For the PCR we used the following conditions: 10 min at 94°C, 35 cycles of 30 s at 94°C, 1.5 min at 60°C, and 1 min at 72°C, and a final incubation of 10 min at 72°C. The PCR amplification products were analyzed by agarose gel electrophoresis.

The expression of the T-DNA insertion affected gene was checked with reverse transcription quantitative PCR (RT-qPCR). Total RNA was isolated from whole roots of twelve 14 days-old plants of the specific T-DNA insertion mutant and cDNA was synthesized as described below for the gene expression analysis. cDNA matching *Arabidopsis thaliana* elongation factor 1-alpha (At5G60390) was amplified as a reference for gene constitutive expression (Czechowski *et al.*, 2004). To quantify the expression level for the gene of interest we used a gene specific forward and reverse primer (Table S1). For the RT-qPCR we used conditions as described below for gene expression analysis. Relative expression ratio between the gene of interest and the reference gene was calculated as described elsewhere (Pfaffl, 2001).

Nematode bioassay

Eggs of *Meloidogyne incognita* were obtained by treating tomato roots infected with *M. incognita* (strain 'Morelos' from INRA, Sophia Antipolis, France) with 0.05% v/v NaOCl for three minutes. Roots were rinsed with tap water and the eggs were collected on a 25 µm sieve. Next, the eggs were incubated in a solution of 3 mM NaN_3 for 20 min while shaking. Thereafter, the eggs were rinsed with tap water and incubated on a 25 µm sieve in a solution of 1.5 mg/ml gentamycin and 0.05 mg/ml nystatin in the dark at room temperature. Hatched juveniles were collected after 4 days and surface-sterilized using 0.16 mM $HgCl_2$, 0.49 mM NaN_3, 0.002% v/v Triton X-100 for 10 min. After surface sterilization, the juveniles were rinsed three times with sterile tap water and transferred to 0.7% Gelrite solution (Duchefa biochemie).

To test the susceptibility of Arabidopsis seedlings, seeds were vapour sterilized (with 0.7 M NaOCl and 1% HCl in tap water) for 5 h and transferred to a 6-well cell culture plate containing Murashige and Skoog (MS) medium (MS with vitamins 4.7 g/L (Duchefa biochemie), 20 g/L sucrose and 5 g/L Gelrite. The 6-well plates were first incubated in the dark at 4°C for 3 days. Thereafter, the seeds were allowed to germinate at 21°C under 16h light / 8h dark conditions in a growth cabinet for seven more days. Individual one-week-old seedlings were subsequently transferred to separate wells in a new 6-well plate containing MS medium. The seedlings were incubated for seven more days at 21°C under a 16h light and 8h dark regime. Next, individual seedlings were inoculated with 180 infective J2s of *M. incognita* per plant and incubated at 24°C in the dark. The number of egg masses per plant were counted six weeks after inoculation by visually inspecting the roots with a dissection microscope. Each Arabidopsis genotype was tested in at least three independent experiments and 18 replicates per experiment. The obtained values were batch-corrected using the following equation: variable corrected = variable + (total mean (variable) - batch mean (variable)). Unless indicated otherwise, the differences in counts per plants were statistically analysed using two-way ANOVA and post-hoc Tukey HSD test in R (version 3.0.2, www.r-project.org).

To collect roots of infected and non-infected Arabidopsis seedlings for gene expression analyses with microarrays and RT-qPCR, seeds were treated as described above for the susceptibility test. Four 7 day old seedlings were transferred to 12 cm square plates containing MS medium, which were placed vertically in a growth cabinet for 7 days at 21°C under a 16h light and 8h dark regime. Next, individual seedlings were inoculated with 180 infective J2s of *M. incognita* per plant and incubated horizontally at 24°C in the dark. Samples of twelve whole root systems were collected at the time of inoculation, at 7 days after inoculation with *M. incognita*, and at 7 days after being mock-inoculated. The samples were stored at -80 degrees Celsius until further use.

Root phenotype

Arabidopsis seedlings were allowed to germinate and grow for 14 days on MS medium as described above for the susceptibility test. To determine the total root length and number of root tips of the seedlings, the complete plants were transferred from the media onto a plastic tray with water. Next, the leaves of the seedlings were removed and the roots were spread out over the surface of the tray. The total root length was measured using WinRHIZO package for Arabidopsis (EPSON perfection V800, WinRHIZO pro2015, Regent Instruments Inc.). The number of root tips was counted manually based on the scan that was made using WinRHIZO. Differences in the total root length per seedling in cm and number of root tips were statistically analysed with a two-way ANOVA and post-hoc Tukey HSD test in R ($p<0.05$).

Gene expression analysis

Expression analysis for gene of interest was performed on the stored root samples produced during the nematode infection study. Whole root systems were cut from aerial parts of the seedlings and snap frozen in liquid nitrogen. Total RNA was isolated from whole roots of twelve 14-days-old plants of *dsc1-1*, *wrky19-1* and *bat5-2* and Col-0 wildtype. The frozen root systems were homogenized using TissueLyser (Qiagen) two times for 30 seconds. Total RNA was extracted from 100 mg of the homogenate with the Maxwell Plant RNA kit (Promega Corporation) using the Maxwell 16 Robot (Promega Corporation) according to the manufacturer's protocol. The amount of total RNA per sample was determined by spectrophotometer ND-1000 (Isogen Life Science).

For quantitative transcription PCR (qRT-PCR) first strand cDNA was synthesized from total RNA using Superscript III first strand synthesis system (Invitrogen) according to manufacturer's protocol. Samples were analysed by quantitative PCR using Absolute SYBR Green Fluorescein mix (Thermo Fisher Scientific). cDNA matching *Arabidopsis thaliana* elongation factor 1 alpha was amplified as a reference for constitutive expression using primers as indicated in table S1 (Czechowski *et al.*, 2004). To quantify the expression level for the gene of interest specific gene primers were used (Table S1). For the qRT-PCR 5 ng cDNA was used with the following conditions: 15 min at 95°C, forty cycles of 30 s at 95°C, 30 s at 62 °C, and 30 s at 72°C, and a final incubation of 5 min at 72°C. Relative expression ratio between the gene of interest and the reference gene was calculated as described elsewhere (Pfaffl, 2001). Relative expression ratio was statistically analysed for significance compared with a student t-test (P-value<0.05).

For microarray analysis, approximately 200 ng of total RNA of each sample of Col-0, *dsc1-1* and *wrky19-1* were used for gene expression analysis on an *Arabidopsis* V4 Gene Expression Microarray (4x44K, Agilent Technologies). The probes were re-blasted against TAIR11 using the BLASTN function of the command line blast tool (version 2.6.0, win64). The default settings were used and the top-hit was used as probe annotation. Probes with multiple hits were censored (Camacho *et al.*, 2009). The total RNA was fluorescently labelled for two-colour microarray analysis and subsequently used for hybridization to the probes on the slides according to the manufacturer's protocols (QuickAmp Protocol, Agilent Technologies). Binding of fluorescent RNA to the probes on the microarray was measured with a High-Resolution C Microarray Scanner and Feature extraction Software (Agilent). Two sets of data were generated: a set for comparison between Col-0 and *dsc1-1* and a set for the comparison between Col-0 and *wrky19-1*. The different sets for Col-0 contained different experimental samples. Each sample had at least three biological replicates.

Microarray analysis

After scanning, the spot intensities of the microarrays were not background corrected (Zahurak *et al.*, 2007). Gene expression profiles were normalized using the Loess (within array normalization) and the quantile method (between array normalization) (Smyth & Speed, 2003) in the Bioconductor Limma package (Ritchie *et al.*, 2015). The normalized intensities were log2-transformed for further analysis.

A linear model was used to identify differentially expressed genes in a side-by-side comparison. The following treatments were compared: day-0 control Col-0 versus mutant, day 7 control Col-0 versus mutant, day 7 infected Col-0 versus infected mutant, and day 7 control Col-0 versus infected Col-0. Each treatment consisted of three biological replicates. We used the linear model

$$E_i = T_i + \text{error}$$

where the log2-normalized expression (E) of spot i (i in 1, 2, ..., 45220) was explained over treatment (T). Afterwards, the obtained significances were corrected for multiple testing using the FDR procedure in p.adjust (Benjamini & Hochberg, 1995). All microarray data were deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6897.

Results

Multiple TIR-NLR proteins in a QTL for susceptibility

In our previously published GWA study of the susceptibility of Arabidopsis to *M. incognita* we identified a single nucleotide polymorphism (SNP) marker on chromosome 4, which was closely linked to two genes with similarity to TIR-NLR-type immune receptors (Warmerdam *et al.*, 2019). This SNP marker (138442) was located inside an exon of *BILE ACID TRANSPORTER 5* (*BAT5*; At4G12030) and causes non-synonymous mutation (Val to Ala) close to amino

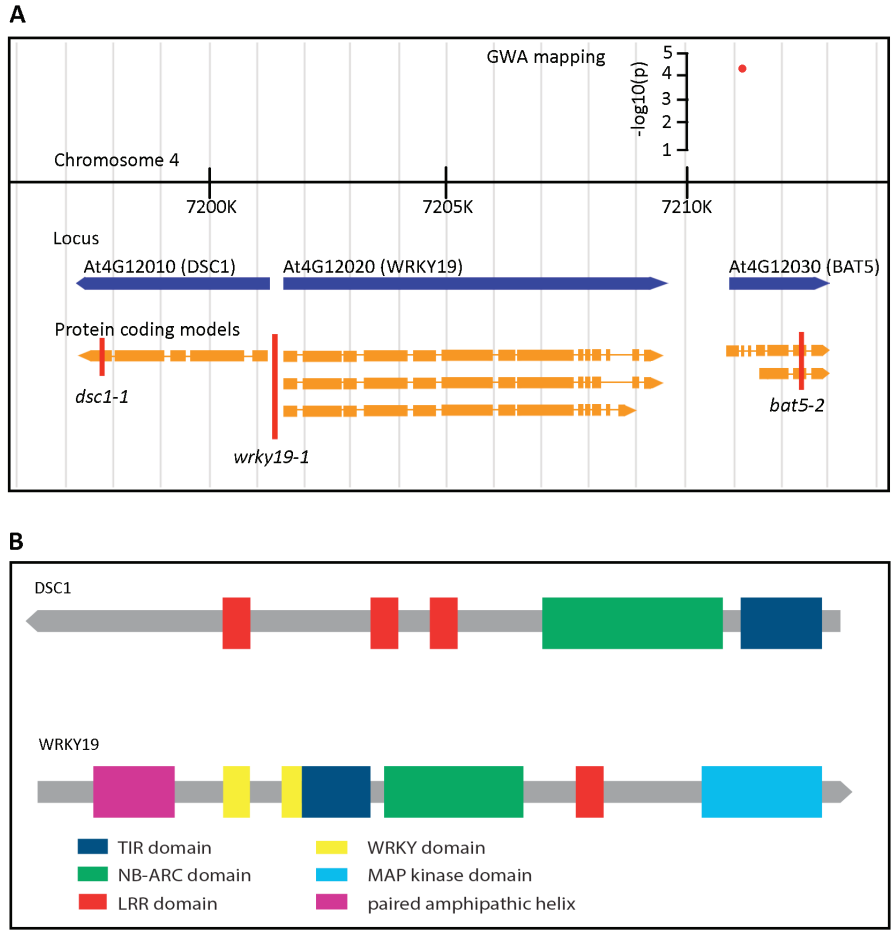

Figure 1. Genomic orientation of BAT4, WRKY19 and DSC1 with protein domains present in WKRY19 and DSC1. A. Representation of the genomic region around significantly associated SNP marker 138422. The red dot represent the SNPs with the corresponding –log10(p) score from the genome wide association mapping. The blue arrows represent the predicted genes. Transcript deriving from these genes are indicate in orange, with rectangles marking the protein coding exons. The red vertical line indicate a T-DNA insert with the corresponding name. B. Schematic representation of protein domains present in At4G12010 (DSC1) and At4G12020 (WRKY19). Coloured blocks represent the different domains present in the protein sequence.

terminus of the protein (Fig. 1A). However, directly upstream of *BAT5* are *DOMINANT SUPPRESSOR of Camta3 NUMBER 1* (DSC1; At4G12010) and *MITOGEN-ACTIVATED PROTEIN KINASE-WRKY19* (WRKY19; At4G12020), the products of both of which include TIR, NB-ARC and LRR domains (Fig. 1A and B). *BAT5*, *DSC1* and *WRKY19* are all within 10kb of SNP marker 138442 and could therefore be causal for the effect on susceptibility to *M. incognita* associated with this marker.

BAT5 is not causally linked to susceptibility of Arabidopsis to *M. incognita*

Since SNP marker 138442 is located inside the coding sequence of *BAT5*, we first tested if this gene is required for susceptibility of Arabidopsis to *M. incognita*. Thereto, we inoculated *in vitro* grown plants of the homozygous Arabidopsis T-DNA insert line *bat5-2* with infective J2 of *M. incognita*. The *bat5-2* mutant harbors a T-DNA insertion in the second last exon, resulting only in a slight reduction of mRNA levels of *BAT5* (Fig. 1A; Fig. S1A and B). However, as the insert in *bat5-2* disrupts the open reading frame in *BAT5*, mRNAs are probably not translated into a functional protein. Nonetheless, six weeks after inoculation the number of egg masses produced by *M. incognita* per plant was not significantly different between *bat5-2* and wildtype Arabidopsis plants (Fig. 2). We also investigated the root architecture of the *bat5-2* mutant line at the time of inoculation as this may affect the susceptibility of Arabidopsis for *M. incognita*, but we observed no significant difference in the number of root tips per plant for the *bat5-2* mutant compared to the wildtype Arabidopsis (Fig. S2). Altogether, our results provide no evidence for a significant role of BAT5 in susceptibility of Arabidopsis to *M. incognita*.

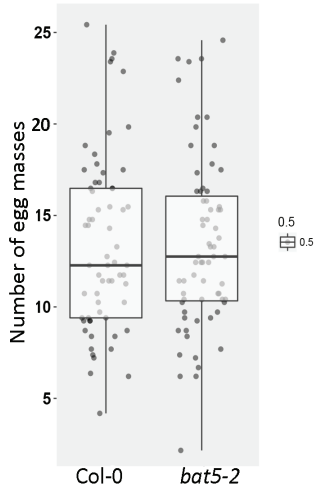

Figure 2. Susceptibility of homozygous T-DNA insert mutants *bat5-2* to *M. incognita*. (a) Number of egg masses per plant at 6 weeks post inoculation on *bat5-2* and wild-type Arabidopsis plants. Bars reflect the averages and standard error of the mean of three independent experiments (n > 18 per experiment). Data was statistically tested for significance with ANOVA with post-hoc Tukey test ($p<0.05$).

DSC1 and _WRKY19_ may both regulate susceptibility of Arabidopsis to _M. incognita_

To study a possible role of *DSC1* and *WRKY19* in susceptibility of Arabidopsis to *M. incognita*, we also tested the homozygous Arabidopsis T-DNA insertion lines *dsc1-1* and *wrk19-1* in nematode infection assays. The T-DNA insert in *dsc1-1* is located in the last exon of *DSC1*, which causes a complete knock-out of gene expression (Fig. S1). In contrast, the T-DNA insert in *wrky19-1* is located in the putative promotor regions of both genes, which leads to strong upregulation of *WRKY19* expression and a small but significant down-regulation of *DSC1* (Fig. S1D). At seven days after inoculation with *M. incognita*, we observed a significantly higher number of juveniles inside roots of the *dsc1-1* mutant plants compared to wildtype Arabidopsis plants (Fig. 3A). The number of juveniles inside the *wrky19-1* overexpressing mutant was also slightly, but not significantly, higher as compared to wildtype Arabidopsis plants (Fig. 3E). However, it should be noted that we also observed a significant smaller number of root tips per plant for both the *dsc1-1* and *wrky19-1* mutants compared to wildtype Arabidopsis plants at the time of inoculation (Fig. S3). When we corrected our data for this difference in root architecture, the number of juveniles per root tip was significantly higher in both *dsc1-1* and *wrky19-1* mutant lines as compared to wildtype Arabidopsis plants (Fig. 3B and F). Likewise, at six weeks after inoculation the number of egg masses per plant and the number of egg masses per root tip was significantly higher in both the *dsc1-1* knock-out mutant line and *wrky19-1* overexpressing mutant line as compared to wildtype Arabidopsis (Fig. 3C, D, G, and F).

Our phenotype data with the *wrky19-1* mutant line suggested that quantitative differences in expression levels of *DSC1* and *WRKY19* could influence both root development and susceptibility to *M. incognita*. We therefore investigated if the expression of *DSC1* and *WRKY19* is also regulated during root development in wildtype Arabidopsis seedlings and in association with nematode infections using quantitative reverse transcription PCR with gene specific primers (Fig. 4). Interestingly, both *DSC1* and *WRKY19* were upregulated in non-infected roots of Arabidopsis seedlings at seven days after the time point of inoculation. In contrast, we only observed a significant down-regulation of *WRKY19* in nematode-infected roots at the same time point after inoculation with *M. incognita*. *DSC1*, but also *BAT5*, was not significantly regulated in association with nematode-infections in wildtype Arabidopsis seedlings at seven days after inoculation with *M. incognita*.

DSC1 and _WRKY19_ regulate gene expression during _M. incognita_ infection

To gain more insight in the possible role of *DSC1* in susceptibility of Arabidopsis to *M. incognita*, we analyzed the transcriptome of whole roots of the *dsc1-1* mutant line and wildtype Arabidopsis seven days after inoculation with *M. incognita* using Arabidopsis gene expression microarrays. As expected, in non-infected roots of the *dsc1-1* mutant expression

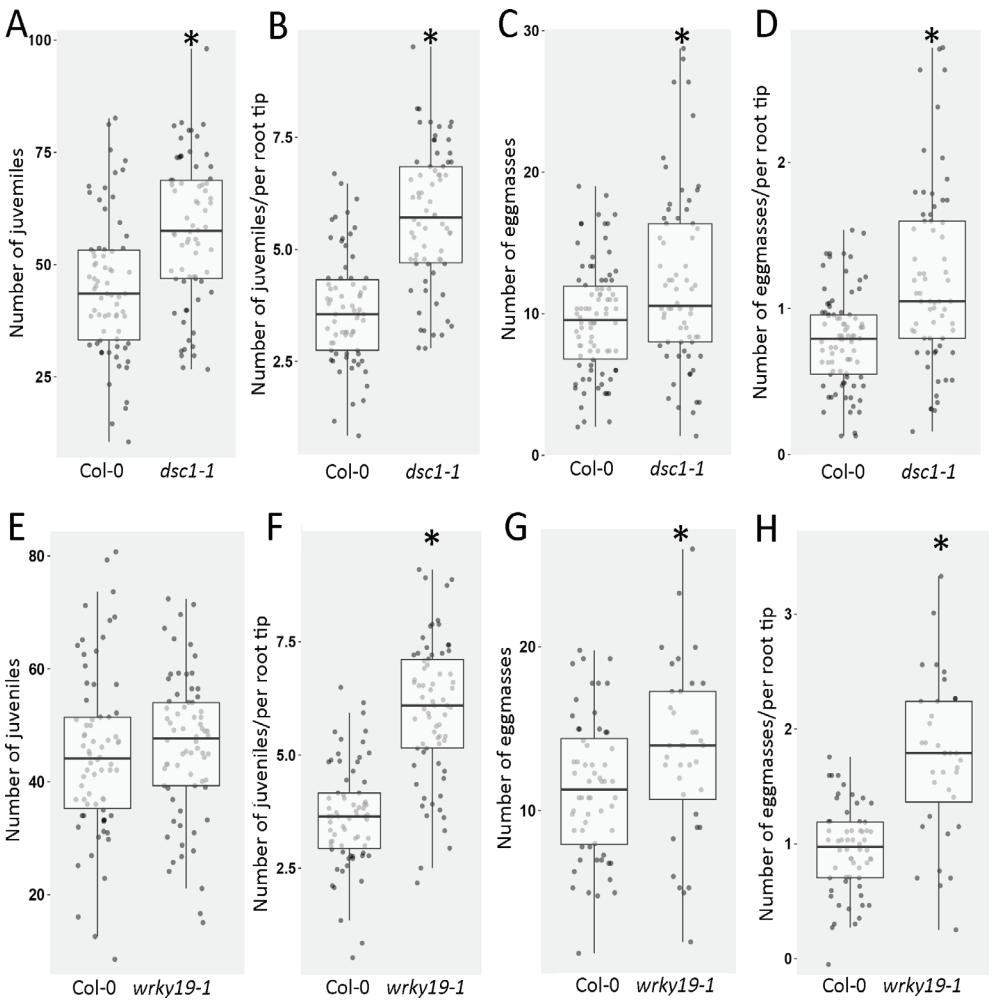

Figure 3. Susceptibility of homozygous T-DNA insert mutants *dsc1-1* and *wrky19-1* of Arabidopsis to *M. incognita*. (A,E) number of juveniles per plant at 7 days post inoculation on dsc1-1, wkry19-1 and wildtype Arabidopsis. (B,F) number of juveniles per plant at 7 days post inoculation on dsc1-1, wkry19-1 and wildtype Arabidopsis corrected for the number of root tips at the start of the infection. (C,G) Number of egg masses per plant at 6 weeks post inoculation on *dsc1-1, wrky19-1* and wildtype Arabidopsis plant. (D,H) Number of egg masses per plant at 6 weeks post inoculation on *dsc1-1, wkry19-1* and wildtype Arabidopsis plants corrected for the number of root tips at the start of the infection. Boxplot represent data of three independent experiments (n >12 per experiment). Data was statistically tested for significance with ANOVA with post-hoc Tukey HSD (* p<0.05).

of *DSC1* was absent, but to our surprise no other genes were differentially expressed in a comparison with non-infected roots of wildtype Arabidopsis plants (at -log10(P)>3.5). However, we observed the differential expression of 221 genes in a comparison between nematode-infected roots of the *dsc1-1* mutant and wildtype Arabidopsis plants at seven days after inoculation (Fig. 5A). To determine which genes are mostly affected by the mutation in *dsc1-1* we focused on genes that were either standing out because of exceptionally large change in expression level (i.e., mean normalized change in probe intensities < -0.3 or > 0.3), because of high statistical support of the changes (-log10(P)>6.5)), or both (Fig. 5A). Applying these criteria resulted in a list of twelve differentially regulated genes in the absence of *DSC1*, including *DSC1,* several of which have been linked to response to abiotic and biotic stress (Table 1, Fig. 5A).

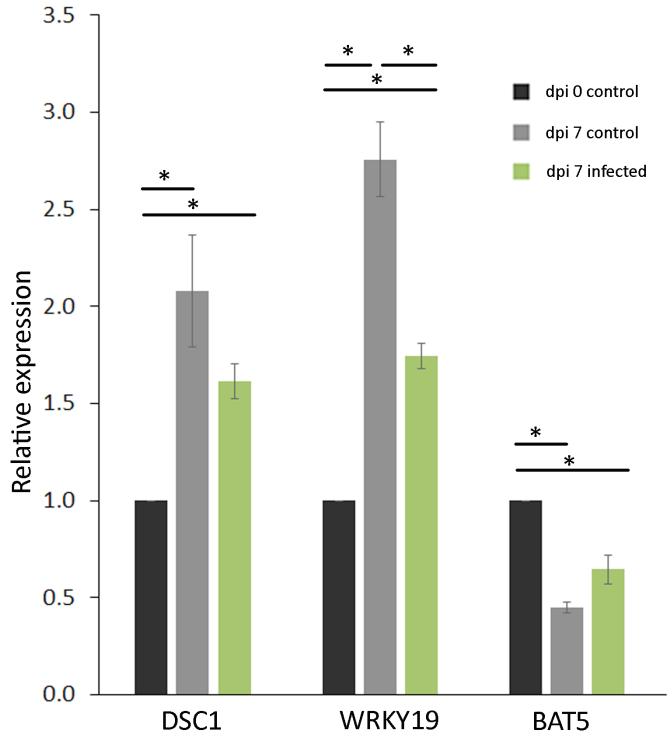

Figure 4. Relative expression of *DSC1, WRKY19* and *BAT5* in roots of *A. thaliana* with and without *M. incognita***.** Data is shown for whole roots collected at the time of inoculation with *M. incognita* (0 days control), for whole roots collected at 7 days after mock-inoculation (7 days control) and 7 days after inoculation with *M. incognita* (7 days infection). Data is represented as comparison against the expression level at 0 days control. Data is based on three independent experiments with three technical replicates per experiment. Error bars represent standard error of the mean. Data was analyzed with a student t-test (*=p <0.05).

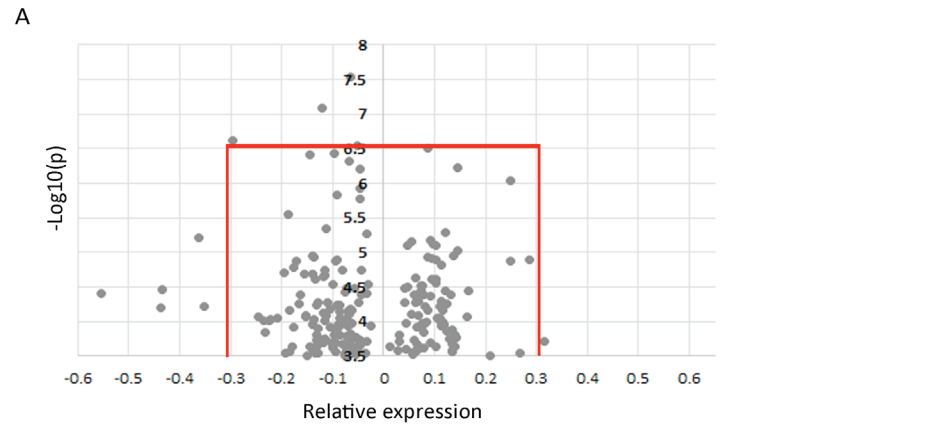

Figure 5: Differential expression analysis of *dsc1-1* and *wrky19-1* compared to Col-0 at 7 days after *M. incognita* infection. (A) 221 genes differentially expressed in *dsc1-1* compared to Col-0. The red lines indicate the threshold for significance of 7 and effect size of 0.3. (B) 1710 genes differentially regulated in *wrky19-1* compared to Col-0. The red lines indicate the threshold for significance of 7 and effect size of 0.7. (C) Venndiagram indicating the comparison between differently expressed genes in *dsc1-1* and *wrky19-1* 7 days after infection with *M. incognita*.

To identify genes regulated in association with *WRKY19*, we also analyzed the transcriptome of whole roots of the *wrky19-1* mutant in the absence and presence of the *M. incognita*. At 7 days after the time of inoculation, we found no differentially expressed genes between non-infected root of the *wrky19-1* mutant and wildtype Arabidopsis plants (threshold for significance -log10(P)>3.5). The expression of *WRKY19* was slightly but not significantly upregulated in the *wrky19-1* mutant line as compared to wildtype Arabidopsis (significance -log10(P)=1.67; relative expression 0.22). However, in nematode-infected roots we detected 1,710 differently expressed genes in a comparison between *wrky19-1* and wildtype Arabidopsis plants 7 days after inoculation with infective juveniles of *M. incognita* (Fig. 5B). Notably, the expression of DSC1 was significantly reduced in the *wrky19-1* mutant (significance -log10(P)=5.2; relative expression -0.31). To determine which genes are mostly affected in *wrky19-1* we focused on genes that were above the threshold of significance of -log10(P)>6.5 or had a relatively large change in relative expression (relative expression < -0.3 or > 0.3). In total 253 differentially expressed genes matched these criteria (Table S2). Over 80% of the top 15 most significantly regulated genes or those with largest relative expression have a W-box motif ((T/A) TGAC(T/A)) present in the promotor region (Table 1). Most of the genes in this subset have been linked to biotic and abiotic stress responses. For instance, the gene with by far the highest relative expression in *wrky19-1* is *ILITHYIA* (ILA, At1G64790), which encodes a HEAT-repeat protein required for basal defense to *Pseudomonas syringea* (Monaghan & Li, 2010).

Table 1: Differentially expressed genes in *dsc1-1* and *wrky19-1* compared to Col-0 at 7 days post inoculation with *M. incognita*. For each gene the level of significance and relative expression is stated, and the presence of a WRKY domain in the promotor region.

Gene ID	Significance	Relative expression	Gene description	WRKY domain in promotor	Associated with
Regulated in *dsc1-1*					
AT4G12010	10.71	-2.50	Dominant suppressor of CAMTA3 1 (DSC1)	N	Defense response (Lolle *et al.*, 2017)
AT1G28040	7.55	-0.07	Ring/U-box family protein	-	Ubiquitination (Moon *et al.*, 2004)
AT2G38380	7.08	-0.12	Peroxidase superfamily protein	Y	Stress response (Kim *et al.*, 2012)
AT3G52970	5.21	-0.36	Cytochrome P450, family 76, subfamily G, polypeptide 1	Y	
AT5G06905	4.46	-0.44	Cytochrome P450, family 93, subfamily D peptide 1	Y	
AT4G29690	4.41	-0.56	Alkaline-phosphatase-like family protein	Y	Metabolic changes (Goda *et al.*, 2004)
AT1G68040	4.22	-0.35	S-adenosyl-L-methionine-dependent methyltransferase superfamily protein	N	
AT5G06900	4.21	-0.44	Cytochrome P450, family 712, subfamily A polypeptide 2	Y	
AT1G56280	3.72	0.32	drought-induced 19 (Di19)	N	Stress response (Liu *et al.*, 2013)
Regulated in *wrky19-1*					
AT2G38330	9.00	0.18	Mate Efflux family protein	Y	Pathogen response (Ascencio-Ibáñez *et al.*, 2008)
AT5G51860	8.53	0.09	K-box and MADS box transcription factor family protein (AGL72)	Y	Floral transition (Dorca-Fornell *et al.*, 2011)
AT5G45380	8.41	-0.09	DEGRADATION OF UREA 3 (DUR3)	Y	Nitrogen uptake (Kojima *et al.*, 2007)
AT1G34320	8.11	-0.18	PSK simulator 1 (PSK1)	Y	Growth (Stührwohldt *et al.*, 2014)
AT5G14470	7.96	-0.19	Galactokinase 2 (GALK2)	Y	Stress response (Zhao *et al.*, 2013)
AT5G63730	7.53	-0.29	ARIADNE 14 (ARI14)	Y	Ubiquitination (Mladek *et al.*, 2003)
AT5G46470	7.45	-0.11	Resistant to P. Syringae 6 (RPS6)	Y	Defense response (Kim *et al.*, 2009)

Table 1 continued

Gene ID	Significance	Relative expression	Gene description	WRKY domain in promotor	associated with
AT5G38320	7.30	-0.30	hypothetical protein	N	
AT4G32890	7.25	0.23	GATA transcription factor 9 (GATA9)	Y	
AT4G36540	7.23	0.13	BR enhanced expression 2 (BEE2)	Y	Shade avoidance (Cifuentes-Esquivel *et al.*, 2013)
AT4G05020	7.18	-0.16	NAD(P)H dehydrogenase B2	Y	Stress response (Smith *et al.*, 2011)
AT1G09770	7.15	0.13	Cell division cycle 5 (CDC5)	N	Defense response (Monaghan *et al.*, 2009)
AT1G12440	7.12	0.17	A20/AN1-like zinc finger family protein	Y	
AT3G23840	7.02	-0.20	HXXXD-type acyl-transferase family protein (CER26-LIKE)	Y	Fatty acid metabolism (Pascal *et al.*, 2013)
AT1G64790	2.84	1.19	ILITHYIA (ILA)	Y	Plant immunity (Monaghan & Li, 2010)
AT4G26010	3.68	-0.73	Peroxidase super family protein	Y	Metabolic changes (Goda *et al.*, 2004)
AT5G38910	3.34	-0.74	RmLC-like cupins superfamily protein	Y	Stress response (Swindell, 2006)
AT3G47340	3.28	-0.71	glutamine-dependent asparagine synthase 1 (ASN1)	Y	Metabolic pathways (Gaufichon *et al.*, 2017)
AT5G08250	3.09	-0.76	Cytochrome P450 superfamily protein	N	

Table 2. 17 genes regulated by *DSC1* and *WRKY19* seven days after inoculation with *M. incognita.*

Gene code	Gene name	Gene description	Associated with
AT4G12010	DSC1	Suppressor of camta 3 1	Defense response (Lolle *et al.*, 2017)
AT4G08390	SAPX	Stromal ascorbate peroxidase	oxidative stress (Vishwakarma *et al.*, 2015)
AT4G27550	TPS4	Trehalose phosphatease/synthase 4	Sucrose signaling (Delorge *et al.*, 2015)
AT4G36290	MORC1	R-protein-interacting-protein	Transcriptional regulation during defense (Bordiya *et al.*, 2016)
AT5G44920	TIK	TIR-KASH protein	defense response (Meyers *et al.*, 2002)
AT3G17810	PYD1	Pyrimidine 1	Seed production (Cornelius *et al.*, 2011)
AT3G20210	AEP4	Asperaginyl endopeptidase 4	Seed development (Nakaune *et al.*, 2005)
AT5G18050	SAUR22	Small auxin up RNA 22, auxin activated signaling pathway.	Auxin signaling (Spartz *et al.*, 2012)
AT4G01460		Basic Helix-loop-helix DNA binding superfamily protein	Transcription regulation (Heim *et al.*, 2003)
AT2G45620	URT1	UTP:RNA URIDYLYLtransferase 1	mRNA metabolisme (Zuber *et al.*, 2016)
AT4G32850	NPAP	nuclear polymerase IV	Transcription regulation (Kappel *et al.*, 2015)
AT4G38800	MTN1	Arabidopsis methylthioadenoise nucleasidase 1	Vascular development (Waduwara-Jayabahu *et al.*, 2012)
AT4G18670		Leucine-rich repeat Family protein	Unknown
AT3G30290	CYP702A8	Cytochrome P450 family 702, subfamily A polypeptide 8	Unknown
AT3G27180		s-adenosyl-L-methionine-dependent methyltransferases superfamily protein	Unknown
AT1G66890		50S Ribosomal-Like protein	Unknown
AT4G33800		Hypothetical protein	Unknown

Discussion

Previously, we mapped a QTL on chromosome 4 of Arabidopsis linked to reproductive success of *M. incognita* harboring two TIR-NLR proteins (Warmerdam *et al.*, 2019). Although the SNP marker that identified this locus was located in *BAT5*, we have found no further evidence that allelic variation in this gene could be causal for variation in the number of egg masses per plant of *M. incognita* at six weeks after inoculation of Arabidopsis seedlings (Fig. 2). Others have shown that BAT5 is associated with jasmonic acid-dependent signaling and wound responses (Gigolashvili *et al.*, 2009), both of which are also relevant processes in the context of *M. incognita* infections (Cooper *et al.*, 2005; Zhou *et al.*, 2015; Holbein *et al.*, 2016). Nevertheless, our data of the bioassays with the *bat5-2* knock out mutant makes it unlikely that BAT5 plays a significant role in regulating susceptibility of Arabidopsis to *M. incognita*.

After eliminating *BAT5* as a candidate susceptibility factor for *M. incognita* infections in Arabidopsis, we focused on the TIR-NLR-encoding genes *DSC1* and *WRKY19* to explain the phenotypic variation associated with this locus. We showed that the loss of *DSC1* expression in the *dsc1-1* Arabidopsis mutant significantly increased the number of juveniles per plant shortly after inoculation and the number of egg masses at the end of the life cycle of *M. incognita* (Fig. 3). This demonstrates that allelic variation linked to *DSC1* may indeed contribute to the phenotypic variation in the susceptibility of Arabidopsis to *M. incognita* (Warmerdam *et al.*, 2019)

Less straightforward is the interpretation of the data from our nematode infections assays with the *wrky19-1* Arabidopsis mutant. The T-DNA insert in *wrky19-1* is located 191 base pairs upstream of the transcription start site of *WRKY19* and 71 base pairs upstream of the transcription start site of the reversely oriented *DSC1* gene. Our quantitative reverse transcription PCR suggested that this T-DNA insert decreases expression of *DSC1* but increases expression of *WRKY19* (Fig. S1). However, we found that only *DSC1* expression is weakly but significantly reduced in the *wrky19-1* mutant in the microarray analysis of the complete transcriptome of *wrky19-1* (Fig. 5B). Regardless of the absence of a strong up- or downregulation of either *DSC1*, *WRKY19* or both, we observed a significant phenotype in root architecture and susceptibility to *M. incognita* for *wrky19-1*.

We therefore used gene expression microarray analyses to further investigate possible regulatory networks underlying the enhanced susceptibility of both *dsc1-1* and *wrky19-1* Arabidopsis mutants to *M. incognita*. Overall, we observed only a small overlap (17 genes) in the sets of differentially expressed genes in nematode-infected roots of *dsc1-1* and *wrky19-1* (Fig. 5C). Despite this small overlap in commonly regulated genes, we noted that both sets are enriched for genes with a regulatory W-box in their putative promoter sequences (Table 1). The W-box is thought to be the consensus of the DNA binding site of WRKY domains of WRKY transcription factors (Ciolkowski *et al.*, 2008). The overrepresentation of

the W-box could indicate that the regulation of these genes is indeed under control of the WRKY domain in WRKY19. However, as we have not observed a major change in *WRKY19* expression in nematode-infected roots of the *wrky19-1* mutant as compared to wildtype Arabidopsis this needs further investigation.

Another striking observation is the number of differentially expressed genes in nematode-infected roots of both *dsc1-1* and *wrky19-1* related to responses to abiotic stress (Table 1). This is in line with data from our earlier multi-trait genome wide association study showing that the susceptibility of Arabidopsis to *M. incognita* cross-correlates with responses to osmotic stress (Thoen *et al.*, 2017). Likewise, we have recently shown that the transcription factor *ERF6* which functions as a mediator of abiotic stress is also required for susceptibility of Arabidopsis to *M. incognita* (Warmerdam *et al.*, 2019). Altogether, these findings suggest that the ability to mitigate abiotic stress is one of the key regulating factors in susceptibility of the Arabidopsis to *M. incognita*.

The fact that many of the genes differentially regulated in the *dsc1-1* and *wrky19-1* mutants have been linked to plant defense and responses to abiotic stress before might not be surprising. It is clear that nematode-infections are likely causes of stress in Arabidopsis roots. Furthermore, it has already been shown that DSC1 functions in plant immunity (Lolle *et al.*, 2017). Nevertheless, to the best of our knowledge this is the first time that DSC1 can be linked to immunity to plant parasitic nematodes. DSC1 is a dominant suppressor of the calmodulin-binding transcription factor CAMTA3, which regulates resistance to various pathogens (Du *et al.*, 2009; Shen *et al.*, 2015). The loss of DSC1 could increase the activity of CAMTA3 in nematode-infected roots of the *dsc1-1* mutant, leading to suppression of defense responses. However, further investigations are needed to test this model.

Although, we cannot directly pinpoint the probable cause for the enhanced susceptibility of the *wrky19-1* mutant to *M. incognita*, our analyses of the transcriptome of nematode-infected roots of this mutant revealed a remarkably strong upregulation of the defense related gene *ILITYHIA* (*ILA*; Fig. 5B). *ILA* encodes a HEAT repeat protein, which is required for basal defense, resistance mediated by a subset of CC- and TIR-NLR proteins, and systemic acquired resistance against *Pseudomonas syringae (Monaghan & Li, 2010)*. *ILA* has not been linked to susceptibility of Arabidopsis to nematodes before, but the relative expression level of this gene in the microarray analyses is so high (relative expression of 1.19) that it could in fact be causal to the increased susceptibility of the *wrky19-1* mutant to *M. incognita*.

TIR-NLR genes can confer dominant disease resistance to Arabidopsis (Narusaka *et al.*, 2009), but our data on the role of *DSC1* and *WRKY19* in nematode-infected roots does not point into that direction. First of all, the relatively low level of significance and small effect size of SNP marker 138442 do not fit the typical dominant phenotype of a major resistance based on TIR-NLRs. Second, the differences in reproductive success of *M. incognita* on the *dsc1-1* knock-out mutant and the *wrky19-1* overexpressing mutant as compared to wildtype

Arabidopsis plants were significant, but relatively small, and unlike major disease resistance responses. We therefore conclude that *DSC1* and *WRKY19*, either separately or together as a pair, do not confer major resistance against *M. incognita* to Arabidopsis, but are probably involved in basal immunity.

A role for DSC1 and WRKY19 in basal immunity is consistent with observations by others that *DSC1* transcript levels increase upon application of SA or flg22 (Meyers *et al.*, 2003) and that *WRKY19* is thought to be an early component in regulatory networks of PTI (Birkenbihl *et al.*, 2018). Likewise, other TIR-NLR proteins have been found to contribute to basal defense in Arabidopsis against *Pseudomonas syringae* (TN13) and a necrotrophic fungus (RLM3) (Staal & Dixelius, 2008; Roth *et al.*, 2017). Furthermore, the head-to-head genomic orientation of *DSC1* and *WKRY19* could indicate that they form an immune receptor pair (Meyers *et al.*, 2003; Narusaka *et al.*, 2009; Cesari *et al.*, 2014). So far, other *R*-gene pairs have been identified in Arabidopsis consisting of two tightly linked NLR coding genes located in a similar head-to-head tandem arrangement (Cesari *et al.*, 2014). For instance, the genomic organization of *DSC1* and *WRKY19* pair shows much similarity with that of *RRS1* and *RPS4* suggesting that they may act as a TIR-NLR pair in plant immunity (Meyers *et al.*, 2003; Narusaka *et al.*, 2009; Cesari *et al.*, 2014). This is further supported by the similarities in protein architecture including the presence of functional domains required for immune receptor activity (Ma *et al.*, 2018). Here, we provide first evidence for a functional role of *DCS1* and *WRKY19* in basal plant immunity to a plant pathogen as a TIR-NLR gene pair. It will be interesting to investigate whether DSC1 and WRKY19 form indeed a functional protein complex and how this complex contributes to basal immunity in plants to root-knot nematodes. Moreover, it provides novel leads to breed for improved crop resistance to this major polyphagous root-feeding parasite.

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Supplemental information

Table S1: Overview of primers used in RT-qPCR and ofr confirmation of T-DNA insert

Identifier	Forward	Reverse
Expression of		
At4G12010 (DSC1)	TCGTGGGTCATGCTCGAATC	ACTCGGTATTTCAGTGGCCG
At4G12020 (WRKY19)	AATGTCCCTCTGGCGAACTC	CAGTACACCCAAGGCTCCAT
At4G12030 (BAT5)	CGAGTTGCGAATTTCCCACG	GCTCGTTTGAGTCAGATTCGG
At4G12040 (SAP7)	TCCGACTTTTCTGTTGAAGGTGA	CATGTTGGATGGTGACCCGA
At4G12050 (AHL26)	AAGATGAAGAACAAAATTCCAGCTT	CTAGCATGGAAAGGAGGAGGA
At5G60390 (EF1a)	GAGTACCACCTTTGGGACG	TTGGGTCCTTCTTGTCCACG
T-DNA insert		
DSC1 wildtype	TTAAGCGGAAACAACATCGAG	ACAACCTGGTTCTTCACCACC
dsc1-1 allele	ATTTTGCCGATTTCGGAAC	ACAACCTGGTTCTTCACCACC
WRKY19 wildtype	TTCATCAACAAGTTTGGCCTC	CCAATCATCATTCATAACCGG
wrky19-1 allele	ATTTTGCCGATTTCGGAAC	CCAATCATCATTCATAACCGG
BAT5 wildtype	TTCTTTCACATGGTTCAAGCC	ACAGCCGACCATAAACAACAG
bat5-2 allele	ATTTTGCCGATTTCGGAAC	ACAGCCGACCATAAACAACAG

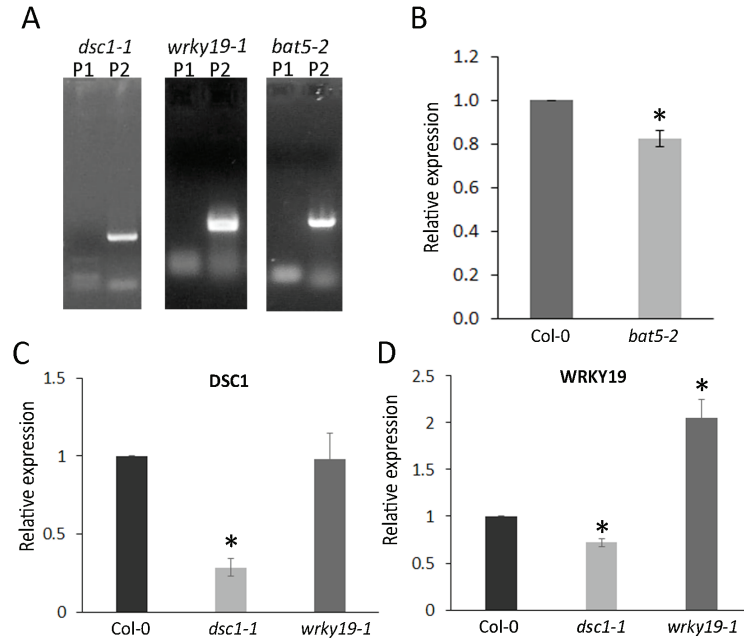


Figure S1: Confirmation of T-DNA insert in line *bat5-2, dsc1-1 and wrky19-1* with PCR. A, allele specific PCRs on genomic DNA isolated from each Arabidopsis mutant line. PCR amplification products using primer combinations for only the wildtype gene allele (P1) and for the wildtype allele including the T-DNA insert (P2). B-D, Relative gene expression of the genes harbouring the T-DNA insert in the mutant lines as compared to the wildtype Arabidopsis Col-0 using quantitative RT-PCR on roots of 14-day old seedlings. B, represents the relative gene expression of *BAT5* in *bat5-2*. C, represents the relative gene expression of DSC1 in dsc1-1 and wrky19-1. D, represents the relative gene expression of WKRY19 in *dsc1-1 and wrky19-1*. Data (B-D) was generated with three independent biological replicates with three technical replicates each.

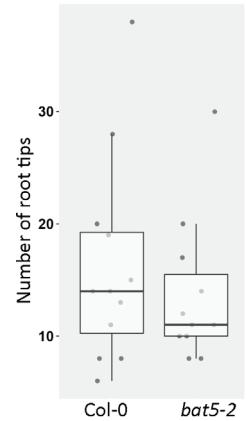


Figure S2: Number of root tips for *bat5-2* on 14 day old seedlings. Statistically tested with ANOVA and post hoc Tukey test (p= 0.05). Data represents two biological replicates.

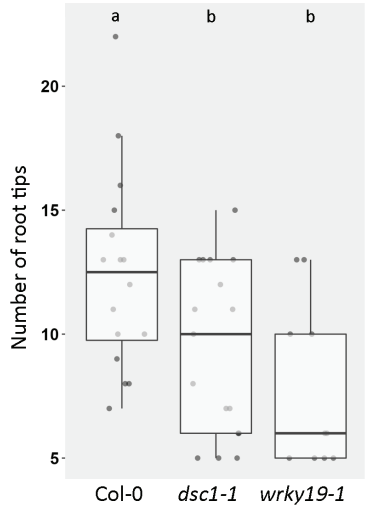


Figure S3: The number of root tips for *dsc1-1* and *wrky19-1* on 14 day old seedlings. Statistically tested with ANOVA and post hoc Tukey test (p= 0.05); letters determine the group based on the level of significance. Data represents three biological replicates.

Table S2: 245 regulated genes in wrky19-1 during *M. incogntia* infection with significance >7 or with relative expression of <-0.3 or > 0.3

Gene ID	Significance	Relative expression
AT2G38330	9.00	0.18
AT5G51860	8.53	0.09
AT5G45380	8.41	-0.09
AT1G34320	8.11	-0.18
AT5G14470	7.96	-0.19
AT5G63730	7.53	-0.29
AT5G46470	7.45	-0.11
AT5G38320	7.30	-0.30
AT4G32890	7.25	0.23
AT4G36540	7.23	0.13
AT4G05020	7.18	-0.16
AT1G09770	7.15	0.13
AT1G12440	7.12	0.17
AT3G23840	7.02	-0.20
AT5G08250	3.09	-0.76
AT5G38910	3.34	-0.74
AT4G26010	3.68	-0.73
AT3G47340	3.28	-0.71
AT1G34510	3.09	-0.67
AT3G62680	3.51	-0.67
AT4G25820	4.05	-0.65
AT2G44925	3.02	-0.65
AT3G12700	2.86	-0.62
AT5G22555	3.26	-0.61
AT2G39010	3.87	-0.60
AT5G57625	4.36	-0.60
AT4G02270	2.98	-0.59
AT2G43580	5.29	-0.59
AT5G57625	3.59	-0.58
AT2G26560	4.19	-0.57
AT1G62980	3.81	-0.57
AT1G29020	3.68	-0.57
AT5G17820	4.10	-0.54
AT4G25220	3.20	-0.54
AT2G46740	3.26	-0.54
AT4G40090	2.96	-0.53

Table S2: continued

Gene ID	Significance	Relative expression
AT2G43590	3.48	-0.53
AT2G26560	4.46	-0.52
AT5G20790	4.72	-0.52
AT3G09925	3.48	-0.52
AT2G43590	2.89	-0.52
AT1G02360	5.05	-0.51
AT3G03530	3.88	-0.51
AT3G30775	2.88	-0.51
AT5G62110	2.71	-0.50
AT2G24720	3.24	-0.50
AT1G78000	3.37	-0.49
AT5G26300	3.27	-0.49
AT3G16530	2.87	-0.49
AT1G01680	3.60	-0.48
AT4G30320	4.07	-0.48
AT1G30870	4.07	-0.47
AT4G09110	4.41	-0.46
AT1G66200	4.39	-0.46
AT5G39580	3.02	-0.46
AT1G11655	3.27	-0.46
AT3G48640	3.81	-0.46
AT5G24210	2.84	-0.46
AT1G24020	3.98	-0.46
AT5G54040	2.87	-0.46
AT3G60330	3.50	-0.45
AT4G16260	2.94	-0.45
AT3G12540	3.99	-0.45
AT1G34520	3.13	-0.45
AT4G32810	3.20	-0.45
AT3G49960	2.96	-0.45
AT2G25240	3.51	-0.45
AT1G32350	4.31	-0.45
AT2G31425	2.99	-0.44
AT5G16980	2.85	-0.44
AT3G13610	6.66	-0.44
AT4G29180	4.01	-0.44
AT1G02220	2.92	-0.44
AT3G10710	3.30	-0.44
AT5G43520	3.88	-0.43
AT4G28940	3.95	-0.43
AT3G48850	4.13	-0.43
AT1G48260	3.62	-0.43
AT5G25260	4.72	-0.43
AT4G16260	2.65	-0.43
AT1G66200	2.89	-0.42
AT1G60050	2.66	-0.42
AT3G46280	2.87	-0.42
AT1G51880	3.04	-0.42
AT4G33020	3.78	-0.42
AT3G16150	3.01	-0.42
AT1G24020	4.69	-0.42
AT5G42510	4.02	-0.42
AT1G24020	3.49	-0.42
AT2G20520	3.51	-0.42
AT4G10510	3.73	-0.41
AT4G36820	5.02	-0.41
AT5G24210	2.74	-0.41
AT5G39050	3.49	-0.41
AT1G13110	2.88	-0.41
AT2G21900	2.85	-0.41
AT1G74790	3.50	-0.41
AT3G44550	4.32	-0.41
AT1G02220	3.20	-0.41
AT5G49350	3.59	-0.41
AT3G14770	3.02	-0.41
AT3G22370	2.68	-0.41
AT1G74000	3.31	-0.40
AT3G12700	4.31	-0.40
AT5G20860	3.27	-0.40
AT2G43510	3.24	-0.40

Table S2: continued

Gene ID	Significance	Relative expression
AT4G16260	3.30	-0.40
AT3G12700	3.36	-0.40
AT2G20520	2.98	-0.40
AT4G26790	4.44	-0.40
AT3G46270	3.98	-0.40
AT5G50260	2.74	-0.39
AT5G19790	3.88	-0.39
AT5G12420	4.56	-0.39
AT4G13890	2.96	-0.39
AT3G47780	2.89	-0.39
AT1G48930	3.20	-0.39
AT1G20180	3.95	-0.39
AT4G02700	3.23	-0.38
AT5G02230	5.73	-0.38
AT5G46950	2.85	-0.38
AT4G34930	2.77	-0.38
AT3G45130	6.59	-0.38
AT2G02300	3.33	-0.38
AT5G44550	3.37	-0.37
AT3G47480	3.77	-0.37
AT1G32940	4.13	-0.37
AT4G12550	3.76	-0.37
AT5G41040	5.08	-0.37
AT4G09100	3.84	-0.37
AT5G48180	2.96	-0.37
AT5G50200	4.07	-0.37
AT1G47890	4.06	-0.37
AT1G05880	3.21	-0.37
AT1G79330	2.85	-0.37
AT3G48640	4.31	-0.37
AT5G05500	3.83	-0.36
AT4G12545	3.85	-0.36
AT2G29620	3.13	-0.36
AT4G25790	4.53	-0.36
AT1G34050	3.20	-0.36
AT5G61010	3.50	-0.36
AT2G05910	3.26	-0.36
AT3G22565	4.21	-0.36
AT1G11190	2.78	-0.36
AT4G37290	3.90	-0.36
AT1G68450	3.12	-0.36
AT5G58840	3.61	-0.36
AT2G20825	2.71	-0.36
AT1G02575	2.69	-0.36
AT1G01560	3.20	-0.36
AT3G05155	4.05	-0.35
AT5G41570	2.74	-0.35
AT3G15700	2.84	-0.35
AT1G65670	2.93	-0.35
AT3G49580	3.62	-0.35
AT1G01750	4.38	-0.35
AT3G55130	3.04	-0.35
AT1G44970	3.40	-0.35
AT3G51350	2.88	-0.35
AT2G28700	2.95	-0.35
AT1G17615	3.91	-0.35
AT4G25110	2.93	-0.35
AT5G01490	3.91	-0.35
AT5G48850	3.28	-0.35
AT1G74460	3.25	-0.35
AT1G51620	3.02	-0.35
AT3G52820	3.21	-0.35
AT1G08790	5.86	-0.35
AT5G59680	2.80	-0.34
AT5G22460	2.84	-0.34
AT1G55020	2.85	-0.34
AT4G26790	2.99	-0.34
AT4G26790	4.12	-0.34
AT5G50750	3.49	-0.34
AT1G04220	2.81	-0.33

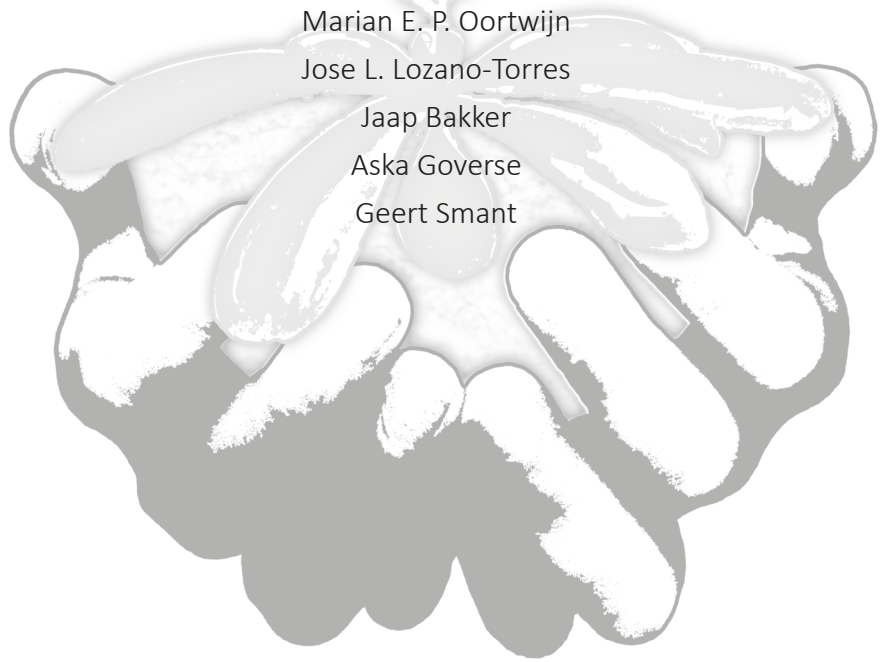
Table S2: continued

Gene ID	Significance	Relative expression
AT1G08050	3.96	-0.33
AT3G04720	3.65	-0.33
AT5G53870	3.06	-0.33
AT1G53270	3.55	-0.33
AT3G19615	2.66	-0.33
AT1G51800	3.07	-0.33
AT2G28160	3.18	-0.33
AT1G70410	3.09	-0.33
AT4G24140	4.44	-0.33
AT4G25310	3.28	-0.33
AT1G54540	4.35	-0.33
AT3G12540	3.92	-0.33
AT4G12010	5.20	-0.32
AT5G58860	2.84	-0.32
AT1G05560	2.78	-0.32
AT3G24290	2.77	-0.32
AT2G36295	2.73	-0.32
AT3G62590	4.77	-0.32
AT5G65980	4.36	-0.32
AT5G64100	3.78	-0.32
AT2G43820	3.26	-0.32
AT4G30140	4.67	-0.32
AT5G13580	3.14	-0.32
AT1G71140	4.17	-0.31
AT1G78990	3.61	-0.31
AT2G33710	2.90	-0.31
AT4G18250	2.77	-0.31
AT5G59130	4.23	-0.31
AT5G06720	3.08	-0.31
AT5G49340	3.45	-0.31
AT2G26410	3.17	-0.31
AT4G35420	4.76	-0.31
AT4G25030	3.10	-0.31
AT3G63380	3.65	-0.31
AT4G16920	3.41	-0.31
AT2G23130	5.12	-0.31
AT1G57630	2.99	-0.31
AT2G44260	3.16	-0.31
AT4G24310	2.73	-0.31
AT1G29000	3.06	-0.31
AT1G56010	2.81	-0.31
AT3G62730	3.96	-0.30
AT3G51360	3.23	-0.30
AT5G20860	3.18	-0.30
AT2G18480	3.64	-0.30
AT4G25190	3.37	-0.30
AT2G41540	3.25	-0.30
At1G64790	2.84	1.19
AT1G66870	2.81	0.62
AT3G48740	2.72	0.58
AT3G27940	3.18	0.54
AT5G23660	2.77	0.53
AT4G08290	3.71	0.48
AT5G48110	3.23	0.45
AT2G22980	3.57	0.45
AT5G42200	6.13	0.42
AT5G45310	2.80	0.38
AT3G59480	3.18	0.38
AT5G42200	2.95	0.38
AT3G56210	2.78	0.37
AT1G74500	3.07	0.36
AT4G01330	3.34	0.36
AT2G14210	3.06	0.35
AT4G37260	3.36	0.35
AT4G07825	6.42	0.34
AT4G14270	2.66	0.32
AT2G22990	2.66	0.32
AT1G19610	3.20	0.32
AT5G25620	3.13	0.31

Chapter 5

The two closely linked RING-variant domain and F-box domain-containing proteins regulate susceptibility of Arabidopsis to *Meloidogyne incognita*

Sonja Warmerdam
Nina Guarneri
Mark G. Sterken
Casper C. van Schaik
Marian E. P. Oortwijn
Jose L. Lozano-Torres
Jaap Bakker
Aska Goverse
Geert Smant



Abstract

Recently, we resolved the polygenic architecture of susceptibility of Arabidopsis to the root-knot nematode *M. incognita* using genome-wide association mapping. In total, we identified 19 quantitative trait loci (QTL) in the genome of Arabidopsis significantly associated with the number of egg masses produced by *M. incognita* per plant seven weeks after inoculation. Here, we report on the characterization of one of the QTLs located on chromosome five of Arabidopsis harboring the RING-variant domain containing protein RU-BOX1 and two novel F-box domain-containing proteins named FFBD1 and FRNI1. Previously, we showed that FRNI1 regulates susceptibility of Arabidopsis to *M. incognita*, but a possible involvement of RU-BOX1 and FFBD1 in susceptibility conferred by this locus had not been excluded. In this study, we found that at 7 days after inoculation the number of invasive juveniles in an *RU-BOX1* overexpressing mutant line was higher than in wildtype Arabidopsis plants, whereas at the same time point we observed less juveniles in an *FFBD1* knock-out mutant line. This demonstrated that both genes could play a role in the susceptibility of Arabidopsis during host invasion by *M. incognita*. However, the final reproductive rate of *M. incognita* was only significantly different in the *RU-BOX1* overexpressing mutant as compared to wildtype Arabidopsis plants at 6 weeks after inoculation. Furthermore, this *RU-BOX1* overexpressing mutant also responded differently to exogenous application of gibberellic acid, while only a *FRNI1* knockout mutant line showed a different response to indole-acetic-acid treatment as compared to wildtype Arabidopsis. Altogether our findings show that multiple genes linked to a single QTL can contribute to the quantitative variation in susceptibility of Arabidopsis to *M. incognita* possibly through different hormonal pathways.

Introduction

The sedentary plant parasitic nematode *Meloidogyne incognita* is one of the most devastating pests in modern agriculture (Jones *et al.*, 2013; Bebber *et al.*, 2014). Second stage juveniles (J2s) of *M. incognita* penetrate the cortex of host plant roots at the base of the elongation zone. The J2s enter the vascular cylinder via the quiescent center in the root tip and migrate further into the differentiation zone where they establish a permanent feeding site (Caillaud *et al.*, 2008; Gheysen & Mitchum, 2011; Mantelin *et al.*, 2015). For the initiation of the permanent feeding site, the J2s redirect the differentiation and growth of vascular cells into large transfer cell-like units that are commonly referred to as giant cells. The exact molecular mechanisms underlying this dedifferentiation of host cells is not well understood. After establishing a permanent feeding site, the J2s feed on host cell cytoplasm for several weeks during which they undergo several molts to finally enter the adult stage. Adult females reproduce by mitotic parthenogenesis and deposit their eggs held together in a gelatinous matrix on the surface of the root (Gheysen & Mitchum, 2011; Kyndt *et al.*, 2013). The reproductive success of *M. incognita* is a measure for the susceptibility of a host plant, which is a complex trait and involves many different plant genes (Warmerdam *et al.*, 2018).

The genome of Arabidopsis includes five regions that predicted proteins harboring a RING-variant domain (RINGV; OrthoDB), none of which has been linked to susceptibility to nematodes before. The RINGv motif folds into a C4HC3-type RING-CH finger found in a number of membrane-associated E3 ligases (Dodd *et al.*, 2004; Metzger *et al.*, 2014). The RING-variant domain consists of approximately 50 residues and is thought to determine the substrate specificity of the E3 ubiquitin ligase protein. Although ubiquitination activity has been demonstrated for several mammalian membrane-associated E3 ligases their precise functions are still not well understood (Dodd *et al.*, 2004; Bauer *et al.*, 2017). To the best of our knowledge, the five orthologous RINGv-containing proteins in Arabidopsis have yet not been extensively studied and their functions are therefore also not known.

In stark contrast with the small number of predicted RINGv-domain containing proteins, Arabidopsis harbors about 700 genes encoding F-box proteins. The F-box is a conserved domain consisting of 40-50 amino acids (Kipreos & Pagano, 2000). Most F-box proteins are also thought to play a role in determining substrate specificity of E3 ubiquitin protein ligase complexes (Gagne *et al.*, 2002; Xu *et al.*, 2009). Various F-box subunits in E3 ligase complexes are involved in linking specific perception of phytohormones to intracellular protein ubiquitination and degradation (Shabek & Zheng, 2014). For instance, auxin binds to the F-box containing protein receptor TIR1 and promotes the ubiquitination and degradation of the Aux/IAA family of transcriptional repressor proteins during auxin response (Mockaitis & Estelle, 2008). Jasmonic acid signaling is dependent on the F-box containing receptor complex COI1 which regulates the ubiquitination and degradation of the JAZ class of

transcriptional repressors (Chico *et al.*, 2008). And lastly, the F-box containing receptor GID1 is thought to regulate the degradation of DELLA transcriptional repressors upon perception of gibberellic acid (Schwechheimer, 2008). However, it should be noted that these examples represent a small minority of the F-box proteins in Arabidopsis of which a specific function is known (Lechner *et al.*, 2006; Shabek & Zheng, 2014).

Earlier work has demonstrated the involvement of two different F-box genes in susceptibility of Arabidopsis to *M. incognita*. Knock-out mutants and lines stably overexpressing the Kelch-domain containing F-box protein 39 (AtKFB39) revealed that this protein has large impact on the susceptibility of Arabidopsis to *M. incognita* (Curtis *et al.*, 2012). AtKFB39 regulates the biosynthesis of phenylpropanoids by altering the stability of phenylalanine ammonia lyases (Zhang *et al.*, 2015), but it is not clear if alterations in this process are also underlying the effect of AtKFB39 on nematode susceptibility. More recently, we have shown that the F-box/Ribonuclease inhibitor-like protein FRNI1 regulates the reproductive success of *M. incognita* in Arabidopsis (Warmerdam *et al.*, 2018). However, the molecular processes underlying the role of FRNI1 in susceptibility of Arabidopsis to root-knot nematodes are also not clear.

The locus harboring the *FRNI1* gene was identified by genome-wide association (GWA) mapping of the reproductive rate of *M. incognita* in Arabidopsis (Warmerdam *et al.*, 2018). Five single nucleotide polymorphisms (SNPs) located in the putative promoter region of *FRNI1* on chromosome 5 were significantly associated with the number of egg masses of *M. incognita* per plant. However, more recently we discovered another SNP significantly associated with reproductive rate of *M. incognita* further upstream of *FRNI1*, which was closely linked to two neighboring genes encoding a RING-variant domain containing protein (RU-BOX1) and an F-box domain containing protein (FFBD1), respectively. Here we demonstrate that RU-BOX1 and FFBD1 also regulate the infection process of *M. incognita* . Furthermore, we identified that *RU-BOX1* is linked to response upon gibberellic acid treatment, while *FRNI1* is linked to response upon indole-acetic-acid treatment as compared to wildtype Arabidopsis. Altogether our findings show that multiple genes linked to a single QTL can contribute to the quantitative variation in susceptibility of Arabidopsis to *M. incognita* possibly through different hormonal pathways. Together, these data add to the complexity of the genetic architecture of susceptibility of Arabidopsis to root-knot nematodes at this locus.

Materials and methods

Plant material

The following homozygous Arabidopsis T-DNA insertion mutant lines were obtained from the Nottingham Arabidopsis Stock Centre (Alonso et al., 2003): SALK_061933C (*ru-box1-1*) with a T-DNA insert in the promotor region of At5G18760, SALK_016393C (*ffbd1-1*) with a T-DNA insert in At5G18770 and SALK_050274C (*frni1-1*) with a T-DNA insert in At5G18780. The T-DNA insert lines were all generated in the background of Col-0 (N60000), which was used as wildtype Arabidopsis throughout this study.

The T-DNA line *frni1-1* was previously confirmed as knock-out (Warmerdam et al., 2018). The presence and homozygosity of the T-DNA insert in lines *ru-box1-1* and *ffbd1-1* was checked by PCR on genomic DNA isolated from leaf material of twelve seedlings (Warmerdam *et al.*, 2018). To determine if the wild type allele was present in the T-DNA mutant we used a different combination of primers for each line (Table S1). To determine if the T-DNA insert was present we used a gene specific primer and the T-DNA-insert specific Lbl3.1 primer (Alonso et al., 2003). For the PCR we used the following conditions: 10 min at 94 °C, 35 cycles of 30 s at 94°C, 1.5 min at 60°C, and 1 min at 72°C, and a final incubation of 10 min at 72°C. The PCR amplification products were analyzed by agarose gel electrophoresis.

The affected gene by the T-DNA insertion was checked with reverse transcription quantitative PCR (RT-qPCR). Total RNA was isolated from whole roots of twelve 14-days-old plants of the specific T-DNA insertion mutant as described below for the RT-qPCR analysis with specific forward and reverse primers (Table S1). Relative expression ratio between the gene of interest and the reference gene was calculated as described elsewhere (Pfaffl, 2001).

Nematode bioassay

Eggs of *M. incognita* were obtained by treating tomato roots infected with *M. incognita* (strain 'Morelos' from INRA, Sophia Antipolis, France) with 0.05% v/v NaOCl for three minutes. Roots were rinsed with tap water and the eggs were collected on a 25 µm sieve. Next, the eggs were incubated in tap water with 3 mM NaN3 for 20 min while shaking. Thereafter, the eggs were rinsed with tap water and incubated on a 25 µm sieve in tap water with 1.5 mg/ml gentamycin and 0.05 mg/ml nystatin in the dark at room temperature. Hatched juveniles were collected after four days and surface-sterilized using 0.16 mM HgCl2, 0.49 mM NaN3, 0.002% v/v Triton X-100 for ten minutes. After surface sterilization, the juveniles were rinsed three times with sterile tap water and transferred to 0.7% Gelrite solution (Duchefa biochemie).

To test the susceptibility of Arabidopsis seedlings, seeds were vapour sterilized (with 0.7 M NaOCl and 1% HCl in tap water) for five hours and transferred to a six-well cell culture

plate containing Murashige and Skoog (MS) medium (MS with vitamins 4.7 g/L (Duchefa biochemie), 20 g/L sucrose and 5 g/L Gelrite). The 6-well plates were first incubated in the dark at 4°C for three days. Thereafter, the seeds were allowed to germinate at 21°C under 16/8 hour light/dark conditions in a growth cabinet for seven days. Individual one-week-old seedlings were subsequently transferred to separate wells in a new six-well culture plate containing MS medium. The seedlings were incubated for seven more days at 21°C under a 16/8 hour light/dark regime. Next, individual seedlings were inoculated with 180 infective J2s of *M. incognita* per plant and incubated at 24°C in the dark.

The number of juveniles were counted seven days after inoculation. Prior to counting, the juveniles were stained with acid fuchsin. Roots were cleaned and incubated in 16.8 mM NaOCL for five minutes, and thereafter in tap water for ten minutes. Next, the roots were transferred into an acid fuchsin solution (0.2M acid fuchsin and 0.8% glacial acetic acid in tap water) and heated in a microwave oven for 30 seconds. After cooling, the roots were transferred into 40% glycerol and the number of juveniles were counted by visually inspecting the roots with a dissection microscope. The number of egg masses per plant were counted six weeks after inoculation by visually inspecting the roots with a dissection microscope.

Each Arabidopsis genotype was tested in at least three independent experiments and 18 replicates per experiment. Experimental effects were corrected with the following equation: variable corrected = variable + (total mean (variable) - batch mean (variable)). Unless indicated otherwise, the differences in counts per plants were statistically analysed using two-way ANOVA and post-hoc Tukey HSD test in R (version 3.0.2, www.r-project.org).

Gene expression analysis with RT-qPCR

To collect roots of infected and non-infected Arabidopsis seedlings for gene expression analyses with RT-qPCR, plants were grown as described for the *M. incognita* infection assay. Samples of twelve whole root systems were cut from aerial parts of the seedlings and snap frozen in liquid nitrogen. Total RNA was isolated from whole roots of *ru-box1-1*, *ffbd1-1* and Col-0 wildtype of 14-day-old seedlings and 21-day-old seedlings with and without *M. incognita*. The frozen root systems were homogenized using TissueLyser (Qiagen) two times for 30 seconds. Total RNA was extracted from 100 mg of the homogenate with the Maxwell Plant RNA kit (Promega Corporation) using the Maxwell 16 Robot (Promega Corporation) according to the manufacturer's protocol. The amount of total RNA per sample was determined using the spectrophotometer ND-1000 (Isogen Life Science). First strand cDNA was synthesized from total RNA using Superscript III first strand synthesis system (Invitrogen) according to manufacturer's protocol. Samples were analysed by quantitative PCR using Absolute SYBR Green Fluorescein mix (Thermo Fisher Scientific). cDNA matching

Arabidopsis elongation factor 1 alpha was amplified as a reference for constitutive expression using primers as indicated in table S1 (Czechowski et al., 2004). To quantify the expression level for the gene of interest specific gene primers were used (Table S1). The RT-qPCR was performed with the following conditions: 15 min at 95°C, forty cycles of 30 s at 95°C, 30 s at 62°C, and 30 s at 72°C, and a final incubation of 5 min at 72°C. Relative expression ratio between the gene of interest and the reference gene was calculated as described elsewhere (Pfaffl, 2001). Relative expression ratio was statistically analysed for significance compared to sample at dpi 0 with a Student t-test (P-value<0.05).

IAA and GA treatment

Arabidopsis seedlings were allowed to germinate and grow for seven days on MS medium as described above for the susceptibility test. After seven days, the seedlings were transferred to square plates with MS20 media with the addition of 0, 1, 2.5, 5 and 10 µM Indole-3-acetic acid (IAA, Duchefa biohemie) or 0, 5 and 10 µM GA 4+7 (Duchefa biochemie). Each plate harboured four seedlings, two of the wildtype Col-0 and two of the T-DNA mutant line. The square plates were placed vertically in 16/8 hours light/dark regime for seven days. To determine the total root length of the seedlings treated with GA, the square plates were scanned with a resolution of 600 dpi (Epson perfection V800). The total root length was measured using WinRHIZO package for Arabidopsis (WinRHIZO pro2015, Regent Instruments Inc.). The number of root tips for the treatment with IAA were counted manually with the use of a dissection microscope. Unless indicated otherwise, three biological replicates were performed of each experiments. Experimental effects were corrected with the following equation: variable corrected = variable + (total mean (variable) - batch mean (variable)). Differences in the total root length per seedling in cm or number of root tips were statistically analysed with a two-way ANOVA and post-hoc Tukey HSD test in R (P-value<0.05).

Promoter GUS analyses

The promotor region of *FRNI1* and the promotor with gene region of *FRNI1* were amplified by PCR from Col-0 genomic DNA using Fusion High-Fidelity PCR (Thermo Fisher scientific) with designed primers (Table S1). The PCR fragment was cloned into pDONR221 (Thermo Fisher scientific) with overnight BP reaction using BP clonase II enzyme mix (Invitrogen) and transformed into *Escherichia coli*. The entry vector was recombined with the pKGWFS7.0 (plant systems biology, Ghent, Belgium) through LR reaction with LR Clonase II enzyme mix (Invitrogen) and transformed into *Agrobacterium tumefaciens* strain GV3101. Sequence integrity was verified by sequencing the cloned products. Arabidopsis ecotype Col-0 plants were transformed by floral dipping (Clough & Bent, 1998) with *Agrobacterium tumefaciens* harbouring the pFRNI1::GUS or the pFRNI1::FRNI1-GUS plasmid.

Seeds of the F1 generation were surface sterilized and the population was screened on MS20 gelrite plates with kanamycin (50 mg/l) and cefotaxin (100 mg/l). Resistant seedlings were transferred after ten days to MS20 medium and 20 day-old plants were transferred to the greenhouse to produce seeds.

Arabidopsis seedlings (F2) were grown and infected as described for the nematode infection assay. After seven days of infection the 21-day-old seedlings were individually placed in an Eppendorf tube and treated with 90% cold acetone and incubated for 15 minutes at -20°C. After incubation the roots were rinsed three times with PBS and incubated at 37°C for two hours in substrate solution (9 ml PBS pH 7.2, 1 ml 3mM K3Fe(Cn)6, 3 mg X-GLcA (Duchefa biochemie)). After incubation, the substrate was removed and the plant material was stored in 70% ethanol at 4°C. Images were made with Axiocam MRc5 (Zeiss) on a dissection microscope.

Results

Recently, we reported on a QTL on chromosome 5 of Arabidopsis marked by four closely linked SNPs significantly associated with the reproductive rate of *M. incognita* (Warmerdam *et al.*, 2018). By lowering the threshold for significant associations (to -log10 (P)>4) (Warmerdam *et al.*, 2019), we identified three additional SNP markers for susceptibility of Arabidopsis to *M. incognita* linked to this QTL. Interestingly, SNP marker Ch5.172110 located at position 6261603 is located within 2 KB of the previously identified SNPs (Fig. 1). SNP Ch5.172110 is located in the first intron of At5G18770 encoding a F-box/FBD-like domain containing protein hereafter named *FFDB1*. The predicted FFBD1 protein consist of an amino terminal F-box domain (PF00646 in PFAM database), a central Leucine-Rich Repeat domain (PF07723), and an F-box-like domain (PF08387) located more at the carboxy terminus. SNP marker Ch5.172110 may also reside in the promotor region of At5G18760, which is located in the reverse orientation on the opposite strand at this locus. The predicted protein encoded by At5G18760 is classified as a RING/U-box superfamily protein (hereafter named RU-BOX1) harboring a variant of the C3HC4 RING-type zinc finger domain (RING-variant; PF12906). Based on the location of SNP Ch5.172110, we concluded that both *FFDB1* and *RU-BOX1* could be functionally linked to the effect on *M. incognita* susceptibility associated with this marker.

To first determine whether *FFBD1* and *RU-BOX1* could play a role in the reproductive success of *M. incognita* in Arabidopsis, we tested the homozygous T-DNA insertion mutant lines *ffbd1-1* and *ru-box1-1* in nematode infection assays. The *ffbd-1* mutant harbors an T-DNA insert in the second exon resulting in a complete knock-out of *FFBD1* expression, while the expression of *RU-BOX1* in this mutant was not affected (Fig. 1 and S1). The *ru-box1-1* mutant

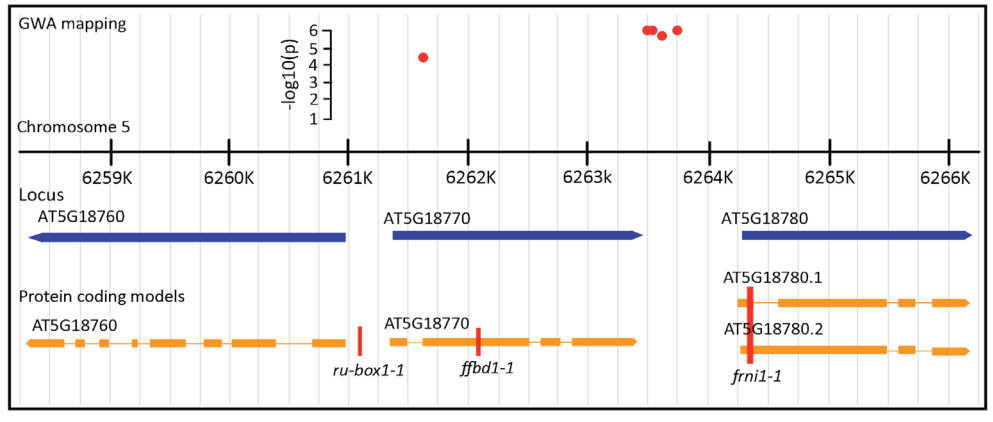


Figure 1: Overview of the genomic region of QTL2 located on chromosome 5. The red dots represent the significantly associated single nucleotide polymorphisms (SNPs). The blue arrows indicate the predicted genes. Transcripts deriving from these genes are indicated in orange, with rectangles presenting exons. The red vertical lines indicate T-DNA insertions with the corresponding name for the T-DNA insertion mutant line.

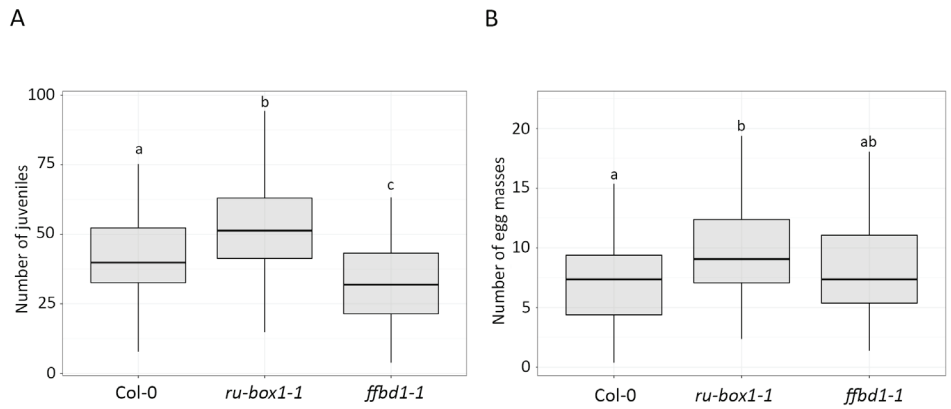


Figure 2: Susceptibility of homozygous T-DNA mutant *ru-box1-1* (*RU-BOX1*) and *ffbd1-1* (*FFBD1*) of Arabidopsis to *M. incognita*. A, Number of juveniles present in the roots counted 7 days post inoculation with *M. incognita*. Juveniles were visualized with acid fuchsin staining. B, Number of egg masses present in the roots counted 42 days post inoculation with *M. incognita*. (A,B) Data was obtained from three independent experiments with n>50. Different lower-case letters indicate statistical difference determined by ANOVA with post-hoc Tukey HSD (P-value<0.05)

harbors a T-DNA insert in the shared promotor region of *FFBD1* and *RU-BOX1,* but only increases the expression of *RU-BOX1* (Fig. 1 and S1). Seven days after inoculation with *M. incognita*, we observed a significantly lower number of juveniles in the roots in the *ffbd1-1* knock-out mutant compared to the wildtype Arabidopsis plants, whereas the number of juveniles inside roots of the *ru-box1-1* overexpressing mutant was significantly higher (Fig. 2A). This demonstrates that both genes are probably involved in the establishment of an infection by *M. incognita* in Arabidopsis. However, at 6 weeks after inoculation we only found a higher number of egg masses per plant on the *ru-box1-1* overexpression mutant as compared to the wildtype Arabidopsis plants (Fig. 2B). Based on these data we conclude that *RU-BOX1* is more likely to contribute to the effect size associated with SNP Ch5.172110.

To determine whether *FFDB1* and *RU-BOX1* are involved in the establishment of an infection with *M. incognita*, we analyzed the transcriptional regulation of both genes by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in whole Arabidopsis roots 7 days after inoculation with infective juveniles of *M. incognita*. We also included samples of mock-inoculated Arabidopsis roots to assess whether *FFDB1* and *RU-BOX1* undergo significant developmental regulation in roots in absence of root-knot nematodes. Both *RU-BOX1* and *FFDB1* were indeed significantly upregulated in non-infected plants at 7 days post inoculation compared to root samples collected at the day of inoculation (Fig. 3). However, only *FFDB1* was significantly repressed in plants infected with *M. incognita* (Fig. 3). We therefore conclude that *FFDB1*, but not *RU-BOX1*, is transcriptionally regulated in association with *M. incognita* infections in Arabidopsis roots 7 days post inoculation.

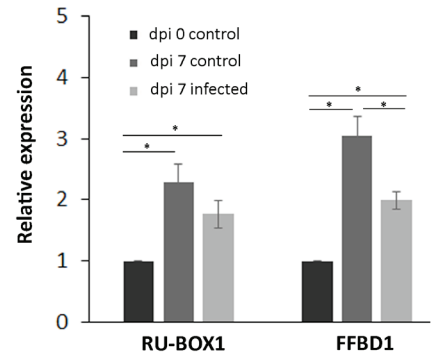


Figure 3: Relative expression of *RU-BOX1* and *FFBD1* in roots of wildtype Arabidopsis Col-0 plants upon infection with *M. incognita*. Data reflect gene expression levels determined with reverse transcription quantitative PCR in whole roots collected at the time of inoculation with *M. incognita* (dpi 0 control), at 7 days after inoculation without *M. incognita* (dpi 7 control) and at 7 days after inoculation with *M. incognita* (dpi 7 infected). The bars represent average values relative to the expression at 0 dpi. The data was based on three independent biological samples with three technical replicates per sample. Error bars represent standard error of the mean. Asterisks indicate statistical differences per comparison as determined by ANOVA with post-hoc Tukey's HSD (P-value<0.05).

FRNI1 is expressed in the feeding site of *M. incognita*

To gain more insight in the spatial expression of *RU-BOX1*, *FFBD1* and *FRNI1*, we set out to generate promotor glucuronidase (GUS) fusion constructs to make transgenic Arabidopsis lines stably expressing these reporter genes. Unfortunately, for the *RU-BOX1* gene we could not obtain a correct PCR product, while for the *FFBD1* gene we were unable to produce stable transgenic Arabidopsis lines expressing the construct. However, we managed to generate multiple transgenic Arabidopsis lines harboring constructs of the *FRNI1* promotor region driving the expression GUS (pFRNI1::GUS) and the *FRNI1* promotor region driving the expression of a translational fusion of FRNI1 and GUS (pFRNI1::FRNI1-GUS). We first monitored GUS expression in the pFRNI1::GUS and pFRNI1::FRNI1-GUS Arabidopsis lines in 21 day old plants (Fig. 4A). We observed GUS expression in the vascular bundle in the oldest leaf and in the roots. In the roots of the pFRNI1::GUS line, GUS expression was observed in the vascular bundle in fully differentiated region of the root, but not in the elongation zone, differentiation zone or root tips. Furthermore, accumulation of GUS was also visible at sites in roots with lateral root primordia. Similar observations were done with the Arabidopsis lines expressing the pFRNI1::FRNI1-GUS construct albeit that the overall GUS expression levels seem to be lower and more confined to older parts of roots.

Infection with *M. incognita* on Arabidopsis plants harbouring pFRNI1::GUS and pFRNI1::FRNI1-GUS showed a similar expression pattern as in 21 days old plants without M. incognita. However, a particularly high level of GUS expression was observed inside the galls induced by *M. incognita* in nematode-infected roots of pFRNI1::GUS and pFRNI1::FRNI1-

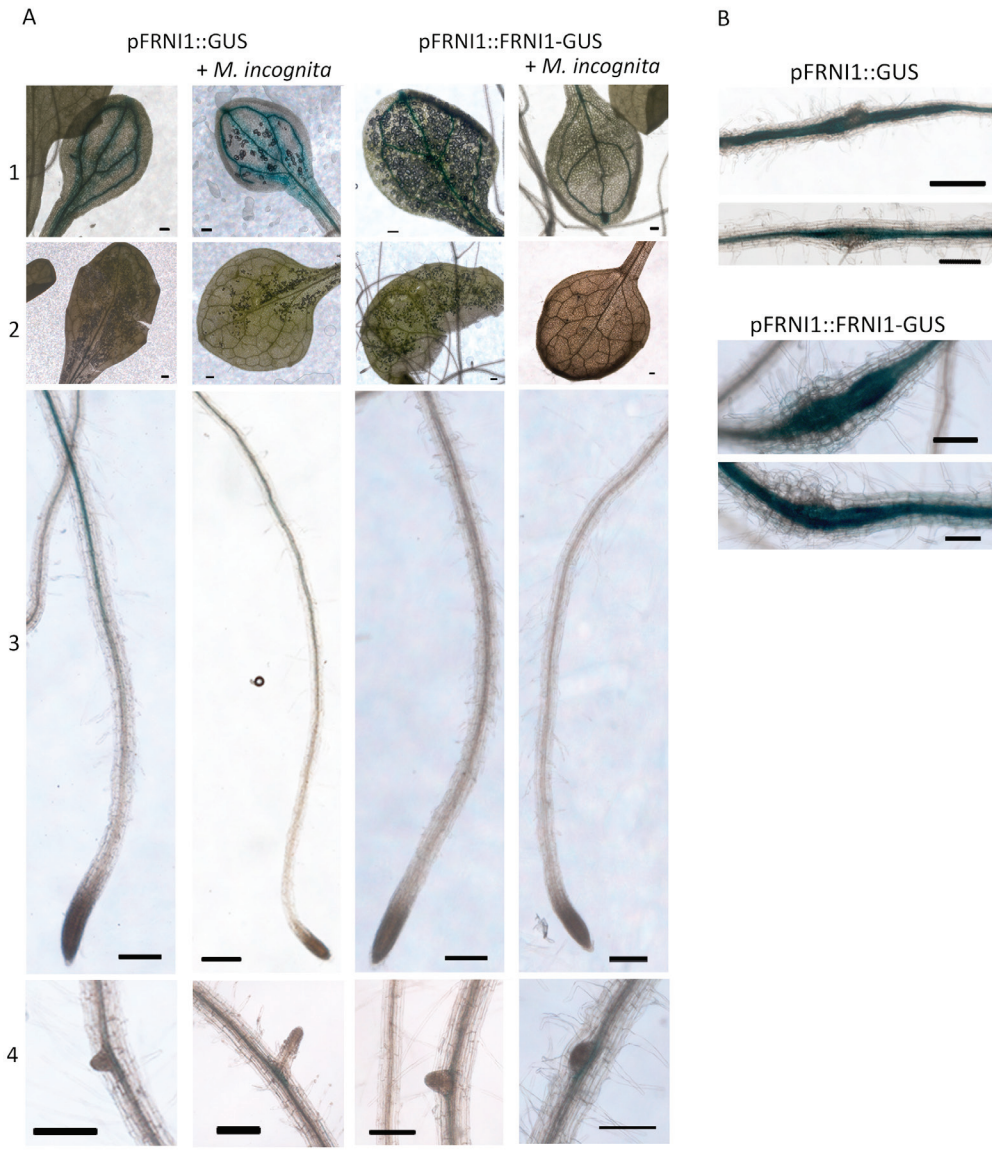


Figure 4. Expression of the *FRNI1* fused to GUS in 21-days-old seedlings with and without *M. incognita* infection. A, expression of pFRNI1::GUS (promotor of *FRNI* fused to GUS) and expression of pFRNI1::FRNI1-GUS (promotor and gene of *FRNI1* fused to GUS) in different plant parts with and without *M. incognita* infection. 1, oldest leaf. 2, youngest leaf. 3, root. 4, root primordia. B, Expression of pFRNI1::GUS and pFRNI1::FRNI1-GUS in feeding sites of *M. incognita*. Black bar represent 200 uM.

GUS lines. Likewise, the vascular tissue in the vicinity of the galls also showed a high level of GUS expression (Fig. 4B). Based on these observations, we conclude that *FRNI1* is expressed inside the vascular bundle of Arabidopsis roots and that its expression is locally induced in and around the infection site of *M. incognita*.

RU-BOX1 and FRNI1 are involved in responses to plant hormones

Several F-box domain containing protein are involved in perception and signaling of plant hormones (Lechner *et al.*, 2006), which are also known to regulate susceptibility of plants to nematode infections (Kyndt *et al.*, 2013). During nematode infection there is a strong upregulation of genes involved the gibberellic acid (GA) biosynthesis (Klink *et al.*, 2007; Kyndt *et al.*, 2012). To assess whether *FRNI1*, *FFBD1*, and *RU-BOX1* are involved in perception and signaling of GA, we monitored the root growth of seedlings of the *ru-box1-1*, *ffbd1-1* and *frni1-1* mutant lines while being exposed to various concentrations of GA in the growth medium. Seven days after the transfer of seedlings to hormone-containing growth medium we observed a significant increase in root length for the *ru-box1-1* mutant as compared to wildtype Arabidopsis at 5 µM GA (Fig. 5A). In contrast, the root length of *ffbd1-1* and *frni1-1* mutants was not significantly different as compared to the wildtype Arabidopsis plants at any of the GA concentrations (Fig. 5B and C). These results indicate that the regulation of susceptibility of Arabidopsis to *M. incognita* by RU-BOX1 may involve the plant hormone GA.

Likewise, to assess whether *FRNI1*, *FFBD1*, and *RU-BOX1* are involved in perception and signaling of indole acetic acid (IAA), we monitored the formation of the root tips in seedlings of the *ru-box1-1*, *ffbd1-1* and *frni1-1* mutant lines while being exposed to various concentrations of IAA in the growth medium. Seven days after the transfer of seedlings to hormone-containing growth medium, we observed a small decrease in the number of root tips per plant for all three mutant lines as compared to wildtype Arabidopsis at most concentrations of IAA (Fig. 6A, B, and C)). However, only the difference between the *frni1-1* mutant line and wildtype Arabidopsis at 5 µM IAA was statistically significant (Fig. 6C). These results indicate that the regulation of susceptibility of Arabidopsis to *M. incognita FRNI1* may involve the plant hormone IAA.

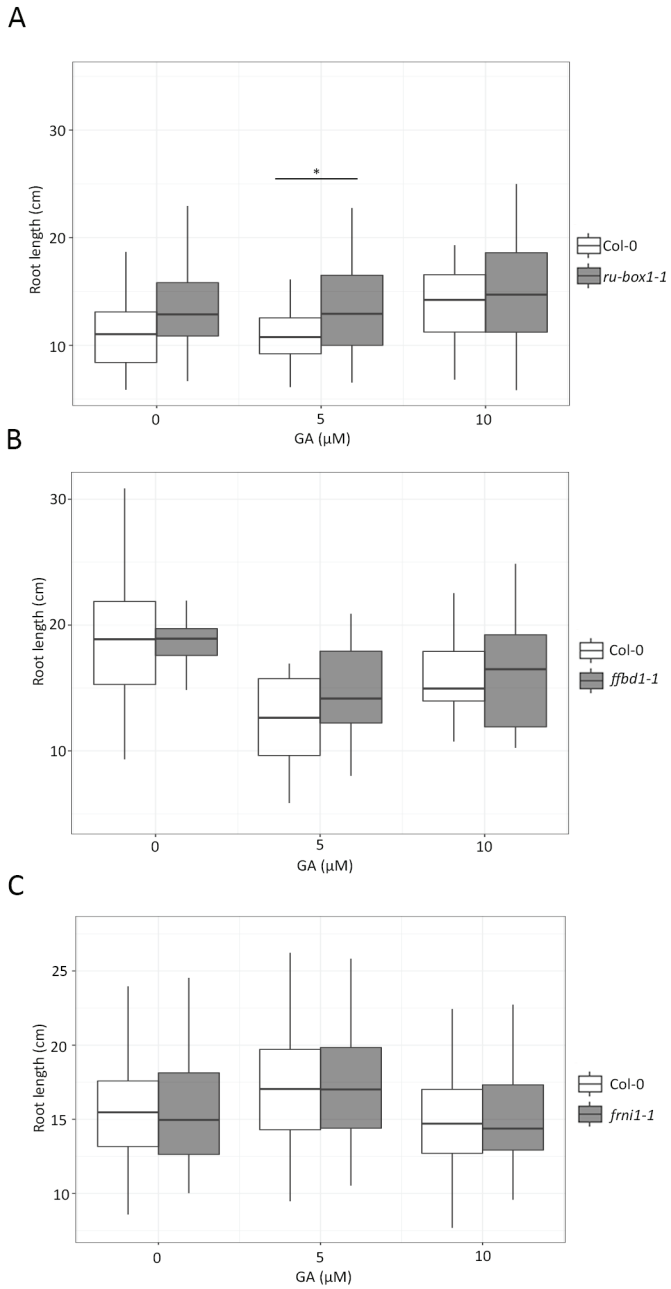


Figure 4. Root length of Col-0, *ru-box1-1, ffbd1-1* and *frni1-1* upon treatment with Gibberellic acid (GA). A, root length of Col-0 and *ru-box1-1*. B, Root length of Col-0 and *ffbd1-1*. C, root length of Col-0 and *frni1-1*. Root length measured in cm for treatment with GA concentration 0, 5 and 10 µM. Data was obtained from three independent experiments with a total of n>30. Data was statistically analysed using ANOVA and post-hoc Tukey HSD (*P-value<0.05).

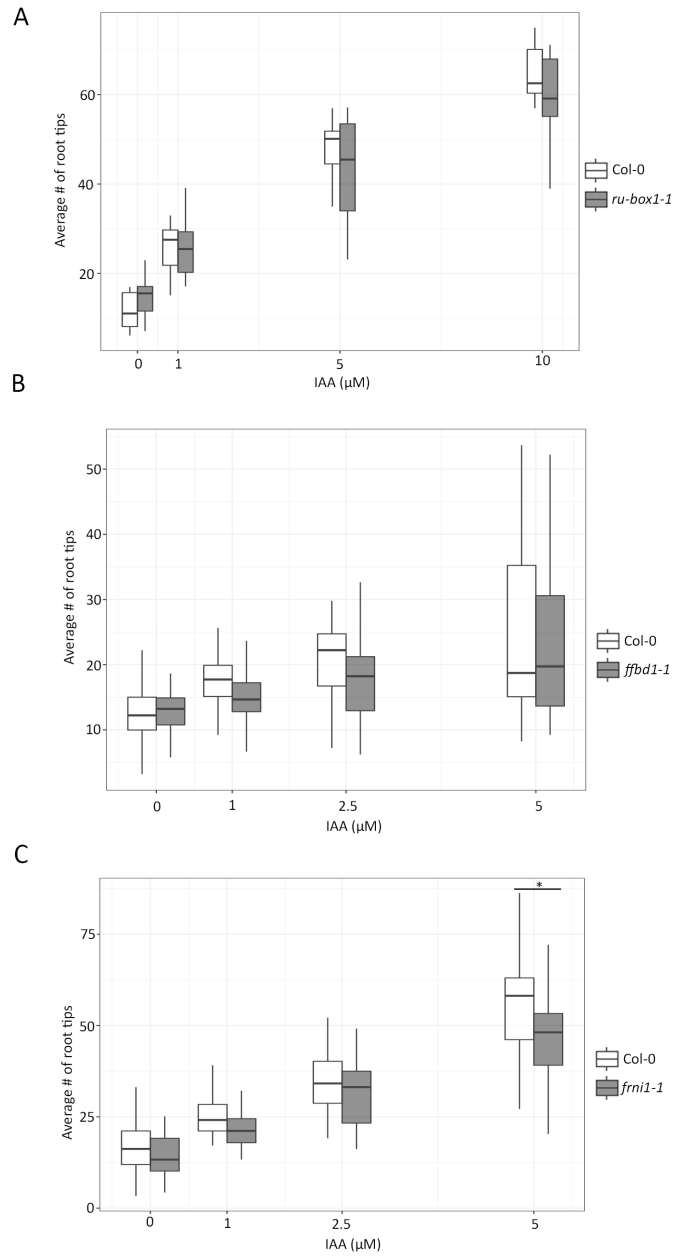


Figure 5. Number of root tips for Col-0, *ru-box1-1*, *ffbd1-1* and *frni1-1* upon auxin (IAA) treatment. A, root tips of Col-0 and *ru-box1-1,* data was obtained from 1 biological experiment with n=10. B, root tips of Col-0 and *ffbd1-1,* data was obtained from three biological experiments with n>30. C, root tips of Col-0 and *frni1-1,* data was obtained from tree biological experiments with n>30. Root tips counted by eye with a dissection microscope upon treatment with different concentrations of IAA. Data was statistically analysed with ANOVA and post hoc Tukey's HSD test (* P-value<0.05).

Discussion

Recently, we resolved the polygenic architecture of susceptibility of Arabidopsis to the root-knot nematode *M. incognita* using genome-wide association mapping to 19 quantitative trait loci (QTL; (Warmerdam *et al.*, 2019)). In this study we further characterized of one the QTLs located on chromosome five of Arabidopsis harboring the RING-variant domain containing protein RU-BOX1 and the two F-box domain-containing proteins FFBD1 and FRNI1. FRNI1 had already been identified as co-regulator of susceptibility of Arabidopsis to *M. incognita*, but the possible involvement of RU-BOX1 and FFBD1 could not be excluded. Furthermore, after relaxing the criteria for significant associations between SNPs and the number of egg masses of *M. incognita*, we discovered an additional marker for this locus suggesting that RU-BOX1 and FFBD1 might indeed contribute to the susceptibility of Arabidopsis to *M. incognita* (Fig. 1). We therefore set out to investigate the role of *RU-BOX1* and *FFBD1* in the susceptibility of Arabidopsis to *M. incognita.*

Our mutant analyses suggest that both *RU-BOX1* and *FFBD1* could play a role in susceptibility during host invasion and early stages of feeding site development in Arabidopsis root infected by *M. incognita* (Fig. 2). The fact that the expression of *FFBD1* in Arabidopsis roots is down-regulated during *M. incognita* infection at 7 days after inoculation lends further support to this statement (Fig. 3). However, the number of egg masses produced on the *ffbd1-1* knock-out mutant line at the end of the life cycle of *M. incognita* was not different from wildtype Arabidopsis plants. This suggests that FFBD1 could only be important for the establishment of a feeding site shortly after host invasion or for the attraction of *M. incognita*. Apparently, this gene does not seem to be critical for the reproduction in female nematodes that have successfully established a feeding site, including those that have been lagging behind due to the lack of FFBD1 activity. In conclusion, given that a complete knock-out of *FFBD1* expression did not significantly alter the number of egg masses of *M. incognita*, it is not likely that this gene has contributed to the quantitative variation in susceptibility we observed in our GWAS panel of 340 Arabidopsis natural inbred lines (Warmerdam *et al.*, 2018).

In contrast, the impact of the overexpression of *RU-BOX1* on the susceptibility of the Arabidopsis *ru-box1-1* mutant to *M. incognita* seems to persist until the production of offspring by adult females. This suggests that part of the effect on susceptibility associated with the SNP markers at this locus could be caused by quantitative variation in expression levels of *RU-BOX1*. Further research using a full knock-out mutant of *RU-BOX1* may reveal to which extent this gene is required for successful reproduction of the *M. incognita*. Nonetheless, our current data indicates that two closely linked genes within a single locus - *RU-BOX1* and *FRNI1* - regulate susceptibility of Arabidopsis to *M. incognita*.

The altered root growth response of the *RU-BOX1* overexpressing mutant to GA suggests that the enhanced susceptibility associated with this gene may involve GA signaling or perception. However, it should be noted that our observations do not follow a classical dose-response curve as the difference in root growth response with wildtype Arabidopsis plants is only significantly different at one intermediate concentration of GA (Fig. 5A). Furthermore, the *ru-box1-1* overexpression mutant already seems to have slightly, but not significantly, larger total root length in the absence of GA in our experimental setup. Nonetheless, the predicted protein encoded by *RU-BOX1* includes a RING/U-box E3 domain which could function as an E3 ubiquitin ligase within an SKP, Cullin, F-box protein containing complex in GA signaling (Chen & Hellmann, 2013). This could be similar to the F-box containing receptor GID1 which is thought to regulate the degradation of DELLA transcriptional repressors upon perception of GA (Schwechheimer, 2008). Furthermore, a role for RU-BOX1 in mediating GA signaling and responses would also fit well with earlier observations of a strong upregulation of genes involved the GA biosynthesis during nematode infections (Klink *et al.*, 2007; Kyndt *et al.*, 2012). However, additional research using a full knock-out *RU-BOX1* mutant is needed for more conclusive evidence for a connection between RU-BOX1 and GA signaling, and responses in nematode-infected roots of Arabidopsis.

The trend in number of root tips per plant in response to indole acetic acid in the growth media was more consistent throughout the range of concentrations for all three Arabidopsis mutants tested in this study. However, only the difference in the number of root tips per plant between *FRNI1* knockout mutant and the corresponding wildtype Arabidopsis plants was statistically significant at the highest concentration (Fig. 6C). This may suggest that FRNI1 could regulate susceptibility of Arabidopsis to *M. incognita* through auxin signaling and response pathways. Auxin has been shown to play an important role in the development of the feeding site induced by plant parasitic nematodes. During *M. incognita* infection auxin levels increase in the feeding site (Hutangura *et al.*, 1999; Karczmarek *et al.*, 2004) and absence of several auxin transporters disrupts feeding site formation of *M. incognita* (Kyndt *et al.*, 2016).

Our data of the spatial expression of *FRNI1* matches to some extent earlier observations on the accumulation of auxin in and around nematode-induced feeding sites (Kyndt *et al.*, 2016). Furthermore, both *FRNI1* expression and auxin accumulation occurs at sites where lateral roots are formed, which further points at a connection between *FRNI1* mediated susceptibility to nematodes and auxin signaling and responses in Arabidopsis. The lateral root formation pathway is known to be necessary for *M. incognita* to establish a feeding site (Cabrera *et al.*, 2014). Additional experiments could be done on lateral root formation in absence and presence of FRNI1 and auxin to find further evidence for the connection of FRNI1 auxin in lateral root formation.

In conclusion, our data shows that three genes linked to one QTL play a role in susceptibility of Arabidopsis to *M. incognita*, which reveals another layer of complexity to the polygenic architecture of this trait. Previously, we also characterized QTL12 that harbours 2 functional

related genes (Warmerdam, submitted) indicating that it is likely that more QTLs associated with susceptibility to M*. incognita* harbour multiple genes linked to susceptibility to *M. incognita*. Furthermore, in view of our earlier discoveries of *BRASSINAZOLE-RESISTANT 1* (*BZR1*; (Warmerdam *et al.*, 2018)) and *ETHYLENE RESPONSE FACTOR 6* (*ERF6*; (Warmerdam *et al.*, 2019)) as candidate susceptibility genes together with our current findings, we expect that much of the allelic variation in the Arabidopsis genome linked to altered susceptibility to *M. incognita* involves different hormonal pathways.

Acknowledgements

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Supplemental information

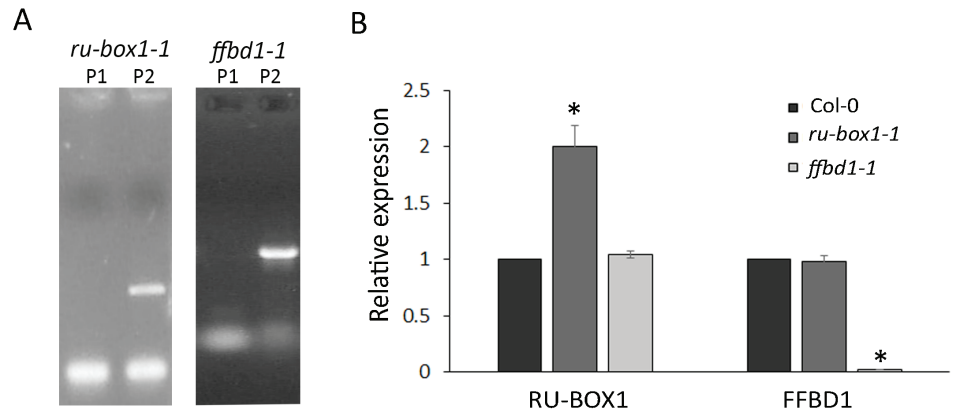

Figure S1: Confirmation of homozygous functional T-DNA insert in *ru-box1-1* and *ffbd1-1*. A, segregation PCR according to SALK guidelines on 1% agarose gel. P1 is the primer combination for wild type allele detection, P2 is primer combination for T-DNA allele detection. B, Relative expression of *RU-BOX1* and *FFBD1* in the T-DNA lines *ru-box1-1* and *ffbd1-1*. Data represents three biological replicates with each three technical replicates. Error bars represent standard error of the mean. Data was analysed with ANOVA post hoc Tuckey HSD (* P-value<0.05).

Table S1: Primers used for RT-qPCR, PCR and cloning.

Identifier	Forward	Reverse
Expression of		
At5G18760 (*RU-BOX1*)	CAGCAGAGTTCAGAGCCACA	TGAAAATGGCGCTGCAACAA
At5G18770 (*FFBD1*)	AACTTGTGCGGCTTTCATCG	CACTACACCAAGACACGGCT
At5G60390 (*EF1a*)	GAGTACCCACCTTTGGGACG	TTGGGTCCTTCTTGTCCACG
T-DNA insert		
RU-BOX1 wildtype	CTTCCACTTGGCTGAGATCTG	AAAGCAATCCCGAAACCTAAC
ru-box1-1 allele	ATTTTGCCGATTTCGGAAC	AAAGCAATCCCGAAACCTAAC
FFBD1 wildtype	CTCTCAAGAACGATTCGCAAC	CAGAATTTTGGAGAGGGGAAG
ffbd1-1 allele	ATTTTGCCGATTTCGGAAC	CAGAATTTTGGAGAGGGGAAG
GUS construct		
FRNI1::GUS Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCTGAAAGTCTAAAAATGTGGGATATGT	
FRNI1::GUS Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTATCAAGACTGAAATACAAATGACCA	
FRNI1::FRNI1-GUS Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCTGAAAGTCTAAAAATGTGGGA	
FRNI1::FRNI1-GUS Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAGAATGACTAATTGACACAAACT	

Chapter 6

General discussion

Introduction

The root-knot nematode *Meloidogyne incognita* is currently ranked as the most invasive plant disease-causing agent (Bebber *et al.*, 2014). For decades, root-knot nematode infestations have been controlled by the application of chemical pesticides. However, most pesticides against root-knot nematode infestations face regulatory bans due to their human and environmental toxicity. Therefore, control of root-knot nematode infestations is now more reliant on crops that harbour major resistant genes (*R*-genes) against root-knot nematodes (Wesemael *et al.*, 2011). For instance, most commercial tomato varieties (*Solanum lycopersicum*) carry the dominant *R*-gene *Mi-1.2* introgressed from the wild tomato species *Solanum peruvianum*. Unfortunately, two natural phenomena currently threaten the use of dominant resistance against root-knot nematodes. First, the widespread dispersal of resistance-breaking nematode populations across major tomato producing regions has turned them into a major concern for growers (Semblat *et al.*, 2000; Davies & Elling, 2015). Secondly, dominant tomato resistance genes against root-knot nematodes are temperature sensitive and rising soil temperatures by global warming may render them ineffective (Jacquet *et al.*, 2005). Thus, to control the root-knot nematode *Meloidogyne incognita* in agriculture new sources of resistance are necessary.

The root-knot nematode *M. incognita* is an obligate biotroph that feeds on living plant cells. Soil-born second stage juveniles (J2s) of *M. incognita* invade the root just above the elongation zone. Subsequently, they migrate intercellularly through the root cortex into the vascular cylinder from below. Inside the vascular cylinder, the J2s establish a permanent feeding structure consisting of several giant cells (Gheysen & Mitchum, 2011; Escobar *et al.*, 2015). Feeding on giant cells enables J2s to moult three times into the adult female stage, while remaining attached to the permanent feeding structure. After a couple of weeks, the adult female will produce offspring as an aggregate of eggs held together by a gelatinous matrix. The exact molecular mechanism underlying the cellular transformation of vascular parenchyma into giant cells is not well understood. However, it is clear that formation of the feeding site involves alterations in a wide range of fundamental molecular and cellular processes, including plant cell wall modification, cell cycle regulation, epigenetic control of gene expression, and cytoskeletal rearrangements (Kyndt *et al.*, 2013). Transcriptomic analysis of giant cell-enriched tissue of roots infected with root-knot nematodes shows that feeding site formation is associated with the differential regulation of more than 1000 genes (Jammes *et al.*, 2005; Fuller *et al.*, 2007; Barcala *et al.*, 2010). The large number of genes regulated in association with feeding site formation suggests that the formation of a feeding site by *M. incognita* is a highly polygenic trait. This highly polygenic trait provides the opportunity to identify less conducive alleles of genes causal for susceptibility (*S*-genes) to *M. incognita* as alternative to major *R*-genes.

The overall objective of this thesis was to identify novel *S*-genes for *M. incognita* in *Arabidopsis thaliana* using a genome-wide association (GWA) mapping strategy. Overall, we showed that GWA mapping makes it possible to resolve the genetic architecture underlying the reproductive rate of *M. incognita* in Arabidopsis, by identifying seven *S*-genes linked to the reproductive rate of *M. incognita* in Arabidopsis. This thesis provides an approach where natural variation is used to identify *S*-genes to create durable resistance against nematodes (Figure 1). In this general discussion I will discuss why *S*-genes can be used as a new source to create resistance against nematodes. I will address the use of GWA mapping to unravel the genetic architecture of nematode-plant interactions. Furthermore, I will elaborate on the identification of specific *S*-genes and their implications for our understanding plant-nematode interactions. Finally, I will discuss how allelic variation in S-genes can be used to create resistance against nematodes.

Susceptibility genes as alternative to major resistance genes

Allelic variation in plant genes enabling initiation, expansion, and maintenance of giant cells may translate into quantitative variation in susceptibility to nematode infections. Less conductive alleles of these *S*-genes could be an alternative approach to make plants more resistant to nematode infections independently of major *R*-genes (Pavan *et al.*, 2010; Schie & Takken, 2014). As a theoretical concept, *S*-genes are defined as host plant genes being required for compatible plant-pathogen interactions. By extension, loss-of-function alleles of *S*-genes may limit the ability of a pathogen to cause disease (Panstruga, 2003; de Almeida Engler *et al.*, 2005). *R*-genes can be overcome by selection of virulent pathogen populations whereas resistance by loss of function of *S*-genes is thought to be more durable as a pathogen would have to overcome a dependency of an essential host factor (Pavan *et al.*, 2010). An example of a long-lasting *S*-gene is the *MLO* gene in barley conferring resistance against powdery mildew (Buschges *et al.*, 1997). The loss-of-function allele of *MLO* was introduced in barley breeding in 1987 and is still providing adequate levels of resistance (Lyngkjær *et al.*, 2000). Similarly, breeding for loss-of-function alleles of *S*-genes in plant-nematode interaction could provide an alternative for the use of R-genes to control nematode infections. However, a possible problem of nematode resistance based on *S*-genes are undesirable pleotropic effects on plant development and growth. Loss of function alleles of *S*-genes can severely alter plant morphology, which makes them not useful for creating resistance to nematodes. Hence, the selection of *S*-genes for breeding purposes should be based on careful functional characterization to assess whether allelic variation in these genes is also associated with undesirable pleotropic effects on other agronomically important traits.

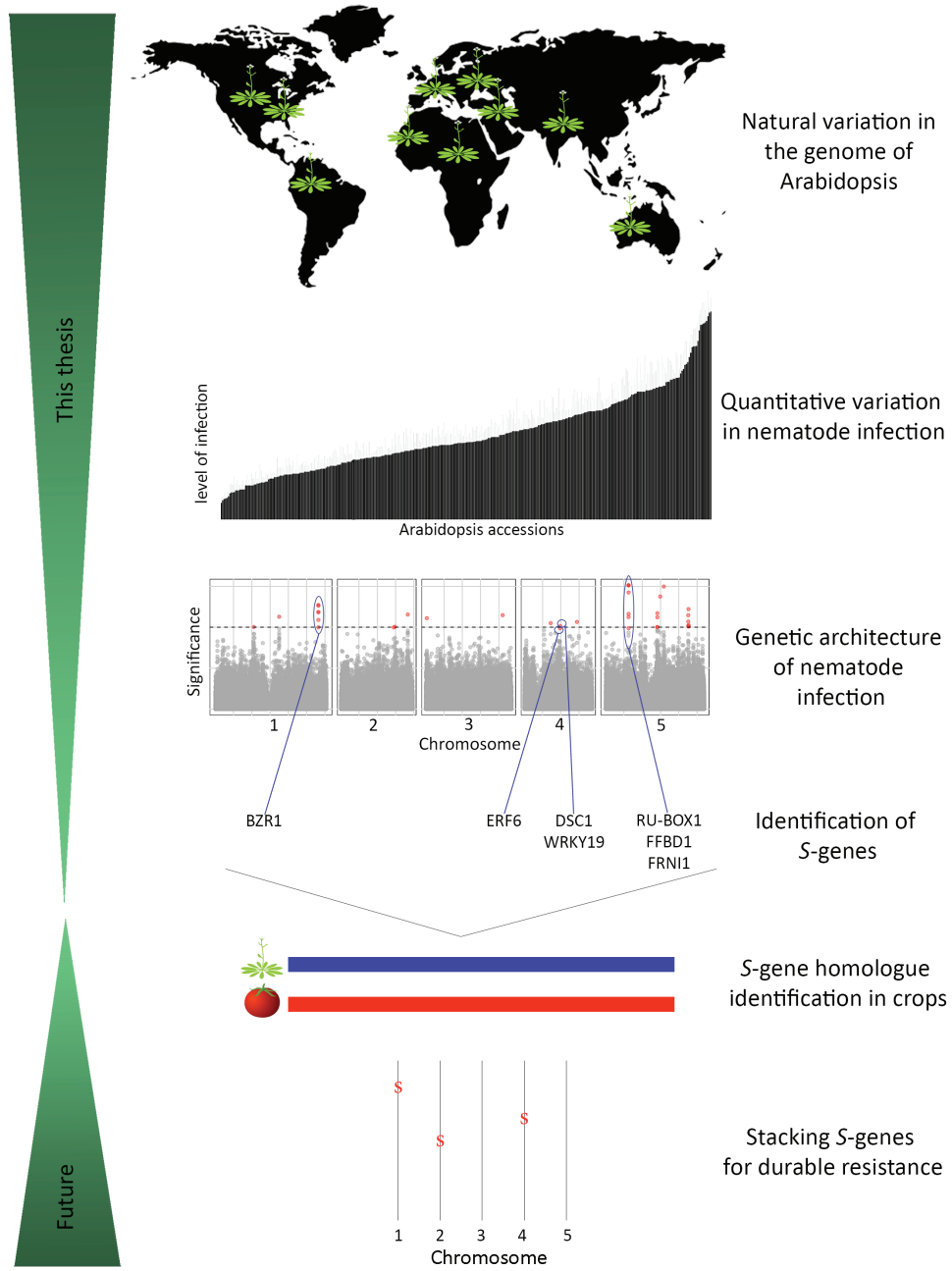

Figure 1. Schematic representation of the approach to identify susceptibility genes (*S*-genes) for *M. incognita* infection in crops. Natural variation in the genome of Arabidopsis plants is used to unravel the genetic architecture underlying susceptibility to *M. incognita* infections in Arabidopsis. This results in the identification of specific *S*-genes that can be used to select for functional homologues in crops to breed for durable resistance against nematodes.

Identification of *S*-genes by forward genetics

A forward genetics approach can be applied to a collection of *Arabidopsis thaliana* natural accessions harbouring quantitative variation in disease susceptibility to identify possible *S*-genes in plant-microbe interactions. The genetic architecture of susceptibility can be resolved by linkage of allelic variation in specific plant genes to pathogen reproduction (Alonso-Blanco & Koornneef, 2000; Koornneef *et al.*, 2004). Narrowing down genome regions causally involved in the variation in susceptibility can be done by quantitative trait loci mapping (QTL mapping) and by genome wide association mapping (GWA mapping) (Doerge, 2002; Koornneef *et al.*, 2004; Bergelson & Roux, 2010; Kloth *et al.*, 2012; Weigel, 2012). QTL mapping typically focuses on genetic diversity in offspring of two parental lines used to create a mapping population. However, the parental lines will only represent a small part of the genetic diversity in a natural gene pool. Nonetheless, QTL mapping has been successfully used to identify several traits in flowering time, plant development, and in resistance to stresses (Kowalski *et al.*, 1994; Jander *et al.*, 2001; McKay *et al.*, 2008; Alonso-Blanco *et al.*, 2009; Jiménez-Gómez *et al.*, 2010). GWA mapping is based on studying associations between a large number of SNPs across a genome and complex traits within a representative set of genetically diverse individuals from a natural population (Zhu *et al.*, 2008). GWA mapping offers several advantages over QTL mapping. It allows for (1) genetic mapping at higher resolution, (2) is less time consuming and requires fewer resources, and (3) it takes into account a larger number of alleles and thus more of the naturally occurring genetic variation (Yu & Buckler, 2006; Bergelson & Roux, 2010). On the other hand, GWA mapping has two major limitations, which may make dissecting of complex traits more difficult (Bergelson & Roux, 2010). It can easily lead to pursuing false positives due to population structure and it will miss rare alleles and weak allele effects due to the insufficient statistical power. So far, genome-wide associations between natural allelic variants and responses to a variety of biotic and abiotic stresses have been mapped successfully onto the genome of Arabidopsis (Kloth *et al.*, 2012; Bac-Molenaar *et al.*, 2015; Kloth *et al.*, 2016; Davila Olivas *et al.*, 2017; Warmerdam *et al.*, 2018; Warmerdam *et al.*, 2019). Recently, multi-trait genome-wide association mapping has revealed cross-correlations between SNPs and resistances to multiple biotic and abiotic stresses in Arabidopsis, including resistance to both osmotic stress and root-knot nematodes (Thoen *et al.*, 2016).

The genetic architecture of susceptibility to *M. incognita* in Arabidopsis

To identify novel *S*-gene candidates for susceptibility to root-knot nematodes in plants with GWA mapping, we first investigated the quantitative variation in reproductive rate of *M. incognita* in 340 natural inbred lines of Arabidopsis. Arabidopsis was used throughout this study because it was thought to lack major *R*-genes against *M. incognita* (Niebel *et al.*, 1994). To measure susceptibility to *M. incognita,* we counted the number of egg masses present in

the roots of Arabidopsis at 7 weeks after inoculation. Our bioassays revealed a surprisingly large variation in reproductive rate of *M. incognita* within the 340 natural Arabidopsis inbred lines (Chapter 2). Initially, we resolved the genetic architecture underlying this large variation in susceptibility to 4 genomic loci when applying a threshold for significance of –Log10(p) =5 in the GWA mapping (Chapter 2). Further characterization of two loci on chromosomes 1 and 5 provided proof-of-principle for using GWA mapping to identify candidate *S*-genes associated with the reproductive rate of *M. incognita* in Arabidopsis. More specifically, allelic variation in *BRASSINAZOLE RESISTANT 1* (*BZR1)* and the F-box family protein *FRNI1* located in these loci were discovered as novel regulators of susceptibility of Arabidopsis to *M. incognita* (Chapter 2 and 5).

Missing heritability

Given that giant cell formation was thought to be a highly polygenic trait, the number of QTLs significantly associated with reproductive rate of *M. incognita* was small. Furthermore, the major-effect SNPs located in the four loci explained 50% of the additive heritable variation in susceptibility observed in the population of 340 Arabidopsis lines. Missing heritability is a common feature of GWA mapping and can be partly explained by the exclusion of alleles that are rare or have a small effects (Yang *et al.*, 2010). Another possibility is the inability to detected allelic variants with a moderate effect on the trait (Caballero *et al.*, 2015). We could resolve the moderate additive effects by reducing the threshold of significant associations in GWA mapping to -log(p)=4, increasing the number of QTLs linked to susceptibility to *M. incognita*. However, by reducing the threshold of significance we also increased the false discovery rate. Nonetheless, at -log(p) of 4 and higher we identified 19 QTLs significantly associated with the reproductive rate of *M. incognita* (Chapter 3). To challenge the risk of pursuing false positives loci, we further characterised a QTL on chromosome 4 with the lowest statistical support and smallest effect size. This locus harbours *ETHYLENE RESPONSE FACTOR 6* (*ERF6*) which was also found to be associated with the reproductive rate of *M. incognita* in Arabidopsis. ERF6 has been identified as mediator in abiotic stress in Arabidopsis before (Dubois *et al.*, 2013) and this thesis provide the first evidence for its association with plant nematode interactions (Chapter 3).

T-DNA insertion mutants versus allelic variation

For this thesis, I mostly used T-DNA insertion mutants to test the contribution of specific plant genes to the susceptibility of Arabidopsis to *M. incognita*. The advantage of using T-DNA knock-out mutants is that it can show if a specific gene is important in susceptibility. Furthermore, loss of susceptibility in a T-DNA knockout mutant could indicate that a gene is mainly involved in plant development and growth and as such it could enable feeding site

initiation, expansion and maintenance. In contrast, a gain of susceptibility phenotype of a T-DNA knockout mutant is more likely to indicate that the gene of interest plays a role in plant immunity. From the view point of generating novel types of resistance based on loss of susceptibility, the second category of genes is more interesting. However, knocking out this type of *S*-genes by a T-DNA insert is more likely to have undesirable pleotropic effects on plant development and growth, complicating the separation of direct and indirect effects on susceptibility. For instance, we observed pleotropic effects on root development leading to a change in root length for the loss of function mutant for RU-BOX1 (Chapter 5).

To reduce the interference of significant pleotropic effects when analysing if a gene is causal for the phenotype linked to a particular locus, it might be more effective to use allelic variants instead of complete knockout mutant lines. To determine whether multiple alleles of *S*-genes differently alter the reproductive rate of *M. incognita,* they will have to be introduced into the same genetic background. Achieving this by traditional transgenesis of alleles is a time-consuming process. Instead, the application of CRISPR/Cas9 technology to introduced different SNPs in a gene of interest may offer a more efficient approach to validate candidate genes from GWA mapping (Ceasar *et al.*, 2016; Curtin *et al.*, 2017).

Genome-wide association mapping and susceptibility to nematodes

Most of the earlier studies to identify plant genes involved in susceptibility of plants to root-knot nematodes have focused on differential expression of genes with various comparative transcriptomics approaches (Jammes *et al.*, 2005; Fuller *et al.*, 2007; Barcala *et al.*, 2010). GWA mapping was used in this thesis to resolve the architecture of the susceptibility of Arabidopsis to *M. incognita*, because it does not result in a strong bias towards genes differentially regulated in association with nematode infections. GWA mapping makes use of genetic differences that explain a variation in a specific trait without considering expression levels of genes. Our data showed that GWA mapping can identify genes that are required for nematode reproduction, but that are not strongly regulated upon nematode infection (Chapter 2, 5). Many genes can be regulated in response to the cellular changes induced by feeding nematodes, but may not necessarily be required for the susceptibility of plants to nematode infections. For instance, we showed that the expression of *GSP1* is affected by *M. incognita* infection, nonetheless functional characterization of T-DNA insert mutant showed that *GSP1* does not contribute to the reproductive rate of *M. incognita* in Arabidopsis (Chapter 2).

So far, GWA mapping has also been used to study the susceptibility of three other plant species to plant parasitic nematodes (Dimkpa *et al.*, 2016; Zhang *et al.*, 2016; Zhang *et al.*, 2017; Anwer *et al.*, 2018). Most of these GWA studies have identified several loci linked to susceptibility, but need further characterization to determine the most likely causal

gene (Dimkpa *et al.*, 2016; Zhang *et al.*, 2016; Zhang *et al.*, 2017). In contrast, a single QTL was identified in a GWA study of Arabidopsis infected with cyst nematodes (Anwer *et al.*, 2018). This QTL harboured the gene *AtS40-3* which was shown to determine the sex ratio of *H. schachtii* on Arabidopsis. Altogether, genome wide association mapping is a powerful technique to identify novel genes associated with susceptibility of plants to parasitic nematodes.

Susceptibility genes and plant hormones

In this thesis, I describe the characterization of seven genes contributing to the susceptibility of Arabidopsis to *M. incognita*, four of which most likely involve plant hormone signalling and responses. Firstly, *BZR1* encodes a transcription factor playing an important role in the brassinosteroid signalling during cell differentiation and cell growth (Chapter 2) (Jaillais & Vert, 2016). BZR1 also regulates the expression of several genes related to auxin biosynthesis and signalling (Sun *et al.*, 2010). Secondly, we have identified two genes, *RU-BOX1* and *FRNI1* in a single locus on chromosome 5, both of which are possibly involved in hormones signalling and responses. For RU-BOX1 we could establish a link with gibberellic acid, while we could link FRNI1 to auxin responses (Chapter 5). Lastly, the transcription factor ERF6 targets genes associated with ethylene and jasmonic acid-dependent responses (Song *et al.*, 2005; Dubois *et al.*, 2015). The exact function of these four *S*-genes in the context of susceptibility of Arabidopsis to *M. incognita* remains unclear, but their link to plant hormone signalling and responses suggests that a significant part of the heritable variation underlying the susceptibility in Arabidopsis to *M. incognita* may involve plant hormones (Gheysen & Mitchum, 2011; Kyndt *et al.*, 2013). This hypothesis can be tested by further investigating the functions of genes located in the 15 QTLs that have hereto not been explored in more detail.

Susceptibility genes and innate immunity

We chose Arabidopsis as model plant to study quantitative variation in susceptibility to *M. incognita* based on the assumption that it lacks major *R*-genes to this nematode species (Niebel *et al., 1994)*. This absence of segregating major resistance genes in Arabidopsis against *M. incognita* was confirmed by the average low effect sizes of SNPs associated with the reproductive rate of *M. incognita* (Chapter 2). Nonetheless, QTL12 on chromosome 4 harbours two genes, *DSC1* and *WRKY19*, with the typical TIR-NLR domain configuration of major *R*-genes (Chapter 3). *DSC1* encodes a typical TIR-NLR immune receptor (Lolle *et al.*, 2017), whereas *WRKY19* encodes a TIR-NLR with an integrated WRKY and MAPx domain at the C-terminus (Rushton *et al.*, 2010). After further characterization with T-DNA mutant lines, we concluded that both genes do not confer major resistance, but rather play a role

in a role in basal immunity against *M. incognita* (Chapter 4). However, the head-to-head genome organisation and specific domain architecture of *DSC1* and *WRKY19* suggests that they could function as TIR-NLR receptor pair (Cesari *et al.*, 2014; Ma *et al.*, 2018). If such is indeed the case then this would be the first discovery of a TIR-NLR pair that functions in basal immunity. Further protein-protein interactions analyses on DSC1 and WRKY19 is needed to test whether they indeed form a functional TIR-NLR pair and how they could control basal immunity against *M. incognita*.

Allelic variation and nematode susceptibility in crops

Allelic variation outside major resistance genes has shown to determine susceptibility in plant-nematode interactions in crops. This variation is either based on the presence of non-synonymous SNPs or on copy number variation. For instance, the presence of two different allelic variants of the papain-like cysteine protease Rcr3 with 6 non-synonymous SNPs results in a major shift in susceptibility of tomato to the potato cyst nematode *Globodera rostochiensis* (Lozano-Torres *et al.*, 2012). Likewise, SNPs at the *Rhg4* locus in soybean contributes to susceptibility to the soybean cyst nematode *Heterodera glycines* (Liu *et al.*, 2012). The *Rhg4* locus encodes a serine hydroxymethyltransferase and the two causal SNPs have a profound impact on both enzyme activity. The *Rhg1* type of resistance to soybean cyst nematode in soybean is linked to copy number variation at this locus on chromosome 18 (Cook *et al.*, 2012). The level *Rhg1*-dependent susceptibility is attributed to the expression levels of three genes, none which shows similarity to major resistance genes. In this thesis, I identified two transcription factors (BZR1 and ERF6) and two putative E3 ligases (RU-BOX1 and FRNI1) as *S*-genes through their linkage with SNPs in the genome of Arabidopsis. It is conceivable that allelic variation in transcription factors can lead to loss of susceptibility through quantitative differences in expression levels of target genes. Similarly, alleles of E3 ligases could result in quantitative variation in susceptibility through changes in substrate specificity and turn-over levels of target substrates. I would therefore recommend to search the genomes of crops for orthologs of these enzymes and transcription factors and test if plants harbouring different alleles of these orthologs also show differences in susceptibility to *M. incognita*.

Future perspectives

To achieve sufficient resolution in GWA mapping of susceptibility to nematodes have to be done on test populations including several hundred different plant genotypes. A major bottleneck in GWA mapping of susceptibility to nematodes in plants are the tedious and labor-intensive bioassays, which involve manual counting of individual nematodes or egg masses in root systems with a dissection microscope. Because of the sheer of size our experiment

(340 Arabidopsis lines) and the fact that the visual inspection of nematode infected roots could not be automated, we needed to separate the phenotypic analysis over more than 20 time-separated batches over a time span of two years. This generated a significant batch effect, which reduced the statistical power of our GWA analyses. This batch effect was among others caused by the preparation of a fresh inoculum out of our propagation cultures for each trial. Furthermore, in spite of using a fully controlled growth cabinet, we also observed seasonal changes in disease susceptibility in Arabidopsis throughout the course of the study. To determine the impact of this batch effect, our phenotypic analysis could be repeated with smaller pre-selected set of plant genotypes in less batches and over a shorter time period. This would also provide an indication of the overall robustness of the data. With the future development of automated phenotyping platforms screening of a large number of plants for nematode infections might at some point also become more feasible, leaving less room for personal interpretation and inconsistencies in handling (Fahlgren *et al.*, 2015).

This thesis demonstrates that GWA mapping of plant-nematode interactions may contribute to the development of resistance against *M. incognita* in plants based on allelic variation of *S*-genes independent of *R*-genes. So far, we have investigated four QTLs located on three chromosomes of Arabidopsis. The fifteen remaining QTLs will likely lead to other novel *S*-genes for susceptibility to *M. incognita* in Arabidopsis. By searching for orthologous genes in other plant species breeders may be able to use the results of this thesis research to generate nematode resistant crops. However, with the proof-of-concept delivered in the thesis a more direct approach could be to employ GWA mapping on a diverse panel of genotypes of a crop species lacking major R-genes. This will probably reveal the genetic architecture of susceptibility of a crop to nematode infection, which can be more easily translated into resistance in cultivars.

To achieve sufficient levels of durable resistance against *M. incognita* it will probably be necessary to introgress loss-of-susceptibility alleles of multiple *S*-genes in crops. As shown in this thesis, the effect size associated with single SNPs and *S*-genes is at best a 20% in loss of susceptibility. However, the cumulative effect of stacking less conducive allelic variants of multiple *S*-genes could result a higher levels of resistance to *M. incognita*. Another benefit of resistance based on multiple *S*-genes is that it will reduce the chance of developing virulent strains by creating a situation where multiple adaptations would be necessary for nematodes to overcome the resistance. With the continuing improvements of the CRISPR/Cas technology for plants it will become less difficult in the near future to introduce allelic variants of multiple *S*-genes in one genetic background of a crop to determine, and to test if stacking of *S*-genes indeed provides an alternative for major *R*-genes to control nematode infections

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Summary

The root-knot nematode *Meloidogyne incognita* is the most invasive plant disease-causing agent threatening global agricultural productivity. The lifecycle of *M. incognita* mainly takes place inside host roots where it forms a permanent feeding site. The formation of the feeding site is damaging to the plant as it is forced to allocate significant amounts of assimilates to the feeding nematode. To control nematode infections, crops can be made more resistant by introgressing major resistance genes (*R*-genes). So far, only the *R*-gene *Mi-1.2* is used in cultivated crops in the defence against *M. incognita*. Resistance based on *Mi-1.2* is currently losing efficacy in the field due to its temperature sensitivity and because of natural selection of virulent nematode populations. This has prompted a search for alternative strategies to develop durable nematode resistant crops. To this end, the research described in this thesis focused on identifying less conducive allelic variants of genes that critically determine susceptibility of plants to *M. incognita* (i.e. *S*-genes).

In chapter 2, we used genome-wide association (GWA) mapping to unravel the genetic architecture of susceptibility of Arabidopsis to *M. incognita*. For the GWA mapping, we quantified the susceptibility of 340 natural Arabidopsis inbred lines which were thought to lack major *R*-genes against *M. incognita*. This led to the identification of four QTLS significantly associated with the reproductive rate of *M. incognita* as a measure of susceptibility of Arabidopsis. Functional characterization of two quantitative trait loci (QTL) revealed a role for BRASSINAZOLE RESISTANT1 (BZR1) and a novel F-box family protein (FRNI1) in the susceptibility of Arabidopsis to *M. incognita*.

However, our first GWA study could only explain 50% of the additive heritable variation within the population of Arabidopsis lines. Therefore, we reanalysed the data using less stringent thresholds in the statistical analysis in chapter 3. This resulted in the identification of 15 additional QTLs collectively explaining 100% of the additive heritable variation in reproductive rate of *M. incognita* in Arabidopsis. To test if we had not merely identified false positives, we functionally characterized one novel QTL with the lowest statistical support and smallest effect size. This resulted in the identification of ETHYLENE RESPONSE FACTOR 6 (ERF6) as co-regulator of susceptibility to *M. incognita* in Arabidopsis. Previously, ERF6 had been linked to mediating abiotic stress responses in Arabidopsis, which suggests that susceptibility to root-knot nematodes involves mitigating abiotic stress.

In chapter 4, we describe a QTL harbouring two genes encoding TIR-NLR-type of immune receptors typically associated with major resistance in plants, named DOMINANT SUPPRESSOR OF Camta 3 NUMBER 1 (*DSC1*) and TIR-NB-LRR-WRKY-MAPx protein (*WRKY19)*. After functional characterization with T-DNA mutant lines, we discovered that both genes do not confer major resistance, but play a role in susceptibility to *M. incognita*

through other mechanisms. Given the head-to-head orientation of *DSC1* and *WRKY19* in the Arabidopsis genome, we suggest that both genes may function as a TIR-NLR immune receptor pair regulating basal levels of immunity to root-knot nematodes.

In chapter 5, we describe a QTL on chromosome 5 harbouring three genes all of which contribute to the quantitative variation in susceptibility of Arabidopsis to *M. incognita*. The three genes encode a RING-variant domain-containing protein RU-BOX1 and two novel F-box proteins, FFBD1 and FRNI1. Further investigation showed that the mechanisms underlying the effects of RU-BOX1 and FRNI1 on susceptibility of Arabidopsis to *M. incognita* may involve gibberellic acid and auxin-mediated signalling and responses.

In conclusion, this thesis demonstrates that Arabidopsis harbours significant quantitative variation in susceptibility to *M. incognita*. This quantitative variation in susceptibility to *M. incognita* gives evidence of the complex genetic architecture of this trait in Arabidopsis, which most likely does not involve segregating major *R* genes. Instead, Arabidopsis harbours allelic variation in genes that critically determine susceptibility independent of plant innate immunity, and that are therefore designated as *S*-genes. This knowledge can be used to identify loss-of-susceptibility alleles of homologous *S*-genes in other plant species for the development of durable resistance against *M. incognita* in crops.

Acknowledgment

Once upon a time there was this girl called Sonja, who really wanted to start a quest at the green castle Radix. With fulfilling a quest as a squire you can become a knight of this castle. During her time as a servant for other squires at the castle, she got really enthusiastic to become a squire herself and fulfill a quest on her own in order to become a knight. It started by asking the King if he had room for another squire under the guidance of one of the landlords. So Sonja went to the King of the castle, Jaap, to ask if she could start her quest at castle Radix. Jaap consulted with his landlords Geert, Arjen, Hans and Jan and the landlady Aska. After some time went by, landlord Geert decided that he had time to assist Sonja as a squire during her quest. Sonja was really happy that Jaap could facilitate the start of her quest with the guidance of landlord Geert. And so on she became a squire of Radix. Sonja started her quest by discovering the world underground in the roots of plants that were attacked by some evil worms. Squire Sonja faced some difficult tasks and had to acquire some new skills, however landlord Geert was always there to help and support when times were difficult. Over time she became more capable and independent as a squire. As the quest passed over the land of landlady Aska, she also provided some help with new insights. With Geert and Aska by her side, Sonja knew she would be able to finish her quest, whatever creature would stand in her way.

A major part of Sonja's quest was about the challenging task to count the number of evil worms on different plants. Gatekeeper Casper was always ready to help en made sure that all necessary tools were provided and he guaranteed that the tasks at hand ran as smooth as can be. Together Sonja and Casper accomplished this challenging task of the quest. Without his help the quest was bound to fail, but due to their teamwork the quest could continue. During the end of the quest, specific tasks got more and more complicated and the help of Mark, one of the knights of Radix, was necessary. With the expertise of Mark, which he gained during his own quest, Sonja was able to get through Statistic-forest and Number-swamp, getting near the finish of her quest.

As a squire you are expected to perform most of the task yourself, therefore it can be very lonely. Sonja however was fortunate that during her quest Amalia, another squire, also started her quest at Radix. During their quests Sonja and Amalia became good friends making their quests less lonely by supporting each other during difficult times. As the quest of Sonja took a few years, more and more squires started their own quest to become a knight at castle Radix. A nice squire family grew with Ava, Jaap-jan, Koen, Katharina, Lisa, Paula, Matthijs, Yiru, Qi, Yuqing and the latest additions Joris and Nina. The squire family together made sure that being a squire is not only hard work but can be fun as well.

There were several occasions that Sonja interacted with the other landlords present at the castle. The interaction with Jan, Hans and Arjen helped Sonja to continue on her quest. Casper wasn't the only gatekeeper she received help from. The other gatekeepers Rikus, Joost, Sven, Hein, Debbie, and Marian were there at the castle to make sure Sonja could have all the tools that she wanted and needed. Beside the Landlords and gatekeepers there were the knights Jose, Ruud, Lotte, Jet, Martijn, Casper Q, and Erik that in one way or another shared expertise or experience with Sonja to help her during her quest. As Sonja was able to learn from the other knights at Radix she shared her knowledge with the servants that passed by. In the years as squire she trained the servants Linde, Mariska, Pascal, Karim, Anne, Louise, Sander, Nina, Annabel, Thomas and Leidy. During their training, all servants provided Sonja with information she needed to continue her quest.

Squire Sonja relied on the Ladies of the castle Lisette, Christel and Manouk for any assistance necessary. During Sonja's quest there was often time for a relaxing walk through the courtyards of the castle to clear her mind and get some fresh air. As walking alone can become dull over time, Ladies Liesbeth and Christel where always happy to accompany Sonja to get a bit of fresh air and share stories.

For Sonja there was also time spend outside the castle in the village around the castle where she was able to escape the heavy squire duties and relax. To fully relax she enjoyed to play a nice game with or without a volleyball with the ladies of "Chaos dames 2". A lot of times and moments were also shared with the " Bakermatties", where new life was an essential subject that was discussed at length. The time in the village with all her friends and "Berenchips" made it possible for Sonja to continue her quest.

Besides the friends in the village, Sonja was well supported by her family. Sonja's dad, mom and Johan were there for all the support that she needed. Her sisters Ilona and Elke provided the moments to escape from the quest and explore other villages. Irma, Marcel, Angela, Chiel and Davy enriched the family of Sonja. She was never alone during her quest, she married her prince Michael. Who provided a stable home for Sonja where she could relax and enjoy life without the pressure of being a squire and finishing the quest. During their times together their lives became enriched with the births of their two beautiful sons Loek and Jess.

After all these years of being a squire, she completed her quest and now is the time for Sonja to become a knight. An accomplishment that wouldn't be possible without the help of all the people from the castle, the village and the family. As a knight Sonja lives happily ever after together with Michael, Loek and Jess, ready for new adventures.

-- THE END --

About the author

Sonja Warmerdam was born in Leiderdorp on 24th of October in 1988. Her primary education was at the Driemaster in Leiderdorp. Her secondary education was performed at the Da Vinci College in Leiden from 2001 to 2007. In 2007 she moved to Wageningen to study Plant science at Wageningen University. In 2010 she completed her BSc. with a specialization in Plant, human and health. In 2012 she graduated her MSc. Plant biotechnology with a specialization in Plant, human and animal health.

In October 2012, Sonja started her PhD at the laboratory of Nematology to study the interaction between Arabidopsis and *Meloidogyne incognita*. This study was part of the consortium "Learning from nature to protect crops". In February 2017 Sonja started a Postdoc position at the laboratory of Nematology to study the interaction between Tomato and *Meloidogyne incognita.* During the time at the laboratory of Nematology Sonja become mother of two sons (March 2015, July 2018).

Since February 2019, Sonja is working as a scientific accessor efficacy of biocides at the Dutch Board of Authorisation of Plant Production Products and Biocides in Ede.

List of publications

Mediator of tolerance to abiotic stress ERF6 regulates susceptibility of Arabidopsis to *Meloidogyne incognita*
Warmerdam, S., Sterken, M.G., Schaik, C.C., Oortwijn, M.E.P., Lozano-Torres, J.L., Bakker, J., Goverse, A., Smant, G. (2019)
Molecular Plant Pathology 20 (1). - p. 137 - 152

Genome-wide association mapping of the architecture of susceptibility to the root-knot nematode *Meloidogyne incognita* in *Arabidopsis thaliana*
Warmerdam, S., Sterken, M.G., Schaik, C.C. van, Oortwijn, M.E.P., Sukarta, O.C.A., Lozano-Torres, J.L., Dicke, M., Helder, J., Kammenga, J.E., Goverse, A., Bakker, J., Smant, G. (2018)
New Phytologist 218 (2). - p. 724 - 737

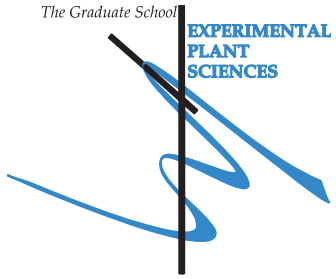
Genetic architecture of plant stress resistance: multi-trait genome-wide association mapping
Thoen, H.P.M., Davila Olivas, N.H., Kloth, K.J., Coolen, S., Huang, P., Aarts, M.G.M., Molenaar, J.A., Bakker, J., Bouwmeester, H.J., Broekgaarden, C., Bucher, J., Busscher-Lange, J., Cheng, X., Dijk-Fradin, E.F. van, Jongsma, M.A., Julkowska, M.M., Keurentjes, J.J.B., Ligterink, W., Pieterse, C.M.J., Ruyter-Spira, C.P., Smant, G., Schaik, C.C. van, Wees, S.C.M. van; Visser, R.G.F., Voorrips, R.E., Vosman, B. Vreugdenhil, D., Warmerdam, S., Wiegers, G.L., Heerwaarden, J. van, Kruijer, W.T., Eeuwijk, F.A. van, Dicke, M. (2017)
New Phytologist 213 (3). - p. 1346 - 1362

Redirection of auxin flow in *Arabidopsis thaliana* roots after infection by root-knot nematodes
Kyndt, T., Goverse, A., Haegeman, A., Warmerdam, S., Wanjau, C., Jahani, M., Engler, G., Almeida Engler, J. De, Gheysen, G. (2016)
Journal of Experimental Botany 67 (15). - p. 4559 - 4570

Apoplastic Venom Allergen-like Proteins of Cyst Nematodes Modulate the Activation of Basal Plant Innate Immunity by Cell Surface Receptors
Lozano Torres, J.L., Wilbers, R.H.P., Warmerdam, S., Finkers-Tomczak, A.M., Diaz Granados Muñoz, A., Schaik, C.C. van, Helder, J., Bakker, J., Goverse, A., Schots, A., Smant, G. (2014)
PLoS Pathogens 10 (12). - 18 p

Education Statement of the Graduate School

Experimental Plant Sciences



Issued to: Sonja Warmerdam
Date: 17 May 2019
Group: Laboratory of Nematology
University: Wageningen University & Research

1) Start-Up Phase	*date*
► **First presentation of your project**	
Identification of susceptibillity factors in Arabidopsis and Tomato to nematode infections	10 Jan 2013
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
Subtotal Start-Up Phase	1.5

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Student day 2013	29 Nov 2013
EPS Get2Gether 2016	28-29 Jan 2016
EPS Get2Gether 2017	9 Feb 2017
► **EPS theme symposia**	
Theme 2 symposium 2013 " Interactions between Plants and Biotic Agents"	24 Jan 2013
Theme 4 symposium 2013 "Genome biology"	13 Dec 2013
Theme 2 symposium 2014 " Interactions between Plants and Biotic Agents"	25 Feb 2014
Theme 2 symposium 2016 " Interactions between Plants and Biotic Agents"	22 Jan 2016
Theme 2 symposium 2017 " Interactions between Plants and Biotic Agents"	23 Jan 2017
► **National meetings (e.g. Lunteren days) and other National Platforms**	
Annual Meeting 'Experimental Plant Sciences' 2013, Lunteren, Netherlands	22-23 Apr 2013
Annual Meeting 'Experimental Plant Sciences' 2014, Lunteren, Netherlands	14-15 Apr 2014
Annual Meeting 'Experimental Plant Sciences' 2016, Lunteren, Netherlands	11-12 Apr 2016
Annual Meeting 'Experimental Plant Sciences' 2017, Lunteren, Netherlands	10-11 Apr 2017
Annual Meeting 'Experimental Plant Sciences' 2018, Lunteren, Netherlands	9-10 Apr 2018
► **Seminars (series), workshops and symposia**	
Symposium: Intraspecific pathogen variation, Wageningen, Netherlands	22 Jan 2013
Symposium: 2nd Annual plenary meeting STW perspectief programme " Learning from nature to protect crops", Wageningen, Netherlands	26-27 Sep 2013
Symposium: How to write a world-class paper, Wageningen, Netherlands	17 Oct 2013
Symposium: STW Perspectief programme "Learning from nature to protect crops: Data base and statistical analyses"	10 Jun 2014
Symposium: 3rd Annual plenary meeting STW perspectief programme " Learning from nature to protect crops", Wageningen, Netherlands	25-26 Sep 2014
Symposium: 'Omics Advances for Academia and Industry: Towards True Molecular Plant Breeding', Wageningen, Netherlands	11 Dec 2014
Symposium: 4th Annual plenary meeting STW perspectief programme "Learning from nature to protect crops", Wageningen, Netherlands	24 Sep 2015
Seminar: Gabino Sanchez Perez. Is your research becoming Digital? Time to call the bioinfomagician!	12 Mar 2013
Seminar: Alain goossens, How jasmonates provide the key to harnass plant chemistry	8 Dec 2015
► **Seminar plus**	
► **International symposia and congresses**	
The XVIth International Congress on Molecular Plant-Microbe Interactions, Rhodos, Greece	6-10 Jul 2014
SPIT meeting, Raleigh, North Carolina	17-18 Sep 2015
32nd Symposium of European society of Nematologists, Braga, Portugal	28 Aug-1 Sep 2016
► **Presentations**	
Poster: Annual Meeting 'Experimental Plant Sciences' 2014	14 Apr 2014
Poster: Annual Meeting 'Experimental Plant Sciences' 2018	9 Apr 2018
Talk: Exploiting natural variation in susceptibility of Arabidopsis thaliana to Meloidogyne incognita to breed broad-spectrum resistance to root-knot nematodes M. incognita. Spring school, Host microbe interactomics	3 Jun 2014
Talk: Natural variation in susceptibility of Arabidopsis thaliana to Meloidogyne incognita. Project meeting STW " Learning from nature to protect crops" project update	13 Mar 2014
Talk: Natural variation in susceptibility of Arabidopsis thaliana to Meloidogyne incognita. 3rd Annual plenary meeting STW perspectief programme " Learning from nature to protect crops" project update	25 Sep 2014

Talk: Natural variation in susceptibility of Arabidopsis thaliana to Meloidogyne incognita. Project meeting STW " Learning from nature to protect crops" project update	29 May 2015
Talk: Natural variation in susceptibility of Arabidopsis thaliana to Meloidogyne incognita: the underlying cause(s). SPIT meeting, Raleigh, North Carolina	17 Sep 2015
Talk:Natural variation in susceptibility of Arabidopsis thaliana to Meloidogyne incognita. Annual meeting "Experimental plant sciences", Lunteren	12 Apr 2016
Talk: Exploiting natural variation in susceptibility of Arabidopsis thaliana to Meloidogyne incognita to breed broad-spectrum resistance to root-knot nematodes. 32nd Symposium of European society of Nematologist, Braga, Portugal	29 Aug 2016
Talk: Genome wide association mapping reveals natural variation in susceptibility in Arabidopsis thaliana to the root-knot nematode Meloidogyne incognita. EPS symposium theme 2	23 Jan 2017
► **IAB interview**	
► **Excursions**	
Subtotal Scientific Exposure	21.9

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Bioinformatics - A user's approach	26-30 Aug 2013
Introduction to R for statistical analysis	21-22 Oct 2013
Spring School Host-Microbe Interactomics	02-04 Jun 2014
Systems Biology: Statistical Analysis of ~Omics Data	15-19 Dec 2014
► **Journal club**	
Participant of literature discussion group at Nematology	2012-2018
► **Individual research training**	
Subtotal In-Depth Studies	7.5

4) Personal Development	*date*
► **Skill training courses**	
PhD competence assesment	26 Mar & 17 Apr 2013
Project and time management	Sep/Oct 2013
Afstudeervak organiseren en begeleiden	Sep/Oct 2013
EPS Expectations day "Comunication and ethics in science", Wageningen, Netherlands	28 Mar 2014
Techniques for writing and presenting a scientific paper	14-17 Oct 2014
Adobe indesign essentials training	29-30 Sep 2015

Scientific publishing	15 Oct 2015
Wageningen University Career day, Wageningen, Netherlands	2 Feb 2016
Scientific artwork with Photoshop and Illustrator	1-2 Mar 2016
Reviewing a scientific paper	22 Sep 2016
Survival guide to peer review	2 Jun 2017
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	6.1

TOTAL NUMBER OF CREDIT POINTS*	**37.0**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

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