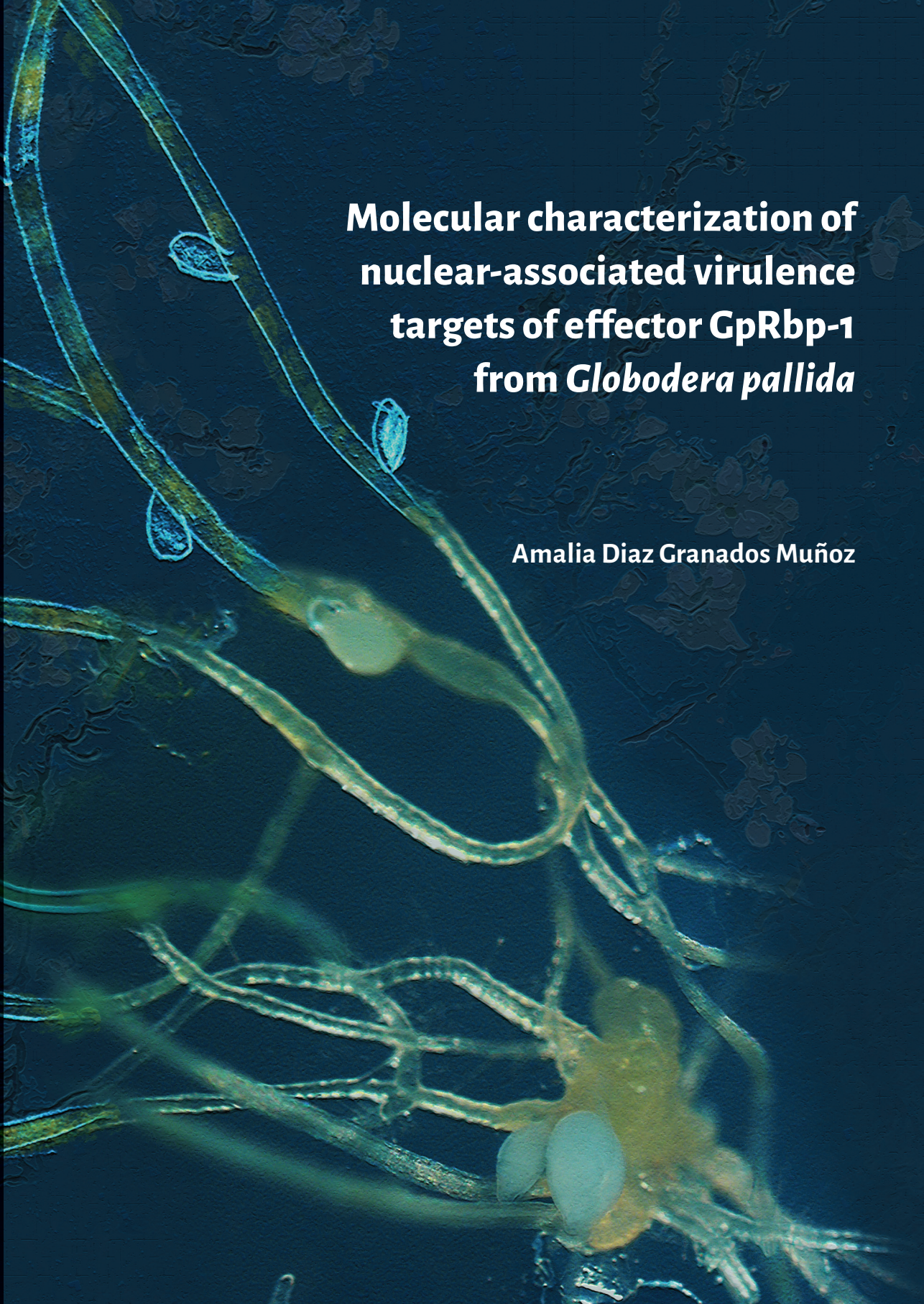

Molecular characterization of nuclear-associated virulence targets of effector GpRbp-1 from *Globodera pallida*

Amalia Diaz Granados Muñoz

Propositions

1. Nematode effector GpRbp-1 supresses salicylic acid-mediated plant immunity through manipulation of SUMO/Ubiquitin ligases. (This thesis).
2. Plant pathogen interactions should be conceptualized as a continuous interface. (This thesis)
3. Pursuing a PhD in Life Sciences is about personal discovery, not scientific relevance.
4. Biologically, gene-edited crops are compatible with organic agriculture.
5. It is shameful that sustainability is marketed as added-value.
6. The female revolution failed by imposing heftier social and economic expectations on women.
7. Scientific writing alienates scientists from society and each other.

Propositions belonging to the thesis entitled

Molecular Characterization of nucleus-associated
virulence targets of effector GpRbp-1 from *Globodera pallida*

Amalia Diaz Granados Muñoz

Wageningen, October 24 2019

Molecular characterization of nucleus-associated virulence targets of effector GpRbp-1 from *Globodera pallida*

Amalia Diaz Granados Muñoz

Thesis committee

Promotor
Dr G. Smant
Associate professor, Laboratory of Nematology
Wageningen University & Research

Co-promotor
Dr A. Goverse
Associate professor, Laboratory of Nematology
Wageningen University & Research

Other members
Prof. Dr G.H.J. Kema, Wageningen University & Research
Dr H.A. van den Burg, University of Amsterdam
Dr V.G.A.A. Vleeshouwers, Wageningen University & Research
Dr S. Mantelin, The James Hutton Institute, Dundee, UK

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Molecular characterization of nucleus-associated virulence targets of effector GpRbp-1 from *Globodera pallida*

Amalia Diaz Granados Muñoz

Thesis

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To my parents

"Dedico esta tesis a mis padres, guardianes celosos de la hogareña heredad y guía inmutable de mi vivir"

- modified from the graduation thesis of Jesús Muñoz Duque, Doctor in Law, 1937

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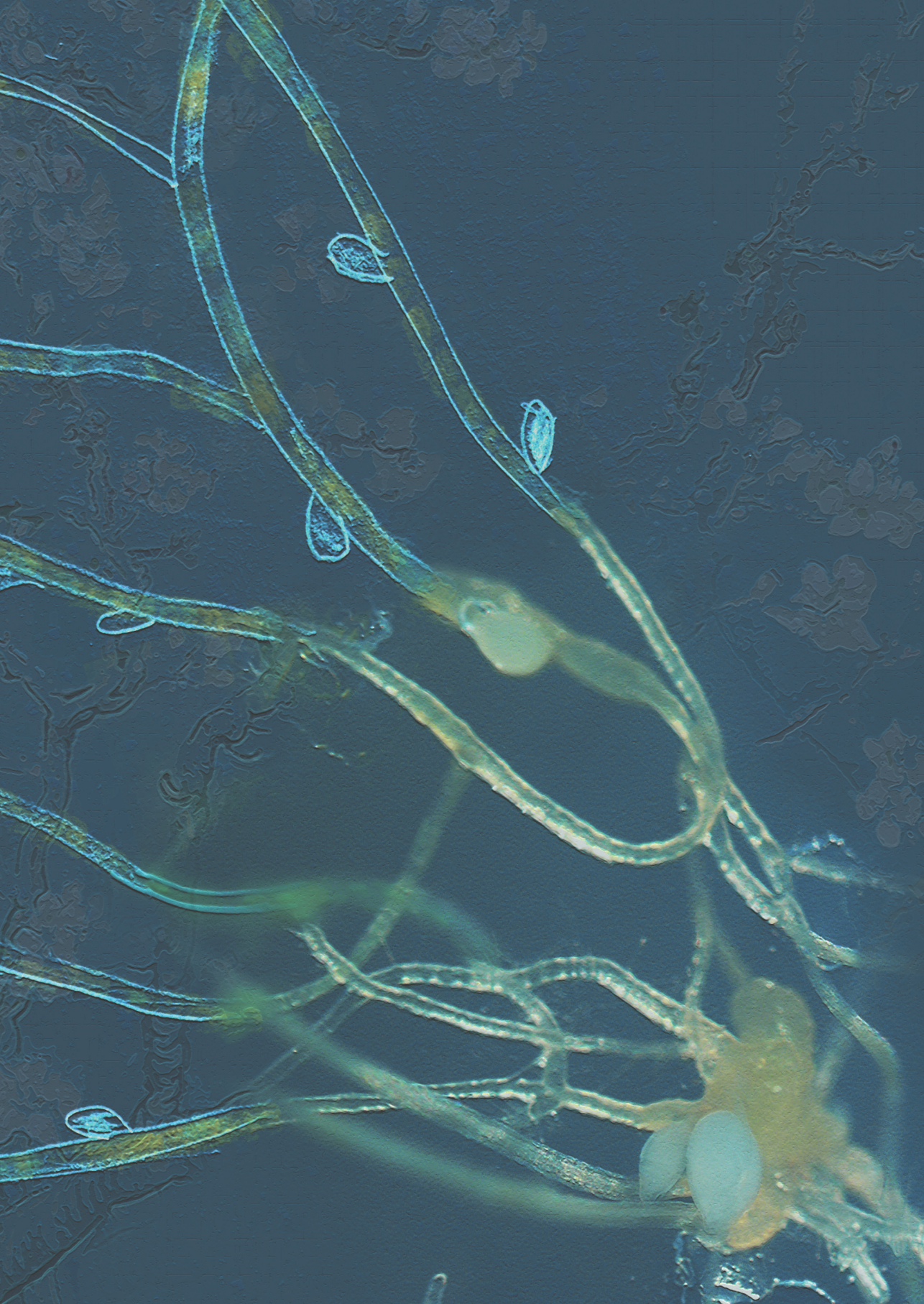

Chapter 1

General Introduction

Amalia Diaz Granados Muñoz

[1] Laboratory of Nematology, Wageningen University, Wageningen, Netherlands

"Nematodes are astonishing organisms.
Despite their deceptively simple morphology...they have been
successful in colonizing an enormous range of environments"
Perry & Moens, 2011

Plant Parasitic Nematodes - Animals Living Inside Plants

Nematodes or round worms are one of the most abundant organisms present in varied environments on the planet (Bohlmann, 2015). Nematodes are believed to have emerged during the Cambrian period, some 550 – 600 million years ago, with the earliest fossilized nematode dated to be circa 396 million years old (Bird *et al.*, 2015). Currently, the phylum *Nematoda* includes over 27,000 taxonomically described species, with an estimate of between 0.5 and 1 million existent species (reviewed in Lambshead & Boucher, 2003; Quist *et al.*, 2015). Phylogenetically, nematodes are divided in 12 clades comprising mostly free-living species feeding on bacteria, fungi, protozoans or algae, but also including parasites of animals, insects, and plants (reviewed in Blaxter & Koutsovoulos, 2015). Morphologically, nematodes are simple animals (e.g. approx. 1000 cells in *Caenorhabditis elegans)*, with an un-segmented vermiform body surrounded by a cuticle which is shed periodically (i.e. ecdysis). Some species change shape in their adult stages as will be illustrated later in this chapter. The body architecture of a nematode can be represented as a "tube inside a tube". The external tube, the hypodermis, acts as a support for the musculature and the nervous system. And the inner tube contains the digestive tract, reproductive system and connections to the longitudinal nerves (Lambert & Bekal, 2002).

Plant parasitic nematodes are microscopic animals that feed from different organs of plants, including roots, leaves, fruit, and bulbs (Perry & Moens, 2011). Plant parasitism is proposed to have evolved independently in at least 4 clades of the phylum Nematoda, with the presence of a protrusible oral stylet as the main morphological characteristic allowing feeding on plant cells (reviewed in Quist *et al.*, 2015; Smant *et al.*, 2018). Different life styles are recognised among parasitic nematodes infecting the roots of their plant hosts (Lambert & Bekal, 2002). Ectoparasitic nematodes are mobile and remain outside of the plant throughout their entire life cycle, being able to switch between hosts. Semi-endoparastic nematodes form a permanent feeding site in their plant hosts, but remain outside of the roots for their entire life cycle (Lambert & Bekal, 2002). Migratory endoparasitic nematodes move inside plant roots and do not form a permanent feeding site. Instead, they draw the cytoplasmic contents of cells and move ahead of the necrotic lesions caused by their feeding (Lambert & Bekal, 2002). Finally, the sedentary endoparasitic nematodes penetrate the roots of plants, establish a permanent feeding site, and remain inside the root for the duration of their life cycle (Lambert & Bekal, 2002).

Sedentary root-knot and cyst nematodes are considered to have the most sophisticated life-style (reviewed in Goverse & Smant, 2014). They establish an intimate interaction with

their hosts, which involves the induction of morphological modifications of the host to support the permanent feeding site. As root-knot nematodes and cyst nematodes depend entirely on the plant, they reside within the roots without ever eliminating their host. Nematodes from these two groups are also considered the most economically damaging plant parasitic nematode species (Jones *et al.*, 2013). Infestation with cyst nematodes results in severely stunted plants with low yields, due to diversion of the nutritional resources of the plant to the root system. Despite a limited autonomous spread, nematodes can be easily transferred by means of agricultural tools, water- or wind-dispersal. Additionally, these parasites can survive up to a few decades in the soil in specialised structures (i.e. hardened cysts). Combined, these factors make nematodes recalcitrant and difficult to eradicate once established in a field (Bohlmann, 2015; Lambert & Bekal, 2002).

Cyst nematodes from the *Globodera* and *Heterodera* genera, go through a series of moults to complete their life cycle (Fig. 1) (Bohlmann, 2015). First, embryos develop into a first-stage juvenile (J1), which moults into an infective juvenile (J2), while still in the egg. Upon the perception of specific hatching factors, mobile infective juveniles hatch from the egg and disperse in the soil to find a suitable host (Perry & Moens, 2011). Hatching factors are mostly root-exudates, but other factors such as carbon dioxide concentration, temperature and pH gradients can help nematodes locate the roots of their host (Perry & Moens, 2011). During migration in the soil, juveniles rely solely on food reserves for energy, and have therefore limited longevity of only a few days. Once a host has been located, the juveniles penetrate the roots, usually in the elongation zone, close to the root tip (Bohlmann, 2015). Root invasion is achieved through stylet thrusts and the use of plant cell wall-degrading enzymes secreted by the nematode (Sobczak & Golinowski, 2011).

Once inside the root, cyst nematodes migrate intracellularly until they reach the vascular bundle or the cortex (Sobczak & Golinowski, 2011). Subsequently, these pathogens probe cells in the cortex, endodermis, or pericycle to find a suitable initial syncytial cell. Migration and proving cause extensive damage, which induces plant responses such as protoplast collapse and callose depositions close to the stylet. Therefore, a suitable cell for initiation of a feeding site is one that does not mount these types of responses during probing (Sobczak & Golinowski, 2011). The initial syncytial cell undergoes drastic modifications to form a mature syncytium. Upon successful initiation of a syncytium, cyst nematodes lose their ability to migrate any further. The feeding site, therefore, becomes their sole nutrient source through the next moults to J3, J4, and adult stages. The induction of a syncytium is pivotal to the sedentary cyst nematodes, because nematode development comes to a halt if the first attempt to establish a feeding site is unsuccessful. Moreover, the availability of nutrients through the syncytium determines sexual differentiation into adult males or females (Bohlmann, 2015). In the event of low nutrient availability, more juveniles differentiate into males. In the adult stage, males regain their vermiform shape and become mobile again to exit the roots and search for females to inseminate. With sufficient resources available, females multiply their size to a round-shaped structure and burst out of the root tissue. Upon insemination of the female and fertilization of oocytes by male sperm, eggs are formed inside the body of the female. The female eventually dies, leaving an egg containing-cyst protected by its former hardened cuticle that can remain attached to the root (Fig. 1) (Bohlmann, 2015).

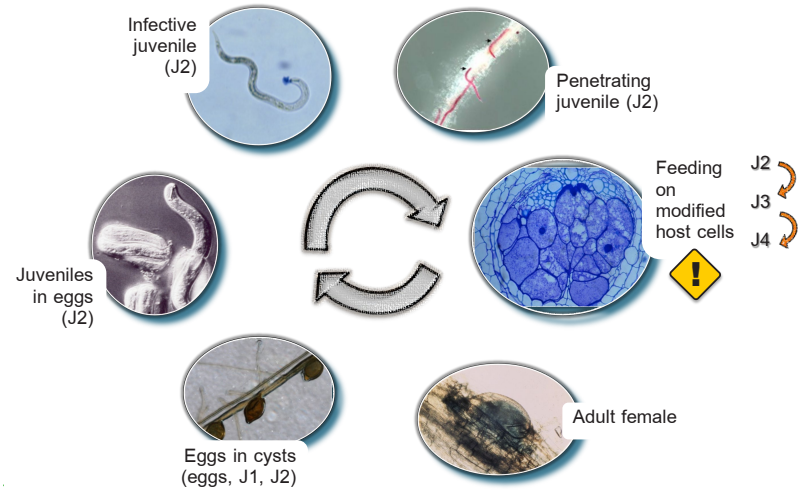

Figure 1. Life cycle of cyst nematodes. Mobile infective juveniles (J2) hatch from eggs and migrate to find a host. After penetration, the juveniles migrate intracellularly in the roots of the host while probing host cells to find a responsive cell to establish a feeding site. The initiation, expansion, and maintenance of the feeding site are essential for the survival of cyst nematodes. The acquisition of sufficient food enables infective juveniles to develop to J3, J4, and adult stage. After insemination and fertilization, females produce offspring that remain inside their body. After death, the outer cuticle of the females hardens into a cyst-shaped container that encloses the eggs. The eggs (along with J1 and J2 stages) remain inside the cyst until conditions are favourable for hatching. The duration of the life cycle depends on the species and environmental conditions, but ranges from 4 to 8 weeks. Modified from (Goverse & Smant, 2014).

The Plant Immune System - Plants Have An Ace Up Their Sleeves

Despite being sessile, plants are continuously involved in interactions with beneficial and detrimental animals, microbes or other plants. Plants have therefore evolved an immune system to sense and differentiate the presence of harmful biotic agents. In addition to recognition, plants are able to mount responses to halt or minimise invasion by these agents. Traditionally, the plant immune system has been conceptualized based on the zig-zag model, which proposed the existence of two layers of plant immunity: PAMP-triggered immunity (PTI) (also referred to as basal immunity) and effector-triggered immunity (ETI) (Jones & Dangl, 2006). This division into two layers hinges on the existence of a set of pathogen and plant molecules, namely Pathogen-Associated Molecular Patterns (PAMPs; later generalised as Danger-Associated Molecular Patterns, DAMPs), effectors and immune receptors, and three immune statuses, namely PAMP/DAMP-triggered immunity, effector-triggered susceptibility and effector-triggered immunity. In a nutshell, the recognition of PAMPs (or DAMPs) by extracellular plant receptors leads to PTI, an immune status supported by specific cellular responses that could halt pathogen invasion. Subsequently, suppression of PTI responses by pathogen-secreted effectors leads to effector-triggered susceptibility (ETS). In addition, a secondary layer of immunity, effector-triggered immunity, is initiated

by intracellular recognition of effectors and leads again to resistance. The dynamic shifting between these immune statuses is therefore conceptualized as a zig-zag between immunity and susceptibility (Jones & Dangl, 2006).

The definitions of PAMP, effector, PTI and ETI have become restrictive in the face of the discovery of numerous new components of the immune system and immunogenic molecules. The most recent proposals, the Spatial Immunity and Spatial Invasion models separate the immune system into two categories, which correspond to the physical separation of the intracellular and extracellular spaces (Kanyuka & Rudd, 2019; van der Burgh & Joosten, 2019). The terminology of both models is slightly different, but they overlap in defining the existence of immunogenic molecules, which can be derived from the pathogen or the plant. Furthermore, these molecules can be recognised in the apoplast or the cytoplasm by immune receptors from the receptor-like kinase (RLK), receptor-like proteins, or nucleotide-binding leucine-rich repeat (NB-LRR) families, respectively. Recognition of the immunogenic molecules activates dedicated signalling cascades and culminates in the deployment of cellular mechanisms to halt pathogen penetration, invasion or establishment (Kanyuka & Rudd, 2019; van der Burgh & Joosten, 2019). While endorsing the ideas of the Spatial Immunity/Invasion models, the use of terminology from the zig-zag model will be retained for the purposes of this thesis. First, because the previous studies underpinning the findings of this work are reported in the framework of the zig-zag model. Second, because specific evolutionary and biological traits of effectors are not fully described by "invasion pattern" or "intracellular immunogenic pattern". Therefore, the use of the term effector is also used throughout this thesis. A more detailed definition of effector is provided in later sections.

Recognition by immune receptors leads to the activation of immune cellular responses (reviewed in Dodds & Rathjen, 2010). These responses include reprogramming of gene expression, callose depositions and a burst of reactive oxygen species. In addition, influx of calcium ions, mitogen-activated protein kinase signalling cascades and hormone-mediated signalling pathways are activated. Finally, in some cases, a type of programmed-cell death known as hypersensitive response is induced (reviewed in Dodds & Rathjen, 2010). Currently, several immune receptors have been shown to mediate resistance to cyst nematodes. Cf-2, an apoplastic RLK receptor confers resistance to *G. rostochiensis* in tomato (Lozano-Torres *et al.*, 2012). Also in tomato, Hero confers resistance to *G. pallida* and *G. rostochiensis* (Ganal *et al.*, 1995). In addition, cytoplasmic NB-LRRs Gro1-4, Hero and H1 (Paal *et al.*, 2004), confer resistance to *G. rostochiensis* in potato (Paal *et al.*, 2004; Rice *et al.*, 1985; Sobczak *et al.*, 2005). Finally, cytoplasmic NB-LRR Gpa2 confers resistance to *G. pallida* in potato (van der Voort *et al.*, 1997).

Downstream of immune signalling, two main phytohormones are recognised as modulators of plant immunity: salicylic acid (SA) and jasmonic acid (JA) (reviewed in Pieterse *et al.*, 2012). The SA and JA pathways are largely recognised to act antagonistically, with the SA pathway being more involved in defense against biotrophic pathogens and the JA pathway, involved in defense against herbivores and necrotrophic pathogens (reviewed in Pieterse *et al.*, 2012). Nevertheless, this antagonism is not reflected during infections with sedentary nematodes. Instead, in the context of cyst nematode infections, both hormones function as

promotors of defense (reviewed in Gheysen & Mitchum, 2019). In particular, SA has been shown to act as a negative regulator of susceptibility (Kammerhofer *et al.*, 2015; Wubben *et al.*, 2008). As discussed in the following section, some nematode effectors supress plant immunity, but the mechanisms underlying suppression of SA-mediated immunity remain widely unexplored (reviewed in Gheysen & Mitchum, 2019).

From Plant Cell To Syncytia – It's All About Effectors

Severe morphological changes occur in plant cells during the expansion of nematode-induced syncytia (reviewed in Kyndt *et al.*, 2013). Initially syncytia expand from the cortex towards the vascular cylinder and then spread further laterally along this vascular cylinder. Expansion of the syncytia involves progressive fusion of neighbouring cells through enlargement of plant cell wall openings and dissolution (Sobczak & Golinowski, 2011). The mitotic cell cycle of plant cells is re-activated during their incorporation into syncytia (de Almeida Engler *et al.*, 1999). Instead of completing the normal mitotic cell cycle, these cells switch to the endocycle resulting in endopolyploidy (de Almeida Engler *et al.*, 1999). At maturity, syncytia are large multinucleated cellular complexes, characterized by large nuclei, a dense cytoplasm rich in small vacuoles, mitochondria, ribosomes, plastids and microtubules and microfilaments (reviewed in Kyndt *et al.*, 2013; Sobczak & Golinowski, 2011). The ultrastructural features of syncytia reflect their high metabolic activity, which is also evidenced in the transcriptional activation of genes involved in biosynthetic and metabolic processes (Szakasits *et al.*, 2009; Walter *et al.*, 2018). Once established, the syncytium will remain active until the nematodes stop feeding (Bohlmann, 2015).

The morphological changes to the plant during penetration, migration and feeding site formation are regulated at the molecular level by interactions of plant cell components and a suite of proteins secreted by the nematode, collectively named effectors (reviewed in Gheysen & Mitchum, 2019; in Smant *et al.*, 2018; and in Vieira & Gleason, 2019). Effectors are active during the different stages of nematode parasitism and are generally produced in the oesophageal glands of the nematodes. The glands are connected to the digestive tract of the system, so effectors are delivered to the host cell through the stylet (reviewed in Gheysen & Mitchum, 2019; inSmant *et al.*, 2018; and in Vieira & Gleason, 2019). For instance, plant cell wall-modifying enzymes such as cellulases and pectin lyases are secreted by the nematode to aid during intracellular migration (reviewed in Wieczorek *et al.*, 2015). Other examples of nematode secreted effectors include the CLAVATA3/EMBRYO SURROUNDING REGION (CLE)-like family of effectors, which are required for infection by various species of cyst nematodes (reviewed in Gheysen & Mitchum, 2019). CLE-like effectors mimic the plant own CLE peptides, which are involved in the maintenance and regulation of differentiation of stem cells (reviewed in Miyawaki *et al.*, 2013). Furthermore, CLE-like effectors can bind the receptors for plant CLE peptides, and are therefore, widely accepted to be involved in the regulation of syncytium formation through an undefined mechanism (reviewed in Gheysen & Mitchum, 2019). Also, effector 30D08 from *H. glycines* and *H. schachtii* translocates to the nucleus and interacts with an auxiliary spliceosomal protein *SMU2* (Verma *et al.*, 2018).

30D08 and *SMU2* are required for virulence of nematodes, and heterologous expression of 30D08 under the *SMU2* promoter results in alternative splicing of plant genes. 30D08 is therefore proposed to interact with SMU2 to modulate alternative splicing in the plant, thereby promoting susceptibility to nematodes (Verma *et al.*, 2018).

The successful establishment of an infection by nematodes requires modulation of the immune system of the plant by effectors. For instance, overexpression of Venom Allergen-like Protein (VAP) effectors from *H. schachtii* supresses the characteristic immune response to bacterial flagellin 22 (Lozano-Torres *et al.*, 2014). The immunosuppression capability of VAPs is further demonstrated by the increased susceptibility of Arabidopsis plants ectopically over expressing *Hs-VAP1* and *Hs-VAP2* to unrelated fungal and oomycete pathogens (Lozano-Torres *et al.*, 2014). Interestingly, the VAP family of effectors is secreted into the apoplast, and suppresses specifically the immune responses activated by extracellular immune receptors (Lozano-Torres *et al.*, 2014). A more recent example of immune-suppressing nematode-secreted effectors is *HgGLAND18* from *H. glycines* (Noon *et al.*, 2016). Transient expression of *HgGLAND18* in the leaves of *Nicotiana benthamiana* promoted invasion by *Pseudomonas syringae* pv. *tomato* and decreased expression levels of marker genes for SA-mediated immunity. This indicates that *HgGLANwD18* is able to suppress basal immunity and SA-mediated immunity in *N. benthamiana* (Noon *et al.*, 2016).

In this thesis I focus on SPRYSEC effectors, a particularly expanded family described from potato cyst nematodes. The structure of SPRYSECs is characterized by an N-terminal signal peptide for secretion and a C-terminal SPRY domain. Interestingly, the SPRY domain lacks a catalytic activity, but has been shown to function as a protein-binding platform when found as part of peptides from other organisms (Woo *et al.*, 2006).

Outline Of The Thesis

SPRYSEC effectors are shown to both activate and supress plant immunity, but their role in virulence in the absence of major resistance genes has not been elucidated (Blanchard *et al.*, 2005; Mei *et al.*, 2018; Qin *et al.*, 2000; Rehman *et al.*, 2009). In this thesis I aimed to characterise the virulence role of SPRYSEC effector GpRbp-1, which is recognised by potato NB-LRR resistance protein Gpa2 in some potato cultivars. GpRbp-1 is proposed to target plant proteins and act as a virulence factor for the nematode in the absence of major resistance genes. Consequently, we aimed to identify plant proteins interacting with GpRbp-1, to characterise the role of such host targets in susceptibility to cyst nematodes, and to evaluate how GpRbp-1 may modify the host targets and their activities to favour nematode virulence (Fig. 2). First, in **Chapter 2** we review recent findings of the function and structure of the SPRYSEC family of effectors from cyst nematodes. There, we propose that members of the SPRYSEC effector family, such as GpRbp-1, confer versatility to the effector repertoire of cyst nematodes and modify the activities of host interactors to promote susceptibility to nematodes. Subsequently, in **Chapters 3, 4,** and **5** we explored the repertoire of host targets of SPRYSEC GpRbp-1 and address questions about the specific role of each target in plant-nematode interactions.

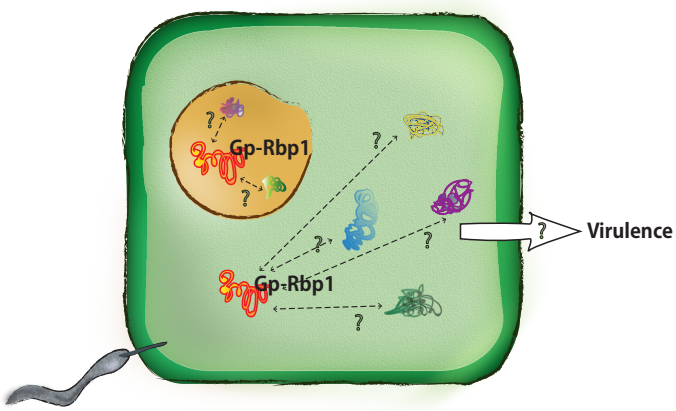

Figure 2. Graphical representation of the main research questions of this thesis. GpRbp-1 is proposed to function as a virulence factor by targeting plant proteins. Therefore, we aimed to identify and characterise host targets of GpRbp-1.

In **Chapters 3** and **4**, an untargeted yeast 2-hybrid screen was used to identify host targets of a virulent (non-Gpa2 eliciting) variant of GpRpb-1. The identification of candidate interactors in a heterologous system (yeast) called for further validation of the individual interactors by *in planta* protein-protein interactions studies. Furthermore, the subcellular localization of the interactors and a possible re-localization due to binding was examined by live confocal microscopy. Finally, a functional role of the GpRbp-1 interactors was assessed by a combination of *in vitro* nematode infection assays and whole transcriptome analyses. The work performed for this thesis made use of two main experimental systems. *Agrobacterium*-mediated transient transformation was used for heterologous expression of the proteins of interest (GpRbp-1 and candidate interactors from potato *Solanum tuberosum*), for protein interaction and live imaging studies. Nematode assays and transcriptomic studies were performed using the *Heterodera schachtii* – *Arabidopsis thaliana* model system, as this platform was more amenable to reverse genetics than the natural host of *G. pallida*, potato. The cyst nematodes *H. schachtii* and *G. pallida* have similar life styles and are assumed to induce similar modifications during the formation of syncytia in their respective hosts.

Chapter 3 describes the identification of E3 ubiquitin ligase UPL3 as a virulence target of GpRbp-1. We further showed that UPL3 from potato localises the nucleus of plant cells, where it co-localizes with GpRbp-1. Additionally, we established that UPL3 has a small effect on susceptibility to cyst nematodes, but a large footprint on the transcriptomic response of the plant to infection by cyst nematodes. The alterations of the nematode-infected plant transcriptome by a mutation of *upl3* suggest that this ligase is involved in the modulation of the plant immune responses.

Similarly, in **Chapter 4** we demonstrate that GpRbp-1 targets the E3 SUMO ligase SIZ1 from potato. SIZ1 has been previously shown to be a nuclear regulator of plant development and immunity (Guo & Sun, 2017; Hammoudi *et al.*, 2018; Lee *et al.*, 2006). In accordance, we show that GpRbp-1 and SIZ1 co-localize and interact in the nucleus of plant cells. Furthermore,

our results suggest that SIZ1 is required for susceptibility to cyst nematodes in *Arabidopsis*. The prominent role of SIZ1 as regulator of SA-mediated plant immunity, suggests that GpRbp-1 may target this ligase to hamper plant immunity. Together the results of Chapters 3 and 4 suggest that GpRp-1 may interact with the post-transcriptional regulatory machinery of the host to modulate plant immune responses.

In **Chapter 5** we took a more targeted approach in studying the role of plant protein RanGAP in susceptibility to cyst nematodes. RanGAP2 is known to be required for immune recognition of GpRbp-1 by resistance protein Gpa2 (Sacco *et al.*, 2009). While the role of RanGAP as immune co-factor has been studied previously, its involvement in nematode virulence remained unexplored. Here, we showed that GpRbp-1 targets both RanGAP2 and its homologue RanGAP1. For RanGAP2, this interaction was mapped to the plant-exclusive WPP domain, which functions as a retention factor for RanGAP in the nuclear envelope. Furthermore, we uncovered that both RanGAP homologues are required for susceptibility to cyst nematodes, with RanGAP1 having a larger effect on susceptibility to cyst nematodes than RanGAP2. Furthermore, our results suggested that binding to RanGAP is not the sole determinant of Gpa2 recognition, as both eliciting and non-eliciting variants from different *G. pallida* populations (Rookmaker and D-383) interact with RanGAP2/1.

Finally, in **Chapter 6** the scientific and practical implications of the main findings of this thesis are discussed. Furthermore, mechanistic models for the virulence function of GpRbp-1, derived from the findings about its host interactors, are proposed.

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Chapter 2

SPRYSEC Effectors: A Versatile Protein-Binding Platform to Disrupt Plant Innate Immunity

Amalia Diaz-Granados[1], Andrei-José Petrescu[2], Aska Goverse[1] and Geert Smant[1]

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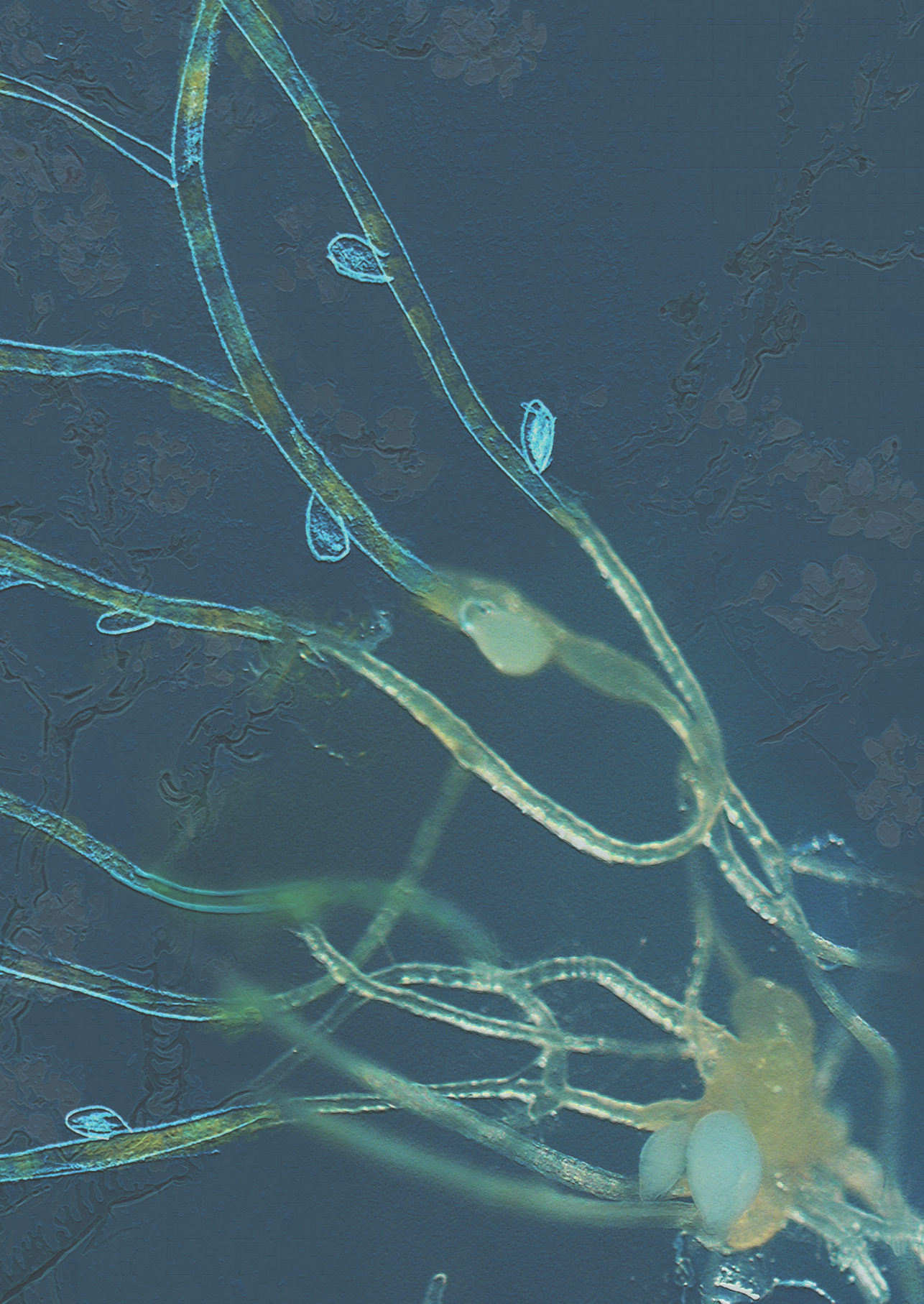
[1] Laboratory of Nematology, Wageningen University, Wageningen, Netherlands; [2] Department of Bioinformatics and Structural Biochemistry, Institute of Biochemistry of the Romanian Academy, Bucharest, Romania



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Abstract

Persistent infections by sedentary plant-parasitic nematodes are a major threat to important food crops all over the world. These roundworms manipulate host plant cell morphology and physiology to establish sophisticated feeding structures. Key modifications to plant cells during their transition into feeding structures are largely attributed to the activity of effectors secreted by the nematodes. The SPRYSEC effectors were initially identified in the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*, and are characterized by a single SPRY domain, a non-catalytic domain present in modular proteins with different functions. The SPRY domain is wide-spread among eukaryotes and thought to be involved in mediating protein–protein interactions.Thus far, the SPRY domain is only reported as a functional domain in effectors of plantparasitic nematodes, but not of other plant pathogens. SPRYSEC effectors have been implicated in both suppression and activation of plant immunity, but other possible roles in nematode virulence remain undefined. Here, we review the latest reports on the structure, function, and sequence diversity of SPRYSEC effectors, which provide support for a model featuring these effectors as a versatile protein-binding platform for the nematodes to target a wide range of host proteins during parasitism.

Introduction

Plant-parasitic nematodes are microscopic roundworms that can infect thousands of different plant species, causing severe damage to food crops all over the world (Gheysen and Mitchum, 2011). Annual crop losses due to nematodes amount to $125 billion per year, but this sum may be an underestimate because of improper identification of nematode infestations (Danchin *et al.*, 2013; Jones *et al.*, 2013). Outbreaks of plant-parasitic nematodes have long been controlled by applications of nematicide chemicals to infested soils. However, recent legal bans on the use of most of these highly toxic compounds have sparked a particular interest in biological factors determining the efficacy and durability of different types of nematode resistance in crops.

So far, most of the research on nematode resistance has focused on the obligate biotrophic cyst nematodes (genera *Globodera* and *Heterodera*) and root-knot nematodes (genus *Meloidogyne*) (Jones *et al.*, 2013). In the early stages of an infection, these endoparasites migrate through the roots until they find a suitable plant cell to initiate a permanent feeding site (Gheysen and Mitchum, 2011). Cyst nematodes induce a syncytium, a large assembly of hundreds of adjacent cells joined by partially degraded cell walls. Root-knot nematodes induce multinucleate giant-cells by stimulating a few cells to undergo multiple rounds of mitosis without cytokinesis. The ontogeny of both syncytia and giant cells involves the regulation of hundreds of different plant genes, many of which are related to plant cell growth, differentiation, and defense. The permanent feeding site functions as the sole nutrient source for the nematodes for several weeks. Failure to establish a permanent feeding site results in an arrest of nematode development, in which the nematode is unable to reproduce and the host plant becomes then effectively resistant to infection (Goverse and Smant, 2014).

The massive molecular and cellular changes associated with permanent feeding site establishment in plants are most likely brought about by nematode-secreted effectors (Gheysen and Mitchum, 2011; Quentin *et al.*, 2013). In other fields of plant sciences the formal definition of effector is limited to proteins that suppress plant defense responses (Hogenhout *et al.*, 2009), but for plant–nematode interactions the term is used more broadly. Nematode effectors are defined as proteins and small peptides with a wide range of molecular functions that either assist in host invasion, modulation of plant immune responses, or initiation and maintenance of the permanent feeding site (Mitchum *et al.*, 2013; Quentin *et al.*, 2013). Plant-parasitic nematodes produce effectors mostly in dedicated esophageal glands. Specific subsets of these single-celled organs are active during different stages in the nematode lifecycle. The subventral esophageal gland cells are more active in migratory pre-parasitic and parasitic stages, secreting proteins required for root invasion and nematode movement inside the host. The dorsal esophageal gland cell specializes in secretion during the sedentary stages, most likely producing effectors involved in feeding site formation and maintenance. However, there is no precise functional boundary between the secretions of the subventral and dorsal esophageal glands. The function of some of the effectors, such as suppression of host defense, can extend throughout various stages of parasitism. By contrast, different

sets of effectors are released to target specific plant cell processes depending on the stage of the infection. Plant-parasitic nematodes deliver the glandular secretions into the plant through a protractible oral stylet. Although this stylet does not seem to penetrate the plasma membrane of host cells, nematodes are able to deliver effectors both into the apoplast and cytoplasm of recipient cells (Mitchum *et al.*, 2013).

A variety of transcriptome and genome analyses have given insight into the diversity and complexity of the large effector repertoires of root-knot and cyst nematodes (Hewezi and Baum, 2012). As the majority of nematode effectors are novel proteins, only a small subset has been functionally well characterized primarily based on initial sequence homology. For instance, host invasion is mediated by a large panel of plant cell wall modifying proteins with striking similarity to bacterial homologs (Davis *et al.*, 2011; Bohlmann and Sobczak, 2014). Likewise, host cell differentiation during the establishment of the permanent feeding site most likely requires the involvement of nematode effectors with sequence similarity to plant CLE peptides (Mitchum *et al.*, 2012). For novel effectors lacking sequence similarity identifying the molecular target in host cells often provides the first concrete lead toward their biological function [e.g., the effector 19C07 of *Heterodera schachtii* (Lee *et al.*, 2011)]. Besides sequence homology and knowledge of host targets, the level of diversity within effector families has also been used to predict their involvement in plant parasitism [e.g., HYP family from *Globodera pallida* (Eves-van den Akker *et al.*, 2014)]. The rationale for focusing on this sequence diversity is the accelerated evolution, which is typically observed in products of gene families operating at plant–pathogen interfaces. In nematodes, as well as in other plant pathogens, many genes encoding effectors harbor highly polymorphic regions and/or variations in copy number resulting from gene duplications and diversifying selection (Hogenhout *et al.*, 2009; Dodds and Rathjen, 2010).

In this review, we focus on recent reports on the diverse roles of secreted SPRY domain-containing proteins (hereafter named SPRYSEC effectors) in plant-nematode interactions. The SPRYSEC effectors were initially identified in the potato cyst nematodes *G. rostochiensis* and *G. pallida*, the genomes of which show remarkable large expansions of SPRY-domain-containing proteins (Cotton *et al.*, 2014; Mei *et al.*, 2015). While the use of the SPRY domain is widespread among eukaryotes, it mostly occurs in association with other functional protein domains (Perfetto *et al.*, 2013). However, the majority of SPRY-containing proteins in potato cyst nematodes do not harbor other functional domains. In the sections below we describe SPRYSEC effectors as selective modulators of plant defense responses mediated by intracellular immune receptors. Based on currently available data we discuss a model in which the versatility of the SPRY domain as protein binding module enables parasitic nematodes to disrupt diverse host protein complexes required for plant innate immunity.

Identification of SPRYSEC effectors in potato cyst nematodes

Before the introduction of new generation sequencing technologies, identifying nematode effectors was a challenging and lengthy process (Davis *et al.*, 2008). In this context, a selective search for nematode proteins that were highly abundant in infective juveniles, were specifically localized to the esophageal glands, and carried a signal peptide for secretion could lead to sound nematode effector candidates.

The application of two differential display approaches using these criteria resulted in the cloning of the first SPRYSEC effectors from *G. rostochiensis* (Qin *et al.*, 2000) and *G. pallida* (Grenier *et al.*, 2002; Blanchard *et al.*, 2005). The genes encoding the SPRYSEC effectors in the two sister species have moderate sequence identity (43.7%) (Blanchard *et al.*, 2005). Further mining of a database with expressed sequence tags of transcripts isolated from (pre-) parasitic juveniles of *G. rostochiensis* resulted in 35 sequence contigs with significant similarity to the original SPRYSEC effector sequences, eight of which contained full length transcripts (Rehman *et al.*, 2009). Recent analyses of the genome sequences of *G. rostochiensis* and *G. pallida* confirmed that the SPRYSEC effectors are members of large, highly diversified gene families (Cotton *et al.*, 2014). The sequence diversity within the SPRYSEC effector families in *G. rostochiensis* and *G. pallida* involves amino acid replacements and significant sequence length variations (Figure 1).

The expression of the SPRYSEC genes in potato cyst nematodes specifically localizes to the dorsal esophageal gland cell (Qin *et al.*, 2000; Blanchard *et al.*, 2005; Rehman et al., 2009). Antisera specific to a conserved peptide sequence in the SPRYSEC effectors is also able to detect these effectors in stylet secretions of infective juveniles of *G. rostochiensis* incubated in root diffusates of host plants (Rehman *et al.*, 2009). However, the delivery of the SPRYSEC effectors to either the apoplast or cytoplasm of host cells has not been conclusively shown. This can be partly explained by the fact that the expression and secretion of the SPRYSEC effectors most likely only takes place during the short transition period from migratory to sedentary second stage juveniles (Rehman *et al.*, 2009).

Proteins with a SPRYSEC architecture seem to be rare in nature. The Pfam protein domain database includes around 9000 SPRY domain-containing proteins (PF00622), fifteen percent of which harbor no other functionally annotated domain(s) while about four percent of the latter are predicted to be secreted. Proteins with SPRYSEC architectures are predicted in different eukaryotes, including a number of pathogens and parasites (e.g., the pea aphid *Acyrthosiphon pisum* pfam J9KHA9, *Clavispora lusitaniae* pfam C4Y7R4 and *Entamoeba histolytica* pfam C4M2H6). Because nematode effectors lack sequence similarity to other proteins with SPRYSEC architectures and because no functions have been assigned to other SPRYSEC proteins, it is not clear if the use of a secreted SPRY domain to promote virulence is exclusive to nematodes.

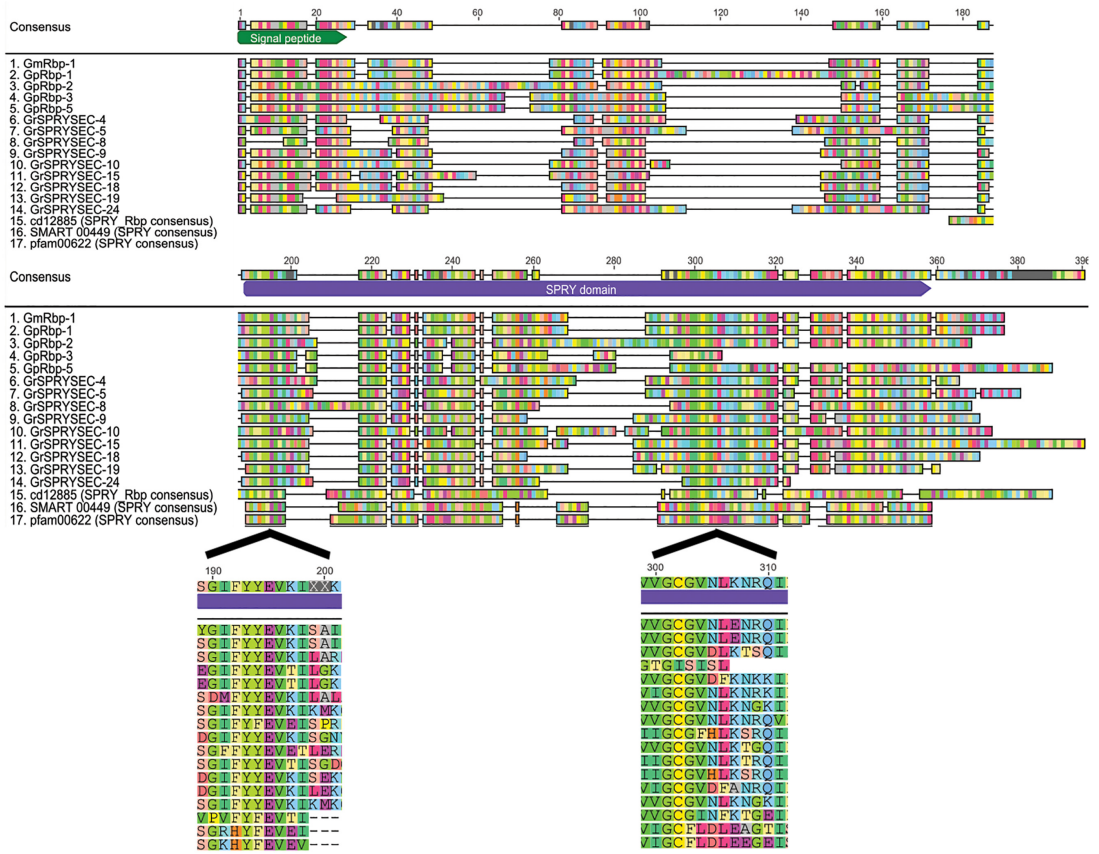

Figure 1. SPRYSEC effectors are variable in sequence and length. An alignment of all full-length SPRYSEC effectors available in the NCBI database shows a high degree of sequence variability among them. Sequences starting with Gm are from Globodera mexicana, Gp are from G. pallida and Gr are from *G. rostochiensis*. The consensus sequences for the SPRY domain from Conserved Domain Database, SMART database and Pfam database are included for reference. The signal peptide and SPRY domain are shown as green and purple blocks in the consensus sequence, respectively. Annotations were done automatically using InterProScan in Geneious 8.1.7 (Quevillon *et al.*, 2005; Kearse *et al.*, 2012). Residues are automatically colored where they are in agreement with the consensus sequence, gray boxes are regions with no agreement with the consensus (Kearse *et al.*, 2012). The enlarged areas show the sequence of the regions where conserved SPRY motifs are found in SPRYSEC effectors.

The SPRY Domain – A Versatile Protein-Binding Platform

The SPRY domain in SPRYSEC effectors was initially characterized as a sequence repeat in tyrosine kinase spore lysis A (splA) from the soil-inhabiting slime mold *Dictyostelium discoideum* as well as in three mammalian ryanodine receptors (Ponting *et al.*, 1997; Rhodes *et al.*, 2005). Concurrently, similar sequence repeats were identified in the product of exon B30.2 in a tripartite motif (TRIM) gene located in the human major histocompatibility complex, which is since then referred to as the B30.2 domain (Vernet *et al.*, 1993). Some aspects of the SPRY and B30.2 domain architectures still remain to be determined with precision. Three sequence motifs (i.e., LDP, YFEVE and LDLE; Figure 1) characterize B30.2/SPRY proteins in protein domain databases, with the LDP being absent in the 'SPRY-only' group (D'Cruz *et al.*, 2013). The SPRYSEC effectors contain highly conserved variations of the YFEVE (YEVK) and LDLE (VNLK) motifs (Figure 1), but not of the LDP motif.

The LDP motif is present in proteins carrying a 60 amino acid extension at the N-terminus of the SPRY domain. This extension is cause for debate about the functional boundaries of the domain. In short, the B30.2 configuration is defined by a SPRY domain and an N-terminal extension, the PRY domain (SM00589, PF13765, cl02686), which was initially suggested as a distinct structural element of the B30.2 domain (Rhodes *et al.*, 2005). 'SPRY-only' proteins also carry N-terminal extensions of 60 amino acids, but these extensions have no significant sequence similarity to the PRY domain. However, the PRY domain and other N-terminal extensions on 'SPRY-only' proteins show remarkable similarity in their predicted secondary structure (Woo *et al.*, 2006). Studies with well-characterized members of the 'SPRY-only' subfamily show that the N-terminal extension is required for the functionality of the SPRY core domain (Woo *et al.*, 2006). Phylogenetic analyses further suggest that the conserved SPRY core is probably the most ancient part of B30.2/SPRY domain architecture (Woo *et al.*, 2006). The PRY domain and other N-terminal extensions are currently considered an integral part of the B30.2/SPRY domain, albeit more evolutionarily diversified than the core SPRY domain (D'Cruz *et al.*, 2013).

The SPRY domains in SPRYSEC effectors carry an N-terminal extension with lengths varying between 60 and 120 amino acids, depending on the SPRYSEC effector variant. These N-terminal extensions have no significant sequence similarity to the PRY domain or other N-terminal extensions known to be associated with SPRY domains. A PRY domain(s) was initially described in the N-terminus of the SPRYSEC effector GpRbp-1 from *G. pallida* (Blanchard *et al.*, 2005; Carpentier *et al.*, 2012). However, current analyses with domain prediction tools no longer identify a significant match between the N-terminus in GpRbp-1 and PRY domains in domain databases (Pfam, SMART, and CDD). Protein structure modeling of the N-terminal region of GrSPRYSEC- 19 from *G. rostochiensis* nonetheless revealed similarities in secondary structure with PRY domain-containing proteins and other "SPRY-only" proteins (Figure 2). Furthermore, two highly conserved residues, a tryptophan and a leucine, are found in the N-terminal extensions of all SPRYSEC effectors studied so far (Figure 3). Other amino acids in a region of 20 amino acids around these two conserved residues also show high levels of conservation. Protein database searches using only this region suggest

that it may be a unique signature sequence of SPRYSEC effectors of nematodes (Figure 3).

There is ample evidence showing that the SPRY/B30.2 domain functions as a versatile platform to selectively mediate physical protein–protein interactions (Woo *et al.*, 2006; Perfetto *et al.*, 2013). For instance, the SPRY domain of Ran-binding protein M (RanBPM) mediates interactions required for the activity of RanBPM as a scaffolding protein (Suresh *et al.*, 2012). The SPRY domain in SPRYSEC effectors is most similar to the SPRY domain of RanBPM from various organisms (Blanchard *et al.*, 2005; Rehman *et al.*, 2009). RanBPM carries other domains named LisH, C-terminal to LisH (CTLH) and C-terminal CT11RanBPM (CRA) domains, which are involved in homodimerization (i.e., LisH) and interactions with targets of RanBPM (e.g., the CT11RanBPM domain) (Suresh *et al.*, 2012). However, the SPRY domain in human RanBPM is sufficient to mediate binding of this protein with the transcription factor p73 (Kramer *et al.*, 2004). Similarly, the SPRY domain is also required for binding of human RanBPM to YEPL5, a regulator of the cell cycle progression and cell growth (Hosono *et al.*, 2010). Furthermore, the SPRY/B30.2 domain has undergone a major expansion in the human genome to facilitate the regulation of a wide range of protein–protein interactions in the innate immune system and in antiviral responses [e.g., TRIM proteins; (Perfetto *et al.*, 2013)]. The exact molecular mechanisms underlying the impact of the SPRY/B30.2 domains on other proteins is not well understood, but they often result in alterations in stability of target protein complexes and receptors by ubiquitination and phosphorylation (Perfetto *et al.*, 2013). It is also not known how the peptide-binding specificity is determined in SPRY/B30.2 domains, although it is evident that particular surfaces contribute significantly more to the overall structural diversity in this domain than others (Woo *et al.*, 2006).

Structural Diversity In SPRY Domains

In crystal structures of SPRY containing proteins the structure of the B30.2/SPRY domain is a compact β-sandwich fold, with two α-helices at the N-terminus (Woo *et al.*, 2006). The β-sandwich is formed by two main layers of β-sheets located in close proximity to each other interacting via a hydrophobic interface. The β-strands are arranged in antiparallel sense and are joined by loops of different lengths that radiate outward from the core sandwich. A structural model of SPRYSEC effectors constructed using as template GUSTAVUS, a SPRY-SOCS box protein from *Drosophila melanogaster*, also predicts a core β-sandwich joined by interspersed flexible loop regions that create exposed surfaces radiating from the β-sandwich core (Rehman *et al.*, 2009).

In the structures of other SPRY-containing proteins highly conserved residues are buried in the core β-sheets of the tertiary structure and therefore are likely required for structural integrity. In comparison, there are no conserved residues in the exposed protein surfaces. This configuration allows the establishment of variable regions in the surface of the SPRY domain that mediate selective protein binding with different targets (Woo *et al.*, 2006). Similarly, mapping of the variable amino acids In different SPRY-containing proteins the two variable surfaces on the surface of the SPRY/B30.2 domain mediate interactions with other proteins (Woo et al., 2006). This enables SPRY-containing proteins, like SPSB2, to function

as E3 ubiquitin ligase, possibly by using one hypervariable region to provide substrate specificity and another to assemble the ubiquitination complex (Kuang *et al.*, 2010). The structural diversity in SPRYSEC effectors is located in multiple predicted exposed hotspots. Thus, a similar model in which a SPRY domain functions as an adapter that joins two host proteins into a complex could apply to SPRYSEC effectors. In a set of SPRYSEC effectors from *G. rostochiensis* the structural diversity concentrates specifically in two surfaces, namely, a hypervariable surface A and a moderately variable alpha helical structure at the C-terminus of the SPRY domain (Figure 2; Rehman *et al.*, 2009). The hypervariable regions in the core SPRY domain of SPRYSEC effectors could thus provide substrate specificity to enzymatically active host proteins. For example, SPRYSEC effectors could bind a host target and hijack the cellular machinery of the host to modify their target in SPRYSEC effectors onto a consensus structural model shows that divergent residues mostly localize to the loops that join the core β-sheets of the SPRY domain. The plant targets of SPRYSEC effectors remain largely unknown. However, it is likely that the hypervariable regions formed by the flexible loops of the SPRY domain determine the binding specificity of the SPRYSEC effectors (Rehman *et al.*, 2009). This concept of a stable scaffold with hypervariable regions in extended loops that determine binding specificity for different targets is reminiscent to that of the complementarity determining regions of lectin-binding proteins and immunoglobulins (Masters *et al.*, 2006; Rehman *et al.*, 2009; Perfetto *et al.*, 2013).

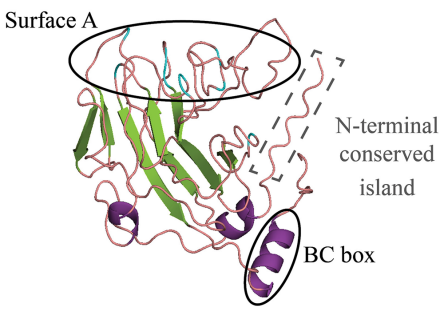

Figure 2. Remote homology-based structural model of GrSPRYSEC4,5,8,9,15,16,18, and 19 from *G. rostochiensis*. A remote homology structural model was built for a consensus of these sequences based on the SPRY protein GUSTAVUS (Rehman *et al.*, 2009). The characteristic SPRY β-sheets are shown in green and α-helices in purple. The flexible loops shown in coral and the residues that are found to be under positive selection are colored blue (Rehman *et al.*, 2009). Surface A and BC box, the most hypervariable regions of the characteristic SPRY domain are encircled in black. A conserved island found to be exclusive for nematode SPRYSEC effectors is encircled by a gray dashed line (see also Figure 3).

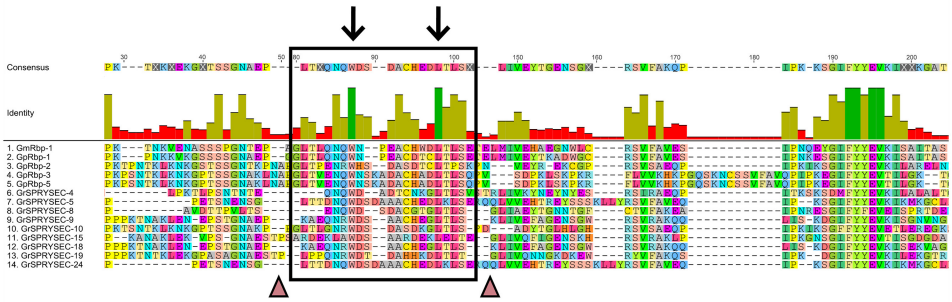

Figure 3. An N-terminal unique identifier for SPRYSECs. The N-terminal region of SPRYSEC effectors shows no homology to proteins in the NCBI non-redundant protein database. The black box shows a region with conserved residues in the N-terminus of SPRYSEC effectors. The arrows show 100% conserved positions. The triangles point to areas where insertions of 30–40 residues are usually present depending on the SPRYSEC variant. These insertions have been manually removed for this figure. Colored residues are in agreement with the consensus sequence, gray boxes are regions with no agreement with the consensus. In the identity graph green indicates 100% identity, gold indicates ranges of identity between 30 and 99% and red indicates less than 30% identity (Kearse *et al.*, 2012).

Genetic Diversity In SPRYSEC Effectors

The relevance of structural diversity in SPRYSEC effectors is also reflected in the large number of gene variants that seem to persist in natural populations of *G. pallida* (Rehman *et al.*, 2009; Sacco *et al.*, 2009; Carpentier *et al.*, 2012). This sequence diversity results from positive diversifying selection, which becomes significant when non-synonymous mutations are favored over synonymous mutations across many generations. Genes participating in the molecular arms race between hosts and parasites typically harbor evidence of positive selection (Jones and Dangl, 2006). For instance, the SPRY domain in TRIM5α proteins that restricts retroviral infections in primates is a hotspot of non-synonymous mutations (Sawyer *et al.*, 2005). Similarly, several amino acid sites mostly located in extended loops that form surface A in the SPRY domain of SPRYSEC effectors in *G. pallida* and *G. rostochiensis* are positively selected (Rehman *et al.*, 2009; Sacco *et al.*, 2009).

The selective forces favoring non-synonymous mutations in SPRYSEC effectors are not fully understood. Changes in amino acid residues that betray the presence of the nematodes to the plant innate immune system can have significant fitness benefits and they seem to contribute to the sequence diversity in SPRYSEC effectors. Position 187 is one of several positively selected sites on the hypervariable surface A of the SPRY domain in GpRbp-1 (Sacco *et al.*, 2009). Multiple variants of the SPRYSEC GpRbp-1 from *G. pallida* carrying a proline at position 187 induce a Gpa2- dependent cell death response in agroinfiltration assays in leaves of *Nicotiana benthamiana* (Sacco *et al.*, 2009). The intracellular NB-LRR immune receptor Gpa2 mediates resistance to specific genotypes of *G. pallida* in potato upon effector recognition (van der Vossen *et al.*, 2000). This characteristic cell death is not observed with nearly identical GpRbp-1 variants carrying a serine at position 187. A single

non-synonymous mutation at this position could lead to loss of recognition of *G. pallida* in potato plants harboring Gpa2 resistance. However, cell death- inducing P187 variants of GpRbp-1 seem to persist in populations of *G. pallida* that break Gpa2 resistance and further research is therefore needed to clarify the role of the P-to-S mutation in (a)virulence.

The persistence of cell death-inducing GpRbp-1 variants in nematode populations suggests that these SPRYSEC effectors do not follow a typical birth-and-death scenario. Birth and death scenarios play out when novel positively selected alleles that are not recognized by plant immune receptors become rapidly fixed, resulting in limited overall sequence diversity of pathogen populations (Nei and Rooney, 2005). This particular outcome contrasts with the extensive sequence diversity among SPRYSEC effectors in populations of *G. pallida* (Jones *et al.*, 2009; Carpentier *et al.*, 2012). Non-synonymous mutations in the SPRY domain may therefore also have been instrumental in the functional diversification of the SPRYSEC effectors. In this context, hypervariable sites in the SPRY domain may reflect the ability of the SPRYSEC effectors to function as versatile protein binding platforms to enable interactions with multiple or variable host targets.

The large expansion of the SPRY-domain containing proteins in the genome of *G. pallida* and *G. rostochiensis* also points at extensive functional diversification of the SPRYSEC effectors (Mei *et al.*, 2015; Eves-van den Akker *et al.*, 2016). Gene duplications and recombinations have resulted in approximately 300 SPRY domain-containing proteins in *G. pallida*. Only 30 of these SPRY domain-containing proteins carry a N-terminal signal peptide for secretion and they are therefore considered SPRYSEC effectors. Interestingly, the expression of the SPRYSEC effectors is restricted to the early parasitic stages, while most of the other SPRY-containing proteins are constitutively expressed throughout different life stages. For comparison, Mei *et al.* (2015) identified far less SPRY domain-containing proteins (<25) in the genomes of *Caenorhabditis elegans*, *Bursaphelenchus xylophilus*, and *Meloidogyne incognita*, none of which harbors a signal peptide for secretion. The function of the large pool of highly homologous SPRY-domain containing proteins in the genome of *G. pallida* remains to be investigated. However, phylogenetic analysis including most of the 300 SPRY domain- containing proteins in *G. pallida* suggests that they might play an important role in maintaining SPRYSEC effector diversity through intergenic sequence exchanges (Mei *et al.*, 2015).

SPRYSEC Effectors Suppressing Plant Innate Immunity

Heterologous expression and identification of host targets of SPRYSEC effectors in plants suggest that they may function as suppressors of innate plant immunity. An important line of defense in plants relies on intracellular immune receptors encoded by host specific resistance (*R*) genes that recognize pathogen effectors and activate effector-triggered immunity (Dodds and Rathjen, 2010). Most intracellular plant immune receptors are NB-LRR proteins composed of a central Nucleotide-Binding domain (also known as NB-ARC), and a C-terminal Leucine-Rich Repeat domain. Two major NB-LRR classes are further distinguished based on N-terminal extensions of either a coiled-coil domain (CC-NB-LRR) or a Toll/interleukin 1-like receptor (TIR-NB-LRRs) (Takken and Goverse, 2012). Activation of NB-

LRRs upon pathogen recognition commonly leads to defense-related programmed cell death in plant cells. For instance, the resistance mediated by the CC-NB-LRR receptor Mi-1.2 in tomato involves a typical defense-related programmed cell death in the permanent feeding site of the root-knot nematode *M. incognita* (Williamson, 1998).

Five members of the SPRYSEC effector family of *G. rostochiensis* selectively suppress the cell death phenotype triggered by a group of closely related CC-NB-LRRs (Postma *et al.*, 2012; Ali *et al.*, 2015). Remarkably, these SPRYSEC effectors also suppress effector-independent cell death induced by autoactive variants of CC-NB-LRR receptors (Postma *et al.*, 2012; Ali *et al.*, 2015). This suggests that SPRYSEC effectors do not disturb effector recognition by NB-LRR receptors, but rather interfere in downstream signaling. However, the cell death mediated by an autoactive form of NRC1, a downstream signaling component of diverse immune receptors, is not suppressed by the GrSPRYSEC-19 effector (Postma *et al.*, 2012). Furthermore, GrSPRYSEC-19 does not suppress the cell death triggered by elicitin INF1 from the oomycete *Phytophthora infestans*, the onset of which is mediated by an extracellular immune receptor in *N. benthamiana*. By contrast, GrSPRYSEC-19 and several other SPRYSEC effectors of *G. rostochiensis* suppress the cell death induced by the NEP1-like protein PiNPP1.1 of *P. infestans* (Ali *et al.*, 2015). Altogether, these data show that several members of the SPRYSEC effector family in *G. rostochiensis* function as selective suppressors of the defense-related programmed cell death. At least two SPRYSEC effectors from *G. pallida* (i.e., GpSPRY-12N3 and Gp-SPRY33H17) also selectively suppress the characteristic cell death induced by Gpa2 (Mei *et al.*, 2015). But, unlike SPRYSEC effectors from *G. rostochiensis*, GpSPRY-12N3 and Gp-SPRY33H17 do not suppress Rx1-mediated cell death. GpSPRY-12N3 and Gp-SPRY33H17 do not suppress cell death activated by TIR-NB-LRR-class immune receptors either. Three other members of the SPRYSEC effector family of *G. pallida* (i.e., GpSPRY-17I9-1, GpSPRY-22E10, and GpSPRY-24D4) lack the ability to suppress cell death induced by either Gpa2 or Rx1 in *N. benthamiana* (Mei *et al.*, 2015)

Defense-related programmed cell death is often associated with disease resistance mediated by CC-NB-LRR-class of plant immune receptors, but it is not a requirement for an effective resistance response (Coll *et al.*, 2011). Nevertheless, all of the SPRYSEC effectors of *G. rostochiensis* that suppress cell death in leaves of *N. benthamiana* also suppress resistance to potato virus X mediated by Rx1 (Ali *et al.*, 2015). Co-expression of the resistance gene *N* and the p50 subunit of the *Tobacco mosaic virus* replicase inhibits the accumulation of PVX coat protein fused to GFP (PVX-GFP) in *N. benthamiana* leaves. Co-infiltration of N, p50, PVX-GFP with various SPRYSEC effectors results in enhanced PVX-GFP accumulation in *N. benthamiana* (Ali *et al.*, 2015). Furthermore, stable overexpression of GrSPRYSEC-19 in the diploid potato line V significantly reduced resistance to the wilt fungus *Verticillium dahliae* (Postma *et al.*, 2012).

Host targets of nematode effectors can provide leads to the molecular mechanisms underlying the phenotypes of these effectors in plants. GrSPRYSEC-19 specifically interacts with the C-terminus of the LRR domain alone (Rehman *et al.*, 2009) and with the full-length protein (Postma *et al.*, 2012) of a member of the SW5 *R* gene cluster in tomato (named SW5F). Other members of this cluster of highly conserved CC-NB-LRR proteins are involved in resistance to tomato spotted wilt virus [TSWV; (Spassova *et al.*, 2001)], but none have been linked to nematode resistance in tomato. The function of SW5F in tomato is not resolved, nor is it clear

if the SW5F gene encodes a functional protein. Mutations that render other members of the SW5 cluster autoactive, do not result in elicitor-independent SW5F-mediated cell death in *N. benthamiana*. It can therefore not be tested if GrSPRYSEC-19 suppresses the induction of cell death mediated by SW5F in absence of a cognate elicitor. The transient co- expression of GrSPRYSEC-19 with SW5F also does not induce cell death in *N. benthamiana*, which makes it less likely that it is an elicitor of SW5F-mediated cell death and resistance.

SPRYSEC Effectors Activating Plant Innate Immunity

At least two SPRYSEC effectors trigger a robust cell death response in transient expression assays in leaves of *Nicotiana* species. First, the SPRYSEC effector GpRbp-1 of *G. pallida* induces a Gpa2-dependent cell death in *N. benthamiana* leaves. Conversely, a distant homolog of GpRbp-1 from *G. rostochiensis* does not induce a Gpa2-dependent cell death response, showing that the recognition of GpRbp-1 by Gpa2 is specific (Sacco *et al.*, 2009). Recognition by Gpa2 is also specific within GpRbp-1 variants in the same species. A single amino acid polymorphism S187P in GpRbp-1 abolishes recognition by Gpa2. Gpa2 is known to interact with RanGAP2, a RanGTP-binding protein involved in the nucleocytoplasmic partitioning and functioning of highly homologous immune receptor Rx1 (Sacco *et al.*, 2007). Transient virus-mediated silencing of RanGAP2 in *N. benthamiana* abolishes the cell death mediated by Gpa2 upon recognition of GpRbp-1 (Sacco *et al.*, 2009). Effector recognition and therefore pathogen detection can occur by direct binding to NB-LRRs, however, most examples characterized until now imply indirect recognition of the effector (Dangl *et al.*, 2013). The requirement of RanGAP2 for Gpa2-mediated cell death could indicate that RanGAP2 is monitored by Gpa2 and serves either as a target, decoy, or bait for GpRbp-1 (Sacco *et al.*, 2009). Any of these cases assumes a direct interaction between RanGAP2 and GpRbp-1. While this interaction remains elusive, artificial tethering of RanGAP2 and GpRbp-1 enhances the cell death response mediated by Gpa2 upon detection of GpRbp-1 (Sacco *et al.*, 2009). Introduction of a non-recognized (S187P) variant of GpRbp1 in an artificially tethered complex does not activate Gpa2-dependent cell death. This shows that the interaction with RanGAP2 is therefore involved in recognition of GpRbp-1 by Gpa2 (Sacco *et al.*, 2009).

The second SPRYSEC effector to trigger a cell death response in transient expression assays is SPRYSEC-15 of *G. rostochiensis* (Ali *et al.*, 2015). Unlike the activation of Gpa2-mediated cell death by GpRbp-1, the molecular underpinnings of this cell death response by GrSPRYSEC-15 in non-host *N. tabacum* are not well understood. Heterologous expression of GrSPRYSEC-15 either from a binary expression vector or as a PVX-GrSPRYSEC-15 amplicon induces cell death. Furthermore, expression as PVX-GrSPRYSEC-15 reduces the systemic spread of the virus in *N. tabacum*. Tobacco plants infiltrated with PVX-GFP show chlorotic lesions consistent with systemic spread of the virus. By contrast, plants with PVX-GrSPRYSEC-15 show no symptoms of viral spread 14 days after infiltration. Notably, transient expression of GrSPRYSEC-15 does not induce a cell death response in *N. benthamiana*. These results suggest that an unknown resistance protein in *N. tabacum* most likely recognizes GrSPRYSEC-15, rendering the recombinant PVX-GrSPRYSEC- 15 virus avirulent (Ali *et al.*, 2015).

Perspectives

The SPRY domain in SPRYSEC effectors may provide potato cyst nematodes with a versatile protein-binding platform that allows them to target variable host proteins. In this context, the diversity in SPRYSEC effectors may reflect the variability in the plant targets of these effectors, but on the other hand it may also reflect changes necessary to avoid recognition by the plant immune system. The only consistent plant phenotypes associated with SPRYSEC effectors so far are suppression and activation of CC-NB-LRR-mediated immune responses. The only confirmed host target of a SPRYSEC effector to date is a CC-NB- LRR protein, the role of which in plant innate immunity needs further investigation. Physical associations between SPRYSEC effectors and CC-NB-LRR proteins would fit both in immune activation and suppression models. In fact, these models are not mutually exclusive as immune suppressing SPRYSEC effectors may compete for binding to CC-NB-LRR receptors with immune activating SPRYSEC effectors (Halterman *et al.*, 2010).

The molecular determinants underlying the binding specificity of SPRY domains in SPRYSEC effectors and how binding could lead to a modification of targeted host proteins remain unknown. A single point mutation in a hypervariable surface of a SPRYSEC effector determines if the effector is recognized by the plant immune system (Sacco et al., 2009). The lack of recognition could be due to interference with the interaction between the SPRYSEC effector and the immune receptor. It is not clear if similar mutations in SPRYSEC effectors have also led to gain of function by acquiring novel affinities for other host targets. Resolving the identity of additional host targets of highly similar SPRYSEC effectors may shed light on binding specificity. Although SPRY domains can confer substrate specificity to enzyme complexes [e.g., E3 ubiquitin ligases; (Kuang *et al.*, 2010)], there is no evidence that the SPRY domain alone exhibits intrinsic catalytic activity. Without known intrinsic catalytic activity, the key to understanding the role of SPRYSEC effectors in nematode virulence is to study alterations of plant native complexes brought about by these effectors. SPRYSEC effectors could act as complex inhibitors either by competitive binding to their plant targets [e.g., bacterial effectors AvrRps4 and HopA1; (Bhattacharjee *et al.*, 2011)] or by mediating post- translational modifications of these targets to prevent formation of a stable native complex in the plant [e.g., bacterial effector HopM1; (Nomura *et al.*, 2006)].

Another important question that remains to be addressed is if only potato cyst nematodes exploit the versatility of the SPRY domain to modify host targets. The large expansion of SPRY domain-containing proteins in nematode genomes could be a tell-tale sign to their importance in nematode–plant interactions. At present, it is not possible to assess if similar expansions of the SPRY domain have occurred in related nematode species, given the availability of the genome sequences of only a small number of plant parasitic nematodes. Homologs of SPRYSEC effectors have not been identified in the genome sequence of the root-knot nematodes (Cotton *et al.*, 2014). Several studies using *de novo* transcriptomics suggest that SPRYSEC effectors might nonetheless be common to different cyst nematodes species and might even be present in migratory plant parasitic nematodes. Entries in non-redundant sequence databases imply that the soybean cyst nematode *H. glycines* harbors at

least three SPRYSEC effectors (Genbank accessions JQ074058.1, HQ123260.1, JQ074057.1). Similarly, the transcriptomes of the cereal cyst nematode *H. avenea* (Kumar *et al.*, 2014) and migratory endoparasitic lesion nematode *Pratylenchus coffea* (Haegeman *et al.*, 2011) also include sequences closely matching SPRYSEC effectors. When the genome sequences of a wider panel of plant parasitic nematodes become available, it will be possible using comparative genomics to assess if SPRYSEC effectors and their extraordinary expansion are clade specific. Furthermore studying the roles of more ancient SPRYSEC effectors can help to characterize the homology between SPRYSEC effectors and RanBPM. Alternatively, identifying and characterizing functional homologs of RanBPM in plant parasitic nematodes can provide clues to the function of SPRYSEC effectors and their evolution.

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Chapter 3

The effector GpRbp-1 of *Globodera pallida* targets a nuclear HECT E3 ubiquitin ligase to modulate gene expression in the host

Amalia Diaz-Granados[1]; Mark G. Sterken[1]; Hein Overmars[1]; Roel Ariaans[1]; Martijn Holterman[1]; Somnath S. Pokhare[2]; Yulin Yuan[3]; Rikus Pomp[1]; Anna Finkers-Tomczak[1]; Jan Roosien[1]; Erik Slootweg[1]; Abdenaser Elashry[2]; Florian M. W. Grundler[2]; Fangming Xiao[3]; Aska Goverse[1]; Geert Smant[1]

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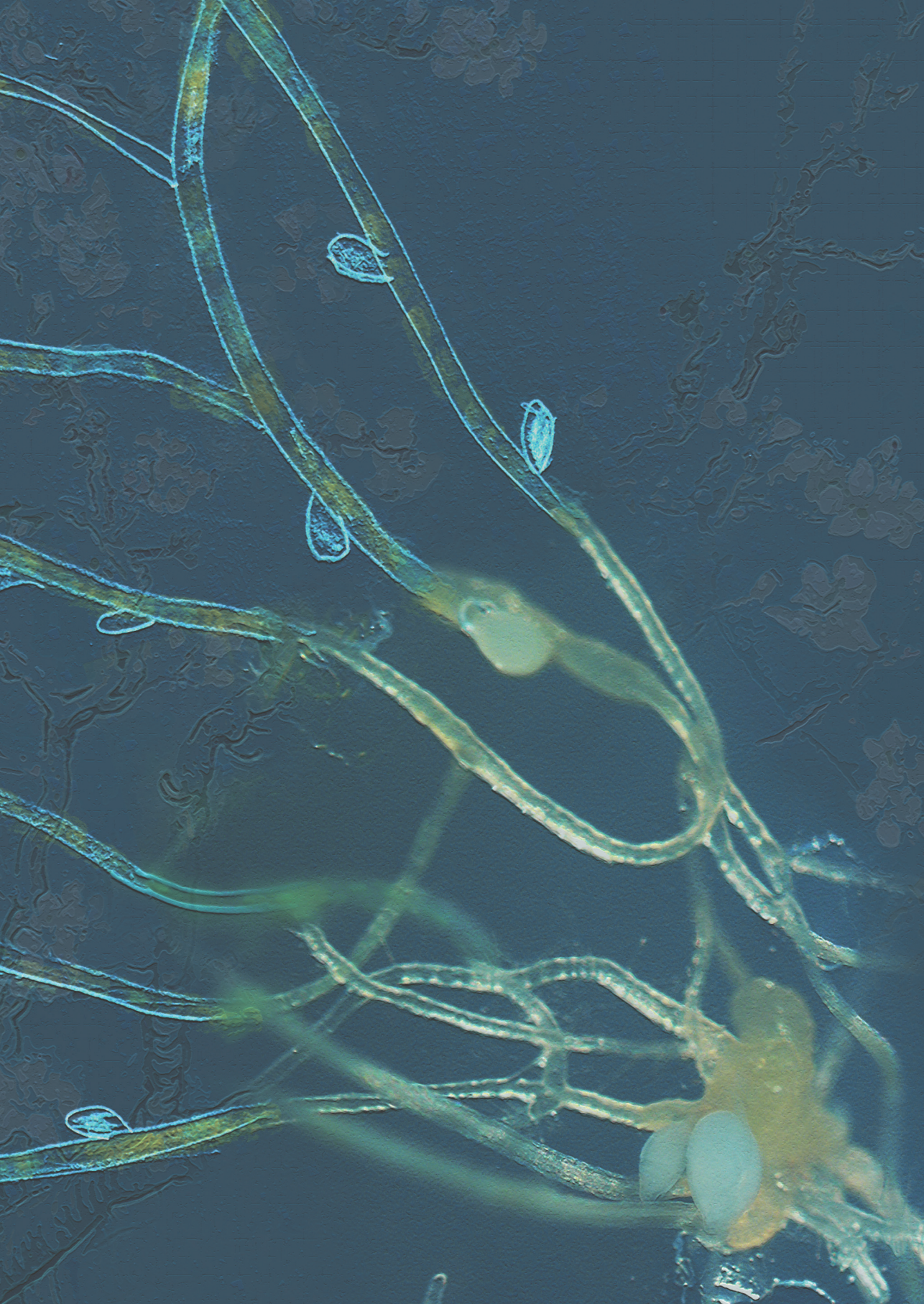
[1] Laboratory of Nematology, Wageningen University, Wageningen, Netherlands;
[2] Department of Molecular Phytomedicine, INRES, University of Bonn, Bonn, Germany;
[3] Department of Plant Sciences, University of Idaho, Moscow, USA.



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Abstract

Plant parasitic nematodes secrete effectors that manipulate plant cell morphology and physiology to achieve host invasion and establish permanent feeding sites. Effectors from the highly expanded SPRYSEC family of potato cyst nematodes have been implicated in activation and suppression of plant immunity, but the mechanisms underlying these activities remain largely unexplored. To study the host mechanisms utilized by SPRYSEC effectors, we identified plant targets of GpRbp-1 from the potato cyst nematode *Globodera pallida*. Here, we show that GpRbp-1 interacts in yeast and *in planta* with a functional potato homologue of the HECT-type ubiquitin E3 ligase UPL3, which is located in the nucleus. Potato lines lacking *StUPL3* are not available, but the Arabidopsis mutant *upl3-5* displaying a reduced *UPL3* expression showed a consistently small but not significant decrease in susceptibility to cyst nematodes. We observed a major impact on the root transcriptome by the lower levels of AtUPL3 in the *upl3-5* mutant, but surprisingly only in association with infections by cyst nematodes. To our knowledge, this is the first example that a HECT-type ubiquitin E3 ligase is targeted by a pathogen effector and that a member of this class of proteins specifically regulates gene expression under biotic stress conditions. Together, our data suggest that GpRbp-1 targets a specific component of the plant ubiquitination machinery to manipulate the stress response in host cells.

Introduction

Plant parasitic nematodes are biotrophic pest organisms posing a serious threat to important food crops like potato, soybean, and rice. Infestation with nematodes is estimated to reduce the world-wide crop yield by 12% (Nicol *et al.*, 2011). Cyst nematodes belonging to the genera *Globodera* and *Heterodera* are among the most destructive nematode species, despite having a relatively narrow host range (Jones *et al.*, 2009). Cyst nematodes have a sedentary lifestyle relying exclusively on nutrients provided by living host cells for their development and survival. At the onset of parasitism, soil-born infective juveniles penetrate the roots of host plants and migrate intracellularly until they settle to establish a permanent feeding site, the syncytium. The flow of nutrients is redirected from the vascular tissue toward feeding nematodes through this host-derived feeding site. Upon the successful establishment of a syncytium, cyst nematodes become sedentary and depend entirely on it throughout their life cycle.

The interaction between cyst nematodes and host plants involves an arsenal of effectors that nematodes secrete into infected root tissue. Nematode effectors modulate plant immunity and promote virulence by manipulating the metabolism and physiology of the plant (Gheysen & Mitchum, 2011, Mitchum *et al.*, 2013, Quentin *et al.*, 2013). Intracellular migration as well as the initiation, establishment and maintenance of the syncytium requires dramatic physiologic and metabolic reprogramming. This reprogramming of host cells is also mediated by nematode effectors (Gheysen & Mitchum, 2011, Mitchum *et al.*, 2013, Quentin *et al.*, 2013). While a large repertoire of effectors is predicted for cyst nematodes, only a small number of effectors have been functionally characterized, often by the identification of their host targets.

Host targets of cyst nematode effectors are (predicted to be) involved at different levels of cellular regulation, including the post-translational level (Hewezi *et al.*, 2016, Juvale & Baum, 2018). Ubiquitination is a mechanism for post-translational regulation in which the small protein ubiquitin is covalently attached to substrate proteins (Sadanandom *et al.*, 2012). Addition of mono- and polyubiquitin can influence endocytosis, protein sorting, and gene expression of the substrate among others (Zhou & Zeng, 2017). Nevertheless, the most prominent role of ubiquitination is to direct proteins for degradation by the 26S proteasome, thereby regulating protein turn-over in the cell (Vierstra, 2009). The hallmark for substrate degradation by the ubiquitin-26S proteasome system (UPS) is the attachment of a chain of four or more ubiquitin subunits interlinked by a conserved lysine in position 48 of the ubiquitin peptide (Vierstra, 2009).

Ubiquitination is pivotal for plant plasticity as it allows specific perception and a rapid response to differing environmental conditions (Sadanandom *et al.*, 2012, Vierstra, 2009). Ubiquitination is also recurrently found to be involved in the responses of plants to biotic stress (Delauré *et al.*, 2008). The plant immune system relies on surface- and cytoplasm-localized receptors to detect attempts of pathogens at invasion and colonization of the plant tissue (Bent & Mackey, 2007). Recognition by either type of receptor results in hormone-

dependent signalling events that ultimately activate defence responses to fend-off pathogen attacks (Pieterse *et al.*, 2012). Ubiquitination has been related to the control of immune receptors as well as hormone-dependent immune signalling in the interactions of several biotrophic pathogens, like bacteria and fungi (Craig *et al.*, 2009, Delauré *et al.*, 2008). Conversely, the ubiquitination machinery of plants can be hijacked or mimicked by pathogens to aid in the infection process, for instance by the Avr3a effector from *Phytophtora infestans,* or by AvrPtoB from *Pseudomonas syringae* (Abramovitch *et al.*, 2006, Banfield, 2015, Bos *et al.*, 2010). Nevertheless, the role of ubiquitination during recognition and responses to nematode invasion remains largely unexplored (Chronis *et al.*, 2013, Hewezi, 2015, Kud *et al.*, 2019).

The importance of ubiquitination as a major regulator of plant responses is reflected in a large portion of plant genes encoding components of the ubiquitination and proteasome machinery. For example, six percent of the genome of *Arabidopsis thaliana* is estimated to encode proteins involved in ubiquitination (Serrano *et al.*, 2018, Vierstra, 2009). Ubiquitination follows an ATP-dependent cascade mediated by three enzymes. E1 ubiquitin-activating enzymes transfer ATP to ubiquitin to activate it (Sadanandom *et al.*, 2012). Subsequently, E2 ubiquitin-conjugating enzymes form a stable intermediate with activated ubiquitin, which is transferred to the final substrate by E3 ubiquitin-ligases (E3 ligases) (Sadanandom *et al.*, 2012). E3 ligases define substrate specificity of the ubiquitination complex and therefore play a central role for the control of this post-translational modification (Mazzucotelli *et al.*, 2006, Shu & Yang, 2017). The 26S proteasome recognises and degrades ubiquitinated substrates to maintain a tight regulation of protein turn-over in the cell (Sadanandom *et al.*, 2012, Vierstra, 2009). Finally, after substrate break-down, ubiquitin subunits are recycled by deubiquitinating enzymes (Sadanandom *et al.*, 2012, Vierstra, 2009). From the enzymes involved in ubiquitination, the E3 ubiquitin ligases are the most abundant in plant genomes. Two E1s, approximately 35 E2s, and 50 deubiquitinating enzymes DUBs are predicted to be encoded in the Arabidopsis genome (Miricescu *et al.*, 2018). In contrast, the Arabidopsis genome harbours over 1500 genes encoding E3 ligases (Miricescu *et al.*, 2018). Plant E3 ligases are divided into families depending on their structure and catalytic mechanism. E3 ligases containing a Homology to E6-AP C-Terminus (HECT) domain are monomeric proteins that bind directly both the E2 and substrate. In this way, HECT E3 ligases serve as an intermediary by forming a covalent bond with the ubiquitin that is later transferred to the final substrate protein (Downes *et al.*, 2003).

GpRbp-1 is a prototypical effector from the potato cyst nematode *Globodera pallida*. It is produced in the dorsal oesophageal gland of the nematode (Blanchard *et al.*, 2005), where part of the salivary secretions of the nematode are produced. Furthermore, it is abundantly expressed in the early parasitic stages of the nematode life cycle. Therefore, GpRbp-1 is believed to play a role during the initiation and/or establishment of syncytia (Blanchard *et al.*, 2005). Additionally, specific variants of GpRbp-1 are recognised by the potato immune receptor Gpa2, leading to cell death upon co-expression by agroinfiltration

in *Nicotiana benthamiana* leaves (Sacco *et al.*, 2009). Interestingly, GpRbp-1 variants remain under positive selection, indicating that at least some members of this effector family play an important role in the virulence of *G. pallida* (Carpentier *et al.*, 2012). GpRbp-1 belongs to the highly expanded SPRYSEC family of effectors in potato cyst nematodes, characterized by an N-terminal signal peptide for secretion and a C-terminal SPRY domain (Ali *et al.*, 2015, Diaz-Granados *et al.*, 2016, Rehman *et al.*, 2009). The N-terminal signal peptide indicates that GpRbp-1 is likely delivered by the nematode to the plant cell where it can interact with plant targets. This may involve the C-terminal SPRY domain which is believed to function as a protein binding platform required for the virulence role of SPRYSEC effectors, as shown for effector SPRY-414-2 from *G. pallida* (Mei *et al.*, 2018).

To elucidate the host mechanisms utilized by GpRbp-1 in virulence of *G. pallida*, we aimed to characterize its molecular targets in host plant cells. Here, we show that the nematode effector GpRbp-1 interacts specifically in yeast and *in planta* with a potato E3 ubiquitin protein ligase (StUPL3). Moreover, we demonstrate by bimolecular fluorescence complementation (BiFC) that this interaction most likely occurs in the nucleus. These data suggest that this HECT E3 ligase may be required for infection by *G. pallida*. Due to the lack of a StUPL3 knock-out mutant in potato, we examined the role of *Arabidopsis thaliana* AtUPL3 in nematode virulence using the beet cyst nematode *Heterodera schachtii,* which exploits the same mode of parasitism as *G. pallida*. We found that inoculation of the Arabidopsis knock-down mutant *upl3-5* revealed in a consistently small, but statistically not significant, reduction of susceptibility to *H. schachtii* as compared to the wild-type plants, suggesting that UPL3 may only have a minor contribution to cyst nematode parasitism. However, a strong transcriptional regulation in Arabidopsis plants was only observed by microarray analysis of nematode-infected *upl3-5* roots. Interestingly, this transcriptional regulation involves genes that are related to stress responses, suggesting that cyst nematodes modulate host gene expression in plant cells through targeting of UPL3 in the nucleus. To the best of our knowledge, this is the first report of a plant-parasitic nematode effector targeting the host ubiquitination machinery by interacting with a plant HECT E3 ligase.

Results

GpRbp-1 interacts with a fragment of Ubiquitin E3 ligase UPL3 from potato

To find plant interactors of GpRbp-1, we performed a yeast two-hybrid screen of a cDNA library of 3.85×10^6 clones obtained from susceptible potato roots infected with *G. pallida*. Using GpRbp-1 from *G. pallida* population Rookmaker as bait, we found two yeast clones harbouring an identical insert sequence of 413 bp which showed the highest similarity to *Arabidopsis thaliana* E3 Ubiquitin protein ligase UPL3 (GenBank accession XP_006359694.1; e-value 2.11287×10^{-42} in BLASTX on the non-redundant database at NCBI). Therefore, we named the interacting fragment StUPL3frag8. In Arabidopsis, UPL3 is composed of an N-terminal Armadillo repeat domain (Pfam16186) and a C-terminal catalytic HECT domain (Pfam00632) (Marchler-Bauer *et al.*, 2017, Marchler-Bauer & Bryant, 2004). StUPL3frag8

localizes to the C-terminal half of UPL3, in the HECT domain of the protein (Fig. 1A-B, Supp. Fig. 1).

To investigate the similarity of UPL3 from *A. thaliana* and potato, we compared the coding and peptide sequence of both genes using a CLUSTALW alignment. We obtained the full-length coding sequence for StUPL3 from the non-redundant nucleotide database of GenBank (XM_015314510). At the nucleotide level, AtUPL3 and StUPL3 are 67% identical (data not shown) and at the protein level they share 70% identity (Supp. Fig. 1). It should be noted that StUPL3frag8 was 98% identical to StUPL3 from NCBI (Fig. 1B, Supp. Fig. 1). Furthermore, we investigated the number of copies of *StUPL3* present in the genome sequence of the doubled monoploid potato genotype DM. To this end, we queried with a BLASTN algorithm the PGSC *S. tuberosum* group Phureja DM1-3 transcripts v3.4 database from the Potato Genomics resource of Michigan University (Hirsch *et al.*, 2014) using AtUPL3 as input. We found two transcripts that match the AtUPL3 coding sequence, transcript PGSC0003DMT400031189 (1189) and transcript PGSC0003DMT400031190 (1190) (e-values $1x10^{-59}$ and $7x10^{-52}$, respectively). Further examination of each transcript indicated that the corresponding genomic sequence is the same for both transcripts (PGSC0003DMG402011946; 11946), indicating that transcripts 1189 and 1190 are segments of the same StUPL3 coding sequence (Supp. Fig. 1A). Most likely, they remain separated in the automatic annotation as individual transcripts due to the presence of a large intron in the genomic sequence. Finally, we analysed the genomic position of the coding sequence 11946 with the SpudDB Genome Browser tool (Hirsch *et al.*, 2014). The genomic region containing sequence 11946 is located in chromosome 10 of the DM potato genotype. These results suggest that StUPL3 is encoded by a single gene that resides on chromosome 10 of the DM potato genotype.

Furthermore, to investigate if StUPL3 affects overall E3 ligase activity *in planta*, we determined whether StUPL3 can promote *in vivo* ubiquitination when expressed in plant cells. To this end, N-terminal HA- or GFP-tagged StUPL3 (HA-StUPL3 or GFP-StUPL3) was co-expressed with HA-tagged ubiquitin (HA-Ub) in *Nicotiana benthamiana* leaves. Total ubiquitination with exogenous HA-Ub was expected as a smear banding pattern in a Western blotting using anti-HA antibodies. Indeed, exogenous HA-Ub can be utilized *in planta* for poly-ubiquitination (Supp. Fig. 1B). Upon expression of StUPL3 the poly-ubiquitination signal was increased, suggesting that StUPL3 has E3 ubiquitin ligase activity *in planta* (Supp. Fig. 1B).

To verify the specificity of the interaction of GpRbp-1 and StUPL3frag8 in yeast by swapping the yeast expression vectors, we co-transformed StUPL3frag08 in the bait configuration and GpRpb-1 in the prey into yeast strain PJ69-4a. Likewise, the nematode effector GrSPRYSEC-19 and its cognate plant interactor Sw5F were used as positive control (Rehman *et al.*, 2009), while StUPL3frag8 was co-transformed with human Lamin C into yeast as negative control. The yeast cells were grown on triple drop-out (-LWH; TDO) media for 6 days after co-transformation, after which individual colonies were re-plated onto fresh TDO media. We obtained several colonies of the co-transformation of StUPL3frag8 with GpRbp-1 and GrSPRYSEC-19 with SwF5, but none with the negative control (Fig. 1C). Therefore, we concluded that GpRbp-1 and a C-terminal fragment of E3 ligase UPL3 from potato specifically interact in yeast.

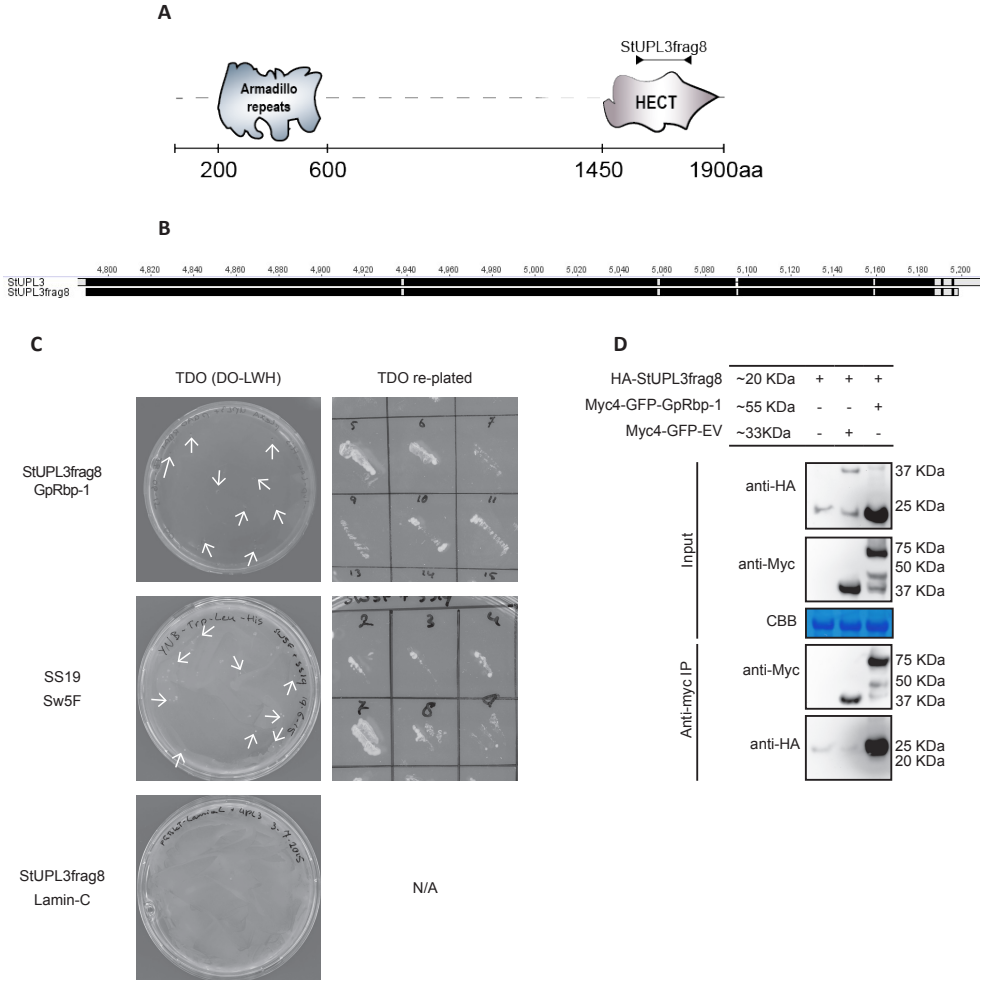

Figure 1. GpRpb-1 interacts in yeast and *in planta* with a fragment of the E3 ubiquitin ligase UPL3 from potato. **A)** Predicted domain architecture of the E3 ligase UPL3 from *S. tuberosum* (StUPL3). The location of the interacting StUPL3frag8 is indicated with an arrow. **B)** Alignment of the coding sequences of the StUPL3DS8 fragment and full length StUPL3 (Suppl. Fig. 1). Identical residues are depicted in black and non-identical residues are shown in grey. The overall sequence identity is 97%. **C)** Directed yeast two-hybrid interaction of StUPL3frag8 and GpRbp-1 in a reverted bait-pray configuration. The interaction between Sw5F and SS-19 is used as positive control (Rehman *et al.*, 2009) and human Lamin C is used as negative control. Yeast were grown on triple drop-out media after transformation (TDO). Colonies were only visible in the positive control and StUPL3frag8/GpRbp-1 interaction plates (arrows). Colonies grown on the TDO selection were re-plated to fresh TDO to confirm positive clones (TDO re-plated). Pictures are taken at 6 and 5 days post-transformation, respectively. **D)** Co-immunoprecipitation of StUPL3frag08 (HA-StUPL3frag8) and GpRbp-1 (Myc4-GFP-GpRbp-1) or empty vector control (Myc4-GFP-EV). Proteins were extracted from *N. benthamiana* leaves 3 days after agroinfiltration.

To independently confirm the interaction *in planta*, we performed co-immunoprecipitation assays with HA-tagged StUPL3frag8 (HA-StUPL3frag8) using Myc-GFP-tagged GpRbp-1 as bait (Myc4-GFP-GpRbp-1) upon co-expression of constructs in leaves of *N. benthamiana*. HA-StUPL3frag8 alone, and also together with a Myc4-GFP vector (Myc4-GFP-EV) as negative controls. HA-StUPL3frag8 was only co-immunoprecipitated by Myc4-GFP-GpRbp-1 (Fig. 1D). We therefore concluded that the C-terminal fragment of UPL3 from potato obtained in the yeast screen is also able to interact specifically with GpRbp-1 *in planta.*

Remarkably, co-expression of StUPL3frag8 with Myc4-GFP-GpRbp1 resulted in differential behaviour of the peptide encoded by the UPL3 fragment on western blot. When StUPL3frag8 was co-expressed with Myc4-GFP-GpRbp-1, it appeared consistently as a more intense band on western blots than when it was expressed with either Myc4-GFP or alone. These observations suggest that the presence of GpRbp-1 alters the expression level or protein stability of the HA-tagged StUPL3 fragment (Fig. 1D). Furthermore, it should be noted that when StUPL3frag8 was co-expressed with the Myc4-GFP-EV an oligomer of approximately twice the molecular weight of the StUPL3fragm8 also appeared on western blots.

GpRbp-1 interacts with full-length StUPL3 *in planta*

Next, we investigated if GpRbp-1 also interacts *in planta* with full-length StUPL3. We obtained the full-length coding sequence for StUPL3 by gene synthesis based on the predicted potato transcript variant X2 (GenBank accession XM_015314510, 6128bp). We first attempted to co-immunoprecipitate HA-StUPL3 in a pull-down assay with Myc4-GFP-GpRbp-1. However, the high molecular weight StUPL3 protein (~210KDa) was only consistently observed on western blots after using destructive protein extraction methods incompatible with co-immunoprecipitation. Therefore, we used bimolecular fluorescence complementation (BiFC) following transient expression in *N. benthamiana* to test the interaction of StUPL3 and GpRbp-1 *in planta*. To this end, the N-terminal half of the fluorescent protein SCFP3A was fused to GpRbp-1 (pN:GpRbp-1) and the C-terminal half of SCFP3A was fused to StUPL3 (pC:StUPL3). As negative control, we co-expressed pN:GpRbp-1 with SCFP3A fused to the viral protein NSs (pC:NSs) and pC:UPL3 with SCFP3A fused to β-glucuronidase (pN:GUS). The characteristic fluorescence of SCFP3A was only reconstituted after co-infiltration of pN:GpRbp-1 and pC:UPL3, indicating that the interaction between GpRbp-1 and StUPL3 brought the N and C halves of CFP in close proximity (Fig. 2 and Supp. Fig. 2). Based on this finding, we concluded that StUPL3 is most likely a target of GpRbp-1 in host plants. As, the signal of reconstituted SCFP3A was only visible in the nucleus of transformed cells, it seemed that the interaction of GpRbp-1 with StUPL3 takes place in this subcellular compartment. Furthermore, the fluorescent signal indicates that the interaction is not evenly distributed throughout the nuclei. We observed a consistent granular pattern of fluorescence throughout the nucleus in addition to discrete globules or speckles with stronger fluorescence than the rest of the nucleus. This fluorescent pattern suggests that the interaction of GpRbp-1 and StUPL3 may be associated with specific structures within the nucleus of the cell.

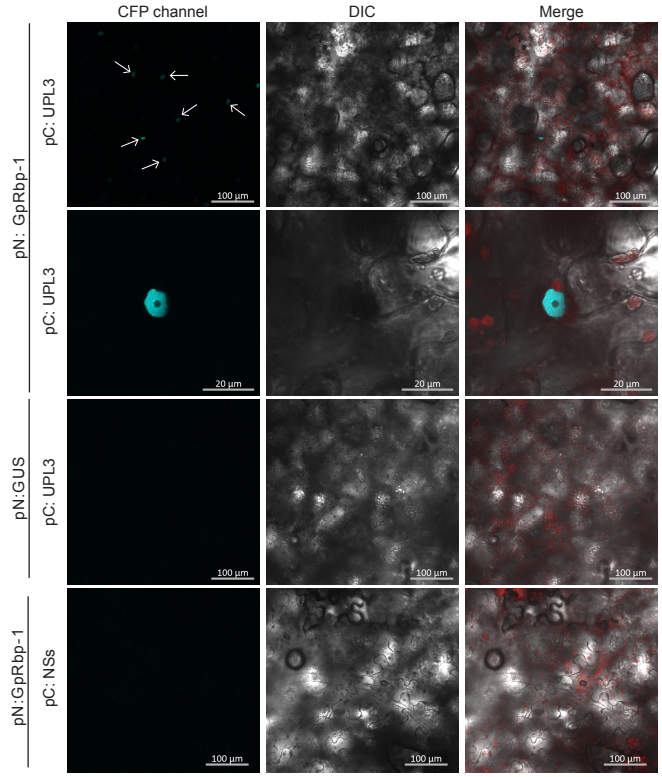

Figure 2. GpRpb1 interacts with full-length StUPL3 in the plant nucleus. Bimolecular fluorescence complementation of sub-fragments of cyan fluorescent protein SCFP3a by co-expression of SCFP3A amino acids 1-173 fused to GbRpb-1 (pN:GpRbp-1) and SCFP3A amino acids 156-239 fused to StUPL3(pC:UPL3). Co-expression of pN:GUS or pC:NSs were used as negative controls. The fusion constructs were agro-infiltrated in leaves of *N. benthamiana*. Images of live cells were taken at 2 dpi and fluorescence was monitored with CLSM. CFP emission is shown in blue, light emission in white in the differential interference contrast channel and chloroplast auto-fluorescence is shown in red in the merge panel. Arrows indicate nuclei with fluorescent signal. Representative images from 2 leaves from 2 plants in 3 independent experiments.

GpRbp-1 and StUPL3 co-localize in the nucleus of *N. benthamiana* cells

Next, we determined the native subcellular localization of StUPL3 *in planta*. First, the localization of StUPL3 was predicted *in silico*, using Plant-mPLoc (Chou & Shen, 2007, Chou & Shen, 2008, Chou & Shen, 2010, Shen & Chou, 2006) and PredictProtein (Yachdav *et al.*, 2014). Both algorithms predicted StUPL3 to be located in the nucleus. Additionally, cNLS mapper (Kosugi *et al.*, 2008, Kosugi *et al.*, 2009a, Kosugi *et al.*, 2009b) predicted a monopartite nuclear localization signal (NLS) composed of RAAKRARVT at position 26 of the amino acid sequence, with score 7, suggesting a partial localization to the nucleus. The same amino acid sequence was identified by LOCALIZER (Sperschneider *et al.*, 2017) as a NLS, together with KKEPPQEKNGSSSKGKGK starting in position 1024 (Supp. Fig. 1A).

Next, we used confocal laser scanning microscopy (CLSM) to evaluate the subcellular localization of N-terminally mCherry-tagged GpRbp-1 (mCh-GpRbp-1) and N-terminally GFP-tagged StUPL3 (GFP-UPL3) when agro-infiltrated in *N. benthamiana* cells (Fig. 3, Supp. Fig. 3). mCh-GpRbp-1 and GFP-UPL3 were infiltrated together or in combination with the corresponding GFP or mCh vectors as negative controls. Individually, GpRbp-1 displayed the same nucleocytoplasmic partitioning that has been reported before (Jones, 2009), whereas GFP-StUPL3 alone showed a specific localisation in the nucleus of individually transformed cells. Moreover, upon co-expression of mCh-GpRbp-1 and GFP-UPL3, the subcellular localisation of both proteins remained largely unchanged. Therefore, we concluded that co-expression did not result in the translocation of either GpRbp-1 or StUPL3, and that they co-localize exclusively in the nucleus, consistent with the results obtained in the BiFC assay. Interestingly, the pattern of GFP-StUPL3 localization resembled the granular distribution observed for the interaction of GpRbp-1 and StUPL3 using BiFC. The fluorescent signal of GFP-StUPL3 was granular throughout the nucleus and was stronger in discrete speckles. Additionally, the granular fluorescence observed for GFP-StUPL3 was not altered by co-expression with mCh-Rbp-1

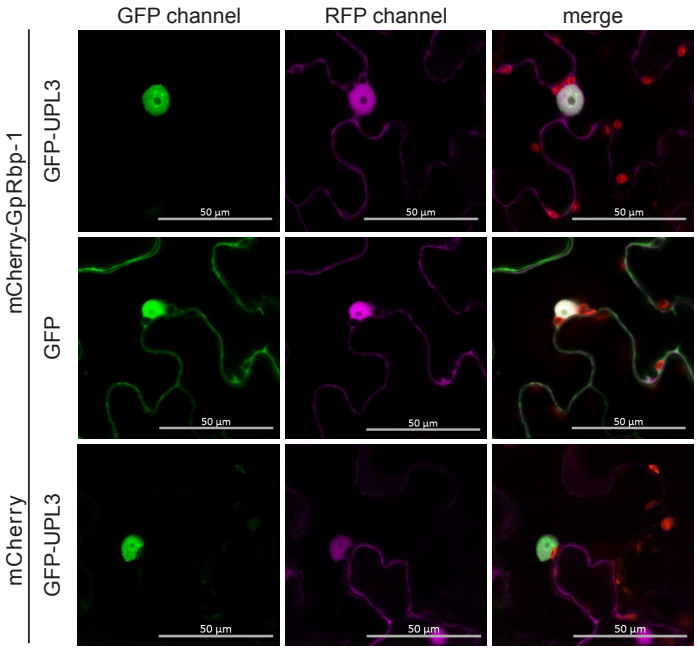

Figure 3. GpRbp-1 and full length StUPL3 co-localize to the nucleus of *N. benthamiana* cells. Live imaging of *N. benthamiana* leaves agro-infiltrated with combinations of protein fusions of green fluorescent protein with StUPL3 (GFP-UPL3), red fluorescent protein mCherry (mCh-GpRbp-1) or GFP and mCh alone. The emission channel for GFP channel is shown in green and the channel for mCherry in purple. Imaging was done at 2 days post-infiltration. Representative images from 2 leaves from individual 2 plants in 3 independent experiments.

UPL3 is involved in cyst nematode infection of Arabidopsis

As stable knock-out or knock-down mutants of *StUPL3* in potato were not available, we further investigated the role of UPL3 during cyst nematode infections in *A. thaliana*. In addition, beet cyst nematode *Heterodera schachtii* is the most-related cyst nematode species to *G. pallida* capable of infecting Arabidopsis. To evaluate the importance of AtUPL3 for the susceptibility of Arabidopsis to *H. schachtii,* we counted the number of nematodes in roots of wild-type Col-0 and homozygous *upl3-5* knock-down mutant plants at two weeks after inoculation (Fig. 4B). The *upl3-5* mutant carried a homozygous T-DNA insertion that does not result in an obvious morphological plant growth phenotype (data not shown). However, we found less nematodes in *upl3-5* mutant plants compared to wild-type plants (7% fewer nematodes; ANOVA, combining replications with a fixed-effect model $p = 0.150$; Fig. 4B). The T-DNA insert in *upl3-5* does not result in a full knock-out of UPL3 (Supp. Table 1), which may lead to an underestimation of the effect of the gene during nematode infection in this mutant. We therefore also analysed the size of the syncytia established by the nematodes and the size of distinguishable females 14 days after inoculation as a parameter for successful establishment of a parasitic relationship with their host. The females and the syncytia formed in the roots of the *upl3-5* mutant seemed slightly smaller compared to wild-type plants (7% and 5%, respectively ; ANOVA, combining replications with a fixed-effect model, $p = 0.075$ and $p=0.466$, respectively; Fig. 4C). Additionally, we investigated if AtUPL3 was regulated at the transcript level in nematode-infected roots. To measure the expression of *AtUPL3*, we performed RT-PCR in *A. thaliana* roots infected with *H. schachtii* or mock infected 2, 7, 10, and 14 days after inoculation. We did not find a significant infection-dependent regulation of *AtUPL3* as compared to reference genes *UBP22* and *UBQ5* (Supp. Fig. 4) (Anwer *et al.*, 2018, Hofmann & Grundler, 2007).

As we suspected that the manipulation of UPL3 by cyst nematodes could have a subtler effect on virulence than the detection power of our bioassays, we performed a whole transcriptome analysis in roots of the *upl3-5* mutant line and wild-type Arabidopsis plants inoculated with *H. schachtii*. To this end, we collected whole roots of *A. thaliana* plants at time of inoculation (0 dpi) and seven days after inoculation with infective J2s of *H. schachtii* or mock inoculation (7 dpi), in four replicates per genotype per treatment. The impact of these conditions on the transcriptome of Arabidopsis was first analysed with principal component analysis. The first two principal components (PCOs) captured 68.0% of the variation and separated the effect of plant development during the seven days after the time of inoculation (PCO 1) and the effect of nematode infection (PCO 2) (Supp. Fig. 5). Next, we tested the number of differentially expressed genes within each condition. Remarkably, the transcriptome in roots of the *upl3-5* mutant and wild-type Arabidopsis plants differed only significantly in the presence of *H. schachtii*. In total, 895 genes were differentially expressed between nematode-infected roots of the *upl3-5* mutant and wild-type Arabidopsis plants at seven days after inoculation (linear model, $p < 0.0001$; false discovery rate [FDR] = 0.0041) (Fig. 5C; Supp. Table 1). In contrast, only 72 genes were differentially regulated between *upl3-5* and wild-type plants at 0 dpi (linear model, $p < 0.0001$; FDR= 0.051), while 53 genes were differentially expressed between mock-infected *upl3-5* and wild-type plants at 7 dpi (linear

model, p < 0.0001; FDR = 0.064). These results showed that despite the lack of a strong effect on female development and syncytium formation, UPL3 regulates plant gene expression in response to root infection by cyst nematodes.

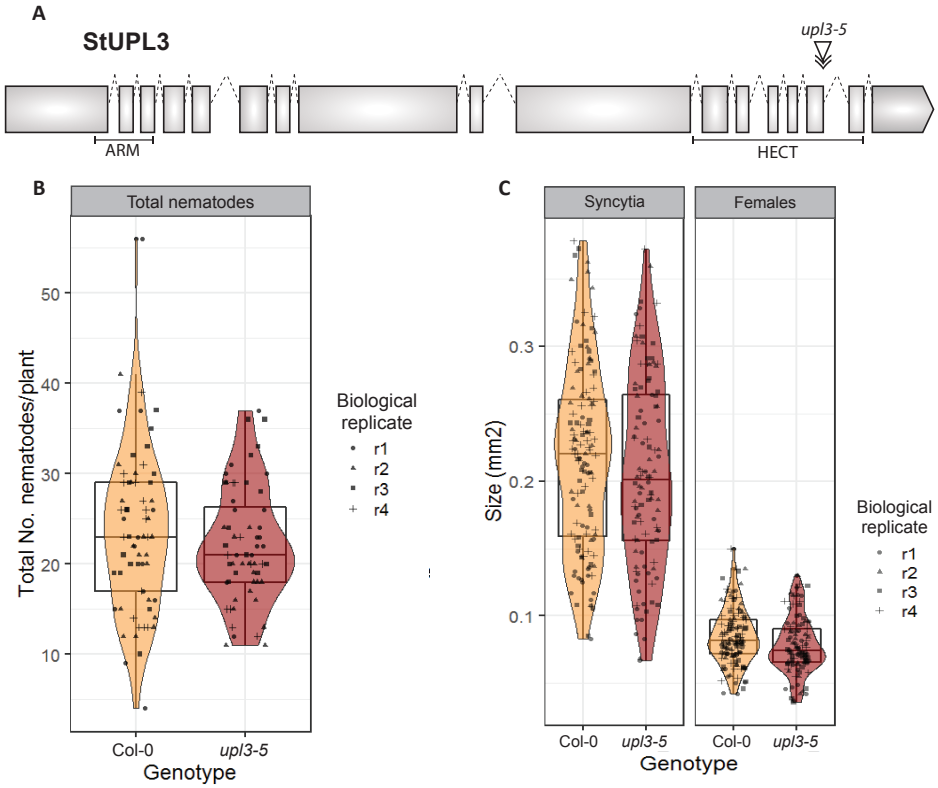

Figure 4. Knock-down of UPL3 in *upl3-5* only slightly reduces susceptibility of Arabidopsis to the beet cyst nematode *H. schachtii*. A) Position of the T-DNA insert in AtUPL3 in Arabidopsis *upl3-5* (triangle). **B)** Total number of nematodes per plant **C)** Average surface area (mm^2) of female nematodes and syncytia present in the roots of *upl3-5* Arabidopsis line and wild-type (Columbia 0), after 2 weeks of infection. Whiskers indicate the quartile (25 or 75%) -/+ 1.5x interquartile range. Results are combined measurements from 4 independent biological repeats. For **B)** $n_{Col\text{-}0}$=63 and $n_{upl3\text{-}5}$= 59. For **C)** $n_{Col\text{-}0}$=127 and $n_{upl3\text{-}5}$=106 for syncytia and $n_{Col\text{-}0}$=129 and $n_{upl3\text{-}5}$=108 for females. Statistical significance of the differences in the amount or size of nematodes infecting the roots of *upl3-5* lines and the wild-type control were established by a fixed model effects (α=0,05).

Genes differentially expressed in association with *upl3-5* are linked to stress responses and metabolism

To identify biological processes that were most likely influenced by the mutation in *AtUPL3* in association with infections by cyst nematodes, we focussed on genes that showed highly significant differential expression between the *upl3-5* mutant and wild-type Arabidopsis plants ($-\log_{10}(p)>6$). At seven days after inoculation, the expression of 131 genes were significantly affected by the mutation in *AtUPL3* (FDR < 0.00029). Enrichment analysis of these 131 genes based on annotation terms showed a significant overrepresentation of 26 categories (Fig. 5D; Supp. Table 2). The categories "Involved in response to salt" (GO:1902074, q= 8.71392E-05) and "Involved in cellular amino acid metabolic process" (GO:0006520, q= 0.000727486) were enriched with the highest statistical significance. The categories "Involved in regulation of transcription, DNA-templated" (GO:0006355) and "Has transcription factor activity, sequence-specific DNA binding" (GO:0003700) contained the highest number of differentially regulated genes. For example, the top 10 most significantly up/downregulated genes include two transcription factors (MYB121 and WRKY59; Table 1.). Finally, from the top 10 most significantly up/downregulated genes the expression of two particular genes appeared to be exceptionally affected in nematode-infected roots of the *upl3-5* mutant line. AT4G07820 was by far the most upregulated gene in the *upl3-5* mutant line (effect size = 2^1.635), with an effect size twice as large as the second most upregulated gene AT3G28345 (effect size = 2^0.797). In contrast, AT3G05950 was the most downregulated gene in the *upl3-5* mutant (effect size =-1.442) by also approximately twice as much as the second most down regulated gene (effect =-0.759). The functions of both genes are not known, but based on sequence similarity AT4G07820 and AT3G28345 are thought to be members of the CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily, and of the RmlC-like cupins superfamily, respectively. It is worth noting that AT4G07820 and AT3G05950 have a highly specific, but common, developmental expression pattern limited to roots of Arabidopsis (Klepikova *et al.*, 2016). Furthermore, co-expression analysis showed a similar pattern of up- or downregulation in most of the experimental conditions currently included in the ePlant database (Supp. Fig. 6) (Waese *et al.*, 2017). This suggests that these two genes may be involved in a gene network co-regulated with UPL3.

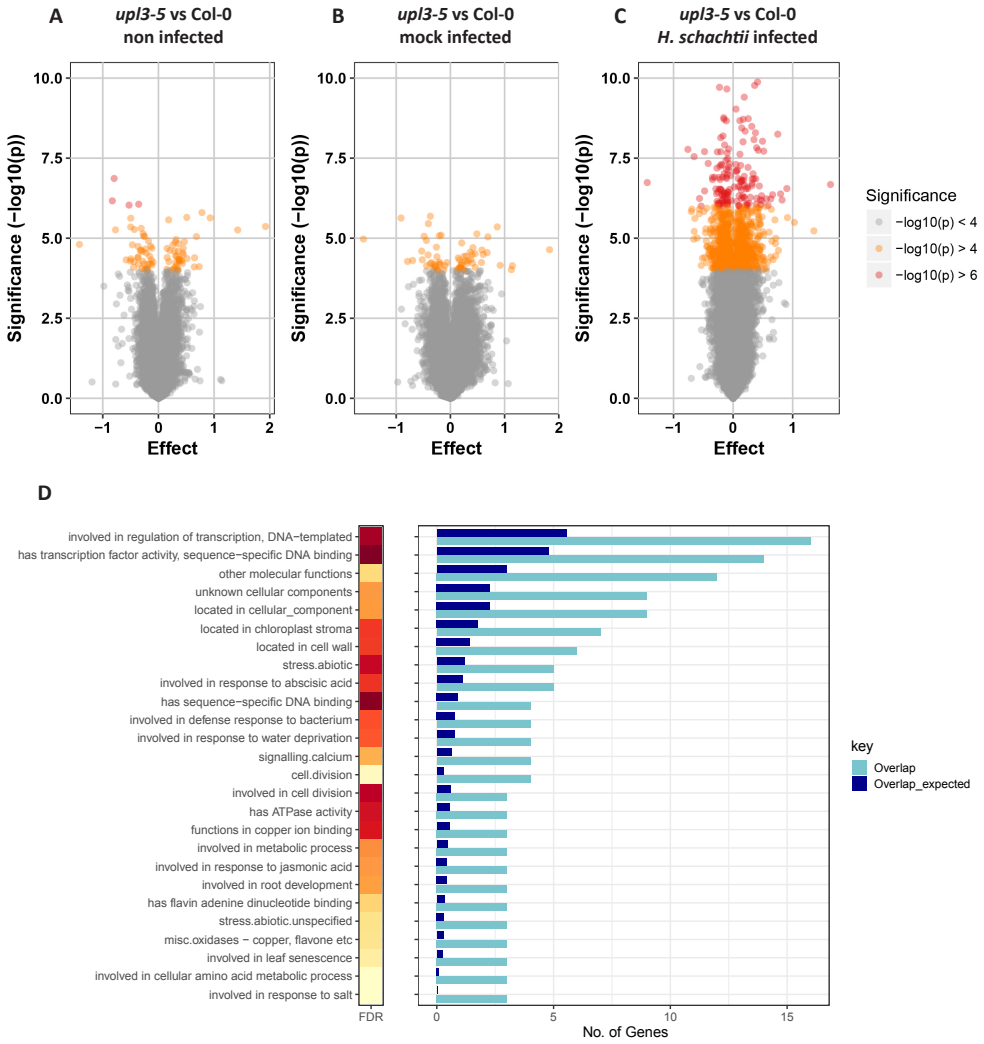

Figure 5. AtUPL3 strongly regulates gene expression in nematode-infected roots of Arabidopsis. A-C) Volcano plots of differential gene expression as determined by micro-array analysis. **A)** Genes differentially regulated in roots of *upl3-5* and wild-type Arabidopsis previous to nematode inoculation. The x-axis shows the relative expression of genes. The y-axis shows the significance of the differences in expression levels. Colours provide a visual aid for the thresholds in the legend. **B)** Genes differentially regulated in mock-infected roots of *upl3-5* and wild-type Arabidopsis plants 7 days after inoculation. **C)** Genes differentially regulated in roots of *upl3-5* and wild-type *Arabidopsis* plants infected with *H. schachtii*, 7 days after inoculation. **D)** Gene Ontology term enrichment analysis (hypergeometric test, FDR correction, q<0.05) of the annotations of the 131 differentially-regulated genes in **C)**. Overlap_expected indicates the number of genes that would be assigned to each category in a random sampling. Overlap shows the number of genes differentially regulated in our dataset.

Table 1. Genes most differentially regulated in *upl3-5* Arabidopsis infected with *H. schachtii*. Top 10 most up- or down-regulated genes in the *upl3-5* mutant categorized by the size of the effect as determined by a linear model. The annotation and gene symbols or names were obtained from the ThaleMine database of Araport (Krishnakumar *et al.*, 2015).

***Downregulated in* upl3-5**				
Gene	***Effect***	***FDR***	***Annotation (ThaleMine)***	***Gene symbol***
AT3G05950	-1.44129	0.000112	RmlC-like cupins superfamily protein	
AT1G29100	-0.75923	2.93E-05	Heavy metal transport/detoxification superfamily protein	
AT1G19250	-0.6548	3.77E-05	flavin-dependent monooxygenase 1	FMO1
AT2G21900	-0.56401	0.000219	WRKY DNA-binding protein 59	WRKY59
AT4G27850	-0.53659	0.000291	Glycine-rich protein family	
AT5G60610	-0.48008	2.94E-05	F-box/RNI-like superfamily protein	
AT4G39740	-0.47864	0.000161	Thioredoxin superfamily protein	HCC2
AT3G27070	-0.41832	5.61E-05	translocase outer membrane 20-1	TOM20-1
AT3G16650	-0.34724	9.37E-05	Transducin/WD40 repeat-like superfamily protein	
AT3G09940	-0.34616	9.86E-05	monodehydroascorbate reductase	MDHAR
***Upregulated in* upl3-5**				
Gene	***Effect***	***FDR***	***Annotation (ThaleMine)***	***Gene symbol***
AT4G07820	1.63498	0.000125	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein	
AT3G28345	0.79684	0.000184	ABC transporter family protein	ABCB15
AT4G16000	0.74938	1.5E-05	hypothetical protein	
AT4G16008	0.6627	0.000186	hypothetical protein	
AT5G05060	0.56052	0.000219	Cystatin/monellin superfamily protein	
AT3G21352	0.51405	2.94E-05	transmembrane protein	
AT4G11211	0.49339	2.01E-05	hypothetical protein	
AT3G30210	0.48315	0.000233	myb domain protein 121	MYB121
AT3G32030	0.47845	0.00019	Terpenoid cyclases/Protein prenyltransferases superfamily protein	
AT4G23670	0.46457	0.000275	Polyketide cyclase/dehydrase and lipid transport superfamily protein	

Discussion

The effector GpRbp-1 is expressed during the onset of parasitism by the potato cyst nematode *G. pallida* and to characterise its role in the virulence of potato cyst nematodes, we aimed to identify the host target(s) of GpRbp-1. Here we show that GpRbp-1 physically interacts in yeast and *in planta* with UPL3, a functional HECT-type E3 ligase from a potato genotype lacking major resistances to *G. pallida*. Additionally, we demonstrated that ectopic StUPL3 and GpRbp-1 co-localize in the nucleus of *N. benthamiana* plants, where they also interact. Our data show for the first time a specific and robust interaction of a pathogen effector with a plant HECT ubiquitin E3 ligase. Together, these results suggest that StUPL3 is a host target of *G. pallida* in nematode parasitism.

Ubiquitination is a post-translational modification that is well established as a key regulator of plant responses during plant-parasite interactions (Banfield, 2015). However, the involvement or recruitment of the ubiquitination machinery during plant-nematode interactions is poorly understood. An example of a nematode effector that may recruit the UPS is effector GrUBCEP12 of *G. rostochiensis* (Chronis *et al.*, 2013). CEP12, a carboxyl extension protein processed from GrUBCEP12 supresses immunity mediated by intracellular immune receptors (Chronis *et al.*, 2013). In addition, effector RHAB1 from *G. pallida* was recently reported to function as a RING-type E3 ligase *in planta* and to promote susceptibility to the nematode (Kud *et al.*, 2019). The recruitment of different components of the ubiquitination machinery by nematodes might suggest that potato cyst nematodes employ a multi-layered strategy to exploit the UPS system of the host. HECT-type E3 ubiquitin ligases have a distinct mechanism of action (Downes *et al.*, 2003), and UPL3 was previously found to co-immunoprecipitate with the HopM1 effector from *Pseudomonas syringae* pv *tomato* (Ustun *et al.*, 2016). Therefore, our results may point toward a previously undescribed strategy to manipulate plant cells by nematodes and other plant pathogens. Recently, UPL3 has been shown to function as a proteasome-associated amplifier of immune responses activated by salicylic acid (SA) (Furniss *et al.*, 2018). Therefore, interaction of GpRbp-1 with StUPL3 may interfere with proteasome-dependent ubiquitination to supress plant immunity.

Potato plants lacking StUPL3 may demonstrate if StUPL3 functions as a virulence target of *G. pallida* in potato roots. However, despite several attempts we have not been able to generate a consistent knock-down of StUPL3 expression in roots using virus-induced gene silencing in potato and tomato roots (data not shown). Hence, to better understand the relevance of UPL3 for nematode parasitism, we focused on the role of the Arabidopsis UPL3 homologue during infection by cyst nematodes. Previously, AtUPL3 was shown to be required for the development of trichomes by acting as an inhibitor of endoreplication (Downes *et al.*, 2003). Moreover, endoreplication is thought to enable the expansion of nematode-induced syncytia (de Almeida Engler *et al.*, 2012). Given this inhibitory role of AtUPL3, we first hypothesised that this ubiquitin ligase might be recruited by cyst nematodes to regulate the endocycle in syncytia. We reasoned that if AtUPL3 indeed functions as a negative regulator of endoreduplication in nematode-induced syncytia, we should find larger syncytia in nematode-infected roots of the *upl3-5* mutant. However, the syncytia established by *H. schachtii* in *upl3-5* mutant plants were slightly smaller than in wild-type Arabidopsis. Based

on the size of syncytia alone we have found no indication that AtUPL3 regulates ploidy levels of syncytial cells. Further direct analysis of the DNA content in syncytial nuclei in the *upl3-5* mutant may provide more conclusive evidence for a role of UPL3 in the regulation of the endocycle in nematode-induced syncytia.

At the transcriptomic level, the subset of differentially expressed genes in nematode-infected roots of *upl3-5* was enriched for genes related to cell division (Fig. 5, Supp. Table 2). Arabidopsis homologues of cell cycle control genes CDC6 and CDC48 were significantly downregulated in nematode infected *upl3-5* as compared to the wild-type (Supp. Table 1). Nevertheless, the specific homologues of CDC6 and CDC48 significantly regulated in our data are not previously described to have a role in the control of endoreplication or mitosis (Supp. Table 1) (Castellano *et al.*, 2001, Copeland *et al.*, 2016, Masuda *et al.*, 2004) Other biological categories found to be enriched in genes differentially regulated in nematode-infected *upl3-5* plants do not indicate further connections to the regulation of the cell cycle or the endoreplication cycle. Altogether, our findings suggest that AtUPL3 does not function as an endocycle regulator during nematode infection in the roots of Arabidopsis.

Our data showed that although *AtUPL3* may not have a significant effect on the establishment of nematode infection in Arabidopsis, female growth and syncytium size, it has a major impact on the transcriptome in nematode-infected roots of Arabidopsis. Interestingly, a parallel can be drawn with the findings of Furniss *et al* (Furniss *et al.*, 2018) where there is a disparity between the relatively small effect of UPL3 on the development of disease symptoms induced by *Pseudomonas syringae* pv. *maculicola* and the major impact on UPL3-dependent transcriptomic modulation by the exogenous application of salicylic acid (SA) to Arabidopsis plants. In both sets of data there is a minor effect of UPL3 in disease resistance, but a large UPL3-mediated transcriptomic response upon the application of an exogenous factor (i.e. SA and nematodes).

Moreover, the set of genes under transcriptional regulation by the combination of UPL3 and nematode infection pointed to stress responses and transcription factor activity. Several genes classified in the Gene Ontology categories of response to both abiotic and biotic stress and transcription factor activity were differentially regulated in *upl3-5* mutant plants in the presence of cyst nematode infections (Fig. 5; Table 1), including some of the top up/downregulated genes. Therefore, UPL3 is likely involved more downstream of syncytium initiation and expansion in host cells, as a magnifier of stress responses which makes host plants more resistant to feeding nematodes. SA is modulated in the early stages of infection with cyst nematodes (Kammerhofer *et al.*, 2015), and it is a negative regulator of cyst nematode susceptibility (Kammerhofer *et al.*, 2015, Wubben *et al.*, 2008, Youssef *et al.*, 2013). Given the role of UPL3 as a magnifier of stress responses to SA, we would expect to find an increase in the number of nematodes infecting the roots of *upl3* Arabidopsis. Therefore, the small decrease in total nematodes infecting the *upl3-5* suggests the existence of mechanisms independent of UPL3, which regulate the cellular components responsible for the SA-mediated inhibition of nematode susceptibility.

Notably, transcription factor MYB121 which is one of the most upregulated genes in *upl3-5* has been found as a high-connectivity regulator of stress response networks mediated by abscisic acid (ABA), in Arabidopsis (Carrera *et al.*, 2009, Nejat & Mantri, 2017) (Table 1). Also,

transcription factor WRKY59, which is one of the most downregulated genes in *upl3-5* plants, is found as a transcriptional target of NPR1 during the establishment of SA-mediated systemic acquired resistance (Wang *et al.*, 2006) (Table 1). This finding is consistent with previous reports where nuclear transcription factors are found as targets of E3 ligases for regulation of plant physiology and immunity (Serrano *et al.*, 2018). Indeed, we found StUPL3 to be specifically located in the nucleus with a granular pattern which resembles the one reported in Arabidopsis for RING-type E3 ligase MIEL1 (Fig. 3) (Marino *et al.*, 2013). MIEL1 promotes the proteasomal degradation of transcription factor MYB96 in the absence of ABA (Lee & Seo, 2016). The degradation of MYB96 attenuates ABA-mediated responses to abiotic stress like drought (Seo *et al.*, 2009). Additionally, transcription factors from different families have been shown to be involved in plant-nematode interactions (Grunewald *et al.*, 2008, Samira *et al.*, 2018, Warmerdam *et al.*, 2019). For example, WRKY23 from Arabidopsis is strongly upregulated in the early stages of infection by root-knot and cyst nematodes (Grunewald *et al.*, 2008). WKRY23 is proposed to be targeted by effectors of *H. schachtii* for establishment of successful feeding sites (Grunewald *et al.*, 2008). Therefore, we hypothesize that UPL3 regulates gene expression at the onset of nematode parasitism through ubiquitination of transcription factors in the nucleus. Furthermore, it is possible that UPL3 targets MYB121 and/or WRKY59 for ubiquitination, thereby regulating the stress responses of the plant. In turn, the role of UPL3 as regulator of these transcription factors could be stimulated or inhibited by nematodes by means of effectors such as GpRbp-1, to promote susceptibility. An alternative explanation could be that GpRbp-1 requires host factors like UPL3 in order to function properly in the plant cell. Additionally, in order to elucidate the role of the interaction of GpRbp-1 and UPL3, it remains to be seen if GpRbp-1 orthologues are present in *H. schachtii* with similar functional roles to GpRbp-1.

In conclusion, our results suggest that nematode effector GpRbp-1 may manipulate the ubiquitin-proteasome machinery of the host to modulate plant immune responses. Upon SA treatment, UPL3 has a large impact on total cellular poly-ubiquitination, suggesting that it may function as a E4 ubiquitin ligase, by “promiscuous” extension of poly-ubiquitination with low substrate specificity (Furniss *et al.*, 2018). Therefore, further characterisation of the substrate(s) for ubiquitination by UPL3, their subcellular localization, and their roles in plant-nematode interactions, will lead to additional knowledge on how sedentary nematodes manipulate the (nuclear) ubiquitination machinery of the host to promote susceptibility.

Materials and Methods

Yeast two-hybrid – library screen

The prey *G. pallida*- infected potato library was generated by Dual Systems Biotech (Switzerland) from grinded roots of potato SH infected with juveniles of *G. pallida* population Pa3-Rookmaker and *G. rostochiensis* population Ro5. Briefly, SH potato were grown on 16 cm square plates containing B5 medium at 20°C in 16 h light/ 8 hours dark conditions. Two weeks after transplant, plantlets were inoculated with ~200 juveniles. Infected roots were collected at 2, 3, 7, 9, 12 and 14 dpi and grinded in liquid nitrogen before shipping. Poly (A) tailing and total RNA isolation were performed by Dual Systems Biotech and a cDNA library consisting of $3{,}85x10^6$ clones with an average insert size of 1.13Kb was constructed. The library yeast two-hybrid screen was performed by Dual Systems Biotech using the DUALhybrid vector system.

Yeast two-hybrid – one-to-one screen

For the reciprocal swap one-to-one yeast two-hybrid the bait GpRbp-1 version 1 from virulent population Rookmaker (Rook1) and prey StUPL3frag8 were exchanged to the prey and bait vectors respectively. GpRbp-1 and StUPL3frag8 were isolated from the Y2H vectors by PCR (Suppl. Table 3). Restriction sites for SfiI or EcoRI and PstI were introduced for restriction-based cloning into pGAD-HA or pLexA respectively. The PCR products were cloned into pCR2.1TOPO following the manufacturer's instructions and digested with SfiI or EcoRI and PstI accordingly. The fragments were re-cloned in frame with the pLexA bait or pGAD-HA prey vectors. PJ69-4a were co-transformed following the transformation protocol of the DUALhybrid system (Dual Systems Biotech). Briefly, yeast cells are initially permeabilized with a polyethylene glycol and lithium acetate mix. Then, cells are heat-shocked at 42°C for 20 minutes in the presence of DMSO. After 1h recovery the cells are plated in minimal SD agar base medium without essential aminoacids (LEU,TRP,HIS) (Triple drop-out; TDO). Transformed cells were incubated at 30°C for 7 days. For confirmation of positive clones, positive colonies were re-plated in fresh TDO plates at 30°C for 5 days. Colonies able to grow after re-plating were considered as true positive colonies. SPRYSEC-19 and its interactor Sw5F (Rehman *et al.*, 2009) were cloned similarly and used as a positive control for the interaction. Auto-activation of the bait and prey vectors was ruled out by co-transformation with empty prey and bait vectors respectively.

Cloning

For co-immunoprecipitation, the interacting fragment StUPL3frag8 was excised from the pGAD-HA prey vector using NcoI and XhoI restriction sites. The NcoI-XhoI fragment includes the StUPL3frag8 with an N-terminal hemagglutinin (HA) tag. The NcoI-XhoI fragment was inserted into vector pRAP digested with NcoI and SalI. In the vector and additional four units of N-terminal HA tag are fused to the HA-StUPL3frag8. The fusion cassette was digested with PacI and AscI and ligated into vector pBIN. In pBIN, the HA5-StUPL3frag8 fusion is under the control of the 35S constitutive promotor. GpRpb-1 version 1 from virulent population

Rookmaker (Rook1) was tagged with an N-terminal fusion of 4 units on the c-myc tag followed by a GFP (Myc4-GFP-GpRbp-1), by ligation of Myc4-GFP isolated as a AscI/BspHI fragment from existing vector pRAP:Myc4-GFP, and GpRbp-1_Rook1 isolated as AscI/NcoI from pre-existing vector pRAP:Rook1. Subsequently the Myc4-GFP-Rook1 cassette was digested from pRAP with PacI/AscI and ligated into the pBINPLUS binary vector (van Engelen *et al.*, 1995) pre-digested with the same combination of enzymes.

The full-length gene of StUPL3 was obtained by synthetic gene synthesis (GeneArt) (Thermo Fisher Scientific, Waltham, Massachusetts) using the potato CDS transcript variant X2 (GenBank accession XM_015314510) into gateway-compatible pMA vector. StUPL3 was synthesized with an additional N-terminal BamHI site and a C-terminal PstI site in order to enable restriction cloning. The internal BamHI and PstI restriction sites were disrupted by introducing silent mutations. The codons were always replaced with ones with similar or higher usage frequency in *Nicotiana benthamiana (Nakamura et al., 2000)*. Full-length UPL3 N-terminally tagged with HA or GFP was obtained by gateway cloning to plant-expression vectors pGWB415 and pGWB425 respectively, (Nakagawa *et al.*, 2007) for interaction and localization studies. mCherry N terminally-tagged GpRbp-1 constructs were generated by ligation of nCherry isolated as a fragment from existing vector pRAP:mCh-PVX_CP106, and GpRbp-1_Rook1 isolated as a SacI/AscI fragment from the vector pRAP:My4-GFP-Rook1 described above. Subsequently the mCh-GpRbp-1_Rook1 cassette was digested from pRAP with PacI/AscI and ligated into the pBINPLUS binary vector (van Engelen *et al.*, 1995) pre-digested with the same combination of enzymes. For Bimolecular complementation, GpRbp-1 was amplified by PCR and cloned into pDONR207 by a BP reaction, following the manufacturer's instructions (Invitrogen). Expression clones of GpRbp-1 were obtained by LR recombination into pDEST`-SCYCE(R)^{GW} BiFC vector (Gehl *et al.*, 2009). Similarly, expression clones of full-length UPL3 (StUPL3) were recombined by LR (Invitrogen, Carlsbad, California) into pDEST-SCYNE(R)^{GW} BiFC vector following the manufacturer's instructions (Gehl *et al.*, 2009).

Expression and detection of recombinant proteins

All proteins were co-expressed by agrobaterium-mediated transient transformation of *Nicotiana benthamina* leaves. All co-expressions are done together with the silencing supressor P19, with a final concentration of OD_{600}=0.5. For co-immunoprecipitation (co-ip), Myc4-GFP-GpRbp-1 was infiltrated at a final concentration of OD_{600}=0.3, StUPL3frag8 was infiltrated at a final concentration of OD_{600}=1.0. For bimolecular fluorescence complementation (BiFC) all constructs were infiltrated at a final concentration of OD_{600}=0.5.

Total protein extracts were prepared by grinding leaf material in protein extraction buffer (20% (v/v) glycerol, 50 mM Tris-HCl pH 7.5, 2 mM EDTA, 300 mM NaCl, 0.6 mg/ml Pefabloc SC plus (Roche, Basel, Switzerland), 2,5% (w/v) polyclar-AT polyvinylpolypyrrolidone (Serva, Heidelberg, Germany), 5 mM dithiothreitol and 0.1% (v/v) Tween20) on ice. Protein extracts were passed through a Sephadex G-25 column (GE Healthcare, Chicago, Illinois) and pre-cleared by treatment with rabbit-IgG agarose (Sigma, 50 µL slurry per 60 µL protein extract). The cleared protein extract was incubated with MACS anti-c-MYC microbeads

(Miltenyi, Bergisch Gladbach, Germany) for 1h at 4°C. Columns were washed with washing buffer (20% (v/v) glycerol, 50 mM Tris-HCl pH 7.5, 2 mM EDTA, 300 mM NaCl, 0.15% (v/v) Nonidet 40 and 5mM dithiothreitol) five times and eluted with pre-heated (95°C) 1X NuPage LDS sample buffer with 0.25 M dithiothreitol**.** The input samples were mixed with 1X NuPage LDS sample buffer with 0.25 M dithiothreitol and incubated at 95°C for 5 minutes. Proteins were separated by SDS-PAGE on NuPage 12% Bis-Tris gels (Invitrogen) and blotted to 0.45 µm polyvinylidene difluoride membrane (Thermo Scientific). Before immunodetection we blocked the membranes for 1h at room temperature in 5% (w/v) powder milk in PBS with 0.1% Tween20. For immunodection we used goat anti-MYC (Abcam, Cambridge, United Kingdom) with horseradish peroxidase-conjugated donkey anti-goat (Jackson ImmunoResearch, Ely, United Kingdom) or horseradish peroxidase-conjugated rat anti-HA (Roche). Peroxidase activity was visualized using SuperSignal West Femto or Dura substrate (Thermo Scientific) and imaging of the luminescence with G:BOX gel documentation system (Syngene, United Kingdom). For protein detection after BiFC, protein extraction, separation and blotting was performed as described above. For immunodetection we used horseradish peroxidase-conjugated mouse anti-FLAG (Sigma-Aldrich, St. Louis, Missouri) or horseradish peroxidase-conjugated rat anti-HA (Roche).

For the *in planta* ubiquitination assay, *Agrobacterium*-infected *N. benthamiana* leaf tissues were collected at 48 hours after infiltration and ground with liquid nitrogen. The fine tissue powder was resuspended with 300 µl of protein extraction buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 5mM EDTA, 2mM DTT, 10% glycerol, 1% polyvinylpolypyrolidone, 1mM PMSF, plant protease inhibitor cocktail (Sigma-Aldrich) and centrifuged at 13,000g/4°C for 15 minutes. Protein samples were separated on 10% SDS-PAGE gels, transferred onto PVDF membrane and probed with anti-HA (Sigma-Aldrich) antibody, followed by anti-mouse secondary antibody (Sigma-Aldrich). Protein signal was detected with ECL Prime (GE Healthcare).

Confocal microscopy

Confocal microscopy was performed on *N. benthamiana* epidermal cells using a Zeiss LSM 510 confocal microscope (Carl-Zeiss) with a 40X 1.2 numerical aperture water-corrected objective. For co-localization studies the argon laser was used to excite at 488 nm for GFP and chlorophyll, and the HeNe laser at 543nm to excite mCherry. GFP and chlorophyll emission were detected through a band-pass filter of 505 to 530nm and through a 650nm long-pass filter, respectively. mCherry emission was detected through a band-pass filter of 600 to 650nm. For BiFC the argon laser was used to excite at 458 nm for SCFP3A. SCFP3A emission was detected through a band-pass filter of 470nm to 500nm and chlorophyll emission was detected through a 615nm long-pass filter.

Plant material and nematode infection in Arabidopsis

Seeds of the homozygous transgenic T-DNA insertion mutant of UPL3 (SALK_116326; *upl3-5*) and Col-0 N60000 Ecotype were obtained from the SALK homozygote T-DNA collection (Alonso *et al.*, 2003). Plants were propagated under standard greenhouse conditions of a 16-h/8-h light/dark regime and 60% relative humidity. For nematode assays, seeds of *upl3-5* and Col-0 were vapour sterilized and sown in Knop's modified medium (Sijmons *et al.*, 1991) in 9mm petri dishes, with two seedlings per plate. Seedlings were grown at 25°C under a 16-h-light/8-h-dark cycle. 10 day-old seedlings were inoculated with 60-70 surface-sterilized *H. schachtii* infective juveniles. After 2 weeks of infection, the number of males and females present in the roots of Arabidopsis plants were counted visually and the size of females and syncytia were calculated with Leica M165C Binocular (Leica Microsystems, Wetzlar, Germany) and the Leica Application Suite software (Leica Microsystems). To combine results from 4 biological replicates, we weighted the measures of association from each replicate by the inverses of their variances. The variance of such weighted average is simply the inverse of the sum of the inverses of the variances which allow standard methods to be used to test for the overall significance at the 5% level of the genotype and the number of nematodes per plant. Such approach correspond to methods to combine studies under a fixed effects model.

Gene expression during nematode infection in Arabidopsis

A. thaliana ecotype Columbia 0 seeds were grown in KNOP media as described above. 2 week-old seedlings were infected with ~100 freshly hatched *H. schachtii* juveniles or mock infected with 0.7% gelrite. Complete root systems of infected and mock infected plants were collected at 2,7,10 and 14 days post infection (dpi). Total RNA was extracted from the complete root systems with the Maxwell-16 instrument according to the manufacturer's manual for the Maxwell-16 LEV Plant RNA kit. Total cDNA was prepared according to the GoScript Reverse Transcriptase instructions with all RNA samples diluted to the concentration of the RNA sample with the lowest yield. RT-PCR was performed with Absolute SYBR Green mix (Thermo Fisher scientific) 1µL forward primer and 1µL reverse primer (5µM), 7µL MQ, 10µL Absolute SYBR Green mix (2X) and 1µL cDNA template adjusted to 10ng/µL. Each sample was processed in triplicate. RT-PCR was run using the following program: Initial denaturation at 95°C for 15 min followed by 40 cycles of amplification at 95°C for 30s, 61°C for 30s ,72°C for 30s and final elongation at 72°C for 5mins with a 0.2 °C ramp melting curve from 72 to 95 in 10s. AtUPL3 expression was quantified using previously described primers (Patra *et al.*, 2013). Relative gene expression was calculated with the Vandesompele method (Vandesompele *et al.*, 2002) using Ubiquitin 5 (UBQ5) (Anwer *et al.*, 2018) and Ubiquitin-specific protease 22 (UBP22) (Hofmann & Grundler, 2007) as reference genes. The experiment was repeated 3 times and the significance of the fold change of the gene expression was determined by a Kruskal-Wallis test (α=0.05).

Microarray analysis

RNA extraction, cDNA synthesis and hybridization

To obtain the root material for microarray analysis, seeds of the transgenic *upl3-5* and Col-0 wild-type (N60000) were vapour sterilized and sown in modified Knop's medium (Sijmons *et al.*, 1991) in 6-well cell culture plates (Greiner bio-one). Seedlings were grown at 21°C under a 16-h/8-h light/dark regime. Two-week old seedlings were infected with approximately 180 surface-sterilized *H. schachtii* juveniles. One week after inoculation the complete root systemsof ~18 *upl3-5* and Col-0 plantlets were harvested and snap-frozen. Root tissue was ground in liquid nitrogen and total RNA was extracted extraction with the Maxwell® 16 LEV plant RNA kit (Promega) in the Maxwell 16 AS2000 instrument (Promega), following the manufacturer's instructions. 4 biological replicates of ~18 plants/sample per condition were generated.

The cDNA and cRNA were prepared using the Two-Color Microarray-Based Gene Expression Analysis, Low Input Quick Amp Labeling kit (Agilent), according to the manufacturer's instructions.

Microarray probe blasting

The Arabidopsis V4 Gene Expression Microarray (4x44K, Agilent Technologies) probes were blasted against the TAIR11 genome of A. thaliana using the blastn function of the command line blast tool (v. 2.6.0+, win64), using the default settings. The top-hit was used as probe annotation. If probes had multiple hits, they were censored (Camacho *et al.*, 2009).

Scanning and normalization

After hybridization, microarrays were scanned using an Agilent high resolution C scanner. The scans were extracted using feature extract (version 10.7.1.1) and data was normalized in R (v. 3.4.2) using the Bioconductor Limma package (Ritchie *et al.*, 2015). The data was not background corrected before normalization (as recommended by) (Zahurak *et al.*, 2007). Within-array normalization was done with the Loess method and between-array normalization was done with the Quantile method (Smyth & Speed, 2003). After normalization the data was batch corrected per condition (mock infected day 0, mock infected day 7, and infected day 7) to remove environmental variation linked to when the biological replicate was performed. Thereto we log2 transformed the intensities and corrected by

$$E_{i,j} = I_{i,j} - \left(\overline{I}_{i,batch} - \overline{I}_{i,total}\right)$$

where E is the batch corrected expression of spot i (1, 2, ..., 45220) of sample j (1, 2, ..., 24) and I is the log2 transformed normalized intensity.

Statistical analysis of differentially expressed genes

Variation attributable to the different conditions was assayed using principal component analysis. For this we transformed the expression data to mean-centred values, by

$$R_{i,j} = \log_2 \frac{E_{i,j}}{\overline{E}_i}$$

where R is the log2-transformed mean-centred expression of spot i of sample j, and E is the batch corrected expression. These values were used as input for the principal component analysis, which was conducted in R.

Differences between genotypes within each condition were tested using a linear model

$$E_{i,j} = G_j + e$$

where E is the batch corrected expression, G is the genotype (either col-0 or *upl3-5*), and e is the error term. The obtained significances were corrected for multiple testing using the Benjamini-Hochberg method, as implemented in the p.adjust function in R (Benjamini & Hochberg, 1995, Team, 2014). To assess the differentially expressed genes per condition, we took a permissible threshold of –log10(p) > 4 (FDR = 0.051, FDR = 0.064, and FDR = 0.0041 for mock infected 14-days old seedlings, mock infected 21-days old seedlings, and *H. schachtii* infected 21 days old seedlings respectively. However, when ascertaining the biological functions affected, we took a more strict threshold, namely –log10(p) > 6 (FDR = 0.010 and FDR = 0.00029 for mock infected 14-days old seedlings and *H. schachtii* infected 21-days old seedlings respectively). This more strict threshold allows us to focus on the genes that are highly differentially expressed between col-0 and *upl3-5* upon *H. schachtii* infection.

Enrichment analysis

For enrichment analysis we mined the following TAIR11 databases: Gene ontology, Gene ontology slim, gene classes, and phenotypes (Berardini *et al.*, 2015, Lamesch *et al.*, 2012). In addition the MapMan gene ontology database (TAIR10) was used (Thimm *et al.*, 2004). Enrichments were calculated using a hypergeometric test, as provided in R (phyper). Overlaps were calculated based on gene identifiers, not on spots. In other words, if a gene was significant for multiple spots, all spots were counted as one. After calculating significances, the lists were filtered on three criteria: (i) the classification group should consist of at least 3 genes, (ii) the number of overlapping genes should be at least 3, and (iii) the significance corrected for multiple testing (FDR) should be $q < 0.05$.

Data availability

The data was submitted to ArrayExpress, under code E-MTAB-7968.

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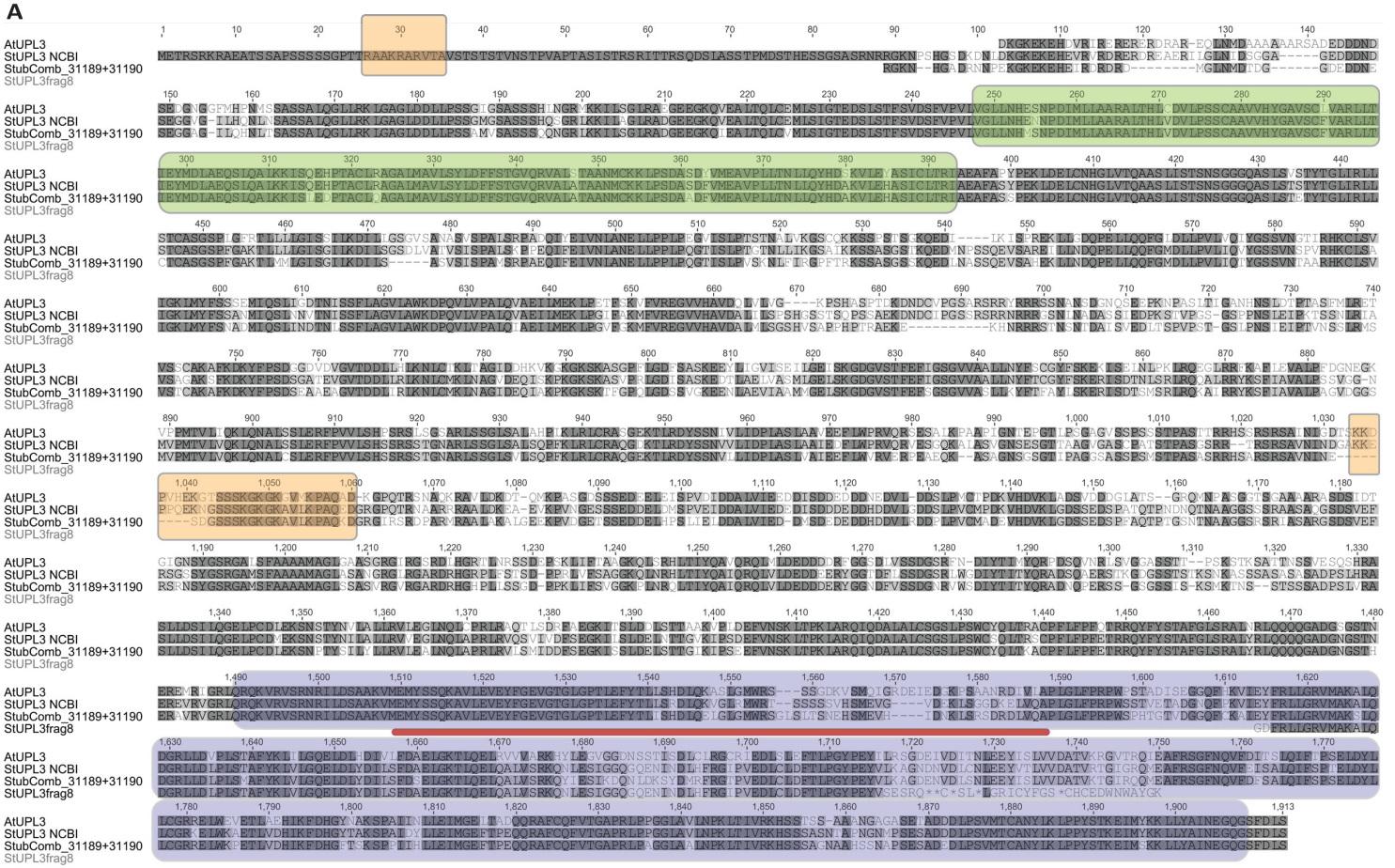
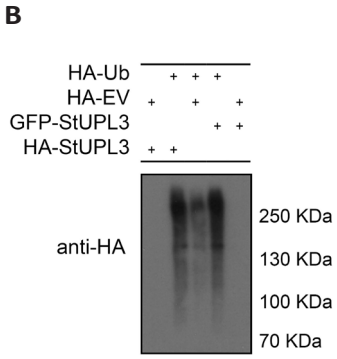
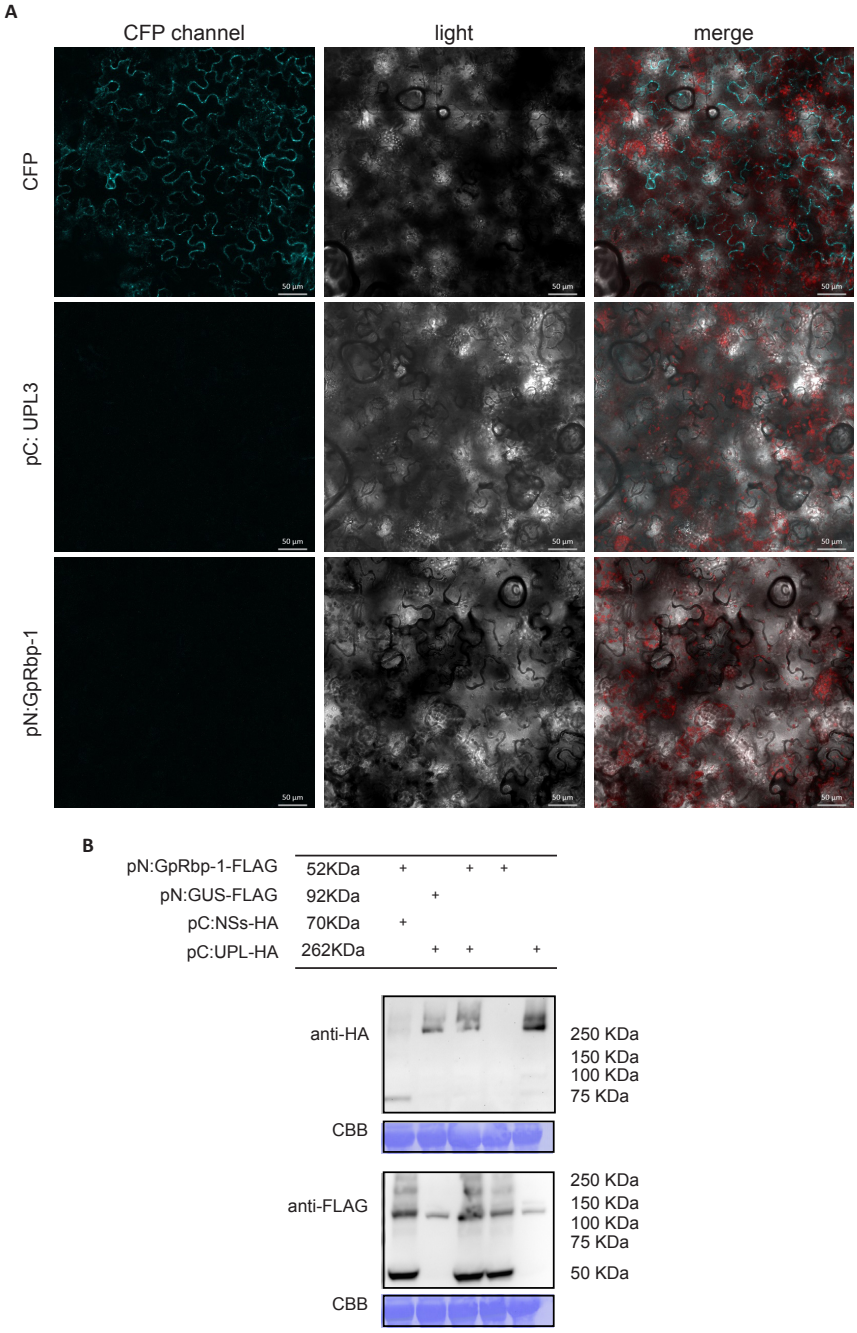
Supplemental information



Supplemental figure 1. Characterization of the potato homolog of UPL3 (*Continued in next page*)

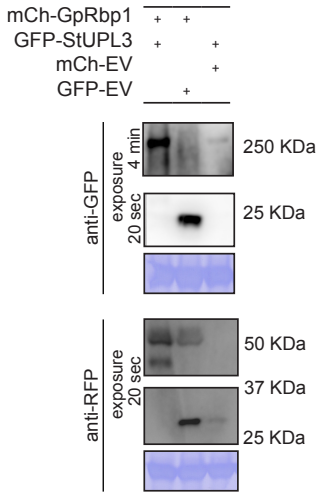


Supplemental figure 1. Characterization of the potato homolog of UPL3. (A) Protein sequence alignment of full length AtUPL3 and StUPL3. Protein sequences were obtained by translation in frame +1 of: the AtUPL3 CDS (AtUPL3; AT4G38600), the predicted StUPL3 promotes poly-ubiquitination in planta. E3 ubiquitin-protein ligase from GeneBank (StUPL3 NCBI; XM_015314510), the combination of Spubdb transcripts PGSC0003DMT400031189 and PGSC0003DMT400031190 (StUPL3Comb) (Hirsch *et al.*, 2014) and the yeast interacting fragment (StUPL3frag8) . The alignment was made with the CLUSTALW plugin of Geneious (version 8.1.9) with cost matrix BLOSUM. Identical amino acids are highlighted in dark grey. Predicted NLS are shown with a yellow box, the armadillo repeats domain is indicated by a green box, the HECT domain is shown with a blue box and the E2 interaction site is shown with a red bar. B) StUPL3 promotes poly-ubiquitination *in planta*. *A. tumefaciens* GV2260 strains harboring the epitope-tagged ubiquitin (HA-Ub) or StUPL3 (GFP-StUPL3 or HA-StUPL3) as indicated combination were infiltrated into *N. benthamiana* leaves at a concentration of OD600 = 0.2. *A. tumefaciens* containing the empty vector (EV) was used as a control. 48 hours after Agrobacterium infiltration, proteins were extracted for Western blotting assay using α-HA antibody to determine poly-ubiquitination, which appears as a smear banding pattern.

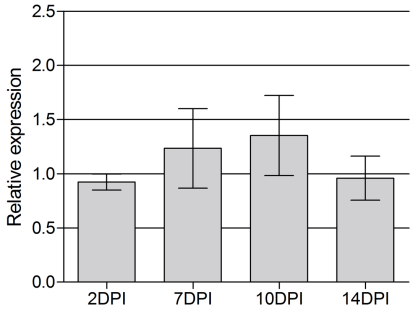


Supplemental figure 2. Individual Bimolecular fluorescence complementation protein fusions do not emit fluorescence, despite appropriate expression *in planta*. A) Live imaging of individual N-CFP (pN) or C-CFP (pC) fusions to StUPL3 and GpRbp-1. A CFP transformation is shown for comparison of the confocal microscopy settings. **B)** Western blot detection with anti-FLAG (pN constructs) and anti-HA (pC constructs).

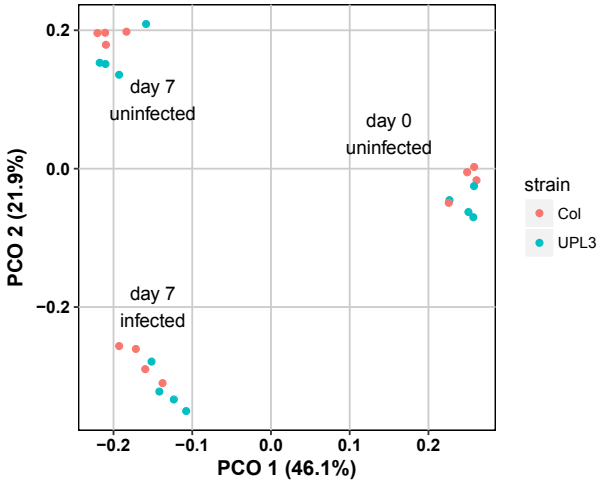
Supplemental Table 1. A list of all the genes significantly regulated in *upl3-5* plants. The treatment column indicates which comparison was made. The significance column gives the significance of the difference as determined by the linear model, the effect column the size of the difference (log2-units; negative values are lower expressed in col-0, positive values higher expressed in col-0). The significance_FDR column lists the q-values as determined by Benjamini-Hochberg correction. The columns thereafter list properties of the genes detected by the spots. Spots with no associated gene either are technical spots or have significant blast-hits with multiple different genes. If this is the case, it is mentioned in the comments. The genes are selected by the-log10(p)<4 (yellow) or –log10(p)<6 (red) thresholds. Available at: https://drive.google.com/open?id=1P2qzPZtfLHbRDHqQcMwrDRjqU7ygYl6-



Supplemental figure 3. Fluorescent fusions of StUPL3 and GpRbp-1 are expressed by agro-infiltration. Western blot detection of fusions of fluorescent proteins green fluorescent protein with StUPL3 (GFP-UPL3), red fluorescent protein mCherry (mCh-GpRbp-1) or GFP and mCh alone. Western blot is performed with anti GFP and anti RFP antibodies.



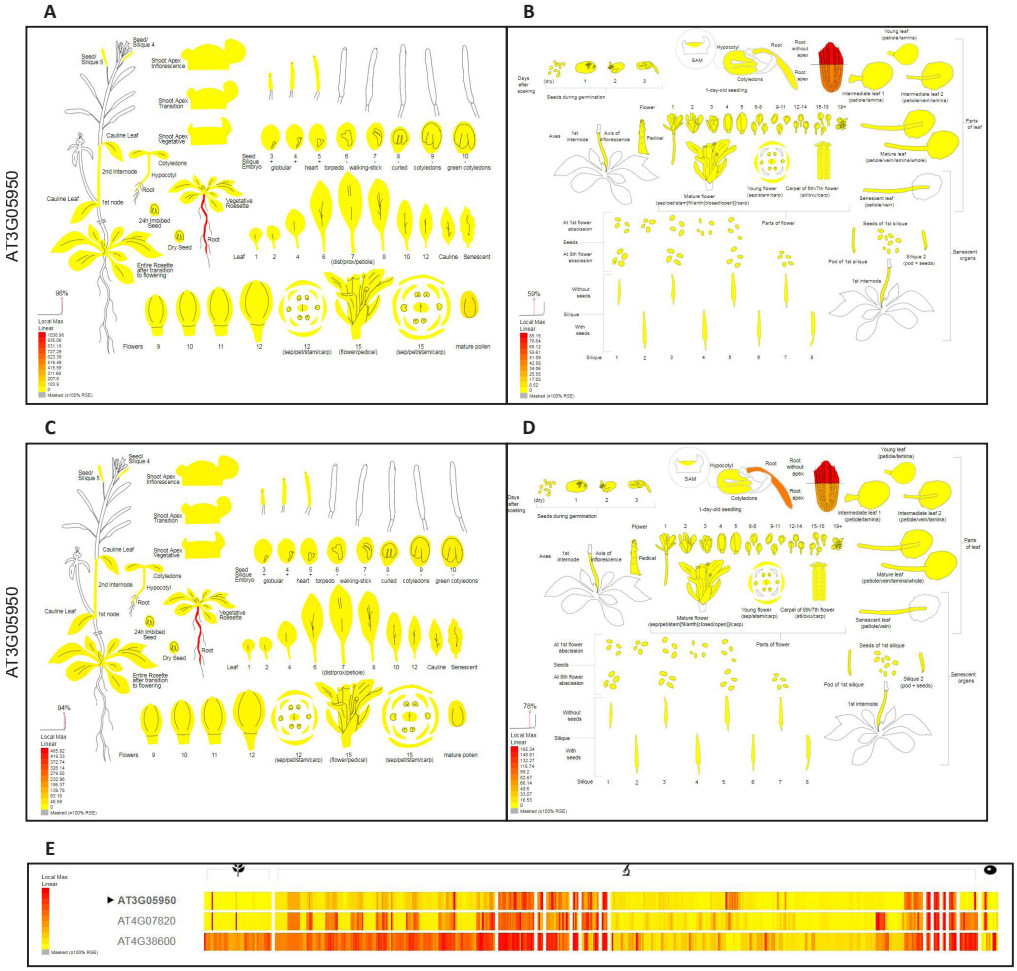
Supplemental figure 4. AtUPL3 is not regulated during cyst-nematode infection in the roots of *A. thaliana*. Expression of AtUPL3 was quantified by RT-PCR in *A.thaliana* roots infected with cyst nematode *H. schachtii*. The ratio of AtUPL3 expression was normalized to the geometric average of ubiquitin 5 (Anwer *et al.*, 2018) and ubiquitin carboxyl-terminal hydrolase 22 (Hofmann & Grundler, 2007) using the Vandesompele method (Vandesompele *et al.*, 2002).



Supplemental figure 5. Principal component analysis of gene expression profiles of *upl3-5* and wildtype Arabidopsis plants before infection, and 7 days after (mock) infection. The first principal component PCO 1 captures 46.1% of the variation and separates the age of the Arabidopsis seedlings. The second principal components PCO 2 captures 21.9% of variation and separates infected from uninfected samples.

Supplemental Table 2. Enrichment analysis of the 131 differentially expressed genes between Col-0 and *upl3-5* upon infection with *H. schachtii*. Groups found to be enriched in the genes that are differentially regulated in *upl3-5* during nematode infection. The groups are categorized by the significance of the enrichment (significance FDR). The annotation database, number of genes present per group in the database, the overlap expected by random and the calculated overlap are also shown.

Group	*Category code*	*Significance FDR*	*Annotation source*	*Genes in group*	*Overlap expected*	*Overlap*
involved in response to salt	GO:1902074	8.71E-05	Gene_ontology	10	0.028969483	3
involved in cellular amino acid metabolic process	GO:0006520	0.000727	Gene_ontology	34	0.098496241	3
cell.division	31.2	0.00204	MapMan	101	0.292591774	4
involved in leaf senescence	GO:0010150	0.005662	Gene_ontology	87	0.252034498	3
misc.oxidases- copper, flavone etc	26.7	0.008817	MapMan	103	0.29838567	3
stress.abiotic.unspecified	20.2.99	0.009391	MapMan	105	0.304179567	3
other molecular functions	-	0.011626	Gene_ontology_slim	1033	2.992547545	12
has flavin adenine dinucleotide binding	GO:0050660	0.012812	Gene_ontology	118	0.341839894	3
signalling.calcium	30.3	0.018617	MapMan	220	0.637328616	4
involved in root development	GO:0048364	0.021469	Gene_ontology	149	0.43164529	3
located in cellular_component	GO:0005575	0.022641	Gene_ontology	787	2.279898275	9
unknown cellular components	-	0.022641	Gene_ontology_slim	787	2.279898275	9
involved in response to jasmonic acid	GO:0009753	0.022641	Gene_ontology	152	0.440336134	3
involved in metabolic process	GO:0008152	0.024246	Gene_ontology	156	0.451923927	3
involved in response to water deprivation	GO:0009414	0.03003	Gene_ontology	265	0.767691287	4
involved in defense response to bacterium	GO:0042742	0.030794	Gene_ontology	268	0.776382132	4
located in cell wall	GO:0005618	0.033407	Gene_ontology	491	1.422401592	6
located in chloroplast stroma	GO:0009570	0.033613	Gene_ontology	610	1.767138434	7
involved in response to abscisic acid	GO:0009737	0.034296	Gene_ontology	382	1.106634233	5
functions in copper ion binding	GO:0005507	0.038452	Gene_ontology	190	0.550420168	3
has ATPase activity	GO:0016887	0.039555	Gene_ontology	192	0.556214065	3
stress.abiotic	20.2	0.041423	MapMan	406	1.176160991	5
involved in cell division	GO:0051301	0.043112	Gene_ontology	200	0.579389651	3
involved in regulation of transcription, DNA-templated	GO:0006355	0.045089	Gene_ontology	1921	5.565037594	16
has sequence-specific DNA binding	GO:0043565	0.048085	Gene_ontology	313	0.906744803	4
has transcription factor activity, sequence-specific DNA binding	GO:0003700	0.049089	Gene_ontology	1647	4.771273773	14



Supplemental figure 6. The top 2 most down/up regulated genes by size of the effect in nematode-infected upl3-5 Arabidopsis show a similar pattern of expression. A) Developmental expression of a predicted member of RmIC-cupin superfamily (AT3G05950) and **C)** predicted CAP-superfamily member (AT4G07820) (Schmid *el al.*, 2005, Waese *el al.*, 2017). **B**) RNA-seq based expression of a predicted member of RmIC-cupin superfamily (AT3G05950) and **D)** predicted CAP-superfamily member (AT4G07820) (Waese *el al.*, 2017, Klepikova *el al.*, 2016). **E)** Heatmap of expression of a predicted member of RmIC-cupin superfamily (AT3G05950), a predicted CAP-superfamily member (AT4G07820) and AtUPL3 (At4G38600) across 350+ samples from the ePlant collection (ePlant HeatMap Viewer tool and references therein) (Waese *el al.*, 2017). Colors are drawn according to the local maximums.

Supplemental Table 3. List of primers and sequences mentioned in the text.

Cloning primers	
Primer name	***Sequence***
GpRbp-1 Forward	5-GGGCCATTACGGCCCAACTCGCTCGCCCAATGGAG-3
GpRbp-1 Reverse	5-GGGCCGAGGCGGCCCGGCCCATTATAAATTCTCG-3
StUPL3frag Forward	5-GAGAGGAATTCGGGGATTTCAGATTGCTAGG-3
StUPL3frag Reverse	5-GAGAGCTGCAGCTCACAGTGGCATTAACTACC-3
SS19 Forward	5-GGGAGGAATTCATGAGTGCTAGCGAGCAAAAGC-3
SS19 Reverse	5-GAGAGCTGCAGTCAAAATGGGCCAAAGTTCGC-3
SW5F Forward	5-GGGCCATTACGGCCCGGCACGAGGTATCAAGGAG-3
SW5F Reverse	5-GGGCCGAGGCGGCCCTACCATCCCTGTATTATAC-3
RT-PCR primers	
Primer name	***Sequence***
At_qPCR_UPL3_001 F	5-ACTCGAACTGCTTTTGGGTTG-3 (Patra *et al.*, 2013)
At_qPCR_UPL3_001 R	5-TTCGGGATACACGGACTTTC-3
At_qPCR-UBP22_001_F	5-ACAACATATGACCCGTTTATCGA-3 (Hofmann & Grundler, 2007)
At_qPCR-UBP22_001_R	5-TGTTTAGGCGGAACGGATACT-3
At_qPCR-UBQ5_001_F	5-GTTAAGCTCGCTGTTCTTCAGT-3 (Anwer *et al.*, 2018)
At_qPCR-UBQ5_001_R	5-TCAAGCTTCAACTCCTTCTTTC-3
Other sequences	
StUPL3frag8	GGGGATTTCAGATTGCTAGGGCGTGTGATGGCGAAAGCACTTCAAGATGGACGGCTTTTGGATCTC CCTCTGTCAACTGCATTTTACAAGCTTGTTCTTGGCCAAGAGCTTGATCTGTATGATATTCTTTCTTT TGATGCTGAGTTAGGCAAGACTTTGCAAGAGTTGCAAGCTCTTGTTAGCCGTAAACAAAATCTAG AATCAATTGGAGGCCAGGGACAAGAGAACATTAATGATTTGCATTTTCGTGGGATCCCAGTTGAGG ATCTTTGCTTAGACTTCACACTCCCAGGCTATCCTGAATATGTTTCTGAAAGCAGGCAATGATAATGT TGATCTTTGTAACTTGGAAGAATATGTTACTTTGGTAGTTAATGCCACTGTGAGGACTGGAATTGGG CGTACGGGAAATA

Chapter 4

SIZ1 is a nuclear host target of the nematode effector GpRbp-1 from *Globodera pallida* that acts as a negative regulator of basal plant defense to cyst nematodes

Amalia Diaz-Granados[1]; Mark G. Sterken[1]; Jarno Persoon[1]; Hein Overmars[1]; Somnath S. Pokhare[2]; Magdalena J Mazur[3]; Sergio Martin-Ramirez[1]; Martijn Holterman[1]; Eliza C. Martin[4]; Rikus Pomp[1]; Anna Finkers-Tomczak; Jan Roosien[1]; Abdenaser Elashry[2]; Florian Grundler[2]; Andrei J Petrescu[4]; Jaap Bakker[1]; Geert Smant[1]; Aska Goverse[1]

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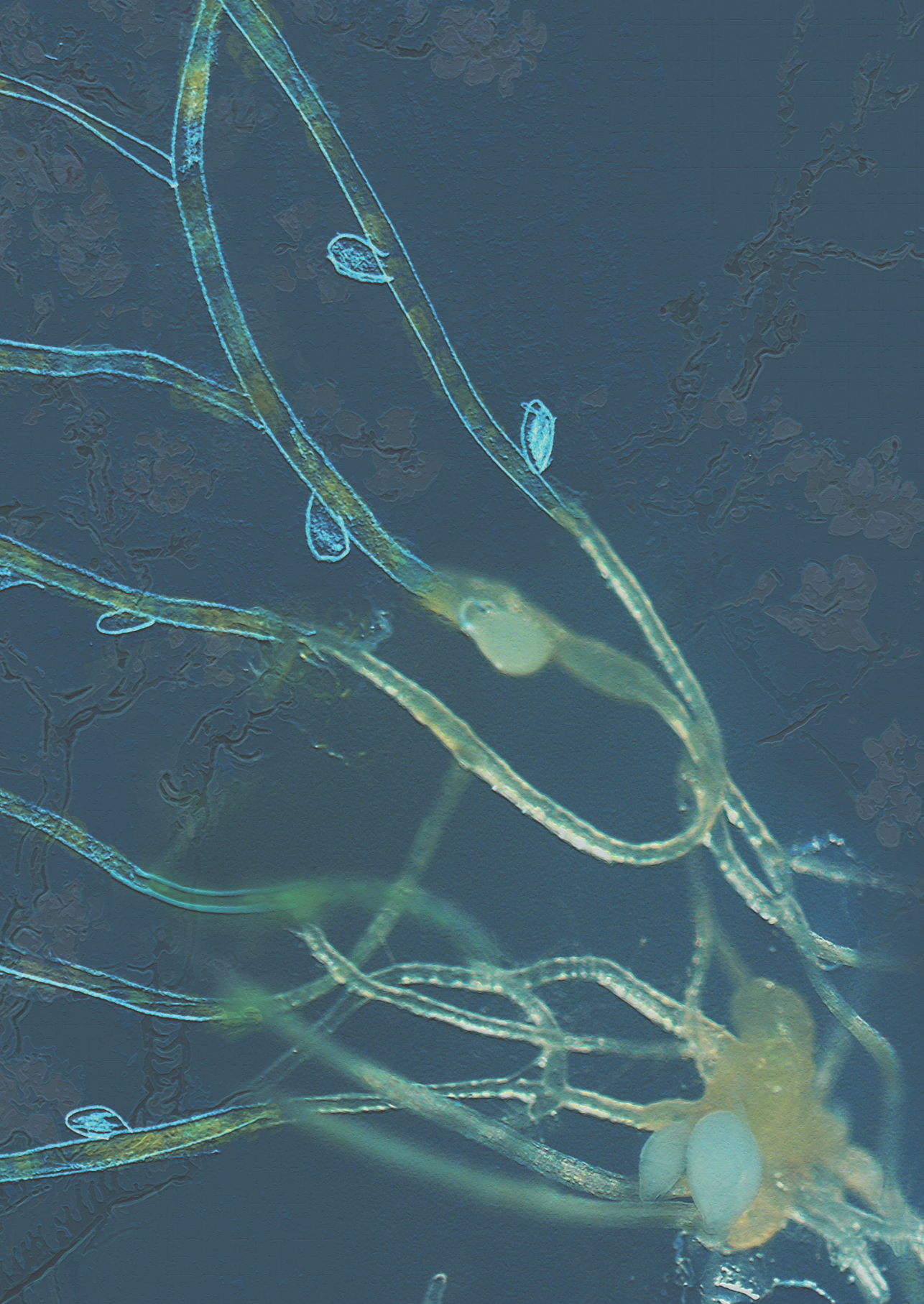
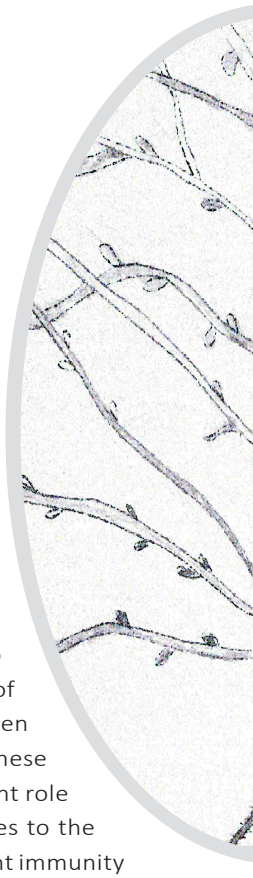
[1] Laboratory of Nematology, Wageningen University, Wageningen, Netherlands;
[2] Department of Molecular Phytomedicine, INRES, University of Bonn, Bonn, Germany;
[3] Laboratory of Virology, Wageningen University and Research, Wageningen, The Netherlands;
[4] Department of Bioinformatics and Structural Biochemistry, Institute of Biochemistry of the Romanian Academy, Bucharest, Romania.





4

Abstract

Soil-borne cyst nematodes are obligatory sedentary parasites that cause severe losses to cultivation of major crops such as potato and soybean. Cyst nematodes establish specialised permanent feeding sites within the roots of their host by manipulating plant morphology and physiology through secreted effectors. Here we identified host targets of effector GpRb-1 and studied their roles in plant-nematode interactions. GpRbp-1 was found to interact in yeast and *in planta* with the potato and Arabidopsis homologues of Siz/PIAS-type E3 SUMO ligase SIZ1. Our results are the first report of a pathogen effector that targets the master regulator SIZ1 in plant cells. Additionally, these findings provide the first evidence that E3 SUMO ligases may play an important role in plant-nematode interactions. The interaction of GpRbp-1 and SIZ1 localizes to the plant nucleus, suggesting that the nuclear functions of SIZ1 as regulator of plant immunity and physiology may be modulated by GpRbp-1. Furthermore, nematode infection assays and transcriptomic profiling indicate that SIZ1 is required for susceptibility to cyst nematodes. Based on the prediction of SUMO acceptor and interaction sites in GpRbp-1, a model is proposed in which the effector may recruit SIZ1 to be SUMOylated for full functionality in host cells.

Introduction

Plant parasitic nematodes are small round worms that infect the underground parts of their plant hosts. In agricultural settings, nematode infections cause yearly losses in the order of $157 billion (Abad *et al.*, 2008) and it is expected that the rate of nematode infections will increase due to a warmer global climate (Bebber *et al.*, 2013). Cyst nematodes are sedentary endoparasites that penetrate and invade the roots of several major food crops from the Solanaceae family as well as cereals, soybean and sugar beet. Cyst nematodes persist in the soil in recalcitrant cysts that contain hundreds of eggs. In the presence of a host plant, infective juveniles (pre-parasitic J2) hatch from the eggs and penetrate the roots by means of an oral needle-like protractible structure, the stylet. Upon penetration, parasitic-J2 migrate intracellularly in the root until they find a suitable cell for establishment of a permanent feeding site and become sedentary. The characteristic permanent feeding site of cyst nematodes, a so called syncytium, is the sole nutrient source that sustains the nematode through three subsequent life stages i.e. J3, J4 and adult (Kyndt *et al.*, 2013). Eggs develop inside the body of adult females which eventually die and convert into a hardened cyst.

The interaction with the host plant is vital for the completion of the nematode's life cycle and it is largely mediated by a suite of effectors secreted by the nematode. However, only a limited set of effectors has been functionally characterized thus far (Juvale & Baum, 2018). Effectors are produced in two oesophageal glands and secreted selectively throughout the different life stages of the nematode to play different roles during the infection process (Hussey, 1989). For example, several plant cell wall-degrading enzymes are secreted by nematodes at the onset of parasitism to modify or degrade plant cell walls, thereby facilitating intracellular migration (Reviewed in (Wieczorek *et al.*, 2015)). Also, a number of effectors that mediate reprogramming of the plant cells are required for the initiation, establishment and maintenance of the syncytium (Gheysen & Mitchum, 2011; Mitchum *et al.*, 2013; Quentin *et al.*, 2013). At the molecular level, nematode secreted effectors function by modifying, competing with, or mimicking the roles of plant structures, genes or proteins. One strategy is the post-translational regulation of host proteins, either directly or indirectly through their interaction with host targets involved in post-translational modification (PTM) (Juvale & Baum, 2018).

Post-translational modifications constitute a powerful tool for functional regulation of proteins in eukaryotic cells (Spoel, 2018; Walsh *et al.*, 2005). These regulatory mechanisms rely most often on reversible modifications of peptides and allow a rapid response to variable environmental cues, without requiring gene synthesis (Spoel, 2018). There are different types of post-translational modifications, including the addition of polypeptides onto specific target proteins. The most widely recognised polypeptide addition is ubiquitination, the attachment of several subunits of ubiquitin to target proteins which often function as a molecular marker for protein degradation (reviewed in (Sadanandom *et al.*, 2012; Smalle & Vierstra, 2004)). More recently, an additional small peptide was described (SUMO; Small Ubiquitin-like Modifier) that bears close structural similarities to ubiquitin and can also be conjugated onto target proteins (Matunis *et al.*, 1998). Opposite to ubiquitination, SUMOylation (addition of SUMO) results in variable cellular fates for the target protein. For instance, SUMOylation can alter the subcellular

localization, the enzymatic activity or the protein-interaction properties of a target protein (Kurepa *et al.*, 2003; van den Burg *et al.*, 2010; Augustine & Vierstra, 2018; Verma *et al.*, 2018).

The cellular machinery for SUMOylation is largely conserved among eukaryotes, and in plants it is best characterized by studies in the model plant *Arabidopsis thaliana*. Four SUMO isoforms SUMO 1/2/3/5 from *A. thaliana* are shown to be functional, with SUMO1 and 2 as the prevalent isoforms serving as substrate for SUMOylation (Kurepa *et al.*, 2003; van den Burg *et al.*, 2010). SUMOylation of target substrates is catalysed by a chain of reactions similar to that of ubiquitination (Kurepa *et al.*, 2003; van den Burg *et al.*, 2010). First, the precursor of SUMO is matured by Ubiquitin-Like Proteases (ULPs) and it is then activated by heterodimeric E1-activating enzymes composed by subunit SAE2 and either SAE1a or SAE1b subunits. The activation of SUMO results in its attachment to the E2 SUMO conjugating enzyme SCE1, which then catalyses the conjugation of SUMO onto an acceptor lysine commonly within the motif ΨKxE in the target protein (Rodriguez *et al.*, 2001)2001. Two E3 ligases, SIZ1 and HYP2 seem to act as enhancers of the activity of the E2 conjugating enzyme (Ishida *et al.*, 2012). Finally, SUMOylation can be reversed by an isopeptidase activity of the SUMO-activating ULPs (Yates *et al.*, 2016).

A large amount of evidence places SUMOylation at the nexus of plant responses to (a) biotic stress (Elrouby *et al.*, 2013; Miller *et al.*, 2013). For instance, the abundance of SUMO conjugates increases when plants are subjected to heat shock or chemical exposure, including hydrogen peroxide, copper, and ethanol (Chen *et al.*, 2011; Kurepa *et al.*, 2003). Additionally, Arabidopsis mutants of the different components of the SUMO machinery often display phenotypes defective in tolerance to abiotic stress or pathogen attack (Ishida *et al.*, 2012; Kurepa *et al.*, 2003; Lee *et al.*, 2006; van den Burg *et al.*, 2010). In particular, the knock-out mutant of the SUMO E3 ligase SIZ1 has a strong pleiotropic phenotype, indicating that SIZ1 plays a prominent role as regulator in the response to several different types of environmental stresses (Lee *et al.*, 2006). In biotic stress, SIZ1 has been shown to be a negative regulator of salicylic acid-mediated defence, i.e. the *siz1-2* knock-out mutant shows increased resistance to infection by *Pseudomonas syringae* pv. *tomato* (Lee *et al.*, 2006). Due to its prominent role as a negative regulator of plant immunity, SIZ1 would be a valuable target for pathogen effectors to modulate plant immunity for successful infection of their host. However, no evidence is provided for this hypothesis yet.

The nematode effector GpRbp-1 belongs to the highly expanded family of SPRYSEC proteins of the potato cyst nematodes *Globodera pallida* and *G. rostochiensis* (Diaz-Granados *et al.*, 2016; Jones *et al.*, 2009; Mei *et al.*, 2015; Rehman *et al.*, 2009). SPRYSEC effectors contain an N-terminal signal peptide for secretion and a C-terminal SPRY domain. The N-terminal signal peptide suggests that SPRYSEC effectors are delivered to the plant cell where they can interact with host proteins. The C-terminal domain, in turn, is proposed to act as a binding platform to mediate interaction with plant target proteins (Diaz-Granados *et al.*, 2016). GpRbp-1 is predominantly expressed during the early parasitic stages of nematode infection, which suggests that it plays a role in early parasitism during the initiation and/or establishment of syncytia (Blanchard *et al.*, 2005). A role of GpRbp-1 in nematode virulence is further supported by signatures of positive selection on GpRpb-1 variants from field populations of *G. pallida*

(Carpentier *et al.*, 2012). The diversification of this effector family is probably due to specific recognition of certain members by the plant immune system, as shown for the potato immune receptor Gpa2. This receptor recognises specific variants of GpRbp-1 and confers resistance to particular populations of *G. pallida* in the field harbouring the corresponding effector variant (Sacco *et al.*, 2009).

To elucidate the role of GpRbp-1 in virulence of *G. pallida*, we aimed to characterize its molecular targets in cells of host plants. We used a combination of protein affinity assays to show that the nematode effector GpRbp-1 interacts specifically in yeast and *in planta* with the SP-RING finger domain of a potato Siz1/PIAS SUMO E3 ligase (StSIZ1). Furthermore, we could demonstrate that this interaction occurs in the nucleus of the plant cell. Similarly, GpRBP-1 was able to interact with AtSIZ1, which prompted us to test the role of SIZ1 in cyst nematode infection by using the *Arabidopsis* mutants *siz1-2* and *siz1-3*. Infection of *in vitro* grown plant resulted in fewer adult nematodes developing on the roots, consistent with the role of SIZ1 as a negative regulator of basal plant defence to biotrophic pathogens (Lee *et al.*, 2006). Additional evidence was obtained by a comparative RNAseq analysis, which shows that the reduction of nematode susceptibility in *siz1-2* plants is likely due to the activation of defence-related pathways by the *siz1-2* mutation. To the best of our knowledge, this is the first report of an effector from any plant pathogen that targets the master regulator SIZ1. Moreover, this study provides first evidence for a functional role of SIZ1-mediated sumoylation in nematode parasitism of plant roots. From our data, a picture emerges in which cyst nematodes target SIZ1 in the nucleus to modulate their host through post-translation modifications. To conclude, possible implications on the modulation of SUMOylation (or SIZ1) in plant cells by cyst nematodes are also discussed.

Results

GpRbp-1 interacts in yeast with a fragment of SUMO E3 ligase SIZ1 from potato

To find plant interactors of GpRbp-1 we performed a yeast two-hybrid screen of a cDNA library obtained from potato (SH) roots infected with the potato cyst nematode species *G. pallida.* We screened a library of $3{,}85 \times 10^6$ clones using a variant of GbRbp-1 from field population Rookmaker (GpRbp-1_Rook-1) as bait. Five yeast clones containing cDNA sequences of 858 - 976bp with identities ranging from 97.5 to 100% were found to interact with bait protein GpRbp-1. To identify the candidate plant target that these clones correspond to, we compared the sequences of all fragments against the UniProtKB/Swiss-Prot non-redundant database using the BLASTX algorithm. All clones showed the highest sequence similarity to *Arabidopsis thaliana* E3-SUMO ligase SIZ1 (e-values 2.11×10^{-42} to 9.6×10^{-116}) (Suppl. Table 1). Among the five yeast clones, there were two pairs with 100% identical sequences within each pair (StSIZfrag10 and StSIZ1frag14; StSIZ1frag49 and StSIZ1frag83). One additional clone contained a fragment that was 87% the length of the fragment contained in the identical clones (StSIZ1frag06). Sequence alignment showed that the clones localized to the C-terminal half of SIZ1 containing a predicted SP-RING finger domain (Fig. 1; Suppl. Fig. 1).

In Arabidopsis, the *SIZ1* gene encodes four protein domains, an N-terminal SAP domain (Scaffold attachment factors SAF-A/B, Acinus, PIAS), PHD (Plant Homeodomain), a PIIT (proline-isoleucine-isoleucine-threonine) motif, and a SP-RING (SIZ/PIAS-REALLY INTERESTING NEW GENE). Additionally, two SUMO Interacting Motif (SIM) domains were encoded by *AtSIZ1* (Miura *et al.*, 2005) (Fig. 1). Finally, AtSIZ1 also contained a nuclear localisation sequence (NLS) in the C-terminal domain of the protein (Fig. 1). We compared the coding and peptide sequences of SIZ1 from *Arabidopsis* and potato to investigate their similarity. The full-length coding sequence for *StSIZ1* was obtained from the non-redundant nucleotide database of GenBank (XM_006340080.2). At the nucleotide level, *AtSIZ1* and *StSIZ* shared ~60% identity and at the protein level they shared ~62% identity (Suppl. Fig. 1). It should be noted that StSIZ1frag14 and StSIZ1frag83 were 97% identical to StSIZ1, and differences were likely due to the differences in potato variety used (Fig. 1B, Suppl. Fig. 1). Additionally, we investigated the number of copies of *StSIZ1* present in the genome sequence of the doubled monoploid potato genotype DM. To this end, we probed the PGSC *S. tuberosum* group Phureja DM1-3 transcripts v3.4 database from the Potato Genomics resource of Michigan University (Hirsch *et al.*, 2014)2014 with a BLASTN algorithm, using AtSIZ as query. Two transcripts (PGSC0003DMT400020963 and PGSC0003DMT400020962; hereafter named 0963 and 0962, respectively) were found corresponding to the same locus in chromosome 11 (PGSC0003DMG400008114), with only transcript PGSC0003DMT400020962 considered to be the representative transcript for the locus (Hirsch *et al.*, 2014)2014. Transcript 0963 is 2396bp long, whereas the GenBank StSIZ1 transcript is 3293bp long. An alignment of the protein products for each transcript shows that the peptide encoded by transcript 0962 (StSIZ SpudDB) shares 98% identity to the StSIZ1 GenBank peptide and encompasses the C-terminal half of SIZ1 protein. These results suggest that StSIZ1 is encoded by a single gene residing on chromosome 11 of the DM potato genotype.

GpRbp-1 interacts with full length StSIZ1 *in planta*

To independently confirm the interaction *in planta*, we used epitope-based co-immunoprecipitation assays. We selected fragments StSIZ1frag14 and StSIZ1frag83 which share a 97% nucleotide identity, differing in ~20 SNPs and 19 nucleotides in length (Suppl. Table 1). StSIZ1frag06 shares 100% identity with StSIZ1frag14 and StSIZ1frag83 (Suppl. Table 1) and was therefore not used to confirm the interaction *in planta*. GpRbp-1 with an N-terminal Myc-GFP tag (Myc-GFP-Rbp1) was co-expressed with the N-terminally HA-tagged fragments StSIZ1frag14 and StSIZ1frag83 (HA-StSIZ1frag14,HA-StSIZ1frag83) by *Agrobacterium tumefaciens* infiltration in *N. benthamiana* leaves. HA-StSIZ1frag14 and HA-StSIZ1frag83 were specifically co-immunoprecipitated by Myc-GFP-Rbp1 and not by Myc-GFP (negative control) captured by magnetic anti-Myc beads (Fig. 1). Therefore, we concluded that GpRbp-1 interacts *in planta* with two fragments corresponding to a sub-region of the SP-RING finger domain of SIZ1 from potato. It is worth noting that after co-immunoprecipitation StSIZ1frag14 and StSIZ1frag83 were detected on western blots as bands migrating approximately 100 KDa higher than the respective bands for the input. This suggests that a complex comprising other peptides may be pulled-down by GpRbp-1.

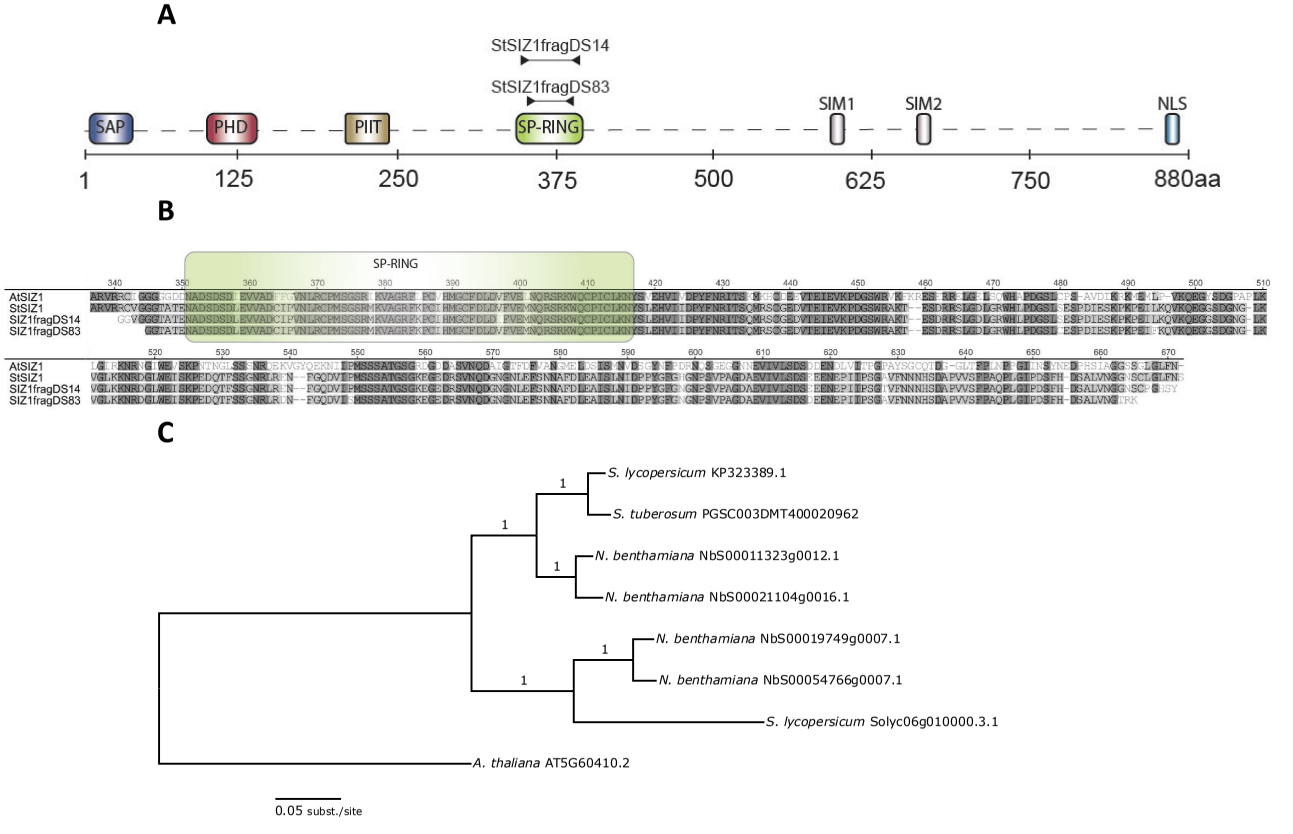


Figure 1. GpRbp-1 interacts with fragments encoding the SP-RING domain of StSIZ1. A) Schematic representation of the domain structure of E3-SUMO ligase SIZ1 from *A. thaliana* (AtSIZ1), indicating the SAP, PHD, PIIT, SP-RING, SIM1 and 2 (SUMO interacting) domains and NLS (Miura *et al.*, 2005; Rytz *et al.*, 2018). Arrows indicate the region where the fragments contained in the yeast clones align to AtSIZ1. **B)** Protein alignment of AtSIZ1(TAIR AT5G60410.2), StSIZ1 (XP_006340142.1) and GpRbp-1 interacting fragments (StSIZ1frag14 and StSIZ1frag83). The alignment was generated using a ClustalW algorithm in Geneious (Geneious version 8.1.9) **C**) (Phylogenetic) tree of StSIZ1 (GenBank XM_015314510.1), predicted NbSIZ1 (Solgenomics Niben101Scf05710g03032.1, Niben101Scf05710g03032.1 and Niben101Scf07109g04008.1), SlSIZ1 (GenBank KP323389.1 and Solyc06g010000.3.1) and AtSIZ1 (TAIR AT5G60410.2). The scale indicates substitutions per site.

To confirm if GpRbp-1 also interacts with full-length SIZ1 from potato, we performed co-immunoprecipitation assays. First, full-length StSIZ1 was obtained by gene synthesis based on the predicted sequence for SIZ1 from potato transcript variant X2 (GenBank code XM_015314510.1 / SpudDb PGSC0003DMT400020962). N-terminally tagged GpRbp-1 (Myc-GFp-Rbp1) was co-expressed with full-length StSIZ1 with an HA-tag (HA-StSIZ1) in *N. benthamiana* leaves by agro-infiltration. Myc-GFP-Rbp1 captured by magnetic anti-Myc beads, co-immunoprecipitated HA-StSIZ1 (Fig. 2). A Myc-GFP (negative control) did not co-immunoprecipitate HA-StSIZ1, indicating a specific interaction of GpRbp-1 with StSIZ1. These results showed that GpRbp-1 was able to interact specifically with StSIZ1 *in planta*.

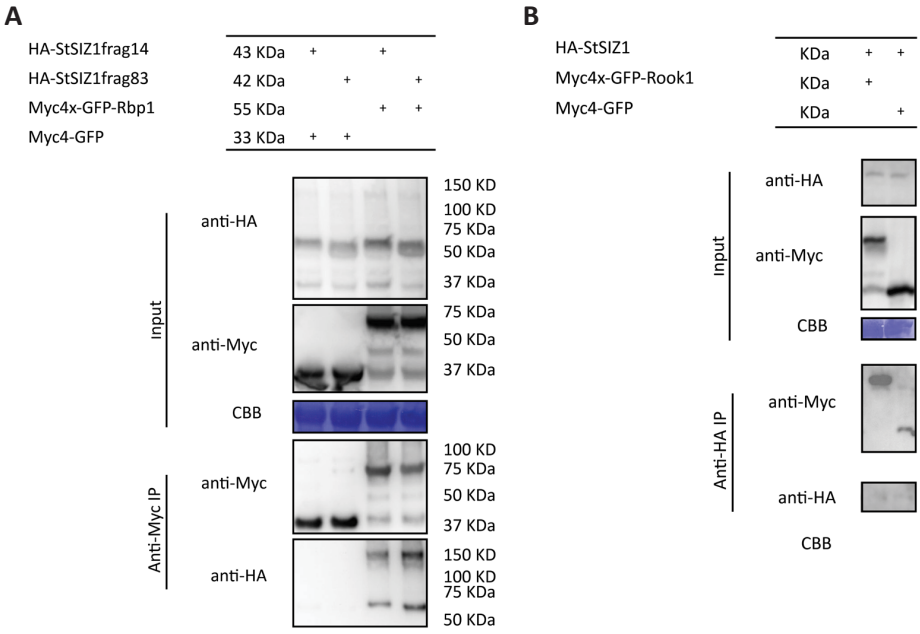


Figure 2. SIZ1 and GpRbp-1 interact *in planta*. A) Co-immunoprecipitation of epitope tagged StSIZ1 fragments (HA-StSIZ1frag14/83) and GpRbp-1 or (Myc4-GFP-Rbp1) or empty vector control (Myc4-GFP-EV). Pull-down of Myc-GFP-Rbp1 with anti-myc agarose beads results in a specific co-immunoprecipitation of SIZ1 fragments StSIZ1frag14 and StSIZ1frag83. Co-expressing proteins were extracted from *N. benthamiana* leaves 3 days after agroinfiltration. Results are representative of 3 biological repeats and all co-infiltrations contain the silencing suppressor p19. **B)** Co-immunoprecipitation of full-length HA-tagged StSIZ (HA-StSIZ1) as in **A).**

StSIZ1 and GpRpb-1 co-localize when expressed *in planta*

A C-terminal nuclear localization signal (NLS) was predicted in StSIZ1. Additionally, SIZ1 from Arabidopsis is exclusively located within the nucleus of cells (Miura, 2005). Therefore, we investigated the localization of StSIZ1 *in planta* by expressing N-terminally GFP-tagged StSIZ1 by transient transformation in *N. benthamiana* leaves. The localization of GFP-StSIZ1 followed a similar nuclear localization as previously reported for AtSIZ1 and the tomato homologue

SlSIZ1 (Fig. 3) (Lee *et al.*, 2006; Zhang *et al.*, 2017). Moreover, we observed that the GFP fluorescent signal was uneven throughout the nucleus, with stronger emission in discrete globules within the nucleus, which was also consistent with the localization reported for AtSIZ1. Additionally, StSIZ1 was co-expressed with GpRbp-1 to evaluate their subcellular localization *in vivo* by confocal laser scanning microscopy (CLSM). Co-transformed mCherry-labelled GpRpb-1 (mCh-Rbp1) and GFP-labelled StSIZ1 (GFP-StSIZ1) in *N. benthamiana* leaves were evaluated. When expressed in combination with free GFP, GpRbp-1 was consistently distributed between the nucleus and the cytoplasm as established previously (Jones *et al.*, 2009)2009. GFP-StSIZ1 localized to the nucleus with higher expression levels in defined nuclear foci when co-expressed with free mCherry. When co-expressed, GFP-StSIZ1 and mCh-Rbp1 co-localized to the nucleus of transformed cells, suggesting that an interaction occurs in the nucleus. Moreover, we concluded that the subcellular localization of GpRbp-1 or StSIZ1 was not altered upon co-infiltration and apparently, is not affected by their complex formation. The fluorescent tags were fused to the N-terminus of GpRbp-1 and StSIZ1 to simulate as closely as possible the configuration of the proteins in the interaction studies. There, the yeast binding domains or epitope tags were also fused to the N-terminus regions of the CDS of the interactors.

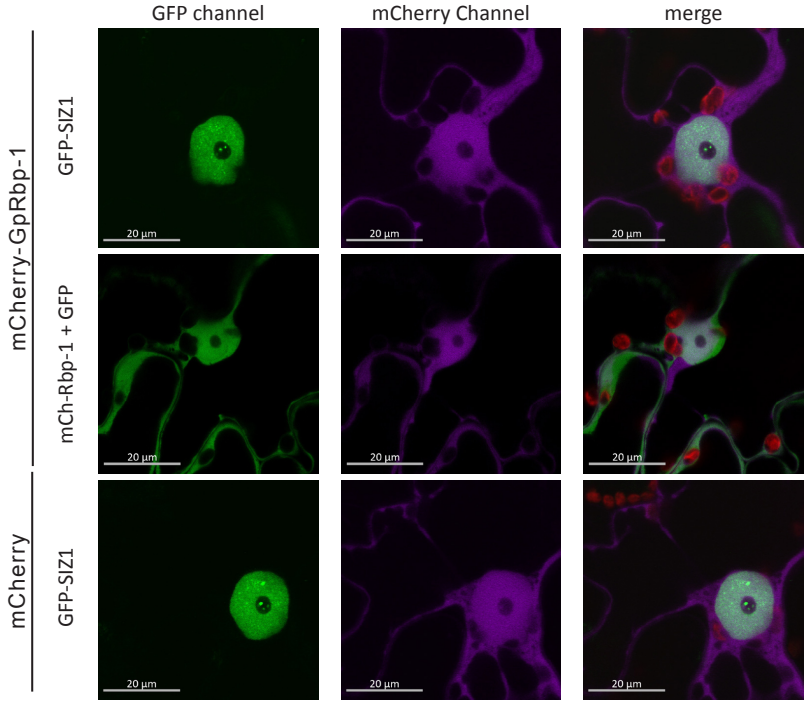


Figure 3. StSIZ1 and GpRpb-1 co-localize to the nucleus of *N. benthamiana* plants. Live imaging of *N. benthamiana* epidermal cells agro-infiltrated with constructs of mCherry tagged GpRbp-1 (mCh-Rbp1) and GFP tagged StSIZ1 (GFP-SIZ1) or GFP and mCh as negative controls. The GFP channel is shown in green and the mCherry in purple. Imaging was performed at 2dpi, images are representative of 3 biological repeats.

GpRbp-1 interacts with StSIZ1 and AtSIZ in the plant cell nucleus

To test if GpRbp-1 indeed interacts with StSIZ1 in the nuclear compartment we performed a bimolecular complementation assay (BiFC). For BiFC, the N-terminal half of the super cyan fluorescent protein SCF3A was fused to GpRbp-1 (pN:Rbp1) and the C-terminal half of SCFP3A was fused to StSIZ1 (pC:StSIZ1). The fluorescent fusions were transiently expressed in *N. benthamiana* by agroinfiltration. pN:Rbp1 was co-infiltrated with the viral protein NSs fused to the C-terminal half of protein SCFP3A (pC:NSs) and pC:StSIZ1 was co-infiltrated with β-glucuronidase fused to the N-terminus of SCFP3A (pN:GUS) as negative controls. The characteristic emission of SCFP3A was only reconstituted when pN:Rbp1 and pC:StSIZ1 were co-expressed. There was no reconstitution of the fluorescent signal of CFP when pN:Rbp1 was co-expressed with pC:NSs, neither by the co-expression of pC:StSIZ1 with pN:GUS. These findings confirmed that GpRbp-1 and full-length StSIZ1 interacted specifically *in planta* (Fig. 4; Suppl. Fig. 1). Interestingly, the fluorescent signal of CFP was only detected in the nucleus of transformed cells, confirming that GpRpb-1 and StSIZ1 only interact within this cellular compartment. Moreover, the observed granular fluorescent pattern suggests that the interaction between GpRpb-1 and StSIZ1 follows specific substructures within the nuclei.

Having confirmed that GpRbp-1 targets StSIZ1 in the nucleus, we wondered if GpRbp-1 was also able to interact with distant homologues of SIZ1 from plant species which were also infected by cyst nematodes like the model species Arabidopsis. To investigate if GpRbp-1 was able to interact with AtSIZ1, we performed similar BiFC assays. N-terminally tagged AtSIZ with the C-terminal half of SCF3A (pC:AtSIZ1) was transiently co-expressed with pN:Rbp-1 in leaves of *N. benthamiana*. The fluorescent signal characteristic of SCF3A was reconstituted when pN:Rbp-1 was co-expressed with pC:AtSIZ1, but not when co-expressed with the negative control pC:NSs or pN:GUS in case of pC:AtSIZ. The re-constituted signal indicating the interaction of pN:Rbp-1 and pC:AtSIZ1 was only visible in the nucleus of co-transformed cells. This shows that effector GpRbp-1 is also able to interact with AtSIZ1 *in planta* and that this interaction was limited to the nuclear cavity.

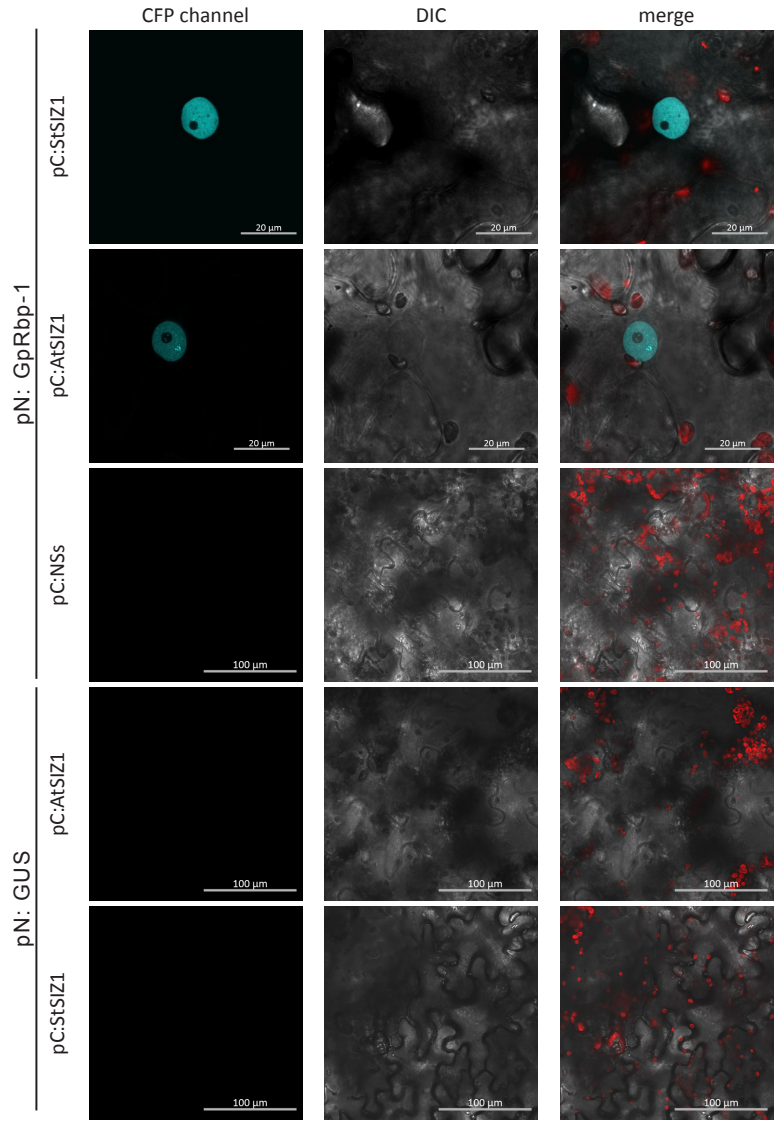


Figure 4. GpRbp-1 interacts with StSIZ and AtSIZ1 in the nucleus *in planta*. Bimolecular fluorescence complementation of N and C-terminal regions of SCFP3a. SCFP3A amino acids 1-173 were fused to GbRpb-1 (pN:Rbp1) and SCFP3A amino acids 156-239 were fused to StSIZ1(pC:StSIZ1) or AtSIZ1(pC:AtSIZ1) were co-infiltrated to *N. benthamiana* leaves. Co-expression of pN:EV or pC:EV were used as negative controls. The CFP emission channel is shown in blue, light emission in white in the differential interference contrast (DIC) channel and chloroplast auto-fluorescence is shown in red in the merge channel. Results are representative of 2 biological repeats and all co-infiltrations contain the silencing suppressor p19.

SUMO-E3 ligase SIZ1 is involved in cyst nematode infection of *A. thaliana*

Based on the selective interaction of GpRbp-1 with both StSIZ1 and AtSIZ1, the role of SIZ1 in nematode infection was further tested *A. thaliana* and the beet cyst nematode *Heterodera schachtii* as a model system. First, we investigated the expression of *AtSIZ1* during cyst nematode infection. We measured the expression of *AtSIZ1* by quantitative RT-PCR in whole-roots of *H. schachtii* or mock-inoculated plants (Columbia-0) at 2, 4, 10, and 14 days post-inoculation (dpi). No differential expression of the *AtSIZ1* transcript upon nematode infection was detected (Suppl. Fig. 4). Furthermore, we visualized the spatio-temporal expression of *AtSIZ1* in nematode-infected *A. thaliana* roots using a SIZ1prom:GFP-GUS construct (Ling *et al.*, 2012). We compared the pattern of GFP fluorescence in SIZ1prom:GFP-GUS plants infected with *H. schachtii* juveniles and in non-infected plants after 2 dpi. Again, the endogenous expression pattern described previously for uninfected SIZ1prom:GFP-GUS plants (Ling *et al.*, 2012) was not modified upon nematode infection (Suppl. Fig. 4). From these data, we concluded that the *AtSIZ1* gene expression is not differentially regulated during cyst nematode infection which is consistent with a regulatory role in post-translational modification of proteins required for nematode parasitism.

To investigate if SIZ1 was involved in nematode infection we challenged *in vitro*-grown *siz1-2* and *siz1-3* knockout *A. thaliana* mutants with *H. schachtii*. These mutant lines carry an independent homozygous T-DNA insertion at different sites of exon 16 of the *AtSIZ1* gene resulting in a knock-out of SIZ1 (Lee *et al.*, 2006; Miura *et al.*, 2005). The homozygosity of the T-DNA insertion was verified using PCR primers designed with the iSect tool from the SALK Institute Genomics Analysis Laboratory (Suppl. Table 6). To examine the importance of AtSIZ1, the total number of nematodes infecting the roots of the mutants and wild-type control (Col-0) were counted at 14 dpi. Furthermore, we discriminated between adult female and male nematodes, as this indicates the nutritional quality of the established infection sites (Anjam *et al.*, 2018; Trudgill, 1967). We observed a significant decrease of 36% and 38% (one-way ANOVA, $p<0.001$ and $p=0.006$, respectively) in the total number of nematodes infecting the roots of *siz1-2* and *siz1-3* mutants as compared to the wild-type plants, respectively (Fig. 6; Suppl. Fig. 5). In addition, the number of female nematodes present in the roots of *siz1-2* and *siz1-3* was reduced by 49% and 33% in the mutants as compared to wild-type plants (one-way ANOVA, $p<0.001$ and $p=0.086$, respectively). A similar effect was observed for the number of males, where decreases of 29% and 41% in *siz1-2* and *siz1-3* plants as compared to the wild-type were found (one-way ANOVA, $p<0.001$ and $p=0.004$, respectively) (Suppl. Fig. 5). Under *in vitro* growth conditions we did not observe an aberrant growth phenotype of the roots in the *siz1-2* or *siz1-3* seedlings, which was consistent with previous reports (Castro *et al.*, 2015; Catala *et al.*, 2007; Miura *et al.*, 2011). Hence, the reduction in susceptibility could be attributed to the *siz1-2* and *siz1-3* mutation and not to differences in the mutant root systems. Therefore, we concluded that SIZ1 plays a role in the susceptibility of *Arabidopsis* to infection by cyst nematodes.

Additional evidence was obtained by investigating the effect of SIZ1 on the size of the feeding site and growth of female nematodes infecting the roots of Arabidopsis. To this end, we measured the surface area of syncytia and females visible in the roots of *siz1-2, siz1-3*,

and wild-type Arabidopsis after two weeks of infection. The size of the syncytia induced by *H. schachtii* in *siz1-2* and *siz1-3* plants was 20% and 7% smaller, respectively, as compared to the wild-type (one-way ANOVA, p<0.001 and p=0.096, respectively) (Fig. 5). The size of the females established in siz1-2 and siz1-3 plants was not significantly different as compared to those developing on wild-type Arabidopsis plants (one-way ANOVA, p=0.335 and p=0.976) (Fig. 5). Together, these results suggest that SIZ1 may not only contribute to the control of the overall infection rate, but might also be required for the expansion of the permanent feeding sites of cyst nematodes in Arabidopsis.

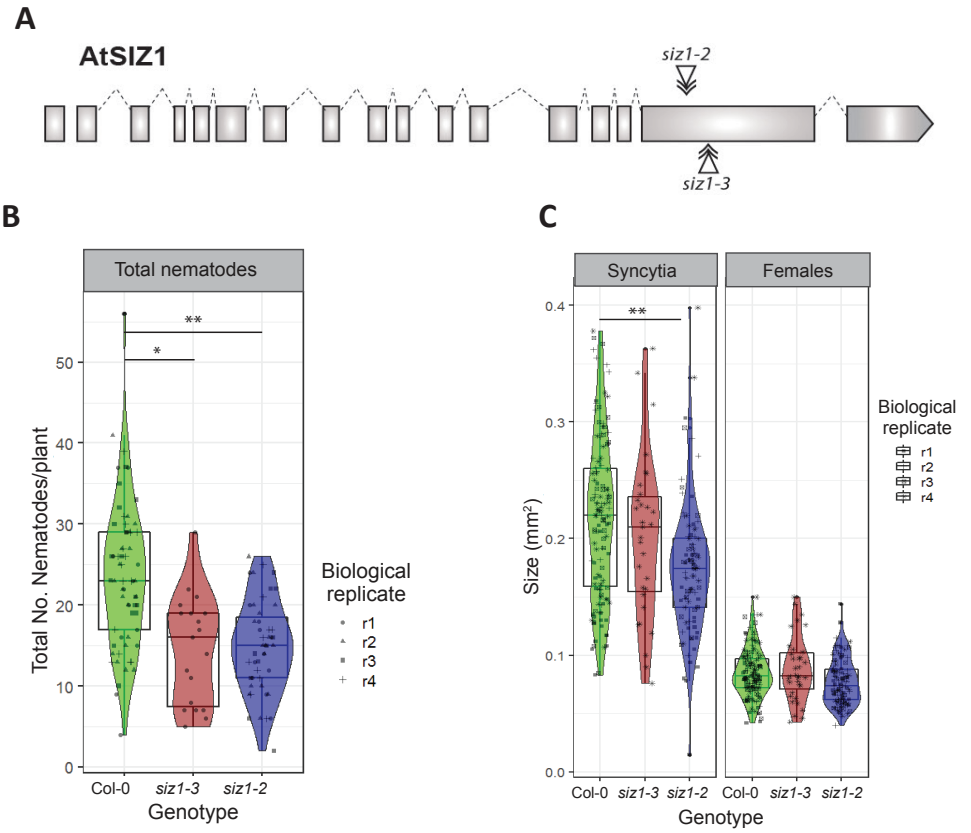


Figure 5. Knock-down of SIZ1 reduces nematode infection in *A. thaliana*. A) Representation of T-DNA insertions in lines *siz1-2* and *siz1-3* in AtSIZ1. B) Average number of nematodes per plant in the roots of *Arabidopsis thaliana siz1-2* (n=47), *siz1-3* (n=19) and the background and wild-type Columbia 0 (Col-0; n=65) at 14dpi. C) Average surface area (mm2) of female nematodes and syncytia present in the roots of *siz1-2* (n=94) and *siz1-3* (n=35) and Col-0 (n= 128) after 2 weeks of infection. Whiskers indicate the quartile (25 or 75%)-/+ 1.5x interquartile range. and violin plots describe the distribution of all data points. Stars indicate a significant statistical difference as determined by a one-way ANOVA, (**p<0.001, *p=0.006). Results are combined measurements from 4 independent biological repeats, using a fixed effects model.

AtSIZ1 may contribute to plant defence to cyst nematodes

To further understand the role of SIZ1 during nematode interactions we performed a whole transcriptome analysis of *siz1-2* and wild-type Arabidopsis roots infected with beet cyst nematodes. To uncouple the effects of a mutated genotype and infection, we isolated whole root RNA from mock-inoculated and cyst nematode inoculated plants for both *siz1-2* and the wild-type plants, 7 days after inoculation (n = 3 replicates for each sample). We observed the overall expression of 13,114 genes in all 12 samples, and principal component analysis (PCA) showed a clear distinction between the *siz1-2* mutant and wild-type (first principal component, 27.1% of variance), and non-infected versus *H. schachtii* infected (second principal component, 13.4% of variance) (Fig. 6) as expected. Interestingly, the non-infected *siz1-2* and infected *siz1-2* samples cluster closer together on the 2nd principal component axis than the non-infected and infected wild-type. This clustering indicates that *siz1-2* plants show less difference upon infection, the impact of the cyst nematodes on the transcriptome is smaller in the *SIZ1* mutant than in the wild-type (Fig. 6). The infection-like transcriptional profile of *siz1-2* is likely reflecting the auto-immune phenotype previously reported for *siz1*-2 and the role of *SIZ1* as negative regulator of SA-mediated stress responses (Lee *et al.*, 2006).

To uncover which genes contributed to the separation of the samples in the PCA, we used a linear model to find 171 genes contributing to the difference between *siz1-2* and wild-type, 29 genes between non-infected and *H. schachtii* infected plants, and 9 genes between *siz1-2* and wild-type upon infection (interaction) (linear model, $p < 0.0001$; $FDR_{genotype} = 0.011$, $FDR_{infection} = 0.064$, $FDR_{interaction} = 0.131$; Fig. 6; Suppl. Table 3; Suppl. Table 4). To examine the hypothesis that nematodes manipulate a specific molecular pathway through SIZ1, we evaluated the gene ontology (GO) annotations of the 9 genes that are differentially regulated by the combination of mutant genotype and infection (Suppl. Table 5). Genes involved in control of the cell cycle (e.g. CYC B2;2), defence (e.g. BAP2) and protein transport (e.g. SLY1) were found in this differentially regulated group. Nevertheless, the small number of genes affected by the combination of the *siz1-2* mutation and the nematode infection (interaction) limits further interpretation of the molecular processes or pathways that nematodes may manipulate through SIZ1. In addition, the strong transcriptional differences between wild-type and *siz1-2* plants prior to infection, supported the notion that a mutation in SIZ1 induces drastic changes in the plant, even more so than infection with cyst nematodes.

To understand further the specific genes that were affected by the *siz1-2* mutation, we performed a gene-enrichment analysis. Thirty gene ontology (GO) terms are significantly upregulated in the *siz1-2* mutant, including “located in cell wall”, “involved in abiotic or biotic stimulus”, “cellular response to ethylene stimulus”, and “functions in carbohydrate binding” (Suppl. Table 5). In contrast, seven gene ontology categories are significantly upregulated in the wild-type, including “functions in sequence-specific DNA binding”, “located in extracellular region”, “involved in response to cold”, and “functions in sequence-specific DNA binding” (Suppl. Table 5). It is worth noting that the GO term most significantly upregulated in *siz1-2* plants is “involved in cellular response to ethylene stimulus”. Likewise, the GO term most significantly upregulated in wild-type plants is “functions in sequence-specific DNA binding” (Suppl. Table 5). These findings further support the existence of an “infection-like”

transcriptional state of the *siz1-2* plants as detected with the PCA analysis due to constitutive activation of defence pathways as previously reported.

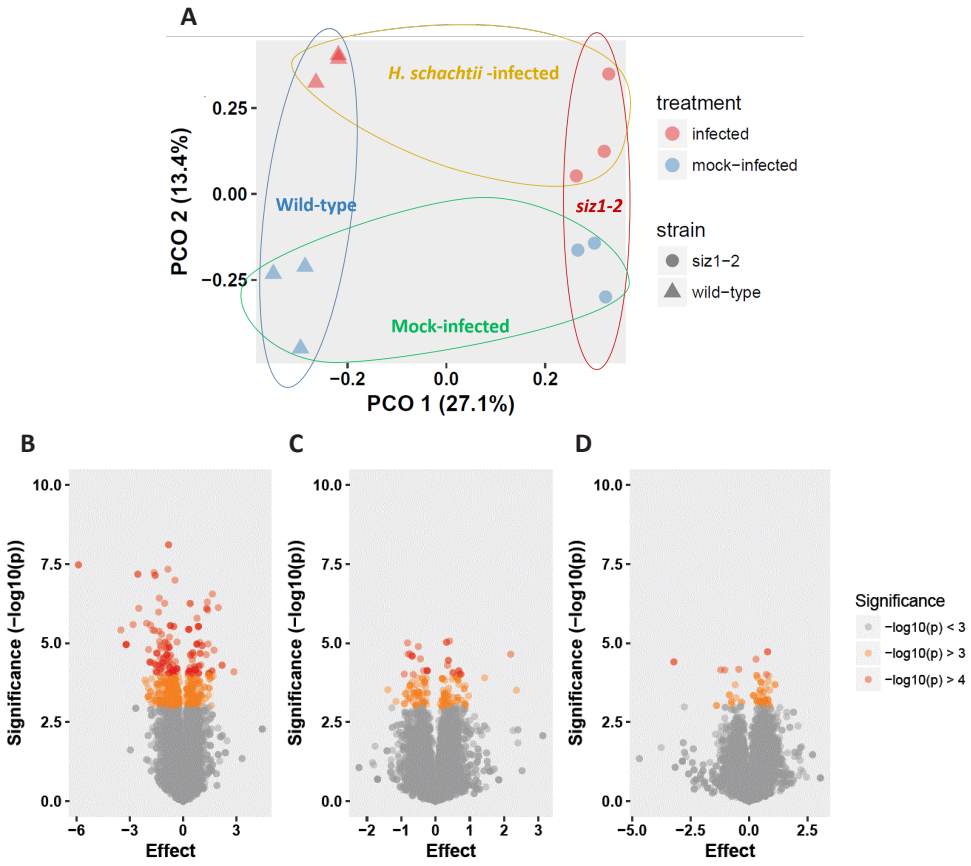


Figure 6. The mutation in *siz1-2* plants has a stronger effect in gene expression than nematode infection. A) Principal component analysis of gene expression profiles of *siz1-2* and wild-type Arabidopsis 7 days after (mock) infection with cyst nematodes. The first principal component PCO 1 captures 27.1% of the variation and separates the Arabidopsis seedlings by genotype. The second principal components PCO 2 captures 13% of variation and separates infected from uninfected samples. **B-D)** Volcano plots of differential gene expression as determined by total RNA RNAseq. The x-axis (effect) shows the relative expression of genes. The values on the y-axis reflect the significance of the differences in expression levels. The colours provide a visual aid for the thresholds mentioned in the legend. **B)** Genes differentially regulated in roots of *siz1-2* and wild-type Arabidopsis, irrespective of infection. **C)** Genes differentially regulated by nematode infection, irrespective of genotype. **D)** Genes differentially regulated by infection with *H. schachtii* in roots of *siz1-2* vs wild-type *Arabidopsis* plants, 7 days after inoculation.

Discussion

GpRbp-1 is an effector secreted by the potato cyst nematode *G. pallida* during the onset of parasitism, presumably to promote nematode virulence. To characterise the virulence role of this effector we aimed to identify the host proteins targeted by GpRbp-1. We found that GpRbp-1 interacts specifically in yeast and *in planta* with the potato homologue of the SUMO E3 ligase SIZ1. Furthermore, evidence from live cell imaging indicates that this interaction was limited to the nucleus of the cell, where GpRbp-1 co-localizes with StSIZ1. In addition, we evaluated the role of SIZ1 during nematode parasitism in the Arabidopsis- *H. schachtii* model system. *In vitro* infection studies in the Arabidopsis *siz1-2* and *siz1-3* mutants show that SIZ1 contributes to infection by cyst nematodes presumably as a negative regulator of plant defence. These results suggest that GpRbp-1 may target SIZ1 to repress plant immunity. This is therefore the first description of a pathogen effector that targets master regulator SIZ1.

Likewise, the involvement of SUMOylation in plant-nematode interactions had not been described previously to the best of our knowledge. In contrast, SUMOylation has been shown to play a role in virulence of other plant pathogens. For instance, proteins from the SUMO machinery are transcriptionally regulated during infection by *Phytophthora infestans* in potato (Colignon *et al.*, 2017). Also, interaction of replication protein AL1 from the geminivirus Tomato Golden Mosaic Virus and the SUMO E2-conjugating enzyme SCE1, is required for viral infection in *N. benthamiana* (Castillo *et al.*, 2004; Sanchez-Duran *et al.*, 2011). Furthermore, the effector XopD from *Xanthomonas euvesicatoria* has SUMO-protease activity, and catalyses the removal of SUMO from the tomato transcription factor SlERF4 to supress ethylene-mediated immune responses (Kim *et al.*, 2013).

The reduced susceptibility of *A. thaliana* mutants *siz1-2* and *siz1-3* to cyst nematode infection (Fig. 5) likely reflects the role of SIZ1 as a negative regulator of plant immunity (Gou *et al.*, 2017; Hammoudi *et al.*, 2018; Lee *et al.*, 2006; Niu *et al.*, 2019). The *Arabidopsis siz1-2* mutant is characterized by a dwarf phenotype associated to increased levels of salicylic acid (SA) (Lee *et al.*, 2006). This increased SA production in *siz1-2* is also associated to an upregulation of pathogenesis-related (PR) genes such as *PR1* and *PR5* (Lee *et al.*, 2006). Additionally, the mutation in *siz1-2* confers resistance to the bacterial pathogen *Pseudomonas syringae* p.v *tomato (Pst)* (Lee *et al.*, 2006). In contrast, the susceptibility of *siz1-2* to the fungal pathogen *Botrytis cinerea* is comparable to that of the wild-type (Lee *et al.*, 2006). Therefore, Lee and co-workers (Lee *et al.*, 2006) proposed that SIZ1 regulates immunity mediated by SA to biotrophic pathogens like *P. syringae,* independent of the jasmonic acid (JA) signalling pathway induced by the necrotrophic pathogen *B. cinerea*. Interestingly, cyst nematodes are also biotrophic pathogens, which trigger local and systemic SA-mediated plant defence responses upon root invasion (Kammerhofer *et al.*, 2015; Lin *et al.*, 2013; Nguyen *et al.*, 2016; Wubben *et al.*, 2008; Youssef *et al.*, 2013). Together, these results suggest that SIZ1 may be required for basal resistance to biotrophic pathogens with different modes of infection above-and belowground.

In line with the auto-immune phenotype of the *siz1-2* mutant (Lee *et al.*, 2006), our gene enrichment analysis shows that stress-related genes are differentially regulated in the

siz1-2 mutant in the absence of nematode infection (Fig. 6; Suppl. Table 4). It should be noted that we did not find elevated levels of *PR-1* or *PR-5* SA-responsive genes in the roots of *siz1-2* or *siz1-3* Arabidopsis. This observation may be due to the differences in growth conditions that may repress accumulation of SA or specific events in the SA-responsive pathway, or to different expression patterns of *PR* genes in roots and shoots. In addition, very few genes were differentially regulated in response to the infection of the mutant, suggesting that nematode infection has a relatively minor effect on transcriptional regulation of plant roots, as compared to the mutation alone (Fig 6; Suppl. Table 4). Therefore, our RNA-Seq data supports the hypothesis that the auto-immune phenotype of the *siz1-2* mutant underlies the mutant's reduced susceptibility to infection by cyst nematodes. From this, a model can be inferred in which the immune-repressive function of SIZ1 in SA-mediated defence responses is enhanced by nematode effectors leading to an increase in the susceptibility of host plants to cyst nematode infections.

In plants, a zinc-finger motif within the SP/RING domain is largely responsible for the nuclear localization of AtSIZ1 as well as the regulatory role of SA accumulation and SA-dependent phenotypes (e.g. dwarfism, resistance to pathogens and thermotolerance) (Cheong *et al.*, 2009). Interestingly, GpRbp1 interacts with a protein fragment comprising this domain of SIZ1, suggesting that it may affect the SUMO E3 ligase activity of SIZ1. Furthermore, the nuclear co-localization and interaction of GpRbp-1 and StSIZ1 seems to follow specific substructures within the nucleus (Figs. 3 and 4). This may point at targeting of StSIZ1 by GpRbp-1 to modify or modulate the nuclear activity of StSIZ. In yeast, the RING domain is necessary to recruit the E2-SUMO complex into a complex with its substrate (Yunus and Lima, 2009). Hence, targeting of the SP-RING finger domain of SIZ1 by GpRbp-1 most likely compromises these SA-related phenotypes. In turn, the characteristic hypervariable region of GpRbp-1 may function as a binding platform to facilitate the targeting of SIZ1 (Diaz-Granados *et al.*, 2016; Rehman *et al.*, 2009).

Additionally, the gene ontology "involved in cellular response to ethylene stimulus" was the enriched category with the highest statistical support. From the differentially regulated genes, LEC1 (lectin-like protein; AT3G15356), FRD3 (Ferric reductase defective 3; AT3G08040), and RBK1 (ROP binding protein kinase; AT5G10520) are grouped in the "response to ethylene stimulus" ontology (Suppl. Table 4). LEC1 has been shown to be transcriptionally regulated in response to several stimuli, including the fungal elicitor chitin and mechanical wounding. The response of LEC1 to chitin is also found in ethylene/jasmonate (ET/JA)-insensitive mutants, suggesting that LEC1 is involved in ET/JA-dependent and independent cellular responses (Seoung Hyun *et al.*, 2009). This finding could indicate that, opposite to previous hypotheses (Lee, 2006), SIZ1 may be involved in regulation of the JA defence pathway through modulation of the JA/ET branch (Pieterse *et al.*, 2012). Moreover, in plant-nematode interactions ethylene can act as a modulator of SA-immunity or as a regulator or cytokinin-dependent susceptibility, and these roles are determined by the activation of specific ethylene receptors (Piya *et al.*, 2019). Therefore, it remains to be determined if SIZ1 acts solely as a regulator of SA-mediated plant immunity in plant nematode interactions.

SIZ1 may also act as regulator of hormone-dependent metabolic processes that influence susceptibility to nematodes. The decrease in the size of the specialised feeding sites (syncytia) induced by nematodes in *siz1-2* plants points to a role of SIZ1 in expansion of nematode-induced feeding sites (Fig. 5). Additionally, the genes differentially regulated by the *siz1-2* mutation in combination with cyst nematode infection may have further roles as regulators of feeding site formation. For example, in addition to ethylene responsiveness, FRD3 is also involved in nutrient homeostasis and iron uptake (Xing *et al.*, 2015). And in turn, RBK1 has been shown to be regulated by pathogen infection (Molendijk *et al.*, 2008), but also to be implicated in auxin-mediated cell expansion (Enders *et al.*, 2017). Both nutrient uptake and cell expansion processes are relevant in the context of nematode feeding sites (reviewed in (Kyndt *et al.*, 2013)). Finally, SIZ1 represses the characteristic root morphological adaptations to phosphate starvation, through the control of auxin patterning (Miura *et al.*, 2011; Miura *et al.*, 2005). Auxin transport and signalling are involved in the proper formation of syncytia, presumably by its role as regulator of plant organogenesis (reviewed in (Gheysen & Mitchum, 2019; Ng *et al.*, 2015)). These findings illustrate how the regulatory network of SIZ1 becomes intricate, with effects on different plant hormones that are implicated in immune responses as well as in cellular modifications related to nematode infection. Ultimately, elucidating the mechanism of action of SIZ1 would require the identification of the molecular targets that are regulated by SIZ1 in plant-nematode interactions. Nevertheless, around 600 proteins are predicted as potential SIZ1-dependent SUMO targets in plants (Rytz *et al.*, 2018). Consequently, a definite mode of action of SIZ1 in plant-nematode interactions will require further dissection through a combination of genetic, biochemical and *in vivo* assays.

Preliminary data also provide evidence for a complementary hypothesis where GpRbp-1 may modulate host cellular processes by recruiting the SUMO machinery of the host. Here, we could show that GpRbp-1 interacts in BiFC with other components of the SUMO machinery, namely SUMO1, 3 and 5 (SUM1, SUM3, SUM5) and the E2 conjugating enzyme SCE1 (Supp. Fig. 6). Interestingly, GpRbp-1 interacts with the SUMOs and SCE1 in the nucleus and cytoplasm, where these proteins localize (Suppl. Fig. 6) (Mazur *et al.*, 2019; Xiong & Wang, 2013). In yeast and mammals, multi-protein complexes including SUMO, E2 (Ubc9), and E3s (PIAS/Nup358) (Mascle *et al.*, 2013; Reverter & Lima, 2005) are required for SUMOylation and the ensuing transcriptional regulation activities of UBC9 and SUMO1 (Mascle *et al.*, 2013; Reverter & Lima, 2005). Similarly, in *Arabidopsis* SUMO, SCE1 and SIZ1 form a ternary complex that is recruited to nuclear bodies (NBs) where COP1 is SUMOylated to regulate the response of the plant to darkness and temperature (i.e. skoto- and thermo-morphogenesis) (Kim *et al.*, 2016; Lin *et al.*, 2016; Mazur *et al.*, 2019; Osterlund *et al.*, 2000; Seo *et al.*, 2003; Yang *et al.*, 2005). Conceivably, the virulence role of GpRbp-1 may be exerted through an influence on the SUMO-SCE1-SIZ1 tertiary complex by stabilisation, for example. In support of this notion, our co-immunoprecipitation assays suggest that intermediate compounds present in a larger complex with StSIZ1frag14 and StSIZ1frag83 are co-pulled down specifically by GpRbp-1 (Fig. 2). The nature of the complex co-pulled down along with StSIZfrag14 and StSIZ1frag83 by GpRbp-1 the remains to be established.

An alternative explanation is that GpRbp-1-like effectors may recruit the SUMO complex

to achieve SUMOylation inside the host cells for full functionality as an effector. Conceivably the SUMOylation of GpRbp-1-like effectors may enhance their stability by competing with ubiquitination (Zheng *et al.*, 2018) or modify their binding patterns (Guo & Sun, 2017; Hansen *et al.*, 2017). This hypothesis is supported by the prediction of consensus SUMO-acceptor (ψKxE) and SUMO-interaction motifs (SIM) in GpRbp-1 (Suppl. Fig. 7), indicating that host-mediated SUMOylation may be relevant for its functioning as an effector. Two SIM are predicted in the N-terminal half of GpRbp-1, in one region that is unique to GpRbp-1 and another present in several Rbp and SPRYSEC sequences (Diaz-Granados *et al.*, 2016). The first SIM, unique to GpRbp-1 falls within a region with low confidence for modelling, so it is difficult to predict in what region of the GpRbp-1 structure it is located. The second SIM (SIM2) localizes to a β-sheet present in the core of the β-sandwich structure that is predicted for GpRbp-1 (Suppl. Fig. 7). In addition, two ψKxE SUMOylation sites are predicted, one inverted in the conserved core of the SPRY domain (ψKxE_inverted) and one in the C-terminus in a motif present only in GpRbp-1 and Rbp-1 from *G. mexicana* (ψKxE_2) (Diaz-Granados *et al.*, 2016) (Suppl. Fig. 7). The "conserved" SIM2 and (ψKxE inverted) sites reside in the β-sheet core of GpRbp-1, and most likely form a binding pocket for SUMO, whereas the unique ψKxE_2 site resides in a C-terminal α-loop and is likely exposed to the solvent (Suppl. Fig. 7). None of these motifs contain residues reported to be under positive selection in *G. pallida* field populations (Carpentier *et al.*, 2012). In this scenario, the lack of SIZ1 in *siz1-2 Arabidopsis* impedes an efficient functioning of GpRbp-1 homologues from *H. schachtii* as a virulence factor. Although the functional homolog of GpRbp-1 of *H. schachtii* is not known, we assume that similar proteins may exist based on the existence of GpRbp-1-like gene transcripts (Fosu-Nyarko *et al.*, 2016) which may exert a similar function in plant parasitism. Moreover, different cyst nematode species share the same mode of parasitism, which results in the formation of typical feeding structures. Therefore, we expect that the underlying molecular mechanisms are conserved among host plant species. This is supported by our observation that GpRbp-1 can also interact with AtSIZ1 (Fig. 4), suggesting that SIZ1 may be a conserved target of cyst nematodes.

Materials and methods

Yeast two-hybrid – library screen

Library preparation was performed as described in Chapter 2. Briefly, A prey library was generated by Dual Systems Biotech (Switzerland) from ground roots of potato SH infected with juveniles of *G. pallida* population Pa3-Rookmaker. Poly (A) tailing, total RNA isolation, cDNA library construction and yeast-two hybrid screening were performed by Dual Systems Biotech.

Cloning

Cloning for co-immunoprecipitation and microscopy was carried out as described in Chapter 2. Briefly, for co-immunoprecipitation, interacting fragments StSIZ1fragDS14, StSIZ1fragDS83

and GpRpb-1 version 1 from virulent population Rookmaker (Rook1) were fused to the respective epitope tags and transferred by restriction enzyme cloning to the pBINPLUS binary vector (van Engelen *et al.*, 1995). For microscopy studies, the full-length gene of StSIZ1 was obtained by synthetic gene synthesis (GeneArt) (Thermo Fisher Scientific, Waltham, Massachusetts) and cloned to appropriate pGWB vectors (Nakagawa *et al.*, 2007) by gateway cloning. The mCherry GpRbp-1 construct was generated by restriction cloning in pBINPLUS (van Engelen *et al.*, 1995). BiFC constructs were generated by gateway cloning to the pDEST-SCYCE(R)^{GW} and pDEST-SCYNE(R)^{GW} vectors (Gehl *et al.*, 2009).

Expression and detection of recombinant proteins

All proteins were co-expressed by Agrobaterium-mediated transient transformation of Nicotiana benthamina leaves. All co-expressions are done together with the silencing supressor P19, with a final concentration of OD600=0.5. Total protein extracts were prepared by grinding leaf material in protein extraction buffer. For co-IP, pull-downs were performed using µMACS anti-c-MYC or anti-HA paramagnetic beads (Miltenyi, Bergisch Gladbach, Germany). Proteins were separated by SDS-PAGE on NuPage 12% Bis-Tris gels (Invitrogen, Carlsbad, California) and blotted to 0.45µm polyvinylidene difluoride membrane (Thermo Fisher Scientific). Immunodection was performed with corresponding horseradish peroxidase-conjugated antibodies. Confocal microscopy was performed on N. benthamiana epidermal cells using a Zeiss LSM 510 confocal microscope (Carl-Zeiss, Oberkochen, Germany) with a 40X, 1.2 numerical aperture water-corrected objective.

Confocal microscopy

Confocal microscopy was performed as described in Chapter 2. Briefly, *N. benthamiana* epidermal cells were examined using a Zeiss LSM 510 confocal microscope (Carl-Zeiss) with a 40X 1.2 numerical aperture water-corrected objective. For co-localization studies the argon laser was used to excite at 488 nm for GFP and chlorophyll, and the HeNe laser at 543nm to excite mCherry. GFP and chlorophyll emission were detected through a band-pass filter of 505 to 530nm and through a 650nm long-pass filter, respectively. mCherry emission was detected through a band-pass filter of 600 to 650nm. For BiFC the argon laser was used to excite at 458 nm for SCFP3A. SCFP3A emission was detected through a band-pass filter of 470nm to 500nm and chlorophyll emission was detected through a 615nm long-pass filter. We also used a CFP marker to calibrate the fluorescence excitation and emission for CFP.

Plant material and nematode infection

Seeds of the homozygous *siz1*-2 were kindly provided by Dr. H. van den Burg (Laboratory for Phytopathology, University of Amsterdam, the Netherlands). Col-0 N60000 wildtype seeds were obtained from the SALK homozygote T-DNA collection (Alonso *et al.*, 2003). For nematode infection, seeds were vapour sterilized and sown in modified KNOP medium (Sijmons *et al.*, 1991) and grown at 25oC under a 16-h-light/8-h-dark cycle. 10 day-old seedlings were inoculated with 60-70 surface-sterilized *H. schachtii* infective juveniles. After

2 weeks of infection, the amount of nematodes present in the roots of Arabidopsis plants were counted visually and the size of females and syncytia were determined as described previously (Siddique *et al.*, 2014). Statistical differences were estimated by one-way ANOVA (α=0,05), using the weighted-inverse variants to combine data from 4 biological replicates.

RNA sequencing

Total RNA extraction

Total RNA extraction was performed as described in Chapter 2. Briefly, seeds of *siz1-2* and Col-0 wild-type (N60000) were vapour sterilized and sown in modified Knop's medium (Sijmons *et al.*, 1991) in 6-well cell culture plates (Greiner bio-one). Seedlings were grown at 21°C under a 16-h/8-h light/dark regime. Two-week old seedlings were infected with approximately 180 surface-sterilized *H. schachtii* juveniles. One week after inoculation the complete root systems of ~18 *siz1-2* and Col-0 plantlets were harvested and snap-frozen. Root tissue was ground in liquid nitrogen and total RNA was extracted with the Maxwell® 16 LEV plant RNA kit (Promega) in the Maxwell 16 AS2000 instrument (Promega), following the manufacturer's instructions. Three biological replicates of ~18 plants/sample per condition were generated.

Count derivation and normalization

Quality checking, removal of adapter sequences, genome mapping and count derivation was performed by a custom-written pipeline. Briefly, read quality was assessed using FASTQC v0.11.5 (Andrews, 2014). Overrepresented adapter sequences, base pairs with a Q-value lower than fifteen in the 5' or 3' and reads shorter than 20bp were removed with cutadapt v1.16 (Martin, 2011). Reads were then mapped to the *A. thaliana* genome TAIR10 with Hisat v2.1.0v (Cheng *et al.*, 2017; Kim *et al.*, 2015);. In all samples, well above 85% of the reads mapped to Arabidopsis (Suppl. Table 2). Obtained SAM files were sorted and converted to BAM files with the help of samtools v1.6 (Li, 2011; Li *et al.*, 2009). From these files FPKM counts of mapped sequences were derived by StringTie (Pertea *et al.*, 2015)2015.

The FPKM-transformed counts were imported in "R" (version 3.4.2, x64) and $C_{i,j} = \log_2\left(FPKM_{i,j}+1\right)$, were $\log_2$ transformed by where C is the $\log_2$-transformed FPKM value for gene i (one of 37217 unique transcripts) of sample j (one of three replicates of wild-type mock infected, wild-type infected, *siz1-2* mock infected, or *siz1-2* infected).

Subsequently, we selected only transcripts that were detected in all 12 samples (C > 0) for further analysis (representing 13114 unique genes). For principal component analysis, we also transformed to data to a $\log_2$-ratio with the mean, by $R_{i,j} = \log_2\left(\frac{FPKM_{i,j}}{\overline{FPKM_i}}\right)$ where R is the $\log_2$-ratio with the mean of transcript i of sample j, and $\overline{\mathrm{FPKM_i}}$ is the mean of the FPKM values for gene i.

Thereafter, both C and R values were batch-corrected by subtracting the mean difference of the batch from the total mean, as follows

$$C_{B,i,j} = C_{i,j} - (\overline{C}_{batch,i} - \overline{C}_{total,i})$$

$$R_{B,i,j} = R_{i,j} - \left(\overline{R}_{batch,i} - \overline{R}_{total,i}\right)$$

where C_B and R_B are the batch-corrected values of gene *i* of sample *j* and $\overline{C}_{batch}$ and $\overline{R}_{batch}$ are the batch averages, and $\overline{C}_{total}$ and $\overline{R}_{total}$ are the averages over the total.

Differential expression analysis

To understand the contributing factors underlying variance in the gene expression data, we first used a principal component analysis on R_B to understand the major sources of variance. Thereto we used the *prcomp* function in "R".

We then applied a linear model to identify genes contributing to the genotype differences, the differences between mock-infection and infection, and the interaction between both variables, by solving $C_{B,i,j} = G_j + T_j + G_j \times T_j + e_j$ where C_B of gene *i* of sample *j* was explained over genotype (G; either wild-type or *siz1-2*) and treatment (T; either mock-infected or infected), the interaction between G and T, and an error-term (*e*). The significances were used to calculate a false discovery rate (FDR) using the *p.adjust* function in "R" (Benjamini & Hochberg, 1995). To make explanatory terms comparable, we applied a single significance threshold of $p < 0.0001$, which corresponded to a FDR of 0.011 for genotype, 0.064 for treatment, and 0.131 for the interaction between genotype and treatment.

The differentially expressed genes ($p < 0.0001$) were used in an enrichment analysis, as described before (Warmerdam *et al.*, 2019). In short, enrichments were calculated by hypergeometric test, using the TAIR11 databases: Gene ontology, Gene ontology slim, gene classes, and phenotypes (Berardini *et al.*, 2015; Lamesch *et al.*, 2012), and the MapMan gene ontology database, based on TAIR10 (Thimm *et al.*, 2004)We filtered groups were fewer than three genes overlapped, and selected significant enriched groups based on a correction for multiple testing (FDR).

Phylogenetic tree

Sequences were aligned in BioEdit v.7.2.6 (Hall, 1999) and a Bayesian tree was created using MrBayes v.3.2.26 (Ronquist *et al.*, 2012). The data set was partitioned according to codon position and the analysis was run for 500,000 generations with a GTR + invariable sites + gamma substitution model using 4 MCMC chains and 4 parallel runs. After checking for conversion with Tracer v.1.7.1 (Rambaut *et al.*, 2018), the burnin was set to 5,000 generations.

GpRbp-1 modelling

The model of GpRbp-1 was built by remote homology modelling, using a similar workflow as previously described (Rehman *et al.*, 2009). Briefly, an initial sequence analysis was performed by identifying specific sequence patterns signatures using InterProScan (Jones *et al.*, 2014) and ScanProSite (De Castro *et al.*, 2006). Consensus profiles for structural feature predictions were obtained using various methods, namely Jpred4 (Drozdetskiy *et al.*, 2015), RaptorX-Property (Wang *et al.*, 2016), SCRATCH (Cheng *et al.*, 2005), PsiPred (Buchan & Jones, 2019)

and Spider3 (Heffernan *et al.*, 2017) (i.e. secondary structure, intrinsically disorder regions and relative solvent accessibility predictions).

The 3D model of GpRbp-1 sequence was built within the interval (aa 61-246) starting from the closest homologues with available crystal structures- namely the IUS-SPRY domain of human RanBP9 (Ran Binding Protein 9, PDB 5JI7) (Hong *et al.*, 2016), mouse RanBP10 (Ran Binding Protein 10, PDB 5JIA) (Hong *et al.*, 2016), and the SPRY domain of human SPRYD3 (SPRY Domain-Containing Protein 3, PDB 2YYO) (Kishishita *et al.*), sharing 31.5%, 30.9% and 20.8% sequence identity respectively with GpRbp-1. The N-terminus region (aa 1 - 60), including the first extended PRY motif was not modeled, as no 3D templates with adequate homology were detected. The model was built using Modeller v9.20 (Webb & Sali, 2014) and further refined by iterative rounds local and global simulated annealing and energy minimization monitored with MolProbity (Williams *et al.*, 2018) until convergence to a Molprobity quality score of 1.11Å from an optimal polypeptide path. The optimized model was further subjected to a 20 ns long molecular dynamics simulation for stability test. Molecular dynamics, simulated annealing and energy minimization stages were performed in explicit solvent in NAMD v2.12 (Phillips *et al.*, 2005) using the CHARMM36M forcefield (Huang *et al.*, 2017), TIP3 water molecules model and a fixed 0.15 M NaCl concentration, with the overall system size summing up to 27050 atoms (from which 2863 atoms correspond to GpRbp-1).

MD simulations were performed at 300 K constant temperature, using a 2 fs timestep, Particle Mesh Ewald full-system periodic electrostatics and periodic boundary conditions, Langevin temperature control and Nosé-Hoover Langevin piston for a constant 1 bar pressure control, as implemented in NAMD v2.12 (Phillips *et al.*, 2005). The stability of the model was investigated by analyzing the potential energy, RMSD (root mean square deviations) and RMSF (root mean square fluctuations) along the simulation trajectory. All trajectory analyses and predictive model figures were obtained using VMD (Humphrey *et al.*, 1996) and PyMOL v2.2.3 (DeLano, 2002).

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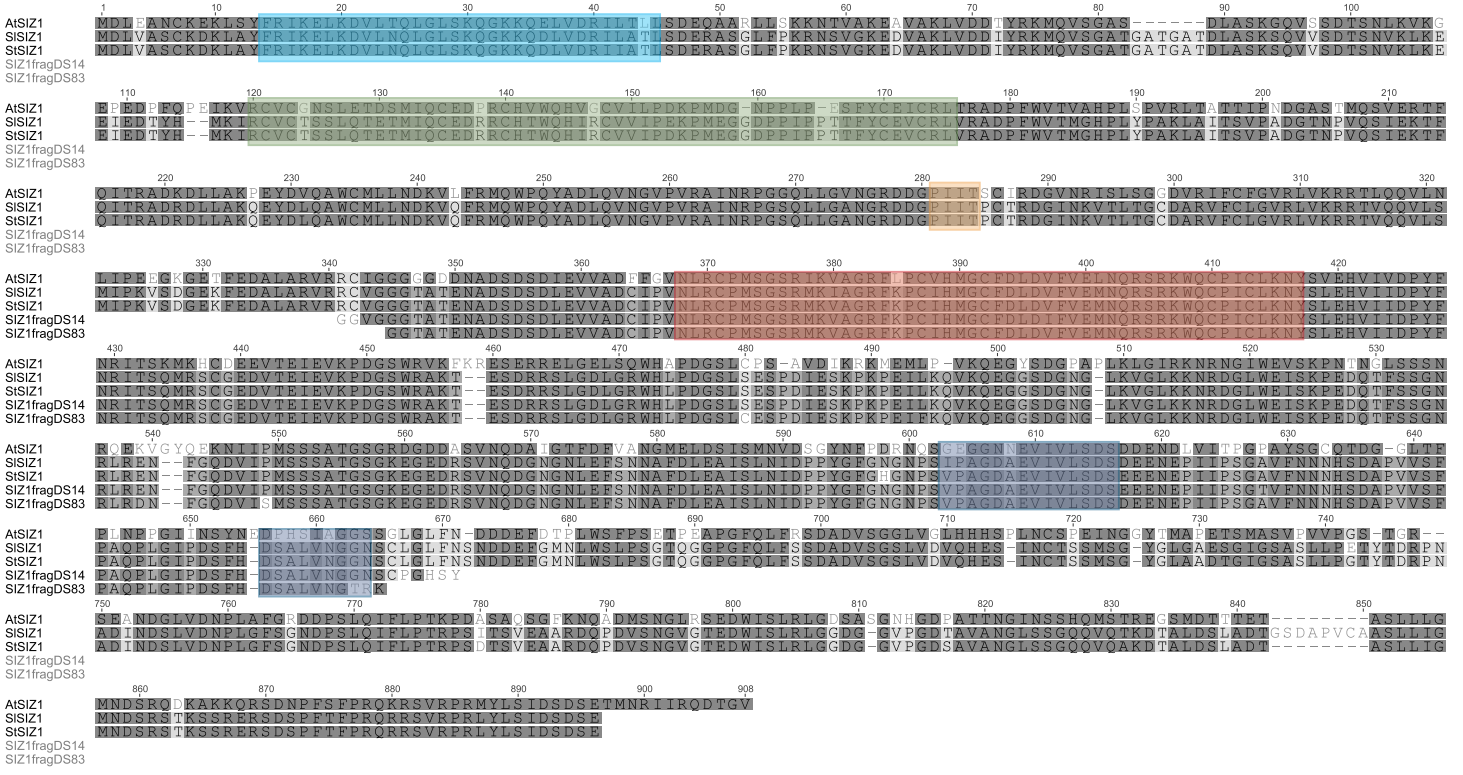
Supplemental information

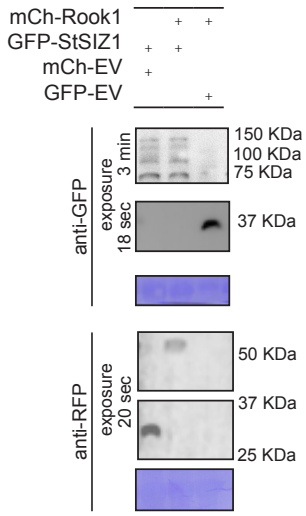
Supplemental table 1. cDNA sequence of StSIZ1 fragments interacting with GpRbp-1 in yeast.

Prey clone	*BLASTX - description*	*BLASTX - eValue*	*Sequence*
06	E3 SUMO-protein ligase SIZ1, Arabidopsis thaliana	8.936E-95	GGGGGGATGAAAGTTGCTGGAAGATTCAAACCTTGTATACACATGGGCTGCTTTGATCTTGACGTCTTTGTTGAAATGAATC AAAGGTCGAGGAAGTGGCAATGCCCTATCTGTCTTAAGAACTACTCTTTGGAGCATGTCATCATAGATCCATATTTCAATCGA ATTACTTCTCAGATGCGTAGTTGTGGAGAAGATGTCACAGAAATCGAAGTGAAACCTGATGGTTCTTGGCGCGCGAAGACA GAATCTGATCGAAGGAGTCTTGGAGATCTTGGACGTTGGCATTTACCTGATGGGTCTCTGTGTGAATCTCCGGATATAGAGT CGAAGCCTAAGCCTGAAATTTTTAAGCAGGTTAAACAGGAAGGAGGCTCTGATGGAAATGGCCTTAAAGTTGGATTGAAGA AGAACAGAGACGGATTGTGGGAAATCAGCAAACCTGAAGACCAGACATTTTCATCTGGAAATAGATTGAGAGATAATTTTG GTCAGGATGTCATTCCAATGAGCAGCAGTGCCACTGGGAGTGGCAAAGAAGGTGAAGATCGCAGTGTTAATCAGGATGGT AATGGGAACCTTGAATTCTCGAACAATGCATTTGATCTTGAGGCCATCTCTCTTAATATTGACCCACCATATGGTTTTGGCAAC GGAAATCCATCGGTACCAGCANGAGATGCTGAAGTTATTGTACTAAGTGATTCTGATGAA
10	E3 SUMO-protein ligase SIZ1, Arabidopsis thaliana	1.057E-107	GGGGGTGTTGGTGGTGGGACAGCCACTGAAAATGCTGATAGCGATAGTGATCTGGAAGTTGTTGCTGATTGTATTCCTGTC AATCTTCGCTGCCCTATGAGTGGTTCAAGGATGAAAGTTGCTGGAAGATTCAAACCTTGTATACACATGGGCTGCTTTGATC TTGACATCTTTGTTGAAATGAATCAAAGGTCGAGGAAGTGGCAATGCCCTATCTGTCTTAAGAACTACTCTTTGGAGCATGT CATCATAGATCCATATTTCAATCGAATTACTTCTCAGATGCGTAGTTGTGGAGAAGATGTCACAGAAATTGAAGTGAAACCTGA TGGTTCTTGGCGCGCGAAGACAGAATCTGATCGAAGGAGTCTTGGAGATCTTGGACGTTGGCATTTACCTGATGGGTCTCTG AGTGAATCTCCAGATATAGAGTCGAAGCCTAAGCCTGAAATTCTTAAGCAGGTTAAACAGGAAGGAGGCTCTGATGGTAATG GCCTTAAAGTTGGATTGAAGAAGAACAGAGACGGATTGTGGGAAATCAGCAAACCTGAAGACCAGACATTTTCATCTGGA AATAGATTGAGAGAAAATTTTGGTCAGGATGTCATTCCAATGAGCAGCAGTGCCACTGGGAGTGGCAAAGAAGGTGAAGA TCGCAGTGTTAATCAGGATGGTAATGGGAACCTTGAATTCTCGAACAATGCATTTGATCTTGAGGCCATCTCTCTTAATATTGA CCCACCATATGGTTTTGGCAACGGAAATCCGTCNGTACCAGCAGAGATGCTGAAGTTATTGTACTAAGTGATTCTGAAGAAGA NAATGAGCCAATTATCCCATCTGGNACTGTCTTTAATAACANCATAGTGATGCACCTGTAGTTTCNTTTTNCAGCTCAAC
14	E3 SUMO-protein ligase SIZ1, Arabidopsis thaliana	4.2979E-97	GGGGGTGTTGGTGGTGGGACAGCCACTGAAAATGCTGATAGCGATAGTGATCTGGAAGTTGTTGCTGATTGTATTCCTGTCA ATCTTCGCTGCCCTATGAGTGGTTCAAGGATGAAAGTTGCTGGAAGATTCAAACCTTGTATACACATGGGCTGCTTTGATCTT GACATCTTTGTTGAAATGAATCAAAGGTCGAGGAAGTGGCAATGCCCTATCTGTCTTAAGAACTACTCTTTGGAGCATGTCAT CATAGATCCATATTTCAATCGAATTACTTCTCAGATGCGTAGTTGTGGAGAAGATGTCACAGAAATTGAAGTGAAACCTGATGG TTCTTGGCGCGCGAAGACAGAATCTGATCGAAGGAGTCTTGGAGATCTTGGACGTTGGCATTTACCTGATGGGTCTCTGAGT GAATCTCCAGATATAGAGTCGAAGCCTAAGCCTGAAATTCTTAAGCAGGTTAAACAGGAAGGAGGCTCTGATGGTAATGGCC TTAAAGTTGGATTGAAGAAGAACAGAGACGGATTGTGGGAAATCAGCAAACCTGAAGACCAGACATTTTCATCTGGAAATA GATTGAGAGAAAATTTTGGTCAGGATGTCATTCCAATGAGCAGCAGTGCCACTGGGAGTGGCAAAGAANGTGAAGATCGC AGTGTTAATCA

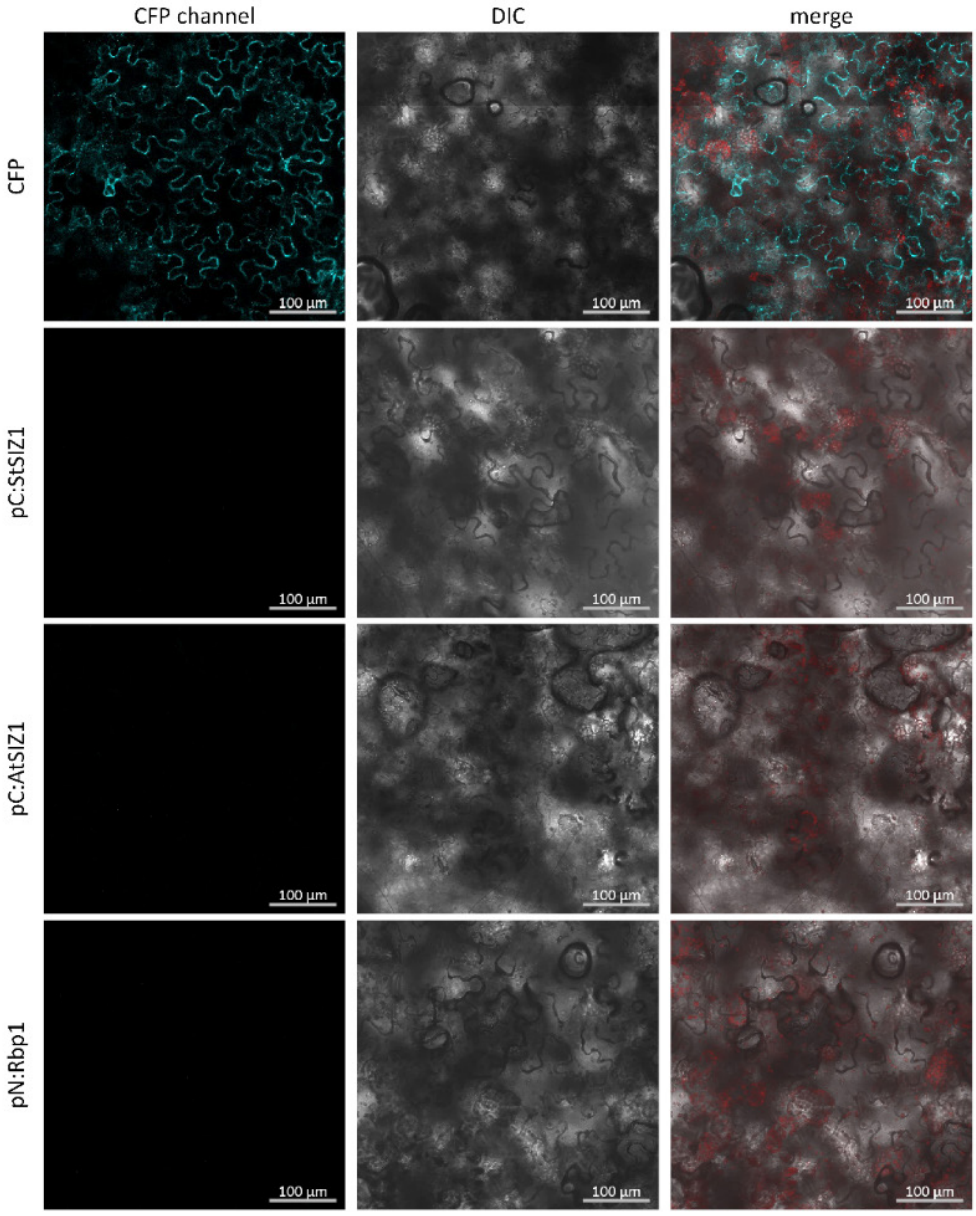
Prey clone	*BLASTX - description*	*BLASTX - eValue*	*Sequence*
49	E3 SUMO-protein ligase SIZ1, Arabidopsis thaliana	9.617E-116	GGGGGGACAGCCACTGAAAATGCTGATAGCGATAGTGATCTGGAAGTTGTTGCTGATTGTATTCCTGTCAATCTTCGCTGCC CTATGAGTGGTTCAAGGATGAAAGTTGCTGGAAGATTCAAACCTTGTATACACATGGGCTGCTTTGATCTTGACGTCTTTGTT GAAATGAATCAAAGGTCGAGGAAGTGGCAATGCCCTATCTGTCTTAAGAACTACTCTTTGGAGCATGTCATCATAGATCCATA TTTCAATCGAATTACTTCTCAGATGCGTAGTTGTGGAGAAGATGTCACAGAAATCGAAGTGAAACCTGATGGTTCTTGGCG CGCGAAGACAGAATCTGATCGAAGGAGTCTTGGAGATCTTGGACGTTGGCATTTACCTGATGGGTCTCTGTGTGAATCTCCG GATATAGAGTCGAAGCCTAAGCCTGAAATTTTTAAGCAGGTTAAACAGGAAGGAGGCTCTGATGGAAATGGCCTTAAAGTT GGATTGAAGAAGAACAGAGACGGATTGTGGGAAATCAGCAAACCTGAAGACCAGACATTTTCATCTGGAAATAGATTGAGA GATAATTTTGGTCAGGATGTCATTTCAATGAGCAGCAGTGCCACTGGGAGTGGCAAAGAAGGTGAAGATCGCAGTGTTAATC AGGATGGTAATGGGAACCTTGAATTCTCGAACAATGCATTTGATCTTGAGGCCATCTCTCTTAATATTGACCCACCATATGGTTT TGGCAACGGAAATCCATCGGTACCAGCAGGAGATGCTGAAGTTATTGTACTAAGTGATTCTGATGAAGAGAATGAGCCAATTA TCCCATCTGGAGC
83	E3 SUMO-protein ligase SIZ1, Arabidopsis thaliana	3.941E-106	GGGGGGACAGCCACTGAAAATGCTGATAGCGATAGTGATCTGGAAGTTGTTGCTGATTGTATTCCTGTCAATCTTCGCTGCCC TATGAGTGGTTCAAGGATGAAAGTTGCTGGAAGATTCAAACCTTGTATACACATGGGCTGCTTTGATCTTGACGTCTTTGTTG AAATGAATCAAAGGTCGAGGAAGTGGCAATGCCCTATCTGTCTTAAGAACTACTCTTTGGAGCATGTCATCATAGATCCATATT TCAATCGAATTACTTCTCAGATGCGTAGTTGTGGAGAAGATGTCACAGAAATCGAAGTGAAACCTGATGGTTCTTGGCGCGC GAAGACAGAATCTGATCGAAGGAGTCTTGGAGATCTTGGACGTTGGCATTTACCTGATGGGTCTCTGTGTGAATCTCCGGAT ATAGAGTCGAAGCCTAAGCCTGAAATTTTTAAGCAGGTTAAACAGGAAGGAGGCTCTGATGGAAATGGCCTTAAAGTTGGAT TGAAGAAGAACAGAGACGGATTGTGGGAAATCAGCAAACCTGAAGACCAGACATTTTCATCTGGAAATAGATTGAGAGATA ATTTTGGTCAGGATGTCATTTCAATGAGCAGCAGTGCCACTGGGAGTGGCAAAGAAGGTGAAGATCGCAGTGTTAATCAGG ATGGTAATGGGAACCTTGAATTCTCGAACAATGCATTTGATCTTGAGGCCATCTCTCTTAATATTGACCCACCATATGGTTTTGG CAACGGAAATCCATCGGTACCAGCNGNGATGCTGAAGTTATTGTACTAAGTGATTCTGATGAAGAGAATGAGCCAATTATCCC ATCTGGAGCTG



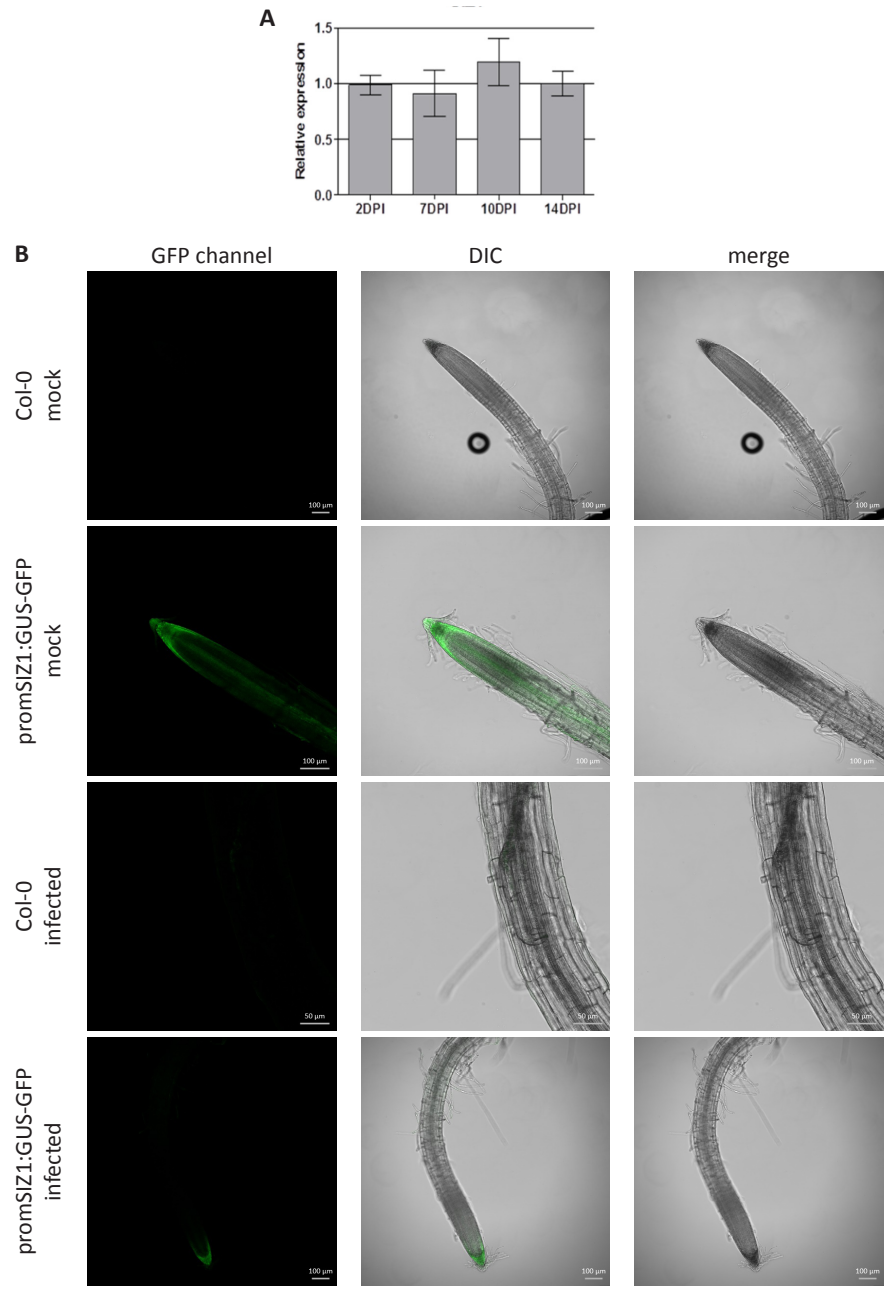
Supplemental figure 1. Full-length alignment of StSIZ1, AtSIZ1, SlSIZ1, StSIZ1frag14 and StSIZfrag83. Protein alignment made with the CLUSTALW plugin of Geneious (version 8.1.9) with cost matrix BLOSUM. The characteristic domains of SIZ1 are shown in colored boxes: the SAP domain in light blue, the PHD domain in green, the PIIT (PINIT) motif in yellow, the SP-RING domain in red and the SIMs in dark blue. Full-lenght proteins were obtained from TAIR, AtSIZ (1009128193) or GenBank StSIZ1 (XP_006340142.1) and SlSIZ1 (KP323389.1). The protein sequences encoded by the yeast-interacting fragments were obtained by automatic translation.



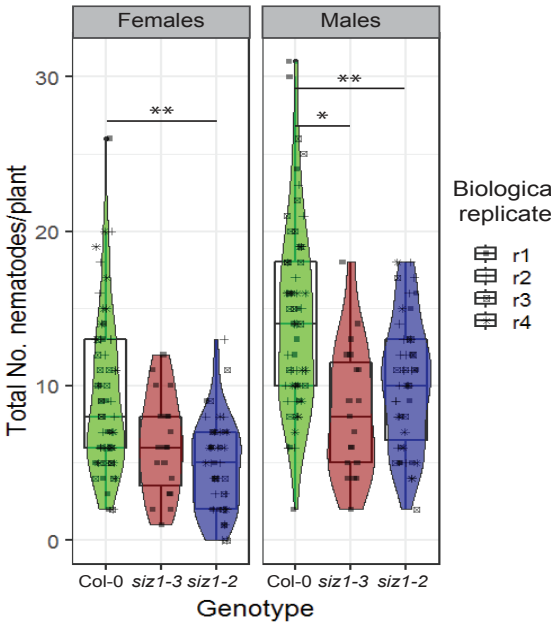
Supplemental figure 2. Fluorescent fusions of GpRbp-1 and SIZ1 are expressed in leaves of *N. benthamiana*. Western blot detection of fusion of green fluorescent protein with StSIZ1 (GFP-SIZ1), red fluorescent protein mCherry (mCh-GpRbp-1) or GFP and mCh alone. Western blot is performed with anti-GFP and anti-RFP antibodies.



Supplemental figure 3. Individual Bimolecular fluorescence complementation constructs do not emit fluorescence when infiltrated individually. Individual N-SCFP3 (pN) or C-SCFP3 (pC) fusions to StSIZ1, GpRbp-1, and. A CFP transformation is shown for comparison of the confocal microscopy settings.



Supplemental figure 4. SIZ1 gene expression during nematode infection of Arabidopsis and potato roots. A) AtSIZ1 expression as quantified by RT-PCR. Relative expression of AtSIZ1 is calculated relative to the geometric mean of reference genes UBP22 (Hofmann & Grundler, 2007) and UBQ5 (Anwer *et al.*, 2018). Error bars indicate the standard error of 3 independent biological replicates. **B)** Visualization of SIZ expression of SIZ1 in the roots of *H. schachtii*-infected *Arabidopsis* using a promSIZ1:GFP-GUS fusion with GFP channel shown in green. Pictures are taken after 2 days of infection.



Supplemental figure 5. Less *H. schachtii* males and females infect the roots of *siz1-2* and *siz1-3 Arabidopsis*. Total amount of females and male nematodes present in the roots of *siz1-2*, *siz1-3* and wild-type (Columbia-0) *Arabidopsis*, after 2 weeks of infection. Whiskers indicate the maximum and minimum data points and violin plots describe the distribution of all data points. Results are combined measurements from 4 independent biological repeats. n_{Col-0}= 65 , n_{siz1-2}=47 and n_{siz1-3}=19. Stars indicate statistical significance of the differences in the amount or size of nematodes infecting the roots of *siz1* lines and the wild-type control, established by one-way ANOVA, (α=0,05).

Supplemental table 2. HiSat mapping of RNAseq reads to Arabidopsis genome.

Number	*Aligned reads (%)*	*Multiple aligned reads (%)*	*Non-aligned reads (%)*	*Sample name*
0	92.76	3.89	3.35	1v2
1	87.83	3.86	8.3	2v2
2	93.33	3.77	2.9	3v2
3	83.68	3.4	12.92	4v2
4	89.92	5.13	4.95	5v2
5	82.08	3.28	14.64	6v2
6	92.32	3.95	3.73	7v2
7	86.23	3.22	10.55	8v2
8	90.44	5.52	4.03	9v2
9	93.66	3.63	2.71	11v2
10	85.71	3.29	11	12v2
11	87.56	4.23	8.21	10v2

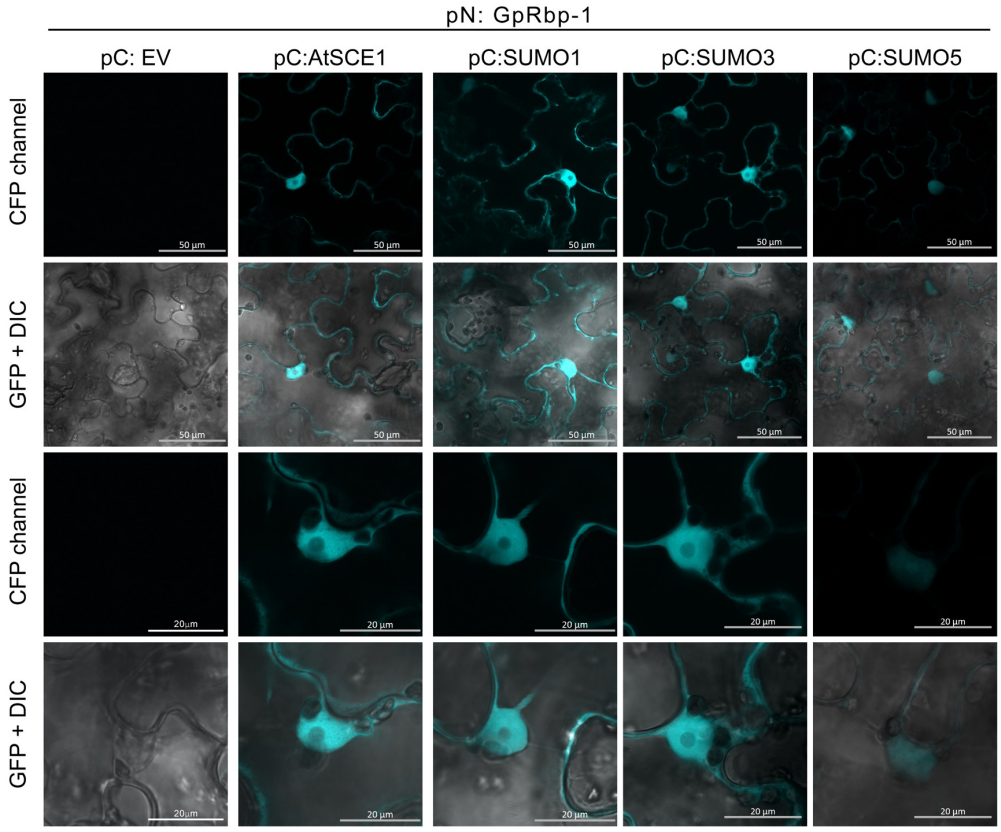
Supplemental table 3. A list of all the genes significantly regulated in *siz1-2* plants. geneID indicates the TAIR ID for each entry; the test column indicates which comparison was made; the significance column gives the significance of the difference as determined by the linear model (with FDR correction?) , the effect column shows the size of the difference in gene expression (log2-units; negative values are lower expressed in col-0, positive values higher expressed in col-0). The significance_FDR column lists the q-values as determined by Benjamini-Hochberg correction. The columns thereafter list properties of the genes. Available at; https://drive.google.com/open?id=1YktRjxXNRkLoFI3ZKyjasnUZU-bZ9g28

Supplemental table 4. Genes significantly regulated by nematode infection and the mutation in *siz1-2* (interaction). The gene, significance FDR and effect are obtained from Supplemental Table 2. The Gene Symbol, Gene full-name and associated GO annotations were obtained from ThaleMine (Krishnakumar *el al.*, 2014).

Gene ID	*Significance FDR*	*Effect*	*Gene symbol*	*Gene full name*	*Biological process*	*Molecular function*	*Cellular compartment*
AT3G26550	0.13113341	0.74965		Cysteine/ Histidine-rich C1 domain family protein	no terms	zinc ion binding	nucleus
AT2G45760	0.11601649	-1.03261	BAP2	BON association protein 2	defense response	no terms	membrane
AT3G05936	0.11601649	-1.206		hypothetical protein			
AT4G35620	0.11601649	-0.43799	CYCB2;2	Cyclin B2;2	cell division, regulation of cell cycle, cyclin-dependent protein serine/ threonine kinase regulator, ell cycle	protein binding, cyclin-dependent protein serine/ threonine kinase regulator activity	nucleus
AT5G40530	0.11601649	-3.21531		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	rRNA processing, transcription DNA-templated, methylation, covalent chromatin modification, methyltransferase activity	methyltransferase activity	nucleus
AT5G40530	0.11601649	-3.21531		Idem	Idem	Idem	Idem
AT2G17980	0.11601649	0.30137	ATSLY1	Sec1/munc18-like (SM) proteins superfamily	protein secretion, protein transport, vesicle docking suring exocytosis, protein transporter activity	protein transporter	cytosol
AT5G56590	0.11601649	0.79666		O-Glycosyl hydrolases family 17 protein	cell wall organization, defense response	glucan endo-1,3-beta-D-glucosidase activity	anchored component of membrane, plasma membrane, extracellular region
AT5G56590	0.11601649	0.79666		idem	idem	idem	idem

Supplemental table 5. Gene Ontology categories enriched in genes differentially regulated either by the mutation in *siz*1-2 Arabidopsis or by infection with *H. schachtii*.

Term	*Direction*	*Annotation*	*Ontology*	*FDR*	*Overlap*	*Overlap expected*	*Genes in group*
Genotype	upregulated in siz1-2	Gene_ontology	located in intracellular membrane-bounded organelle	0.004339	3	0.10608	86
Genotype	upregulated in siz1-2	Gene_ontology	located in vacuole	0.004565	7	0.47981	389
Genotype	upregulated in siz1-2	Gene_ontology	involved in ethylene-activated signaling pathway	0.007004	3	0.12581	102
Genotype	upregulated in siz1-2	Gene_ontology	involved in defense response to bacterium	0.007272	4	0.21092	171
Genotype	upregulated in siz1-2	Gene_ontology	involved in defense response	0.013878	5	0.36263	294
Genotype	upregulated in siz1-2	Gene_ontology	has oxidoreductase activity	0.02262	3	0.19118	155
Genotype	upregulated in siz1-2	Gene_ontology	located in cytosol	0.025492	12	1.45053	1176
Genotype	upregulated in siz1-2	Gene_ontology	involved in response to water deprivation	0.028329	3	0.20722	168
Genotype	upregulated in siz1-2	Gene_ontology	has transporter activity	0.028841	3	0.20845	169
Genotype	upregulated in siz1-2	Gene_ontology	located in apoplast	0.030334	4	0.32316	262
Genotype	upregulated in siz1-2	Gene_ontology	involved in signal transduction	0.033703	3	0.21955	178
Genotype	upregulated in siz1-2	Gene_ontology	functions in zinc ion binding	0.038892	4	0.34783	282
Genotype	upregulated in wild-type	Gene_ontology_slim	extracellular	0.004362	9	0.73792	1032
Genotype	upregulated in wild-type	Gene_ontology_slim	unknown molecular functions	0.024265	13	1.66318	2326
Genotype	upregulated in wild-type	Gene_ontology	functions in sequence-specific DNA binding	0.003638	3	0.09939	139
Genotype	upregulated in wild-type	Gene_ontology	located in extracellular region	0.004785	8	0.61922	866
Genotype	upregulated in wild-type	Gene_ontology	located in membrane	0.007419	8	0.67357	942
Genotype	upregulated in wild-type	Gene_ontology	involved in response to cold	0.010087	3	0.14444	202
Genotype	upregulated in wild-type	Gene_ontology	has molecular_function	0.024225	13	1.66175	2324
Infection	upregulated in infection	Gene_ontology_slim	other binding	0.03602	5	0.56116	3488
Infection	upregulated in infection	Gene_ontology	has protein binding	0.0144	3	0.18325	1139



Supplemental figure 6. Gp-Rbp1 interacts with other members of the SUMO machinery. Bimolecular fluorescence complementation of N and C-terminal regions of SCFP3a. SCFP3A amino acids 1-173 were fused to GbRpb-1 (pN:Gp-Rbp1) and SCFP3A amino acids 156-239 were fused to SUMO1,3,5 or SCE1 (e.g. pC:AtSUM1). The corresponding pN/pC pairs were co-infiltrated to *N. benthamiana* leaves. Co-expression of pN:EV or pC:EV were used as negative controls. The CFP emission channel is shown in blue, light emission in white in the differential interference contrast channel and chloroplast auto-fluorescence is shown in red in the merge channel. Results are representative of 2 biological repeats and all co-infiltrations contain the silencing suppressor P19.

A

Results for putatifs SUMO site

Position K	Sequence	Best PS	Consensus direct			Consensus Inverted		
			Type	PSd	DB Hit	Type	PSi	DB Hit
K11	MESPKPNKKVKGSSSSGNAEP	None	None	None		None	None	2
K108	KESGIFYYEVKISAITASVSI	High	None	None		Strong consensus inv	High	
K123	TASVSIGLATKEMPLDKFVGY	None	None	None		None	None	1
K164	CSHLNKRPFIKVPKFGEGDVV	None	None	None	1	None	None	1
K240	PKSADVIEKLKNENLGS----	Low	Strong Consensus	Low	2	None	None	

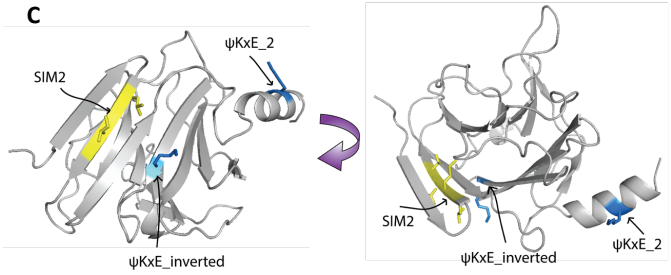
Results for putatifs SIM [PSmax=38.183 | Cut-off=0.000]

Position site	Sequence	Type	a/S stretch	PS	DB Hit
AA 49-52	TLSETERRLMIVEYTKADWA	SIM Type 2	[V][SIM][N]	0.000	
AA 74-77	TLSETERRLMIVEYTKADWG	SIM Type 2	[V][SIM][N]	0.000	

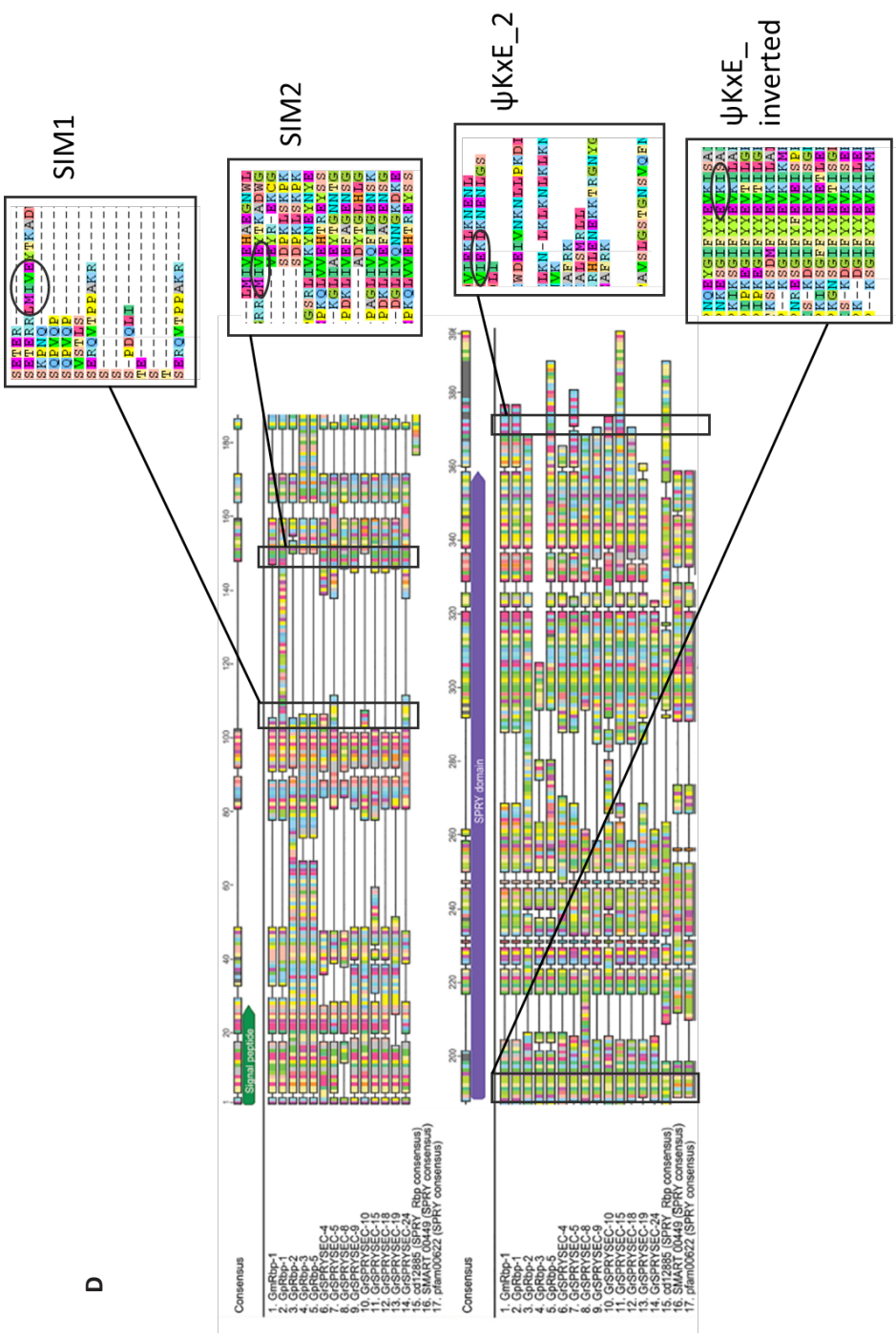
B

Position	Peptide	Score	Cutoff	P-value	Type
49 - 53	LSETERRLMIVEYTKADWA	31.547	29.92	0.14	SUMO Interaction
81	LMIVEYTKADWGCRS	3.348	3.32	0.186	Sumoylation Nonconcensus
219	TVYAPGTKIEANFGP	3.36	3.32	0.109	Sumoylation Nonconcensus
238	KSADVIEKLKNENLG	4.185	3.32	0.044	Sumoylation Nonconcensus
240	ADVIEKLKNENLGS*	26.804	2.13	0.001	Sumoylation Concensus

C



Supplemental figure 7. SUMOylation and SUMO interacting motifs are predicted in GpRbp-1 (*Continues otothe next page*). Lysine residues 108 and 240 of GpRbp-1 are predicted as SUMOylation sites. The aminoacid stretch from position 49 to 53 and from position 74 to 78 of GpRbp-1 are predicted so function as SIMs, using **A)** The Joined Advanced SUMOylation site and SIM Analyser (JASSA) (Beauclair *et al.*, 2015) and **B)** The GPS-SUMO webserver tool (Zhao *et al.*, 2014). **C)** Structural model of GpRbp-1 (grey), with SIM2 (yellow), and ψKxE sites indicated (blue). GpRbp-1 was modelled by remote homology modelling from human RBPM10 (PDB: 5JI7) and mouse RBP9 (PDB: 5J1A).



Supplemental Figure 7. SUMOylation and SUMO interacting motifs are predicted in GpRbp-1. D.) Location of SUMO and SIM sites in SPRYSEC alignment (modified from Diaz Granados, *et al*, 2016).

Supplemental table 6. Primers mentioned in the text.

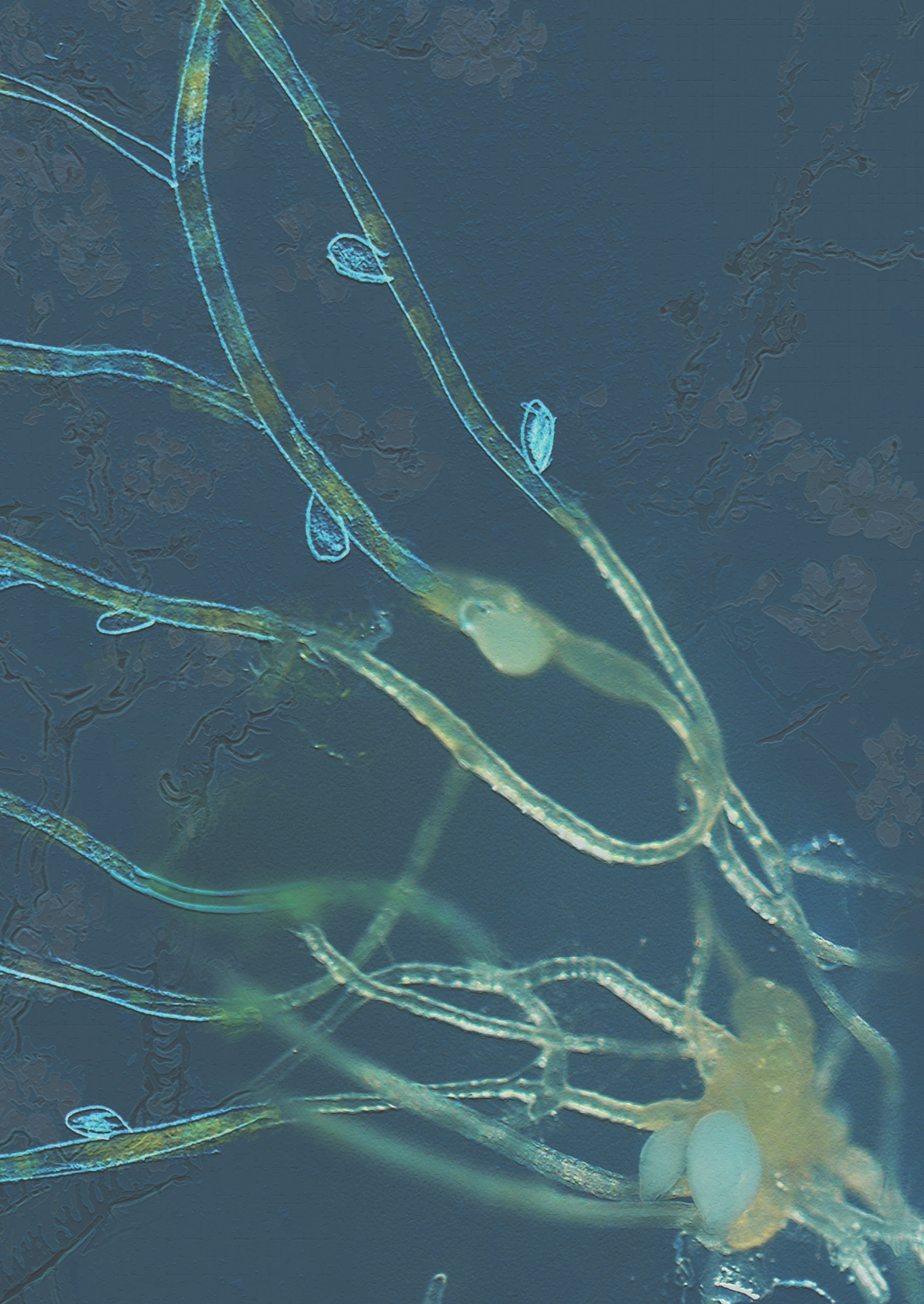
Cloning	
Primer name	***Sequence***
GpRbp-1 Forward	5-GGGCCATTACGGCCCAACTCGCTCGCCCAATGGAG-3’
GpRbp-1 Reverse	5-GGGCCGAGGCGGCCCGGCCCATTATAAATTCTCG-3’
iSALK homozygocity *siz1-2* LP	5-GAGCTGAAGCATCTGGTTTTG-3’
iSALK homozygocity *siz1-2* RP	5-CACGACAGATGAAGCATTGTG-3’
iSALK homozygocity *siz1-3* LP	5-TCCCTCGTAGACATCTGATGG-3’
iSALK homozygocity *siz1-3* RP	5-AAAGAGAGAGTGAGCGAAGGG’-3’
RT-PCR	
Primer name	***Sequence***
At_qPCR_SIZ1_002 F	5-GCTGACGTTTCAGGAGGTTTAGTTG-3’
At_qPCR_SIZ1_001 R	5-GCCTTGTCTTGTCTACTGTCATTCATAC-3’
At_qPCR-UBP22_001_F	5-ACAACATATGACCCGTTTATCGA-3 (Hofmann & Grundler, 2007)
At_qPCR-UBP22_001_R	5-TGTTTAGGCGGAACGGATACT-3
At_qPCR-UBQ5_001_F	5-GTTAAGCTCGCTGTTCTTCAGT-3 (Anwer *et al.*, 2018)
At_qPCR-UBQ5_001_R	5-TCAAGCTTCAACTCCTTCTTTC-3

Chapter 5

RanGAP1 and RanGAP2 are host targets of the nematode effector GpRbp-1 from *Globodera pallida* that are required for cyst nematode virulence

Amalia Diaz-Granados[1]; Erik Slootweg[1]; Somnath Pokare[2]; Casper van Schaik[1]; Jan Roosien[1]; Octavina Sukarta[1]; Rikus Pomp[1]; Abdenaser Elashry[2]; Florian Grundler[2]; Geert Smant[1]; Aska Goverse[1]

[1] Laboratory of Nematology, Wageningen University, Wageningen, Netherlands;
[2] Department of Molecular Phytomedicine, INRES, University of Bonn, Bonn, Germany



5

Abstract

The potato resistance gene *Gpa2* encodes a canonical intracellular CC-NB-LRR immune receptor which detects specific variants of the GpRbp-1 effector secreted by the potato cyst nematode *Globodera pallida*. Effector recognition by Gpa2 is suggested to occur indirectly through the co-factor Ran GTPase Activating Protein 2 (RanGAP2). Using a combination of *in planta* co-immunoprecipitation (Co-IP) and cellular imaging studies, we report that both the eliciting and non-eliciting variants of GpRbp-1 can form complexes with RanGAP2 in plant cells. The conserved WPP domain of RanGAP2 is sufficient for the interaction with these effectors in the cell. Moreover, we show that GpRbp-1 variants also interact with the RanGAP1 homologue. Together, these data suggest that RanGAP targeting by Gp-Rbp-1 is not sufficient for Gpa2 recognition and that instead it may contribute to nematode parasitism. Using Arabidopsis as a model plant, we could demonstrate that both RanGAP homologues are required for susceptibility to cyst nematodes. This supports the role of RanGAP1 and RanGAP2 as functional virulence targets of the nematode effector GpRbp-1. RanGAP2 modulates nucleocytoplasmic transport in interphase and functions as a subcellular marker during mitosis. These functions may be high jacked to promote nematode susceptibility and may represent a new mechanism of parasitism by nematodes.

Introduction

Plants are able to fend off the majority of pathogens by means of a non-adaptive immune system that relies on a suite of specialized immune receptor proteins (Kanyuka & Rudd, 2019; van der Burgh & Joosten). Immune receptors recognize so-called invasion molecules, which are structural features of microbes, plant derivatives from pathogen activity (i.e. DAMPs) (Choi & Klessig, 2016) or pathogen-secreted effectors. Effectors are produced and secreted by pathogens to suppress plant immune responses or manipulate the host cellular machinery in benefit of the pathogen (Varden *et al.*, 2017). The presence or activity of effectors is perceived intra- or extracellularly by immune receptors, resulting in a set of robust defence responses termed cytoplasm-initiated immunity or apoplast-initiated immunity, respectively (Kanyuka & Rudd, 2019; van der Burgh & Joosten, 2019). Immune responses initiated by the recognition of effectors often culminates in a type of programmed-cell death termed hypersensitive response (HR), which may impede further colonization by the pathogen (Jones & Dangl, 2006; Kanyuka & Rudd, 2019; van der Burgh & Joosten, 2019).

Most intracellular plant immune receptors are members of the Nucleotide-binding Leucine-Rich repeat (NB-LRR) receptor family (Dodds & Rathjen, 2010). NB-LRR immune receptors can recognize pathogen effectors by direct physical association. For example, this was reported for the rice CC-NB-LRR (CNL) Pita. Loss of interaction with the *Magnaporthe grisea* effector Avr-Pita, leads to a compromised functionality of the receptor (Jia *et al.*, 2000). However, it seems that the model of direct recognition applies only to a few exceptional cases as described for NB-LRRs from flax (Dodds *et al.*, 2006; Ravensdale *et al.*, 2012). Instead, indirect effector recognition by NB-LRR proteins represents a majority of cases reported to date (Takken & Goverse, 2012). This involves sensing pathogen-induced modifications of effector host targets or their mimics. Indirect recognition enables a single NB-LRR to detect multiple effectors that act on a common host target. Additionally, it is believed that with such surveillance system the plant can circumvent rapidly evolving effectors (Takken & Goverse, 2012).

Gpa2, a single dominant gene located in a small resistance (*R*) gene cluster on chromosome XII of potato (*Solanum tuberosum spp. andigena*), encodes a canonical CC-NB-LRR immune receptor. Gpa2 mediates late resistance in the roots against specific field populations of the potato cyst nematode *Globodera pallida* (van der Voort *et al.*, 1997). Moreover, Gpa2 triggers a specific hypersensitive response in the leaves of *Nicotiana benthamiana* upon recognition of the nematode-secreted effector GpRbp-1, which depends on a single amino acid polymorphism (S187P) in the effector (Sacco *et al.*, 2009). Furthermore, recognition of GpRbp-1 by Gpa2 depends on the co-factor RanGAP2, which interacts with a coiled-coil domain (CC) present in the N-terminus of the receptor (Sacco *el al.*, 2009). Using this first nematode effector-plant immune receptor pair as a model system, we aimed to evaluate the molecular mechanisms underlying RanGAP-mediated detection of GpRbp-1 by Gpa2.

GpRbp-1 is a SPRYSEC effector from the potato cyst nematode *G. pallida*, characterized by a domain architecture composed of an N-terminal signal peptide for secretion and a C-terminal SPRY domain (Ali *et al.*, 2015; Diaz-Granados *et al.*, 2016; Rehman *et al.*, 2009).

The N-terminal signal peptide suggests that GpRbp-1 may be delivered into the plant cell, where it can interact with plant targets. This interaction may be supported by the C-terminal SPRY domain, which is described in other eukaryotes as a versatile protein binding platform (Diaz-Granados *el al.*, 2016; Rehman *el al.*, 2009). GpRbp-1 is produced in the dorsal oesophageal gland of the nematode, and it is more abundantly expressed in early parasitic nematodes (Blanchard *et al.*, 2005). Additionally, GpRbp-1 variants remain under positive selection, indicating that at least some members of this effector family play an important role in the virulence of *G. pallida* (Carpentier *et al.*, 2012). Therefore, GpRbp-1 is proposed to play a virulent role during the initiation or establishment of the characteristic permanent feeding site induced by cyst nematodes, the syncytium (Blanchard *el al.*, 2005).

The underlying mechanism of GpRbp-1 in cyst nematode virulence as well as its recognition by Gpa2 is still unknown. Despite several attempts, direct perception of GpRbp-1 by Gpa2 has never been detected. This is in contrast to the effector SPRYSEC19 from the sibling species *G. rostochiensis*, which binds to the LRR domain of the NB-LRR protein Sw5F (Postma *et al.*, 2012). However, artificial tethering of GpRbp-1 to the Gpa2 co-factor RanGAP2 was shown to enhance the cell death response by Gpa2 in *N. benthamiana*, hinting that RanGAP2 may contribute to GpRbp-1 recognition (Sacco *el al.*, 2009). This prompted us to test whether GpRbp-1 could associate with RanGAP2 in the plant cell. Here, we show that GpRbp-1 can indeed form a protein complex with RanGAP2 *in planta* through binding of the WPP nuclear envelope-targeting domain. Using a combination of co-immunoprecipitation (Co-IP) and advanced imaging studies, we further demonstrate that GpRbp-1 also associates with the homologue RanGAP1. Remarkably, these interactions were observed for both cell death eliciting and non-eliciting GpRbp-1 variants, suggesting that RanGAP1 and RanGAP2 targeting is independent from immune receptor recognition. Therefore, we hypothesized that RanGAP1 and RanGAP2 are targeted to promote nematode virulence. Indeed, knocking down either RanGAP2 or RanGAP1 reduced host susceptibility to the cyst nematode *Heterodera schachtii* in *Arabidopsis thaliana.* From these data we conclude that both RanGAP1 and RanGAP2 function as virulence targets of cyst nematodes in plants. To our knowledge, this is the first study demonstrating the contribution of RanGAP1 and RanGAP2 to the susceptibility of plants to pathogens, including nematodes. The implications of these findings on the role of RanGAP1 and RanGAP2 as virulence factors in cyst nematode parasitism and Gpa2-mediated immunity are discussed.

Results

Cell death eliciting and non-eliciting GpRbp-1 effectors of *G. pallida* interact with full-length NbRanGAP2 *in planta*

Previous works have shown that Gpa2-mediated programmed cell death is enhanced when RanGAP2 is artificially tethered to GpRbp-1 of *G. pallida*, suggesting complex formation between these proteins (Sacco *el al.*, 2009). We therefore aimed to investigate whether RanGAP2 could in fact interact with GpRbp-1 *in planta* by performing a co-immunoprecipitation assay (Co-IP). To that end, full-length RanGAP2 fused to GFP (RanGAP2-GFP) was co-expressed transiently in leaves of *N. benthamiana* upon agroinfiltration with an HA-tagged version of the Gpa2-dependent cell death-eliciting GpRbp-1 variant D383-1 (D383-1-8HA), from *G. pallida* population D383 (Sacco *el al.*, 2009). For pull-downs, RanGAP2-GFP was captured with anti-GFP conjugated paramagnetic beads as bait and the bound proteins were analysed by western blotting (Fig. 1). D383-1-8HA co-immunoprecipitated with RanGAP2-GFP, but not in the absence of a bait protein showing the specificity of the interaction. These findings suggest a physical association between RanGAP2 and D383-1 *in planta*.

We then questioned whether the observed GpRbp-1 interaction with RanGAP2 is restricted to cell death eliciting variants or not. Therefore, the analysis was extended to non-eliciting GpRbp-1 variants. Gpa2 recognition of GpRbp-1 is dependent on the identity of amino acid 187 in the effector sequence (Sacco *el al.*, 2009). Variants with a Proline (P) at this position like D383-1 activate Gpa2, whereas those with a Serine (S) do not. Rook-4 is an S variant derived from the virulent *G. pallida* population Rookmaker (Sacco *el al.*, 2009). To that end, RanGAP2-GFP and Rook-4-8HA were co-expressed transiently in *N. benthamiana* and leaf extracts were subjected to Co-IP assays. Our data indicate that Rook-4-8HA was pulled-down specifically by RanGAP2-GFP (Fig. 1). Interestingly, a stronger band intensity for the non-eliciting Rook-4 variant was consistently observed after Co-IP compared to the eliciting variant D383-1. This suggests that the effector variants may differ in their binding affinity for RanGAP2. Combined, our results demonstrate that GpRbp-1 can indeed target RanGAP2 *in planta* and form a complex as suggested by Sacco *et al* (2009). However, this complex is formed for both the cell death eliciting and non-eliciting GpRbp-1 variants, which shows that the physical interaction alone cannot explain the specificity of GpRbp-1 recognition by Gpa2.

G. pallida GpRbp-1 effectors also interact with RanGAP1 *in planta*

RanGAP2 is closely related to RanGAP1 (66.2% at the amino acid level in *N. benthamiana*), which is shown previously to bind the Gpa2 homologue Rx1 in a yeast-2-hybrid assay (Tameling *et al.*, 2010). Both proteins function as activators of Ran GTPase as part of the nucleocytoplasmic transport cycle and are functionally redundant (Rodrigo-Peiris *et al.*, 2011; Xu *et al.*, 2008). Therefore, we hypothesised that also RanGAP1 could be a host target of the *G. pallida* effector GpRbp-1. To test this, we determined whether our panel of effectors can interact with RanGAP1 using similar *in planta* Co-IP approaches as used for RanGAP2. Pulling down RanGAP1-GFP resulted in the co-immunoprecipitation of D383-1-8HA and Rook-4-8HA

(Fig. 1). No aspecific binding as well as no effect on protein stability was observed by western blotting. Notably, these effectors co-purified with RanGAP1-GFP in lower quantities than when RanGAP2-GFP was used as bait. Our findings suggest that eliciting and non-eliciting effectors of *G. pallida* can also associate with the RanGAP1 homologue *in planta*, albeit to a lesser extent compared with RanGAP2. These data show that also RanGAP1 is a host target of eliciting and non-eliciting GpRbp-1.

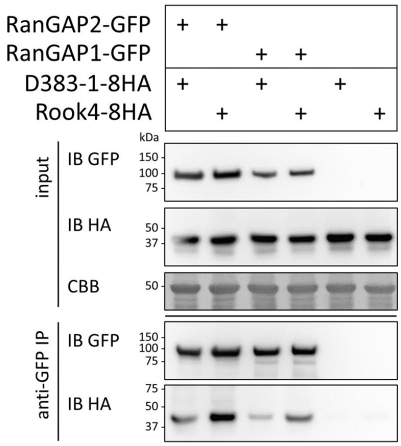


Figure 1. Eliciting and non-eliciting variants of GpRbp-1 interact with RanGAP2 and RanGAP1 *in planta*. Co-IP of full-length NbRanGAP tagged with green fluorescent protein (RanGAP1/2-GFP) as bait and the HA-tagged RBP effector proteins D383-1 and Rook-4 as prey (D383-1-8HA; Rook4-8HA). The baits and preys were co-expressed transiently in *N. benthamiana* leaves and protein extracts were subjected to Co-IP using anti-GFP conjugated beads. The immunoblots (IB) with anti-GFP and anti-HA antibodies of the input material are shown in the top half of the image and the results of co-IP (IP) in the two bottom panels of the figure. A Coomassie brilliant blue (CBB) stained blot serves as loading control for the input material.

The WPP domain of RanGAP2 is sufficient for the interaction with GpRbp-1 *in planta*

Plant RanGAP proteins are characterized by an N-terminal WPP domain, so-called for a conserved Tryp-Pro-Pro motif, which plays a role in the localization of the protein to the nuclear envelope (Rose & Meier, 2001). Therefore, we tested whether the RanGAP2 WPP domain is sufficient for the interaction with GpRbp-1 effectors in plant cells. To test this, co-localization studies were performed in leaves of *N. benthamiana* upon agroinfiltration of a nuclear-targeted WPP construct of RanGAP2 (WPP-NLS-mCh) (Tameling *el al.*, 2010) (Fig. 2). WPP-NLS was co-expressed with GpRbp-1 effectors fused to GFP at their N- and C-terminus (Rook4-GFP-4HA, D383-1-GFP-4HA, 4myc4-GFP-Rook4 and 4myc-GFP-D383-1). These effectors are distributed over both the nucleus and cytoplasm (Jones *et al.*, 2009)2009. It was anticipated that co-expressing WPP-NLS-mCh would shift the distribution of these effectors towards the nucleus, provided that these proteins exist in the same complex. This shift in nucleocytoplasmic distribution can be quantified by determining the fluorescence intensity ratio between the GFP-tagged protein in the nucleus and the cytoplasm (I_N/I_C). Imaging was performed at 2 days post infiltration (2 dpi). Indeed, we could observe relatively higher nuclear intensities for

the C-terminal tagged Rook4-GFP-4HA and D383-1-GFP-4HA upon co-expression with WPP-NLS-mCh as compared to the control (Suppl. Fig. 1). Interestingly, GpRbp-1 variants with GFP attached to their N-terminus did not show a shift in subcellular localization. This suggest that both Rook-4 and D383-1 form a complex with the WPP of RanGAP2, but that the attachment of a GFP tag to the N-terminus hampers this complex formation.

A

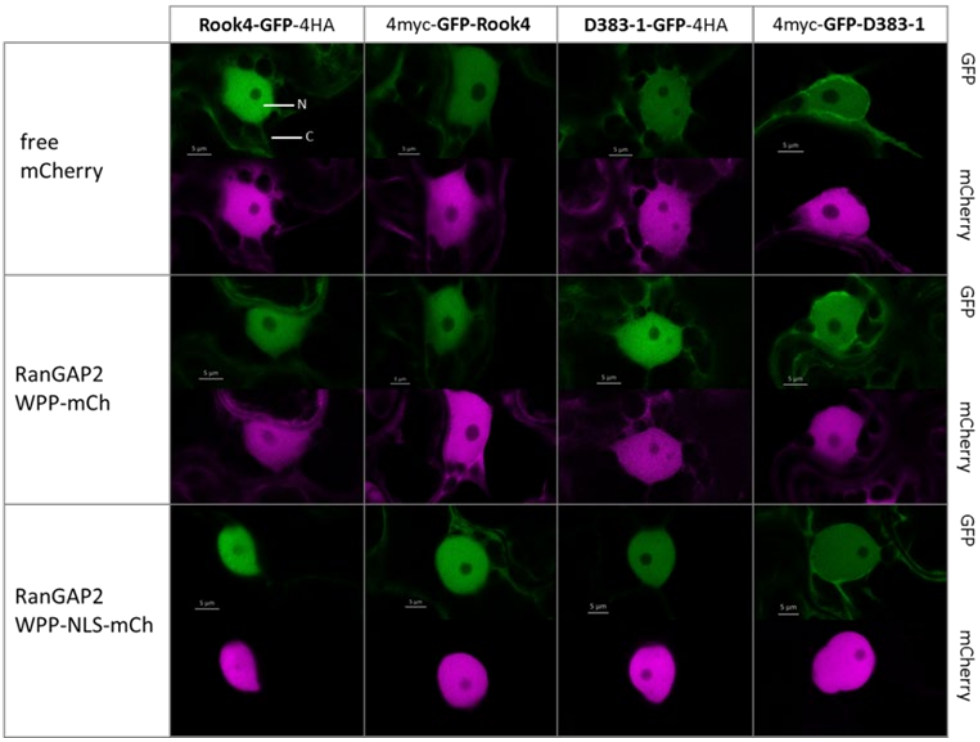


Figure 2. The WPP domain of RanGAP2 is sufficient for the interaction with GpRbp-1 D383-1 and Rook-4 (*Continues to the next page*). C- and N-terminal GFP-tagged GpRbp-1 variants were co-expressed with the WPP domain of RanGAP2 fused to a nuclear localization signal and a modified red fluorescent protein (mCherry) (RanGAP2-WPP-mCherry), to the WPP domain without the NLS motif (RanGAP2-mCherry) or to mCherry as negative control. For each combination six or more cells were imaged using confocal microscopy. **A)** Representative images of each of the combinations of Rook4-GFP-4HA, 4myc-GFP-Rook4, D383-1-GFP-4HA and 4myc-GFP-D383-1 with free mCherry, RanGAP2 WPP-mCherry and RanGAP2-WPP-NLS-mCherry. The fluorescence in the GFP channel is shown in the top panel (in green) and the red fluorescence of mCherry is shown in the bottom panel (in magenta) for each figure. The scale is indicated by a 5 µm scale bar.

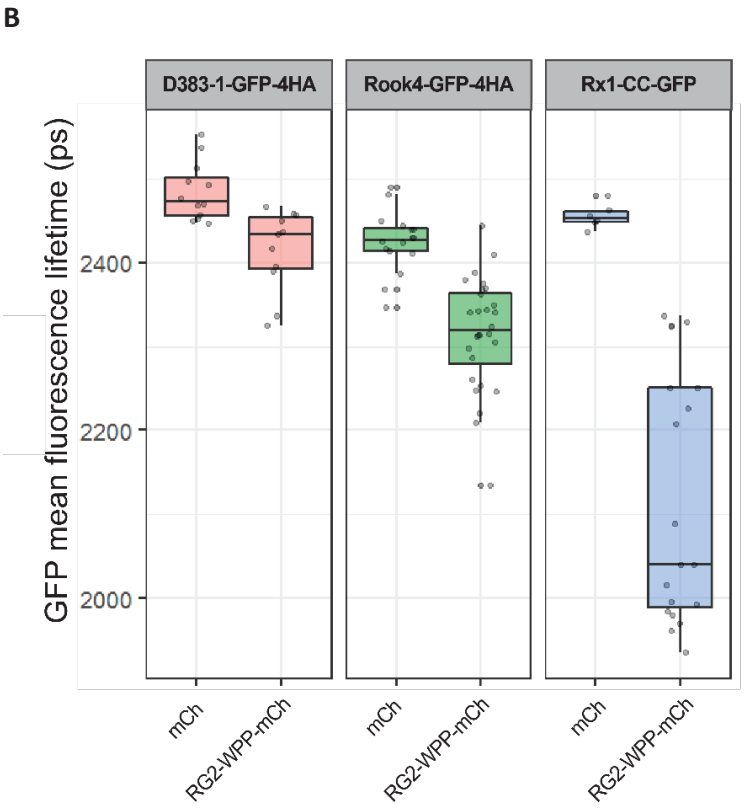


Figure 2. The WPP domain of RanGAP2 is sufficient for the interaction with GpRbp-1 D383-1 and Rook-4. B) The mean fluorescence lifetime of GFP in the cytoplasm of multiple cells was determined (in picoseconds). The decay in lifetime of GFP caused by the strong interaction between the Coiled-coil domain of Rx1 labelled with GFP (Rx1-CC-GFP), was used as positive control to indicate interactions of the WPP domain with GpRbp-1 variants. Whiskers indicate the quartile (25 or 75%)-/+ 1.5x interquartile range.

We corroborated our co-localization data using Förster Resonance Energy Transfer (FRET) between GpRbp-1 labelled with GFP as energy donor and the RanGAP2 WPP domain labelled with a red fluorescent protein (mCherry) as acceptor. Energy transfer can only be detected if the donor and acceptor molecules are in close proximity (less than 10 nm). The occurrence of FRET leads to a reduced fluorescence lifetime of the donor molecule, which can be detected in living cells. Co-expression with the mCherry-tagged WPP-NLS domain resulted in consistent reduction in the fluorescence lifetime of Rook4-GFP-4HA and D383-1-GFP-4HA in comparison to the co-expression of Rook4-GFP-4HA with free mCherry (Fig. 2). Notably, the reduction in lifetime of D383-1-GFP-4HA upon co-expression with WPP-mCh was smaller (59 ps) (Fig. 2), which is in line with the reduced interaction between D383-1-GFP-4HA and RanGAP2-GFP as observed in the Co-IP (Fig. 1). Overall, our findings show that the GpRbp-1 effectors Rook-4 and D383-1 can target the WPP domain of RanGAP2 in living plant cells with different efficiencies and that the interaction depends on accessibility of the N-terminus of GpRbp-1.

RanGAP2 and RanGAP1 contribute to nematode pathogenicity

Given that both RanGAP1 and RanGAP2 are targeted by eliciting and non-eliciting variants of Gp-Rbp-1, we hypothesised that plant RanGAPs could fulfil a broader role that extends beyond a co-factor in pathogen recognition (Hao *et al.*, 2013; Sacco *el al.*, 2009). For instance, effector targeting of host components may be directly used by pathogens to promote infection. To further explore the biological relevance of the interactions observed, we investigated whether RanGAP1 and RanGAP2 contribute to cyst nematode pathogenicity.

To evaluate the role of RanGAP2 and RanGAP1 in potato cyst nematode parasitism, transient gene silencing of RanGAP2, RanGAP1 or combinations thereof was induced in the two host species potato (*S. tuberosum* ssp*. andigena*) and tomato (*S. lycopersicum*) using the TRV-VIGS constructs previously described by Tameling *et al.* (2007; 2010). The sequence homology between the potato, tomato and *N. benthamiana* RanGAP1 and RanGAP2 homologues - on which the constructs are based - ranges from 87 to 94% (Suppl. Table 1), respectively, and are thus sufficient to silence the potato and tomato genes. RanGAP1 was efficiently silenced in tomato, with a relative expression of 0.337, as compared to the expression in GFP-silenced plants (Suppl. Fig. 2). Nonetheless, infection of the roots with *G. pallida* did not result in a difference in the average number of cysts present in the roots of RanGAP1 silenced potato. We also did not observe differences in the number of cysts infecting the roots of potato inoculated with TRV-RanGAP1 and RanGAP2 constructs (Suppl. Fig. 2). Notably, the level of gene silencing achieved locally in the roots may not be not sufficient to show an effect on nematode infection, even when RanGAP1 and RanGAP2 are both targeted. Moreover, RanGAP1 and RanGAP2 gene silencing is most likely not achieved in all nematode infection sites due to the patchy distribution of TRV in the roots potentially masking subtle effects of RanGAP1 and RanGAP2 in cyst nematode parasitism.

Given the lack of more efficient molecular genetic tools in tomato and potato for further analysis, we took an alternative approach to test the contribution of RanGAP2 and RanGAP1 to cyst nematode parasitism in Arabidopsis. RanGAP1 and RanGAP2 are highly conserved across plant species and thought to contribute to the same plant processes. For example, the sequence homology between RanGAP1 and RanGAP2 from Arabidopsis and potato/tomato ranges from 67-70% (Suppl. Table 1). We first analyzed transcript dynamics of *RanGAP2* and *RanGAP1* in the *A. thaliana* wild-type Col-0 challenged with the beet cyst nematode *Heterodera schachtii*. We determined the expression levels of *AtRanGAP1* and *AtRanGAP2* in whole roots of infected and mock-infected plants during different stages of nematode infection at 2, 7, 10 and 14 dpi by qRT-PCR. However, we did not find differential expression of *AtRanGAP1* or *AtRanGAP2* in the measured infection time points (Suppl. Fig. 3). To exclude that this was the result of a dilution effect using whole roots, which may mask transcript dynamics in local infection sites, we also checked changes in RanGAP1 and RanGAP2 gene expression in the NEMAtic database (Cabrera *et al.*, 2014). This revealed that in micro-aspirated feeding cells of 5 and 15 days old a small but significant upregulation of RanGAP1 is detected, whereas no significant transcriptional regulation is found for RanGAP2 in the datasets included in the NEMAtic database. Together, these data suggest that only RanGAP1 may transcriptionally regulated locally in nematode-induced feeding sites. Conversely, under

the experimental conditions of our study and the one of Szakasits *et al.* (2009), it appears RanGAP2 is not transcriptionally regulated during nematode infection.

Next, we challenged the *A. thaliana* mutants *rg1-1* and *rg2-2* (Boruc *et al.*, 2015; Xu *el al.*, 2008) with the beet cyst nematode *H. schachtii*, which has a similar mode of action as *G. pallida* on potato. The mutation in *rg1-1* leads to a full-knock out of RanGAP1, and the mutation in *rg2-2* leads to a knock-down of RanGAP2 levels (Boruc *el al.*, 2015; Xu *el al.*, 2008). We did not observe morphological phenotypes that could interfere with nematode infections (e.g. root length) (Suppl. Fig. 4) (Boruc *el al.*, 2015; Xu *el al.*, 2008). Our data indicate that the total number of nematodes infecting the roots of *rg1-1* and *rg2-2* is significantly lower as compared to the wild-type control (Col-0) after 2 weeks of infection (Fig. 3). We found an average decrease of 21% and 11% in *rg1-1* and *rg2-2* plants compared to the wild-type, respectively (least-square fit, $p<0.001$ for *rg1-1* and $p=0.054$ for *rg2-2*). In cyst nematodes, sex determination is dependent on environmental conditions (Anjam *et al.*, 2018; Trudgill, 1967). Auspicious conditions favour the development of female nematodes. Therefore, we also investigated the proportion of male and female nematodes at 2 weeks post infection. Interestingly, both *rg1-1* and *rg2-2* plants harbour significantly less females than wild-type plants (Fig. 3). Average decreases of 33% and 27% were found for the number of females established in *rg1-1* and *rg2-2* plants compared to the wild-type, respectively (least-square fit, $p<0.001$ for *rg1-1* and $p=0.001$ for *rg2-2*). Similarly, we estimated average decreases of 13% and 1% in the number of males infecting the roots of *rg1-1* and *rg2-2* as compared to the wild-type, respectively (least-square fit, $p=0.027$ for *rg1-1* and $p=0.635$ for *rg2-2*). The size of females and syncytia established in the roots of RanGAP mutant and wild type plants were also quantified. The syncytia established in *rg1-1* and *rg2-2* plants were 8% bigger and 7% smaller than those established in the wild-type, respectively (least-square fit, $p=0.045$ for *rg1-1* and $p=0.131$ for *rg2-2*) (Suppl. Fig. 3). Finally, the females established in *rg1-1* and *rg2-2* plants were 7% and 5% bigger than those established in the wild-type, respectively (least-square fit, $p=0.195$ for *rg1-1* and $p=0.323$ for *rg2-2*). Collectively, the reduction in the total number of nematodes and proportions of females infecting the roots of mutant plants show that both RanGAP homologues are important for successful infection of plant roots by cyst nematodes.

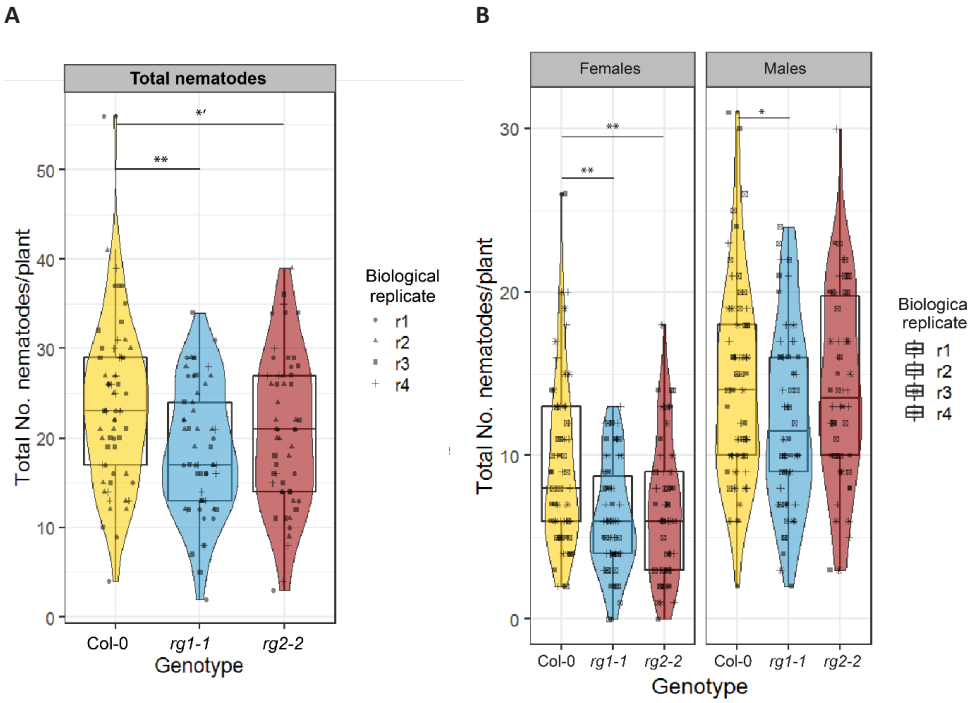


Figure 3. RanGAP1 and RanGAP2 are required for infection and development of *Heterodera shachtii* in Arabidopsis. A) The average number of total or **B)** female and male nematodes per plant in the roots of Arabidopsis after 2 weeks of infection was counted. Boxes indicate the 75th and 25th percentile and whiskers show the 95th and 5th percentile and dots show outlier values. Significant statistical difference as determined by a linear fit model. Data is combined from 4 individual experiments with means weighted by the inverse of the variance of each replicate for **A)** ** p-value <0.001, *' p-value= 0.054 with n_{rg1-1} = 58, n_{rg2-2} = 54 and n_{Col-0}= 65, for **B)** *p-value = 0.027, with n_{rg1-1} = 58, n_{rg2-2} = 54 and n_{Col-0}= 65

Discussion

In this study, we provide evidence for the direct binding of host proteins RanGAP1 and RanGAP2 by the nematode effector GpRbp-1. This demonstrates that RanGAP1 and RanGAP2 are host targets of the potato cyst nematode *Globodera pallida* with a possible role in virulence. Functional support for the role of both RanGAP1 and RanGAP2 in cyst nematode infection was obtained using Arabidopsis mutants *rg1-1* and *rg2-2* infected with the cyst nematode *Heterodera schachtii*. RanGAP1 and RanGAP2 are highly conserved Ran GTPase activating proteins among plant species and our results indicate that these proteins are both required for cyst nematode parasitism of plant roots. To our knowledge, this is the first study demonstrating the contribution of RanGAP1 and RanGAP2 to the susceptibility of plants to pathogens, including nematodes. We also demonstrate that GpRbp-1 interacts with the WPP domain of RanGAP2, which is required for anchoring of these Ran GTPase activating proteins to the nuclear envelope (Rose & Meier, 2001). This suggests that GpRbp-1 targets RanGAP1

and RanGAP2 to modulate the Ran cycle in order to affect the nucleocytoplasmic distribution of proteins in the cell during the onset of parasitism.

In addition, RanGAP2 is also a co-factor of the potato immune receptor Gpa2 and the observed direct interaction between GpRbp-1 and RanGAP2 *in planta* is in accordance to the results of artificial tethering of GpRbp-1 and RanGap2 described by (Sacco *el al.*, 2009). The direct interaction of RanGAP2 with GpRbp-1 supports the idea that plant RanGAP2 may play a role in mediating the indirect recognition of the effector by Gpa2. Therefore, it is possible that RanGAP2 acts as a bait for GpRbp-1 which allows Gpa2 to recognize the effector indirectly, thereby activating the immune receptor according to the bait-and-switch model (Collier & Moffett, 2009). Furthermore, the role of RanGAP2 as a virulence target of cyst nematodes suggests it may function as a classical guardee, being guarded by Gpa2 as a key host component in the cell for the regulation of the nucleocytoplasmic partitioning of proteins (Dangl & Jones, 2001; Van Der Biezen & Jones, 1998). It will be interesting to test these models to resolve the molecular mechanisms underlying GpRbp-1 recognition by Gpa2.

The physical interaction of GpRbp-1 with RanGAP2 is apparently independent from the recognition specificity of the Gpa2 immune receptor, since we observed that both cell death eliciting (D383-1) and non-eliciting (Rook4) variants of the effector interact with RanGAP2 (Fig. 1). The non-eliciting variant (Rook4) appears to be pulled-down to a lesser extent by RanGAP2, and thus may bind this target with a lower affinity. Sequence variations in GpRbp-1 may lead to differences in the protein surfaces available for protein-protein interactions. These variations may thereby affect binding affinity or specificity as is proposed for the SPRYSEC family of effectors (Diaz-Granados *el al.*, 2016). Whether the lower binding affinity of RanGAP2 by variants of GpRbp-1 correlates with differences in pathogenicity or eliciting of Gpa2, warrants further investigation. Additionally, our data show that GpRbp-1 also associates with the RanGAP1 homologue *in planta*, albeit less strongly than with RanGAP2. Hence, the possibility that RanGAP1 may also function as a recognition co-factor of Gpa2, remains to be evaluated. Our protein analyses show that both RanGAP1 and RanGAP2 are expressed to comparable levels, minimizing the likelihood that the observed differences in binding affinity are due to RanGAP1 being present less abundantly (Fig. 1). Presumably, GpRbp-1 effectors have a lower binding affinity to RanGAP1, which is surprising given the degree of conservation shared between the two RanGAP homologues (Hao *el al.*, 2013).

On the other hand, we also found a stronger effect of the depletion of RanGAP1 in susceptibility to cyst nematodes in *A. thaliana*, as compared to RanGAP2. A possible correlation between the strength of interaction and a role in virulence also requires further investigation. In the cell, RanGAP1 and RanGAP2 are reported to be functionally redundant (Rodrigo-Peiris *el al.*, 2011). However, our findings suggest that the RanGAP2 and RanGAP1 homologues may have a yet undefined differing role that nematodes may exploit for their fitness. To probe this idea, we evaluated the total number of nematodes infecting the roots of the viable double-mutant *rg1-1/rg2-2*, expecting to find an additive effect of the mutations in the total infection rate. Surprisingly, the number of cyst nematodes infecting the roots of *rg1-1/rg2-2* is comparable to that of the wild-type (Suppl. Fig. 4). This finding suggests that there is an epistatic interaction of the roles of RanGAP1 and RanGAP2 in infection by cyst

nematodes. Remarkably, the syncytia established on the *rg1-1* mutant are bigger than the ones in the *rg2-2*, suggesting a possible opposite role of these homologues in the formation of the feeding site (Suppl. Fig 3).

RanGAP is involved in the control of nucleocytoplasmic transport by functioning as a GTPase-activating protein of the cargo protein Ran (Ach & Gruissem, 1994; Merkle *et al.*, 1994; Rose & Meier, 2001). The activation of Ran by RanGAP promotes the hydrolysis of RanGTP to RanGDP to increase the concentration of RanGDP in the cytosol. RanGAP together with Ran guanosine exchange factors (RanGEFs) and other Ran-associated proteins maintains a nuclear/cytosolic-GTP/GDP gradient which provides directionality to the exchange of cargo proteins between the nucleus and the cytoplasm (reviewed in Meier & Somers, 2011). Beside the regulation of protein and RNA transport, the Ran cycle is involved in regulation of mitotic processes, for instance the formation of the mitotic spindle and reassembly of the nuclear membrane and nuclear pore complex (Harel *et al.*, 2003; Walther *et al.*, 2003; Zhang & Dawe, 2011). Interestingly, mitosis in cells neighbouring the initial syncytial cell is required for the radial expansion of cyst nematode feeding sites (de Almeida Engler *et al.*, 1999). Considering the fact that cell cycle regulation and mitosis are also involved in cyst nematode pathogenicity, we can predict that nematodes may recruit RanGAP1 and RanGAP2 to modulate cellular division processes for their own benefit.

We provide further insight into the role of RanGAP2 in susceptibility to nematode infection by locating the interaction of GpRbp-1 to the plant-exclusive WPP domain of RanGAP2. The WPP domain is characteristic of a small family of proteins associated to the nuclear envelope and possibly exclusive to plants (reviewed in (Meier *et al.*, 2010)). This domain mediates, together with WPP-interacting proteins (WIPs) and WPP-interacting tail-anchored proteins, the localisation of the RanGAPs to the outer surface of the nuclear envelope (NE) during interphase (Rose & Meier, 2001; Xu *et al.*, 2007; Zhao *et al.*, 2008). During interphase, the activity of the Ran-directed nucleocytoplasmic transport may influence for example hormone-based immune signalling, thereby potentially influencing nematode susceptibility (Gu, 2018). On the other hand, the WPP domain is also a determinant of the subcellular localization of RanGAP at the pre-prophase band during mitosis and to the cell plate during cytokinesis, and therefore may provide a spatial memory during cell division (Jeong *et al.*, 2005; Pay *et al.*, 2002; Rose & Meier, 2001; Xu *el al.*, 2008). Conceivably, targeting of the WPP domain by GpRbp-1-like effectors modifies (either by inhibiting or promoting) the localization of RanGAP during mitosis. Thereby cyst nematode effectors would possibly alter the role of RanGAP during the mitotic cell cycle to support nematode pathogenicity, for example by influencing the mitosis-dependent radial enlargement of syncytia. However, *Boruc et al* elegantly show that association to the NE is dispensable for the role of RanGAP in plant development and mitosis during female gametogenesis (Boruc *el al.*, 2015), where the GAP activity of RanGAP is essential. A plausible alternative hypothesis is that binding of RanGAP by nematode effectors leads to a disruption of the nucleocytoplasmic transport processes required for mounting an immune response in plants (Gu *et al.*, 2016; Rivas, 2012). For instance, this could hamper the as of yet undescribed downstream mechanisms of Gpa2 immune signalling.

In conclusion, our studies reveal additional aspects of the role of RanGAP in effector recognition by Gpa2, and motivate further research into the mechanisms underlying the involvement of RanGAP as a virulence factor in plant-nematode interactions.

Materials and Methods

Cloning

The constructs containing the WPP domain of RanGAP2 fused to red fluorescent protein (mCherry) or to a nuclear localization signal (NLS) and mCherry, were obtained as reported previously (Tameling *el al.*, 2010). GpRpb-1 version 1 from virulent population Rookmaker (Rook1, Pathotype 3) and GpRbp-1 version 1 from avirulent population D383, Pathotype 2) were C-terminally tagged with 8 units of the hemagglutinin tag as mentioned in Chapter 2. N-terminal fusion of 4 units on the c-myc tag followed by a GFP (4myc-GFP-GpRbp-1; 4myc-GFP- D383) were generated as described in Chapter 2. C-terminal fusions of GFP followed by 4 units of the hemagglutinin tag were generated by...

Heterologous expression by *Agrobacterium tumefaciens* transient assay in *N. benthamiana*

Heterologous protein expression was carried out by *Agrobacterium tumefaciens* transient assay (ATTA) in plants, as described previously (Slootweg *et al.*, 2017)2017. Briefly, Agrobacteria strains carrying the expression vectors were grown in YEB medium overnight. Grown bacterial cells were spun down and re-suspended in infiltration medium and optical densities at wavelength 600 nm (OD_{600}) were adjusted to final OD600 values of 0.2 for all constructs in co-immunoprecipitation assays, OD_{600} = 0.2 for all constructs in the confocal assays and OD_{600} = 0.2 for all constructs containing a GFP-tag or OD_{600} = 0.4 for constructs containing a mCherry tag in the FRET-FLIM assays. *A. tumefaciens* suspensions were then infiltrated on the abaxial surface of the leaves of *N. benthamiana* plants using needleless syringes. Infiltrated spots were harvested for protein extraction or examined by microscopy at 2 days post infiltration (dpi).

In planta co-immunoprecipitation and detection of recombinant proteins

Total protein extracts were prepared by grinding leaf material in protein extraction buffer (20% (v/v) glycerol, 50 mM Tris-HCl pH 7.5, 2 mM EDTA, 300 mM NaCl, 0.6 mg/ml Pefabloc SC plus (Roche, Basel, Switzerland), 2% (w/v) polyclar-AT polyvinylpolypyrrolidone (Serva, Heidelberg, Germany), 10 mM dithiothreitol and 0.1% (v/v) Tween20) on ice. For co-immunoprecipitation, protein extracts were passed through a Sephadex G-25 column (GE Healthcare, Chicago, Illinois) and pre-cleared by treatment with rabbit-IgG agarose (Sigma, 50 µL slurry per 60 µL protein extract). The cleared protein extract was incubated with µMACS anti-GFP paramagnetic (Miltenyi, Bergisch Gladbach, Germany) for 1h at 4°C. Columns were washed with washing buffer (20% (v/v) glycerol, 50 mM Tris-HCl pH 7.5, 2 mM EDTA, 300

mM NaCl, 0.10% (v/v) Nonidet 40 and 5mM dithiothreitol) five times and eluted by removing the column from the uMACS collector and adding 45uL of the washing with the washing solution. The input samples were mixed with 1X NuPage LDS sample buffer with 0.25 M dithiothreitol and incubated at 95°C for 5 minutes.

For western blotting, proteins were separated by SDS-PAGE on NuPage 12% Bis-Tris gels (Invitrogen) and blotted to 0.45 µm polyvinylidene difluoride membrane (Thermo Scientific). Before immunodetection we blocked the membranes for 1h at room temperature in 5% (w/v) powder milk in PBS with 0.1% Tween20. For immunodetection rabbit anti-GFP (Abcam, Cambridge, United Kingdom) with horseradish peroxidase-conjugated donkey anti-rabbit (Jackson ImmunoResearch, Ely, United Kingdom) or horseradish peroxidase-conjugated rat anti-HA (Roche) were used. Peroxidase activity was visualized using SuperSignal West Femto or Dura substrate (Thermo Scientific) and imaging of the luminescence with G:BOX gel documentation system (Syngene, United Kingdom).

Confocal laser scanning and FRET-FLIM microscopy

Confocal microscopy was performed on *N. benthamiana* epidermal cells using a Zeiss LSM 510 confocal microscope (Carl-Zeiss) with a 40X 1.2 numerical aperture water-corrected objective. For co-localization studies the argon laser was used to excite at 488 nm for GFP and chlorophyll, and the HeNe laser at 543nm to excite mCherry. GFP and chlorophyll emission were detected through a band-pass filter of 505 to 530nm and through a 650nm long-pass filter, respectively. mCherry emission was detected through a band-pass filter of 600 to 650nm. Nuclear and cytoplasmic fluorescence intensities were quantified using ImageJ(Schneider *et al.*, 2012). For FRET-FLIM analysis, the Förster resonance energy transfer (FRET) between GFP and mCherry was detected via Fluorescent Lifetime Imaging Microscopy. The HYD SMD detector of a Leica SP5 CLSM (Leica, Wetzlar, Germany) was used to measure the emission and fluorescent lifetime of GFP (495-545 nm) and the red fluorescent mCh emission (570-625 nm). The excitation of the GFP chromophore was measured using a white light laser (488 nm). The Time-correlated single-photon counting (TCSPC) was performed using a Becker & Hickl FLIM system FLIM analysis of TCSPC was performed with the B&H SPCImage software (Becker & Hickl GmbH, Berlin, Germany). Images of the fluorescence intensities of GFP and mCh were made with the Leica Application Suite X.

Nematode infection assays in *A. thaliana*

rangap 1-1 (SALK_058630) and *rangap2-2* (SALK_006398) seeds were obtained from the Nottingham Arabidopsis Stock Centre (Scholl et al, 2000) and *rg1-1;rg1-1/rg2-2;rg2-2* were obtained from the group of I. Meier. All *A. thaliana* genotypes used in the experiments are in the Columbia 0 genetic background. The presence of T-DNA inserts in the lines was confirmed by PCR using specific primers designed with the iSect Primers tool of the SIGNAL SALK database (Suppl. Table 2) (Alonso *et al.*, 2003), in combination with the universal LB primer. For nematode assays, seeds were vapour sterilized and vernalized at 4°C in the dark for 4 days to break seed dormancy. After vernalisation the seeds were plated in pairs in 9cm petri dishes containing Knop's modified medium (Sijmons *et al.*, 1991). Plants were grown at 25°C under

a 16h/8h light-dark cycle. 10 day-old seedlings were inoculated with 60-70 surface-sterilized *H. schachtii* infective juveniles. After 2 weeks of infection, the number of males and females present in the roots of Arabidopsis plants were counted visually and the size of females and syncytia were calculated with Leica M165C Binocular (Leica Microsystems, Wetzlar, Germany) and the Leica Application Suite software (Leica Microsystems). To combine results from 4 biological replicates, we weighted the measures of association from each replicate by the inverses of their variances. The variance of such weighted average is simply the inverse of the sum of the inverses of the variances which allow standard methods to be used to test for the overall significance at the 5% level of the genotype and the number of nematodes per plant. Such approach corresponds to methods to combine studies under a fixed effects model.

Gene expression during nematode infection in *A. thaliana*

A. thaliana ecotype Columbia 0 seeds were grown in KNOP media as described above. 2 week-old seedlings were infected with ~100 freshly hatched *H. schachtii* juveniles or mock infected with 0.7% gelrite. Complete root systems of infected and mock infected plants were collected at 2,7,10 and 14 days post infection (dpi). Total RNA was extracted from the complete root systems with the Maxwell-16 instrument according to the manufacturer's manual for the Maxwell-16 LEV Plant RNA kit. Total cDNA was prepared according to the GoScript Reverse Transcriptase instructions with all RNA samples diluted to the concentration of the RNA sample with the lowest yield. RT-PCR was performed with Absolute SYBR Green mix (Thermo Fisher Scientific) 1µL forward primer and 1µL reverse primer (5µM), 7µL MQ, 10µL Absolute SYBR Green mix (2X) and 1µL cDNA template adjusted to 10ng/µL. Each sample was processed in triplicate. RT-PCR was run using the following program: Initial denaturation at 95°C for 15 min followed by 40 cycles of amplification at 95°C for 30s, 61°C for 30s ,72°C for 30s and final elongation at 72°C for 5mins with a 0.2 °C ramp melting curve from 72 to 95 in 10s. Relative gene expression was calculated with the Vandesompele method (Vandesompele *el al.*, 2002) using Ubiquitin 5 (UBQ5) (Anwer *el al.*, 2018) and Ubiquitin-specific protease 22 (UBP22) (Hofmann & Grundler, 2007) as reference genes. The experiment was repeated 3 times and the significance of the fold change of the gene expression was determined by a Kruskal-Wallis test (α=0.05). The NEMAtic database (Cabrera *el al.*, 2014) was queried by introducing the TAIR gene codes for RanGAP1 and RanGAP2 in the manual selection tab.

Virus induced gene silencing in potato or tomato

Constructs used for RanGAP1 and RanGAP2 VIGS silencing in potato and tomato are described previously (Tameling & Baulcombe, 2007; Tameling *el al.*, 2010). Agroinfiltration was performed in a similar way as for the *N. benthamiana* agroinfiltrations (see above). Briefly, bacteria are grown overnight in YEB medium and re-suspended in MMA containing 200µM acetosyringone. Final ODs of a TRV1 and TRV:Rg1.1, TRV:Rg1.2, TRV:RG1+2 or TRV:RG2 mix are adjusted to 0.3 for infiltration in potato and to 0.4 for infiltration in tomato. Potato and tomato plants are grown and maintained in silver sand under standard greenhouse conditions. For nematode infection approximately 1000 eggs or 12.000 eggs of *G. pallida* (Rookmaker) were added to the potato or tomato plants, respectively. Relative gene expression was calculated

with the ΔΔCt method (Schmittgen and Livak, 2008) with RPN7 (Castro-Quezada *et al.*, 2013). For tomato, normalization was done using the geometric mean of reference genes tubulin (Aimé *et al.*, 2013) and MST1.

Acknowledgements

The authors gratefully acknowledge Iris Meier for providing *rg1-1/rg2-2* (SILK) seeds, and Wladimir Tameling or Matthieu Joosten for providing RanGAP-WPP constructs. We would also like to thank Alvaro Muñoz for advice on the statistical method to combine biological replicates and Jasper van Reekum, Miriam Pater and Mandi Ravensbergen for technical assistance. This work also benefited from interactions through the COST Action SUSTAIN FA1208 and was financially supported by NWO project 828.11.002.

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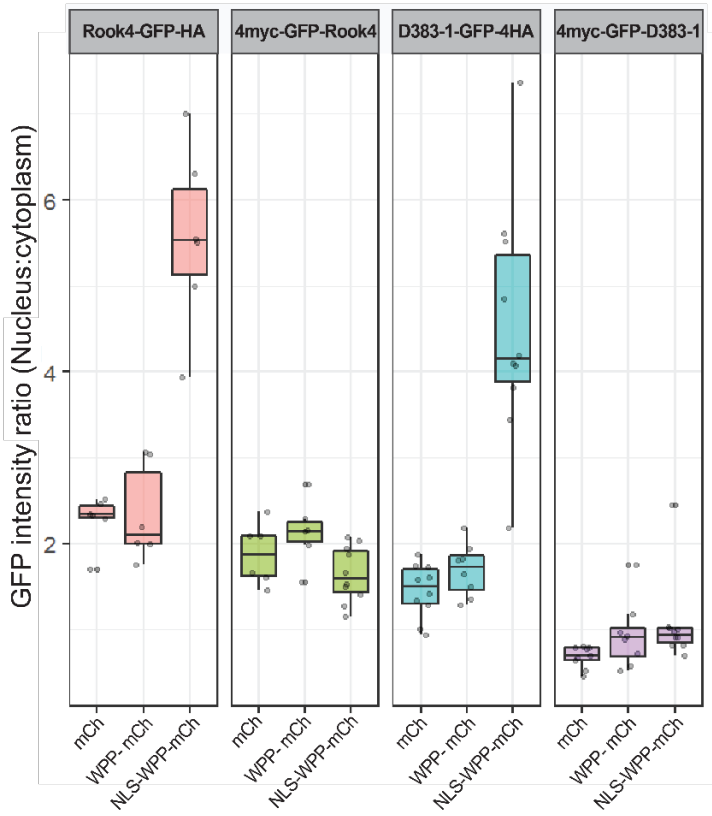
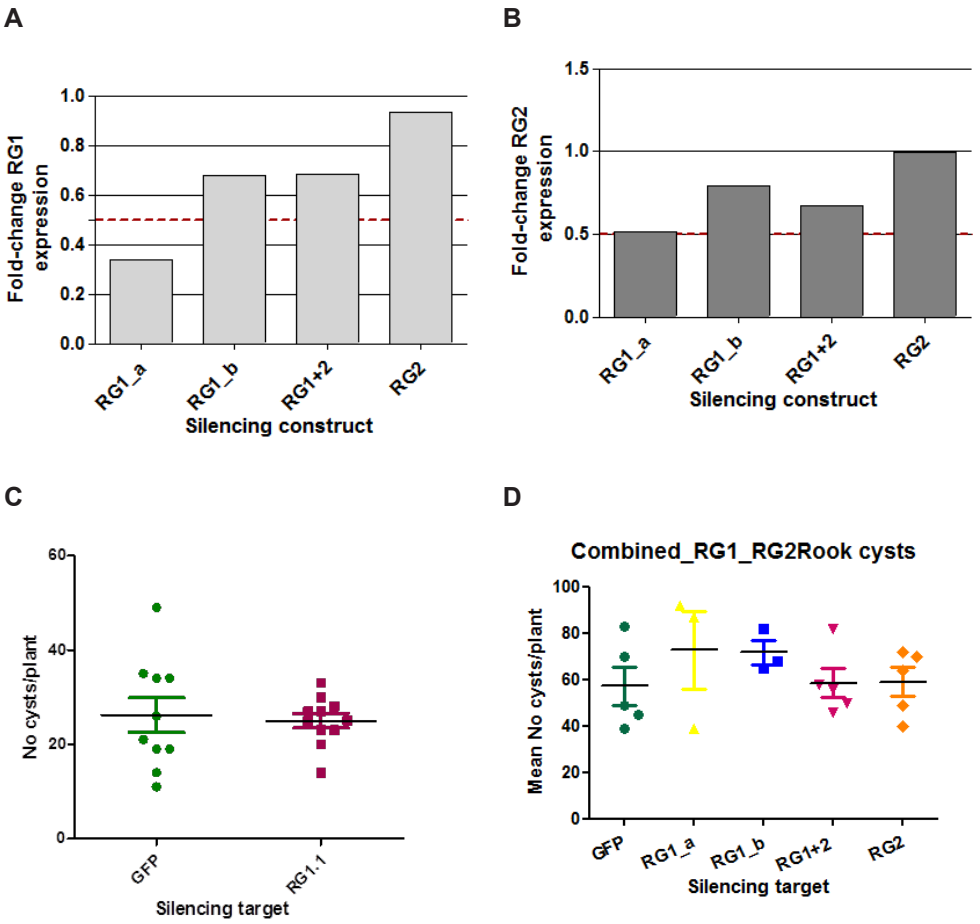
Supplemental information



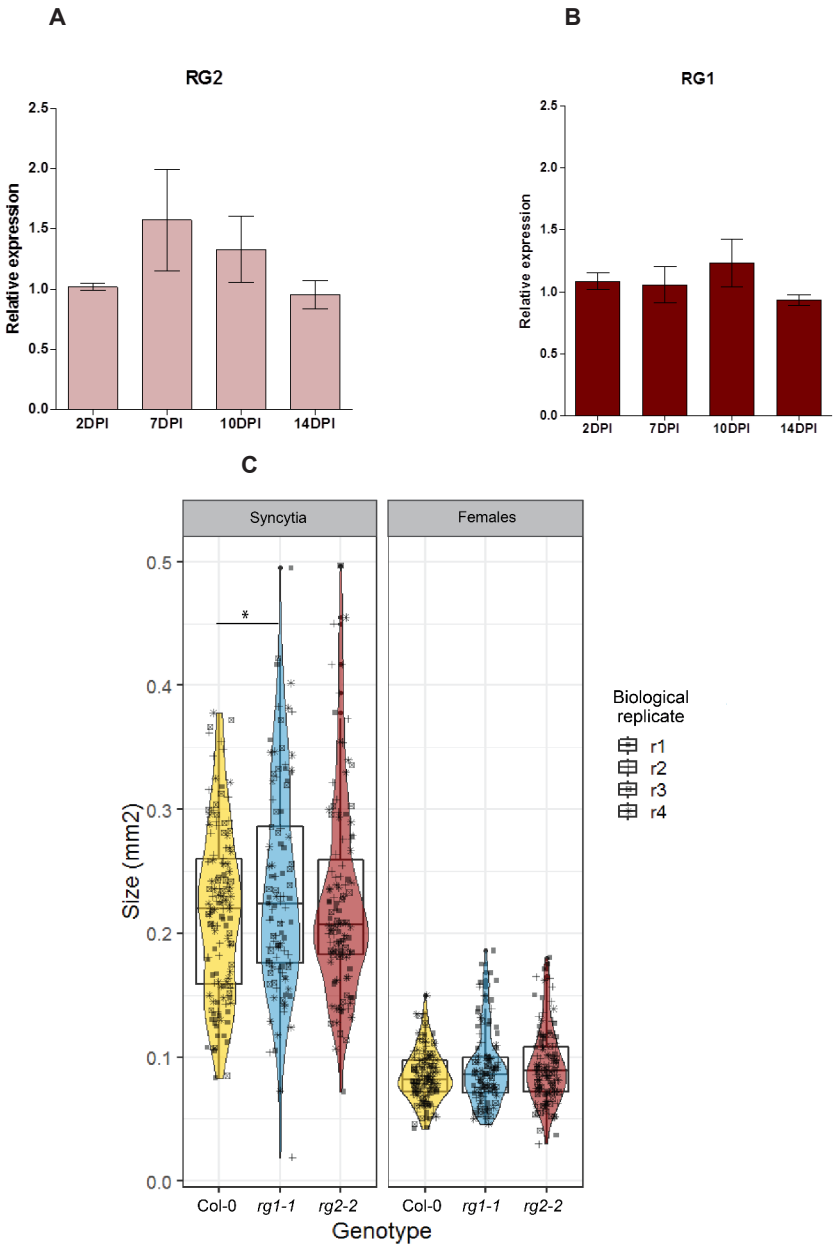
Supplemental figure 1. GpRbp-1 interacts with the WPP domain of RanGAP2. C- and N-terminal GFP-tagged GpRbp-1 variants (Rook4-GFP-4HA/4myc-GFP-Rook4 or D383-1-GFP-4HA/4myc-GFP-D383-1) were co-expressed with the WPP domain of RanGAP fused to a nuclear localization signal and a modified red fluorescent protein (mCherry) (RanGAP2-WPP-mCherry), to the WPP domain without the NLS motif (RanGAP2-mCherry) or to mCherry as negative control. The ratio of the GFP intensity in the nucleus and the cytoplasm was determined for each combination; a higher bar reflects a more pronounced nuclear localization (n ranges from 6 to 10 as indicated in the plots). Whiskers indicate the quartile (25 or 75%)-/+ 1.5x interquartile range.

Supplemental table 1. Identity percentage of RanGAP2 and RanGAP1 sequences from potato, tomato and *N. benthamiana*.

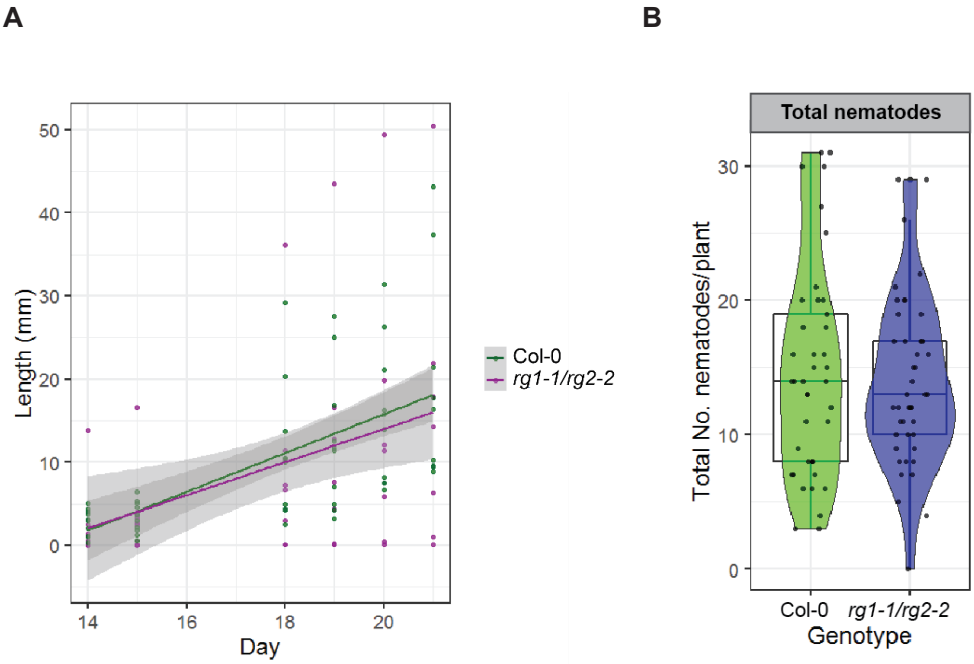
RanGAP1				
%	***S. tuberosum***	***S. lycopersicum***	***N. benthamiana***	***A. thaliana***
S. tuberosum		97.944	92.150	69.951
S. lycopersicum	97.944		84.857	70.195
N. benthamiana	92.150	84.857		69.343
A. thaliana	69.951	70.195	69.343	
RanGAP2				
%	***S. tuberosum***	***S. lycopersicum***	***N. benthamiana***	***A. thaliana***
S. tuberosum		95.683	90.499	67.764
S. lycopersicum	95.683		86.121	67.827
N. benthamiana	90.499	86.121		67.643
A. thaliana	67.764	67.827	67.643	



Supplemental figure 2. Poor silencing of RanGAP1/2 in tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*). Tobacco rattle virus (TRV) carrying guide DNA fragments targeting RanGAP1 (RG1_a and RG1_b), RanGAP2(RG2), both homologues (RG1+2) or green fluorescent protein as negative control was inoculated to the leaves of 10-day old tomato seedlings to induce transient virus-induced silencing of RanGAP1/2. **A)** RanGAP1 or **B)** RanGAP2 expression was measured by quantitative RT-PCR in TRV-infected plants and compared to the expression on TRV-GFP-infected plants. Values are normalized to the geometric mean of reference genes tubulin (Aimé *et al.*, 2013) and MST1. Individual samples are composed of ~10 plants/construct and RT-PCR measurements are performed in triplicate. TRV-mediated silencing was quantified 3 weeks after inoculation in the leaves of inoculated plants. Silencing was efficient for RanGAP1 using construct RG1_a **C)** 3 weeks after TRV infection plants were inoculated with ~12000 eggs of *G. pallida* (Rookmaker) and were grown for 2 months to allow completion of the nematode life cycle. After 2 months of nematode inoculation, cysts were extracted and counted from the complete root systems of plants with efficient RanGAP1 silencing. **D)** a similar set-up was used for TRV-mediated transient silencing in potato, with inoculum being ~1000 eggs. Cysts present in the roots of VIGS-potato were extracted and quantified and no difference was found between mean amount of cysts present in potatoes inoculated with RG1, RG2, RG1/2 and GFP-silencing TRV.



Supplemental figure 3. Expression of A) *RanGAP2* and B) *RanGAP1* in the roots of *H.schachtii*-inoculated Arabidopsis, after 2, 7, 10 and 14 days of inoculation. Expression compared to mock-inoculated plants was determined by quantitative RT-PCR. The relative expression of RanGAP1 and RanGAP2 was normalised to the geometric mean of reference genes Ubiquitin 5 (Anwer *et al.*, 2018) and ubiquitin carboxyl-terminal hydrolase 22 (Hofmann & Grundler, 2007). **C)** Size of female nematodes and syncytia established in the roots of *rg1-1* and *rg2-2*, with Col-0 as wild-type control. Sizes are shown in mm^2. Data from 4 biological repeats is combined, with means weighted by the inverse of the variance of each biological repeat. Stars indicate a significant difference as established by a linear fit, * p-value= 0.015 with n_{rg1-1} = 109, n_{rg2-2} = 80 and n_{Col-0}= 129



Supplemental figure 4. A *rg1-1/rg2-2* (SILK) double mutant is more susceptible to cyst nematode infection than *rg1-1* and *rg2-2* single mutants. A) Quantification of the length of SILK (*rg1-1/rg2-2*) Arabidopsis roots and Col-0 wild type over 6 days of growth (14,15,18,19,20 and 21 days after sowing). Length was quantified using an Epson Perfection v800 photo scanner (Epson) and WinRhizo (Regent Instruments) software of at least 7 plants, per genotype grown *in vitro*. Significant differences were evaluated by one-way ANOVA (p-value= 0.633). **B)** Total amount of *H. schachtii* infecting the roots of *rg1-1/rg2-2* and wild type after (xx) days of inoculation. *nematode* counts. $n_{rg1\text{-}1/rg2\text{-}2=43\ ;\ nCol\text{-}0}=41$. Significant differences were evaluated by one-way ANOVA (p-value=0.686). Data are representative of two biological repeats.

Supplemental table 2. List of primers used.

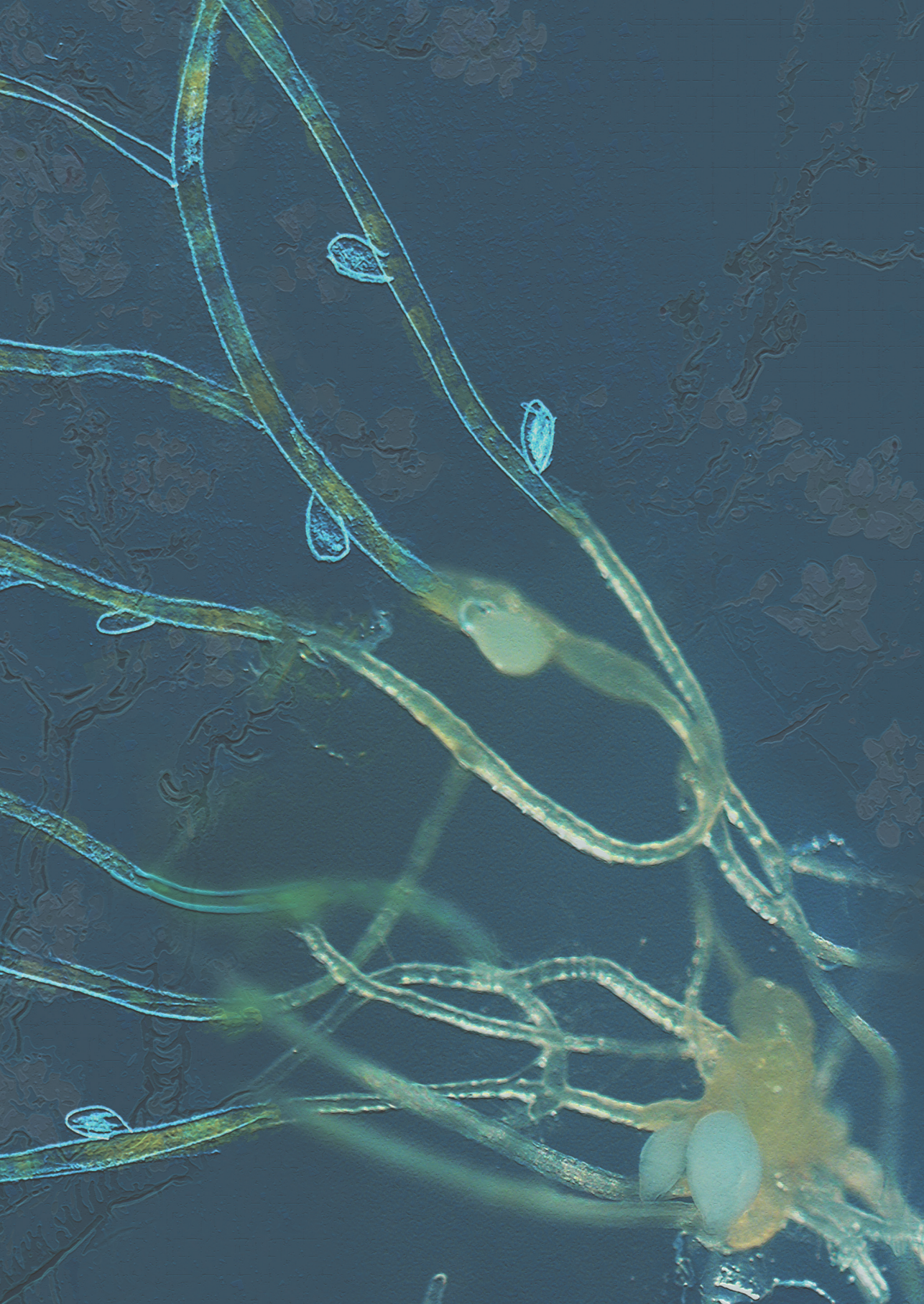
Genotyping	
Primer name	***Sequence***
SALK_058630_LP (*rg1-1*)	5-CAAAGGAACAAGCTTGTCCAG-3
SALK_123512_LP (*rg1-1*)	5-TCCATTTCTCCAACGAATCTG-3
SALK_006398_LP (*rg2-2*)	5-CTCCTCTGATCACAATCGGTC-3
SALK_006398_LP (*rg2-2*)	5-TGGTCTTTGGTTAAGCTACCG-3
RT-PCR primers	
Primer name	***Sequence***
Arabidopsis	
F-qPCR_AtRg1_001 F	5-GTGGAATTGCCCTGGCCAAG-3
R-q1_AtRg1_001 R	5-TGTTTGGAGGCGATGCAAGC-3
F-qPCR_AtRg2_001 F	5-AGGATTCTGTGTCTCCCCGC-3
R-q1_AtRg2_001 R	5-ATAGGCTCAGCGACACGAGC-3
At_qPCR-UBP22_001_F	5-ACAACATATGACCCGTTTATCGA-3 (Hofmann & Grundler, 2007)
At_qPCR-UBP22_001_R	5-TGTTTAGGCGGAACGGATACT-3
At_qPCR-UBQ5_001_F	5-GTTAAGCTCGCTGTTCTTCAGT-3 (Anwer *et al.*, 2018)
Tomato	
Sl_qPCR_Rg1_wo0123-1_F	5-CTTTTCTTCTGCTTTGGACGCC-3
R-q1_NbRg1_wo0124	5-GATTGATAGTACTTTGCTCAAGG-3
SL_qPCR_Rg2_001_F	5-GTTGCCGGAAGAAATGAGGC-3
SL_qPCR_Rg2_001_R	5-GCTCGCTAACAGCATTTGCAG-3
SlTUB_F	5-AACCTCCATTCAGGAGATGTTT-3 Aimé *et al.*, 2013
SlTUB_R	5-TCTGCTGTAGCATCCTGGTATT-3 Aimé *et al.*, 2013
MST1Fw	5-AGTGCAGCTCGTGTATGGTG-3
MST1Rv	5-ATCCAGAAGCACGCCATCT-3

Chapter 6

General Discussion

Amalia Diaz Granados Munoz[1]

[1] Laboratory of Nematology, Wageningen University, Wageningen, Netherlands



Preface

This thesis is contextualised in the understanding, at the molecular level, of the tactics that plant-parasitic nematodes exploit to establish and maintain infection within their plant hosts. These nematodes are recalcitrant parasites that cause agricultural losses in the order of billions of dollars per year (Jones *et al.*, 2013). In this thesis, I focused specifically on the interaction between cyst nematodes and their host plants. Together with root-knot nematodes, cyst nematodes from the genera *Globodera* and *Heterodera*, are some of the most sophisticated plant pests. Cyst nematodes are microscopic soil-borne sedentary endoparasites with a host range that includes economically important crops such as potato, tomato, soybean and beet root. Potato cyst nematodes alone cause an estimated 9% annual production loss in potato cultivation (Jones *et al.*, 2013).

A pivotal adaptation for plant parasitism was the emergence of nematode-secreted effectors (reviewed in Smant *et al.*, 2018). In the field of nematology, effectors are widely recognised as proteins secreted by the nematode through an oral stylet and delivered to the apoplast or cytoplasm of plant cells (reviewed in Vieira & Gleason, 2019). Once delivered to the plant or host cell, effectors interact either directly or indirectly with host molecules, collectively designated host or virulence targets. Through these interactions effectors modulate the biology of plant cells to successfully establish persistent infections (reviewed in Vieira & Gleason, 2019). Therefore, effectors effectively constitute the molecular front-line guiding the delicate interaction between the nematode and its host plant. From the plant point of view, it is important to detect and fend-off nematode attack, a task that is performed through an innate non-adaptive immune system. The immune system of the plant is based on receptors to detect the molecular signals associated with nematode invasion and ensuing cellular reactions that collectively restrain the ability of the nematode to complete its life cycle within the plant. Therefore, host targets and receptors are the plant counterparts in the molecular dialogue of the plant-nematode interaction.

To expand the knowledge of this molecular interaction, we investigated the role(s) of GpRbp-1, a member of the SPRYSEC family of nematode effectors (Blanchard *et al.*, 2005). We hypothesized that GpRbp-1 has a virulence role exerted through direct interactions with plant proteins. To elucidate this virulence role, our main goal was to identify and characterise host proteins that interact directly with GpRbp-1. We combined the use of untargeted and targeted interactomics to expand on the repertoire of host targets of SPRYSEC GpRbp-1 and their role in plant-nematode interactions. To further characterise candidate host targets of GpRbp-1, we validated the interactions *in planta*, examined the *in vivo* subcellular localization of the candidate interactors, and assessed the role of the candidate interactors by infection assays and transcriptomics in a model plant-nematode interaction system.

Overall, by scrutinising the molecular underpinnings of different aspects of the biology of plant-nematode interactions, we have identified a suite of host targets of the nematode effector GpRpb-1. Therefore, the results described here illustrate how the molecular dissection of the different aspects of plant-nematode interactions uncovers specific host proteins required for nematode virulence. In turn, this knowledge provides further insight

into the strategies employed by nematodes to establish themselves within the roots of their plant hosts. Additionally, characterisation of the pathogen and plant macromolecules found in the plant-pathogen interfase provides information required for the design of crop protection strategies. For instance, the virulence targets of nematode effectors could also be considered susceptibility factors, the products of so-called *S*-genes, and may be valuable targets to breed durable crop protection against plant-parasitic nematodes.

Act I: Un-targeted interactomics – introducing "the guests"

Scene I: Post-translational modification as target of nematode effectors

Un-targeted proteomics methods like yeast two-hybrid allow for high-throughput identification of protein-protein interactions *in vivo*. Such approaches have been successfully coupled with genomics, genetics, and computational effector prediction to identify the activities of effectors from several plant pathogens. Recent examples of the use of such methodology can be found in the research that identified and characterized Uvi1 and Uvi2 effectors from the biotrophic fungus *Ustilago hordei* (Ökmen *et al.*, 2018) and in the characterization of effector 4E02 from the beet cyst nematode *Heterodera schachtii* (Pogorelko *et al.*, 2019). For this thesis, we used an yeast two-hybrid cDNA screen to identify host targets of GpRbp-1 (Chapters 3 and 4). Interestingly, this un-targeted approach suggested a direct interaction of GpRbp-1 with plant proteins involved in post-translational modifications (PTMs), such as ubiquitination (Chapter 3) and SUMOylation (Chapter 4). We further validated these interactions *in planta* and evaluated the roles of E3 Ubiquitin-ligase UPL3 (Chapter 3) and SUMO E3-ligase SIZ1 (Chapter 4) in plant-nematode interactions by a reverse genetics screens and transcriptomics. Targeting of E3 ubiquitin ligases and E3 SUMO ligases had not been described previously for nematode effectors.

Since the discovery of enzymatic phosphorylation in the beginning of the 20th century, post-translational modifications have emerged as an important regulatory mechanism deployed in eukaryotic cells (Skelly *et al.*, 2016). These modifications do not require *de novo* protein synthesis, but instead rely on dedicated cellular machineries and substrates to alter the fate of a target protein (reviewed in Serrano *et al.*, 2018). PTMs are particularly relevant for plasticity in plants, allowing them to adapt to changing environments. More recently, the addition of Ubiquitin and other Ubiquitin-like small peptides has been found to regulate several aspects of cellular homeostasis (reviewed in Vierstra, 2012). Covalent conjugation of ubiquitin or small ubiquitin-like modifier (SUMO) results in protein degradation by the ubiquitin-proteasome system (UPS), modification of sub-cellular localization, protein activity or interaction with other proteins (reviewed in Verma *et al.*, 2018; Zhou & Zeng, 2017). Ubiquitination and SUMOylation take place through similar ATP-dependent enzymatic cascades catalysed by three main enzymes. E1 activating enzymes activate mature Ubiquitin/SUMO, which is then transferred to E2 conjugating enzymes. Subsequently, linkage of ubiquitin/SUMO to their target proteins is mediated by E3 ligases (reviewed in Verma *et al.*, 2018; Zhou & Zeng, 2017). In addition, specific ancillary proteins are involved in maturing SUMO and cleaving-off ubiquitin or SUMO from

specific targets (Colby *et al.*, 2006). Thereby, nematodes could potentially manipulate several cellular processes by targeting the plant proteins involved in post-translational modification.

Scene II: Ubiquitination

The involvement of **ubiquitination** in plant-nematode interactions remains largely un-explored. In contrast, several lines of evidence support the involvement of the UPS in infection by bacterial plant pathogens (reviewed in Banfield, 2015). For instance, U-box E3 ligases PUB12 and PUB13 are negative regulators of the immune response initiated by extracellular receptor FLS2, upon perception of bacterial PAMP flagellin 22 (Lu *et al.*, 2011). Additionally, it has been shown that bacterial effectors hijack the UPS of the host to suppress immunity (reviewed in Banfield, 2015). For example, coronatine from *Pseudomonas syringae* binds to F-box E3 ligase COI1 to promote jasmonic acid signalling and ultimately antagonise the immune responses against bacteria mediated by salicylic acid (SA) (Zheng *et al.*, 2012a).

A few studies provide indications that the UPS is also involved in plant-nematode interactions. For instance, effector GrUBCEP12 from *G. rostochiensis* may recruit the UPS to promote nematode virulence (Chen *et al.*, 2013; Chronis *et al.*, 2013). After delivery to the plant cell, GrUBCEP12 is processed into the carboxyl extension protein CEP12 and a single ubiquitin subunit. CEP12 supresses immunity mediated by intracellular immune receptors and basal defense responses induced by bacterial elicitor Flg22 (Chen *et al.*, 2013). Strong downregulation of a specific component of the proteasome suggests that the ubiquitin subunit plays a role by destabilising the proteasome during infection (Chronis *et al.*, 2013). In addition, the effector RHA1B from *G. pallida* was shown to act as a ubiquitin E3 ligase *in planta* and promotes nematode virulence. RHA1B supresses immune signalling in E3 ligase-dependent and independent manners (Kud *et al.*, 2019). Complementarily, we found the first nematode-secreted effector that binds a host protein involved in ubiquitination, E3 ubiquitin-ligase UPL3 (Chapter 3). UPL3 has been previously linked to regulation of endoreplication in trichomes in *Arabidopsis thaliana* (Downes *et al.*, 2003). However, we explored the role of UPL3 during cyst nematode infection using transcriptomics and showed that UPL3 is likely involved in the regulation of immune responses to nematode infection (Chapter 3). Therefore, we proposed that GpRbp-1 targets UPL3 to modulate defence responses mounted by the plant upon cyst nematode infection (Figure 1). A recent report by Furniss *et al* (2018) provides strong support for our hypothesis of the role of UPL3 in plant-nematode interactions by showing that in addition to the regulation of endoreplication, UPL3 is also involved on the regulation of plant immunity in Arabidopsis (Furniss *et al.*, 2018)2018. In the context of immunity, UPL3 functions as a proteasome-bound amplifier of SA-mediated immunity (Furniss *et al.*, 2018)2018. We were not able to assess directly the role of UPL3 during nematode infection of potato, due to a lack of stable *upl3* knock-out potato lines. Nevertheless, we established that UPL3 from *Solanum tuberosum* (StUPL3) has E3 ubiquitin-ligase activity *in planta* (Chapter 3). Therefore, it remains to be tested if knock-out or allelic variants of StUPL3 influence the immune response of potato to cyst nematode infection, and if StUPL3 may be involved in determining the outcome of cyst nematode infection in potato. Finally, a modulatory effect of GpRbp-1 of the E3 ligase activity of StUPL3 also remains to be established.

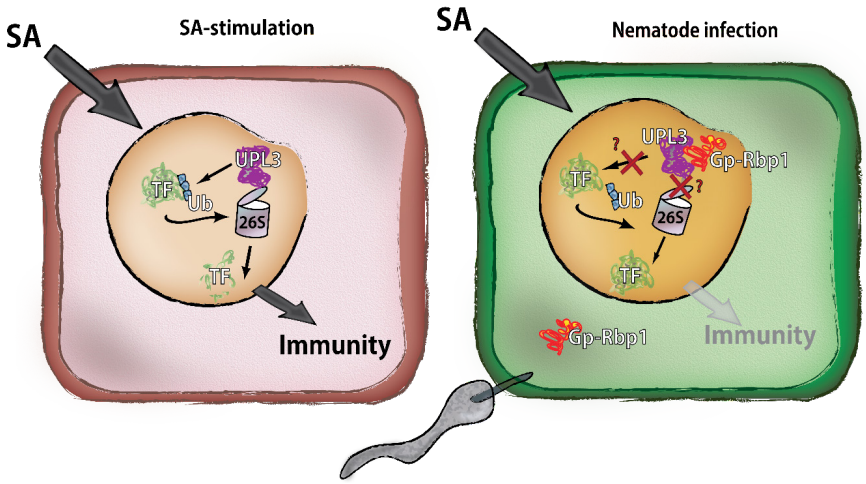


Figure 1. Proposed functional model for the interaction of GpRbp-1 with E3 ubiquitin-ligase UPL3. As established by Furniss, et al (2018), UPL3 is bound to the proteasome and amplifies the immune response mediated by SA through ubiquitination. The targets of this ubiquitination are proposed to be specific transcription factors (Chapter 3) which promote SA-mediated immunity. Interaction with GpRbp-1 may disrupt binding of UPL3 to the proteasome, ubiquitination of transcription factors or both and thereby prevent SA-mediated immunity that may halt nematode infection.

Scene III: SUMOylation

Similarly, the involvement of **SUMOylation** in plant-nematode interactions remained undescribed thus far. SUMOylation is one of the more recently discovered PTM (Kurepa *et al.*, 2003; Matunis *et al.*, 1996; Vierstra & Callis, 1999) and has since gained a lot of attention in the field of cellular and molecular plant biology (reviewed in Augustine & Vierstra, 2018; Verma *et al.*, 2018). Compelling evidence of the developmental relevance of SUMOylation comes from the embryo-lethal phenotypes of mutants of the components of the SUMOylation machinery (i.e. single mutants of the E1 activating enzymes *(sae1* and *sae2)*, the E2 conjugating enzyme (*sce1*), and the double mutants of SUMO (*sumo1 sumo2)* and E3 ligase enzymes (*siz1 mms21*) (Ishida *et al.*, 2012; Saracco *et al.*, 2007; van den Burg *et al.*, 2010). Furthermore, genetic studies with the single mutant of the E3 ligases SIZ1 and MMS21 have led to the identification of SUMOylation as a regulatory mechanism of cell cycle, DNA repair, morphology (e.g root development, photo and thermo-morphogenesis) (Ling *et al.*, 2012; Lin *et al.*, 2016; Ishida *et al.*, 2012; Catala *et al.*, 2007), nutrient homeostasis (e.g. nitrogen, phosphate) (Park *et al.*, 2011; Miura *et al.*, 2005; Castro *et al.*, 2015), hormone signalling (e.g. salicylic acid, auxin, abscisic acid) (Zheng *et al.*, 2012b; Miura *et al.*, 2009; Miura *et al.*, 2011; Lee *et al.*, 2006), tolerance to abiotic stress (e.g. exposure to heat, cold, drought, salinity) (Catala *et al.*, 2007; Chen *et al.*, 2011; Kim *et al.*, 2016b; Miura *et al.*, 2011; Miura & Nozawa, 2014; Miura *et al.*, 2005) and immunity (Gou *et al.*, 2017; Hammoudi *et al.*, 2018; Lee *et al.*, 2006; Niu *et al.*, 2019).

In particular, the E3 SUMO ligase SIZ1 functions as a regulator of several developmental processes in Arabidopsis (Miura *et al.*, 2005; Miura & Nozawa, 2014; Ling *et al.*, 2012; Lin *et al.*, 2016; Ishida *et al.*, 2012; Hammoudi *et al.*, 2018) , rice (Mishra *et al.*, 2018), cotton (Mishra *et al.*, 2017) and tomato (Zhang *et al.*, 2017). In the context of immunity, SIZ1 functions as a negative regulator of SA-dependent immunity in Arabidopsis, inhibiting accumulation of SA-responsive genes by an unknown mechanism (Lee *et al.*, 2006). Furthermore, SIZ1 represses transcriptional corepressor TPR1 and it is proposed that SIZ1-dependent SUMOylation may retain the inactivity of the SNC1/TPR1 immune complex to prevent auto-immunity (Gou *et al.*, 2017; Niu *et al.*, 2019). These regulatory roles could, therefore, constitute an appealing target for pathogen effectors, for example to prevent activation of plant immunity during infection. However, until now targeting of SIZ1 by pathogen effectors has not been reported. Here, we identified the first pathogen effector to target SIZ1 (Chapter 4).

The specific pathway regulated by SIZ1 and which is targeted by nematode effectors remains to be determined. From our results, we could envision three scenarios: targeting of SIZ1 by GpRbp-1 may enhance the role of the E3 ligase as negative regulator of SA-mediated immunity (Lee *et al.*, 2006). This would result in an enhanced susceptibility to nematode invasion as evidenced in our infection assays (Chapter 4). Alternatively, GpRbp-1 may modulate the role of SIZ1 as regulator of phytohormones to favour susceptibility. For example, by manipulating the role of SIZ1 in auxin patterning (Miura *et al.*, 2011), GpRbp-1 may promote the auxin-dependent formation of syncytia (Goverse *et al.*, 2000). Finally, GpRbp-1 may influence the specificity of the SUMOylation machinery to SUMOylate alternative targets, including GpRbp-1 itself. It is possible that upon secretion to the cell, GpRbp-1 recruits the post-translational machinery of the host to acquire modifications it requires for its function, such as SUMOylation (Figure 2). To elucidate further the cellular mechanisms that nematodes explore through targeting of SIZ1, it would be suitable to perform nematode infection assays using mutant plants with combined mutations in *SIZ1* and genes unique to the different molecular pathways that SIZ1 regulates (e.g. *siz1-2 nahG*, which abolishes the SIZ1-dependent hyper-accumulation of SA). Also, it is intriguing to investigate if GpRbp-1 is indeed SUMOylated in plant cells as suggested by the prediction of SUMO acceptor and SUMO-interactor sites in its coding sequence (Chapter 4).

In the context of biotic interactions, SUMOylation is involved in virulence of bacterial, oomycete, fungal and viral pathogens (Castaño-Miquel *et al.*, 2017; Colignon *et al.*, 2017; Kim *et al.*, 2013; Sanchez-Duran *et al.*, 2011). For instance, interaction of geminivirus replication protein AL1 and SCE1 (i.e. E2 conjugating enzyme) is required for viral infection (Sanchez-Duran *et al.*, 2011). Similarly, inhibition of SUMOylation by interference with E1-E2 interactions confers resistance to necrotrophic pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina* (Castaño-Miquel *et al.*, 2017). In addition, our results provide the first evidence Supplemental a role of SUMOylation in plant-nematode interactions (Chapter 4). This finding leads to research questions that will likely provide valuable new insight into the molecular underpinnings of plant-nematode interactions. It remains to be established, for example, if other proteins from the SUMOylation machinery are also required for susceptibility to nematode infection and if they might be targeted by other nematode-secreted effectors.

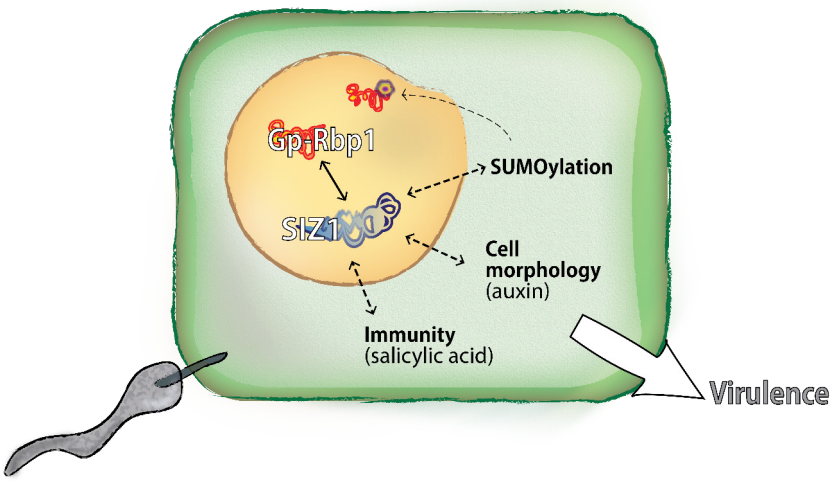


Figure 2. Model of possible outcomes of SIZ1 targeting by GpRbp-1. SIZ1 regulates several processes of the plant cell, including immune responses mediated by salicylic acid (SA) and auxin patterning {Lee, 2006 #39;Miura, 2011 #154}. Targeting of SIZ1 by GpRbp-1 (solid arrow) may influence one or more of these regulatory roles of SIZ1 to promote nematode virulence, as they are implicated in the immune responses against cyst nematodes and in the cellular re-programming required to establish syncytia (dashed arrows). Alternatively, GpRbp-1 require SUMOylation for its function in the plant cell, and may therefore highjack the SUMO E3 ligase activity of SIZ1 to acquire this modification.

Scene IV: The plot thickens

In an additional layer of complexity, mechanisms of PTMs can also interact with each other to fine-tune plant responses to different stimuli. Evidence of cross-talk has been found at the level of the PTM machineries as well as at the substrate level (Skelly *et al.*, 2016; Vu *et al.*, 2018). Ubiquitination and SUMOylation are currently proposed to interact by three different mechanisms: a) antagonistically by competing for the same residue or for separate residues of the same substrate, b) by synergistic feedback, or c) by degron regulation (Skelly *et al.*, 2016; Vu *et al.*, 2018). An example for antagonistic competition is proposed in gibberellin-dependent growth regulation. Here, DELLA proteins are degraded by the proteasome to promote plant growth (Murase *et al.*, 2008; Sun, 2010). On the other hand, salt stress promotes the SUMOylation of DELLA protein RGA, in lysine 65 (K65), thereby promoting its accumulation (Conti *et al.*, 2014). Mutation of K65 to arginine promotes RGA stabilization, suggesting that this residue is also a substrate for ubiquitination (Conti *et al.*, 2014). Therefore, SUMOylation is proposed to impede gibberellin-promoted ubiquitination and degradation of RGA (Vu *et al.*, 2018).

A classic example of a Ubiquitination-SUMOylation synergistic feedback loop is established between E3 ubiquitin-ligase COP1 and E3 SUMO-ligase SIZ1. COP1 regulates photomorphogenesis by promoting the degradation of several transcription factors (Ang *et al.*, 1998). For instance, transcription HY5 which supresses photomorphogenesis (hypocotyl

elongation) in the dark (Osterlund *et al.*, 2000). The E3 ligase activity of COP1 is promoted by SIZ1-mediated SUMOylation, resulting in increased degradation of HY5 (Kim *et al.*, 2016b). In turn, SIZ1-mediated SUMOylation of COP1 is counteracted by COP1-mediated ubiquitination and degradation of SIZ1 (Lin *et al.*, 2016). This SUMOylation-Ubiquitination interplay has an essential role in adjusting photomorphogenesis to changing light conditions during the day (Kim *et al.*, 2016a; Lin *et al.*, 2016).

Finally, the modification of master immune regulator NPR1 provides a good example for degron regulation by cross-talk of PTMs. NPR1 modulates the activities of several transcription regulators to confer immunity, including transcription factors of the TGA and WRKY families (Després *et al.*, 2000; Wang *et al.*, 2006; Zhou *et al.*, 2000). SUMOylation of NPR1 switches the interaction of NPR1 from WRKY repressors to TGA activators, which results in expression of the pathogenesis-related 1 *PR-1* gene (Saleh *et al.*, 2015). In addition, SUMOylation of NPR1 is inhibited by phosphorylation of specific serine residues, creating a PTM-associated regulatory mechanism of NPR1 activity (Saleh *et al.*, 2015). Other examples of cross-talk of ubiquitination and SUMOylation with other PTMs, such as phosphorylation, acetylation and nitrosylation are reported, but are outside of the scope of this thesis.

It is therefore possible that the interaction of GpRbp-1 with UPL3 and SIZ1 (Chapters 3 and 4) is indicative of a cross-talk of SUMOylation and ubiquitination mediated by these two ligases. Investigation of such cross-talk and a possible effect of GpRbp-1 binding, would first require testing if there is a co-dependency of UPL3 and SIZ1 activities. Any hypothesis in this area is highly speculative, but based on our finding that GpRbp-1 interacts with both UPL3 and SIZ1, a few possibilities can be mentioned. For example, to evaluate a hypothesis where SIZ1-dependent SUMOylation influences the attachment of UPL3 to proteasomes (Furniss *et al.*, 2018)2018, it would be relevant to perform protein-binding assays of UPL3 and the proteasome in a plant silenced for SIZ1. In this scenario, GpRbp-1 could act by enhancing the interaction of UPL3 and SIZ1. Alternatively, it is possible that a particular substrate requires ubiquitination and SUMOylation for its activity. GpRbp-1 could interact with UPL3 and SIZ1 to promote such modifications in order to regulate plant immunity or other processes required for establishment of a nematode feeding site. The identification of such substrate would be the biggest challenge in this scenario, but it could be tackled by comparative proteomics in *SIZ1* and/or *UPL3*-silenced backgrounds, in the presence of cyst nematode infection.

To finish, it is worth noting that in the characterization of effector targets, it is common practice to evaluate their roles by pathogen-infection assays on genetic knockout mutants of the host protein. Nevertheless, nematode bioassays produce phenotypic measurements with a high level of biological variation, resulting in wide ranges for statistical estimations of parameters. Consequently, the "infection phenotype" can be a rather crude criterion with which to appraise the subtleties of a biological system, as illustrated by our findings. In Chapters 3 and 4 we used a combination of such infection assays and whole-transcriptome analysis to investigate the roles of UPL3 and SIZ1 in plant-nematode interactions. Interestingly, this combination brought us diverse outcomes. Cyst nematode infection in *upl3-5* roots is only slightly reduced as compared to infection on wild-type *Arabidopsis* (Chapter 3). Normally, this result would have led to us to disqualify UPL3 as a genuine host factor for nematode

susceptibility. However, concomitantly, we also showed a strong response to nematode infection, mediated by *upl3*, at the transcriptomic level (Chapter 3). This disparity illustrates the plasticity or resilience of the plant, where several regulatory layers may compensate for a particular stress, be it pathogen infection or differential functionality of a gene. In contrast, we found a clear decrease in susceptibility to cyst nematode infection in *siz1-2* plants, an obvious indication of the requirement of SIZ1 in plant-nematode interactions (Chapter 4). Nevertheless, using a similar transcriptomic experiment, we could not provide clear evidence for what is the nature of such role of SIZ1. These somewhat opposed outcomes relay the need to combine different biological criteria and experimental designs to characterise the activity or activities of host factors in biotic interactions such as plant-nematode interactions.

Act II: Targeted interactomics – exploring "the host"

Scene I: The art of being indirect

Lacking an adaptive immune system, plants rely on constitutive defences and an inducible innate immune system to fend-off pathogen invasion (Jones & Dangl, 2006). Recently, the "Spatial Invasion/Immunity Model" has been proposed to conceptualize the main components of inducible plant immunity (Kanyuka & Rudd, 2019; van der Burgh & Joosten). Immunity depends on intra- and extracellular receptors (i.e. NB-LRRs, RLKs, RLPs and WAKs) which detect specific molecules related to pathogenic activity. Receptors detect signatures of pathogen invasion (i.e. PAMPs/MAMPs, DAMPs or effectors), and activate signalling pathways which trigger specific cellular mechanisms capable of halting the development of pathogens outside or inside of the plant (Kanyuka & Rudd, 2019; van der Burgh & Joosten).

Intracellular receptors mostly belong to the NB-LRR class and detect directly or indirectly, effectors secreted by plant pathogens (Takken & Goverse, 2012). A majority of the NB-LRR immune receptors described to date recognise their cognate effectors indirectly, requiring additional host co-factors. These co-factors are surveyed by classical NB-LRR receptors and are also targeted by pathogen effectors to either supress plant immunity or modulate the physiology of the host plant to favour pathogen virulence. Recently, the first crystal structure of an immune receptor-cofactor-effector complex was resolved (Wang *et al.*, 2019a). ZAR1 from Arabidopsis forms a constitutive complex with kinase RSK1. This complex indirectly recognizes effector AvrAC from *Xanthomonas campestris*, through co-factor PBL2 (Wang *et al.*, 2015). Uridylation of PBL2 by AvrAC binds and stabilizes the ZAR1-RSK1 complex, inducing a conformational change which activates ZAR1 (Wang *et al.*, 2019b). This conformational alteration leads to oligomerization of the PBL2-ZAR1-RSK1 complex into a pentamer, the so-called ZAR1 resistosome, which is required for disease resistance and the induction of the characteristic hypersensitive response (Wang *et al.*, 2019a; Wang *et al.*, 2019b). Due to the large diversity of NB-LRRs, co-factors and effectors, it remains to be seen if the role of PBL2 as a stabiliser of the ZAR1-RSK1 complex is similar for other virulence targets.

Characterisation of the binary role of effector targets as virulence targets and immune co-factors provides valuable insights into the strategies exploited by pathogens to infect

plants, as well as the strategies employed by plants to prevent infection. Depending on their specific role in susceptibility to pathogens, virulence targets are regarded as guardees, decoys, or integrated decoys (alternatively, sensor domains) of immune receptors (Wu *et al.*, 2015; van der Hoorn & Kamoun, 2008; Van Der Biezen & Jones, 1998; Dangl & Jones, 2001; Cesari *et al.*, 2014). Briefly, guardees are virulence targets which are bound or modified by effectors to promote pathogen virulence, and therefore modify the outcome of infection in the absence of the receptor (Dangl & Jones, 2001; Van Der Biezen & Jones, 1998). Decoys are mimics of virulence targets, which have evolved to lure effectors for immune recognition. Decoys do not function as virulence targets and therefore do not influence the outcome of infection in the absence of the receptor (van der Hoorn & Kamoun, 2008). Decoy domains have been found to be fused to some NB-LRR immune receptors (Cesari *et al.*, 2014). These integrated decoys have a similar molecular role as decoys, but seemed to have evolved by different mechanisms. Since proof is lacking to indicate that integrated decoys do not have a virulence role, they can also be termed sensor domains (Wu *et al.*, 2015). Finally, a "bait-and –switch" model was proposed where baits (guardees or decoys) have an alternative role in immunity by maintaining NB-LRR receptors in an auto-inhibited state (Collier & Moffett, 2009). Binding of the effector to the bait is an initial point of contact with the receptor complex, and subsequently interaction with the LRR is required to activate the receptor and induce immune signalling (Collier & Moffett, 2009). These models have been proposed to explain experimental findings from different plant-pathogen interaction systems, thereby highlighting the variety of mechanisms employed by pathogens and plants for pathogenicity and immune recognition. In the context of immune recognition models, a pressing question is the role of a given virulence target in susceptibility to a pathogen. Therefore, detailed mechanistic studies into these processes reveal further details of pathogenicity and immunity in plant-pathogen interactions.

Gpa2 is an NB-LRR immune receptor which is shown to confer resistance to the *G. pallida* population D383 in the field. Furthermore, recognition of effector GpRbp-1 by Gpa2 upon agroinfiltration of *N. benthamiana* leaves leads to a hypersensitive response characteristic of NB-LRR-mediated immunity (Sacco *et al.*, 2009). Activation of Gpa2-mediated immunity by GpRbp-1 requires the plant protein RanGAP as a co-factor, and a direct interaction of GpRbp-1 with RanGAP2 was previously implied by artificial tethering (Sacco *et al.*, 2009). In Chapter 5, we used a targeted approach to demonstrate that indeed a RanGAP-GpRbp-1 direct interaction occurs in plant cells and assessed the role of RanGAP in plant-nematode interactions. We showed that Gpa2 eliciting and non-eliciting GpRbp-1 variants directly interact with homologues RanGAP1 and RanGAP2, suggesting that binding to RanGAP is not the sole determinant of Gpa2 activation. Furthermore, both RanGAP homologues are required for susceptibility to cyst nematode infection, indicating that RanGAP is a functional virulence target of cyst nematode effectors. Therefore, we can conclude that RanGAP most likely functions as a guardee of Gpa2. Furthermore, RanGAP may function as a bait by maintaining Gpa2 in an inactive state. Conceivably, binding by GpRbp-1 may induce a conformational change in the Gpa2-RanGAP complex which activates Gpa2, similar to the mechanism of the ZAR1-PBL2 resistosome.

Scene II: RanGAP, at the centre of the stage

Homologous plant RanGAP1 and RanGAP2 are essential for plant development, as indicated by lethality of a double knock-out (Rodrigo-Peiris *et al.*, 2011). Several roles of RanGAP have been described, which may be co-opted by cyst nematodes. Phenotypically, plants with a significant decrease in RanGAP1/2 expression levels show deformed cotyledons, have a smaller rosette leaves and upon maturity are bushy, although not shorter than control plants (Boruc *et al.*, 2015)2015. Also, the roots of *rangap1/2* Arabidopsis have irregular cell shapes, with obliquely placed cell-walls and disorganised meristems (Boruc *et al.*, 2015)2015. Some of these developmental phenotypes implicate RanGAP in the regulation of the mitotic cycle in plants, for instance as an essential regulator of mitotic divisions in female gametophytes (Boruc *et al.*, 2015; Rodrigo-Peiris *et al.*, 2011; Xu *et al.*, 2008). On the other hand, in nematode induced syncytia, mitosis is observed in cells neighbouring the initial feeding site, and required for the radial expansion of syncytia (de Almeida Engler *et al.*, 1999). Nonetheless, the mechanisms underlying induction of mitosis in the cells adjacent to the initial feeding cell, remain largely uncharacterized. Therefore, a possible involvement of RanGAP in regulation of mitosis in syncytia warrants further investigation (Figure 3).

In addition, a noteworthy feature of RanGAP are the different cellular regions where it localises throughout the cell cycle, which are proposed to determine its role in regulation of mitosis. During interfase, RanGAP is associated to the nuclear envelope. Throughout mitosis, RanGAP labels the cell division plane (preprophase band) and during cytokinesis, it localizes to the phragmoplast and cell plate (Jeong *et al.*, 2005; Pay *et al.*, 2002; Rose & Meier, 2001; Xu *et al.*, 2008). These subcellular localisations of RanGAP are determined by the WPP domain, a triptophan-proline-proline motif believed to be unique to plant proteins (Jeong *et al.*, 2005; Pay *et al.*, 2002; Rose & Meier, 2001; Xu *et al.*, 2008). Since the WPP is also the domain that mediates interaction of GpRbp-1 with RanGAP (Chapter 5), it is tempting to hypothesize that GpRbp-1 interacts with this domain to interfere with the mitotic roles of RanGAP. However, the mitotic roles of RanGAP are dependent on its GAP activity, while its WPP-dependent subcellular localization seems to be dispensable for them (Boruc *et al.*, 2015)2015. Nevertheless, the conservation of the WPP domain indicates there is a cellular context in which WPP-targeting of RanGAP is necessary for the plant (Boruc *et al.*, 2015)2015. Interaction with pathogens may provide a mitotic requirement for WPP-dependent subcellular localization of RanGAP, and this mechanism may be co-opted by nematodes to promote susceptibility (Figure 3).

On the other hand, RanGAP has a vital role in the regulation of nucleocytoplasmic transport as the retention factor for Ran in the nuclear envelope (Ach & Gruissem, 1994; Merkle *et al.*, 1994; Rose & Meier, 2001). This localization of RanGAP is essential for the establishment of a RanGDP-RanGTP gradient, which drives the transport of molecules to and from the cell nucleus (Ach & Gruissem, 1994; Merkle *et al.*, 1994; Rose & Meier, 2001). Nucleocytoplasmic transport is known to play an important role in virulence of plant pathogens, and it has been mainly implicated in translocation of immune receptors or components of immune signalling pathways (reviewed in Rivas, 2012). Additionally, several pathogen effectors localize to the plant cell nucleus and require the host nucleocytoplasmic transport machinery

for translocation to this compartment (reviewed in Rivas, 2012; Wirthmueller *et al.*, 2013). For example, translocation to the nucleus of TALE effectors, secreted by two *Xanthomonas spp* rice pathogens, is dependent on their interaction with host importins (Hui *et al.*, 2019). Moreover, RanGAP mediates the nucleocytoplasmic distribution required for activation of the immune receptor Rx1 (Slootweg *et al.*, 2010; Tameling *et al.*, 2010). Heterologous expression of GpRbp-1 indicates this effector localizes to the nucleus and cytoplasm of plant cells (Jones *et al.*, 2009)2009. Thus, it is conceivable that targeting of RanGAP by GpRbp-1 could serve the purpose of mediating this characteristic nucleocytoplasmic distribution of GpRbp-1. This mechanism would be complementary to the findings of GpRbp-1 targeting nuclear E3 ligases (Chapters 3 and 4). This supports the need of the effector to be transported to the nucleus of plant cells and most likely relies on the transport systems of the plant for this activity (Figure 3). To elucidate if RanGAP mediates the subcellular distribution of GpRbp-1 and if this activity is dependent on interaction of the effector with the WPP domain, the localization of GpRbp-1 in *rangap* or *wpp*-RanGAP plants remains to be evaluated.

The findings that plant RanGAP is targeted by GpRbp-1 and is required for cyst nematode virulence in Arabidopsis, suggest it is a functional virulence target of nematode effectors (Chapter 5). Therefore, RanGAP is an important player in the interfase between virulence and immunity in plant-nematode interactions. As such, mechanistic details of its role as an immune co-receptor merit further research. In addition, it remains to be established if targeting of RanGAP by GpRbp-1, or other similar nematode effectors, is involved in support of the initiation, establishment and functioning of syncytia or in suppression of plant immunity.

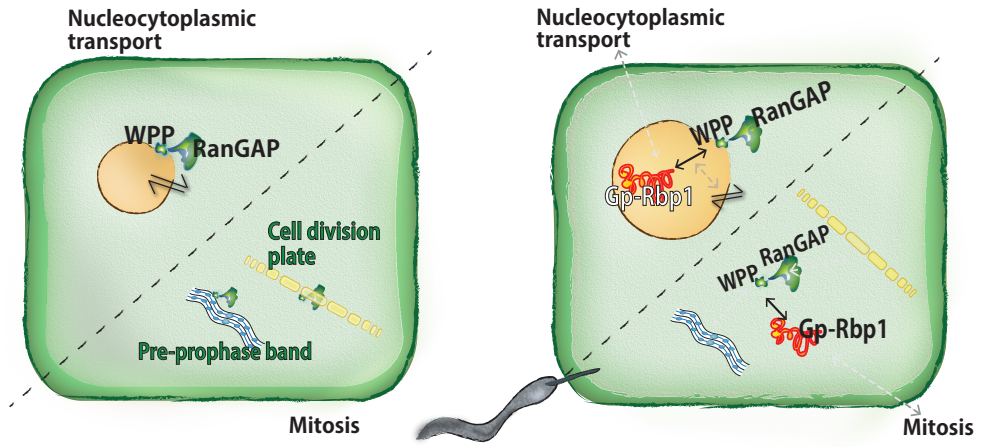


Figure 3. Models for the possible outcome of RanGAP targeting by GpRbp-1. In healthy cells (green), the WPP domain mediates localization of RanGAP to the nuclear envelope during interfase, where it regulates nucleocytoplasmic transport of macromolecules (Pay *el al.*, 2002; Rose & Meier, 2001). During mitosis, the WPP domain localizes RanGAP to the pre-prophase band and to the site of formation of the cell division plate in cytokinesis (Jeong *el al.*, 2005; Xu *el al.*, 2008). In nematode infected cells (red), GpRbp-1 may interact with the WPP domain of RanGAP (black arrows) to modulate any of these subcellular localizations. Thereby, GpRbp-1 may alter cellular nucleocytoplasmic transport or the mitotic cycle (grey dashed arrows) to induce a syncytium/specialized feeding cell.

Intermission: One effector, many targets

The findings described in this thesis also prompt consideration about the conceptual underpinnings of the functions of GpRbp-1 and the SPRYSEC family of effectors. First, the diversity of interactors of GpRbp-1 obtained using untargeted and targeted proteomics may suggest variability in the function of GpRbp-1. In addition to the E3 ligases characterized in this thesis (Chapters 3 and 4), approximately 25 candidate interactors were also identified with a yeast two-hybrid screen using GpRpb-1 as bait. In this case, it would be logical to question why GpRbp-1 interacts with several plant proteins. Indeed, this is not an uncommon finding in effector biology, where large sets of candidate interactors for particular effectors most likely combine technical artefacts, representations of diverse functions of a single effector, or indications of higher order complexes established between the effector and several host proteins. Sticky proteins can be present in a pool of candidate interactors of effectors, for different technical reasons. For instance, due to the loss of compartmentalization in plant cells when proteins are constitutively expressed or expressed in heterologous systems. Alternatively, due to alterations in the electric charge of the protein surface which take place after alternative folding or insertion of affinity tags. Another possible explanation are non-specific interactions with purification matrixes or other proteins (reviewed in Feller & Lewitzky, 2012; Miteva *et al.*, 2013). Therefore, complementary techniques should be used in different biological systems to validate a protein-protein interaction.

Similarly, multiple candidate or genuine interactors should be considered in the context of a dynamic cellular context. The interactors of GpRbp-1 shown in this thesis most likely represent genuine interactors and virulence targets of this effector. This suggests that GpRbp-1 may have different activities in the host cell. Most studies in the effector field focus on the mechanistic details of a binary interaction of an effector an its target, resulting in a conceptual bias implying effectors have unique activities inside the host cell. Nevertheless, it is becoming increasingly clear that single effectors commonly target multiple host proteins and have diverse activities. A good example is provided by the work of Khan *et al* (2018), showing that a majority of bacterial type-III effectors target several host proteins. Furthermore, the common methods implemented to describe protein-protein interactions are effectively snapshots of a cellular status and therefore limited in representing temporal regulation in living cells. Once secreted into the plant cell, effectors most likely interact with different host proteins either one-to-one or in complexes depending on factors such as the infection stage, cellular status (interfase or division), or the age of the host. Therefore, it is likely that the set of GpRbp-1 interactors contains genuine targets that interact separately with GpRbp-1 under specific conditions.

Targeting of multiple host proteins by a single effector is proposed to rely on multiple functional domains within a single effector (reviewed in Khan *et al.*, 2018). This may also be the case for SPRYSEC effectors, which contain hypervariable surfaces and a non-catalytic SPRY domain (Chapter 2). The function of the SPRY domain as a protein-binding module coupled with the presence of a hypervariable region in SPRYSEC effectors likely support the targeting of numerous host proteins and multiple activities of nematode SPRYSEC effectors.

Finally, in this thesis, complementary approaches to unravel the molecular components of the nematode-plant interface from different perspectives, allowed the identification of virulence targets of nematode effector GpRbp-1. From the nematode point of view, identification of effector targets suggests that the virulence role of GpRbp-1 is manipulation of the post-translational machinery of the plant to modulate salicylic acid-dependent plant immune responses (Figure 4). Additionally, from the plant point of view, characterisation of R-protein cofactors, suggests that GpRbp-1 may target nucleocytoplasmic transport of the host to favour virulence (Figure 4). A full mechanistic understanding of the virulence role of GpRbp-1 will require elucidation of several open questions. For instance, it remains to be established how binding by GpRbp-1 influences the roles or (concerted) activities of virulence targets UPL3, SIZ1, and RanGAP. Additionally, it would be interesting to test if SIZ1, UPL3, and/or RanGAP are part of a single complex with GpRbp-1, or interact with the effector individually. Also, it would be valuable to establish if RanGAP regulates the nucleo-cytoplasmic distribution of GpRbp-1 and if this influences or the physiology of the nematode feeding site. Similarly, it should be tested if GpRbp-1 manipulates SA-mediated plant immunity and if this function indeed requires either UPL3, SIZ1, or both. Eventually, it could be clarified if these virulence roles of GpRbp-1 are required for susceptibility of plants to nematodes and if the interactions and roles of GpRp-1 are unique to potato cyst nematodes or a trait common to other cyst nematodes and other plant-parasitic nematodes. For example, a SPRYSEC effector with high sequence similarity to GpRbp-1 is found in *G. mexicana*, however, closely related sequences remained to be fully identified in *G. rostochiensis*, *H. schachtii* and *H. glycines*.

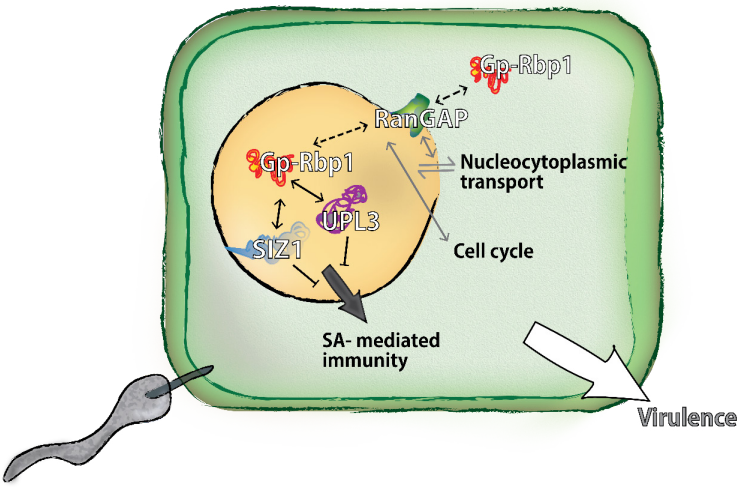


Figure 4. Graphical summary of the findings described in this thesis. GpRbp-1 interacts in the nucleus of plant cells with UPL3 and SIZ1 (black arrows), which may promote nematode virulence by modulation of plant immune responses mediated by salicylic acid. In addition, GpRbp-1 interacts with RanGAP in the nucleus and/or cytoplasm of plant cells (dashed arrows) and may thereby modulate the nucleocytoplasmic transport in plant cells, the mitotic division cycle or both, to promote nematode virulence (grey arrows).

Act III: Molecular encounters in the plant - pathogen interface

Scene I: Of Rs, Ss and effectors

Strategies to develop plant-based crop protection alternatives have mostly relied on the deployment of immune receptor genes to confer resistance to pathogens in the field. Nevertheless, the finding that recessive loss-of-function mutations on non-*R* genes may also confer resistance to plant pathogens, sparked the idea of using these genes as sources for more durable crop protection (Engler *et al.*, 2005; Pavan *et al.*, 2010). Susceptibility genes (*S*-genes) are defined as "dominant genes whose impairment will lead to recessive resistance" (Pavan *et al.*, 2010). Initially, two main mechanisms were proposed to lead to resistance after loss-of-function. Namely, impairment of negative regulators of plant immunity or impairment of genes which are strictly required for a compatible plant-pathogen interaction (Eckardt, 2002; Pavan *et al.*, 2010). For instance, *Mlo* from barley was the first *S*-gene to be described over 75 years ago (Freisleben & Lein, 1942; Jørgensen, 1992), and it is still implemented today to provide resistance to powdery mildew in barley and several other crops including tomato, pepper, wheat and grape (Acevedo-Garcia *et al.*, 2017; Pessina *et al.*, 2016; Zheng *et al.*, 2013). *Mlo* encodes a calmodulin-binding seven transmembrane domain protein, localized to the plasma membrane (Buschges *et al.*, 1997; Kim *et al.*, 2002). Intriguingly, the molecular activity remains uncharacterized, but it is proposed to function as a modulator plant immunity through association with membrane immune receptors of the receptor-like kinase family (reviewed in Acevedo-Garcia *et al.*, 2014). Another *S*-gene, *PMR6* was first described to confer resistance to powdery mildew in Arabidopsis (Vogel *et al.*, 2002). It encodes a pectate lyase and loss-of-function leads to a modified composition of the plant cell-wall and reduced susceptibility to powdery mildew (Vogel *et al.*, 2002). While the mechanism that leads to reduced susceptibility is not elucidated, it is believed to be independent of plant immunity as perception of immune hormones SA and ethylene is not required for reduced susceptibility of *pmr6* plants (Vogel *et al.*, 2002).

After *Mlo* and *PMR6*, several other examples of *S*-genes have been reported to confer resistance to fungal, bacterial, oomycete, viral, and nematode pathogens through the use of –omics technologies (Radakovic *et al.*, 2018; Warmerdam *et al.*, 2019) (reviewed in Hückelhoven *et al.*, 2013; Lapin & Van den Ackerveken, 2013; van Schie & Takken, 2014). For instance, genome-wide association mapping suggested that a RING/U-box E3 ligase and a MYB transcription influence resistance of soybean to cyst nematode *H. glycines* (Zhang *et al.*, 2016). In addition, a similar approach found the ethylene response factor ERF6 to influence susceptibility of Arabidopsis to root-knot nematode *Meloidogyne incognita* (Warmerdam *et al.*, 2019). Interestingly, *erf6* plants are more susceptible to infection, as compared to the wild-type (Warmerdam *et al.*, 2019). This finding challenges previous definitions of *S*-genes, showing that gain-of-function may also lead to a decrease in susceptibility. Therefore, the approach of screening populations for reduced susceptibility only, might overlook host genes that can be exploited by the pathogen to promote virulence.

Interestingly, several *S*-genes are also described to be virulence targets of effectors (reviewed in Hückelhoven *et al.*, 2013; Lapin & Van den Ackerveken, 2013; van Schie & Takken, 2014). For example, the sugar efflux transporters SWEETs are required for phloem loading in the plant and are differentially regulated by fungal and bacterial pathogens (Chen *et al.*, 2010; Chen *et al.*, 2012). In particular, SWEET11 and SWEET14 are targeted by TAL effectors from *Xanthomonas oryzae* pathovar *oryzae* (Chen *et al.*, 2010). Regulation of expression of particular sets of SWEETs is proposed to alter local patterns of sugar efflux to favour nutrient acquisition by pathogens. In this thesis we identify virulence targets of nematode effector GpRbp-1 (Chapters 2, 3 and 4) and thereby provide insight into host proteins, which may contribute to cyst nematode susceptibility in potato, and therefore function as susceptibility factors (products of *S*-genes). Furthermore, effector identification and functional characterisation of their host targets can accelerate the use of effectors as molecular probes to identify and deploy *S*-genes.

Abolishing effector binding or modification might provide an alternative to use *S*-genes as agricultural traits, while avoiding undesired effects of complete genetic loss-of-function. As agricultural traits, *S*-genes may support broad spectrum and durable resistance (lack of susceptibility). The durability of resistance relies on the fact that the evolutionary constraints posed to the pathogen are more difficult to overcome than those imposed by classical resistance mediated by *R*-genes. Nevertheless, difficulties of implementing *S*-genes as agricultural traits arise from possible pleiotropic effects of the mutations of genes which either negatively regulate immunity, or are involved in regulation of plant morphology (reviewed in Hückelhoven *et al.*, 2013; Lapin & Van den Ackerveken, 2013; van Schie & Takken, 2014). Additionally, *S*-genes involved in attraction or accommodation of biotrophic pathogens, might also be required for similar interactions with beneficial pathogens. Such is the case with the enzyme Sit (Sitiens; ABA aldehyde oxidase), which confers loss-of-susceptibility to necrotrophic fungus and bacteria, but leads to increased sensitivity to drought and a deficient interaction with beneficial arbuscular mycorrhizae (Asselbergh *et al.*, 2008; Audenaert *et al.*, 2002; Curvers *et al.*, 2010; Groot & Karssen, 1992; Harrison *et al.*, 2011; Herrera-Medina *et al.*, 2007; Martín Rodriguez *et al.*, 2010; Thaler & Bostock, 2004). In these cases, molecular characterisation of the effector activity is necessary to provide guidelines for strategies to impede effector activity without a disruption of the gene function.

Epilogue: Meeting half-way

With current "omics" techniques large datasets of candidate *S*-genes can be generated relatively fast. The most common current strategies implement Genome-Wide Association studies, to identify the genetic architecture underlying quantitative determination of susceptibility (Jain *et al.*, 2019; Warmerdam *et al.*, 2018). Also, with recent advances in automation of phenotyping, it can be coupled to reverse genetic screens (e.g. the Netherlands Plant Eco-Phenotyping Center). Finally, analysis of differential regulation of transcriptomes or proteomes may also identify genes or gene products relevant for susceptibility.

Similarly, large datasets of predicted effectors and effector targets can be quickly generated with computerised prediction models and high throughput interaction studies, as shown in studies to identify the interactome of *P. syringae* and Arabidopsis (Sahu *et al.*, 2014) and in the identification and validation of effectors and effector targets from the fungus *Melampsora larici-populina* (Petre *et al.*, 2015). In addition, system biology studies suggest that there is a core set of genes which is targeted by effectors from unrelated plant pathogens (Ahmed *et al.*, 2018; Mukhtar *et al.*, 2011; Weßling *et al.*, 2014). These concepts of effector biology can also provide input for the identification and characterisation of "core" *S*-genes. Nevertheless, validation and characterisation of individual *S*-genes, virulence targets, and immune co-receptors still requires the implementation of laborious detailed molecular, biochemical and biological methodologies, creating a bottleneck after candidate gene prediction. Therefore, evaluation of candidate *S*-genes for scientific or breeding purposes can be reinforced by comparison of the large datasets of candidate *S*-genes and candidate effector targets.

Scientifically, we might find substantiation of other principles of effector biology. For example, one could hypothesize that similar to the layers of the plant immune system (i.e. pre-formed defenses, apoplastic-triggered immunity, cytosolic-triggered immunity), there are layers to pathogen manipulation of hosts. For example, core effectors may target a specific subset of plant cell processes, which is likely preserved through evolution and/or shared with the interaction with beneficial microbes. In addition to this core effectors, a Supplemental layer of pathogen effectors might have evolved to protect or support the activities of the core effectors. Either by hindering detection by the immune system, by reinforcing their activities through functional redundancy or by regulating spatiotemporal dynamics of the core effectors within the plant cell. The functional coincidences between effector targets, *S*-genes and immune co-factors also paints a picture of a closely interconnected network of plant and pathogen-derived molecules that interact to mediate the plant-pathogen interaction. This is perhaps an interesting framework to conceptualize these interactions, instead of the current separation of disciplines and terms. So far, the scientific literature describes *S*-genes, virulence targets or immune co-factors somewhat independently of each other, perhaps it would be useful to think about these proteins (or other macromolecules) as the components of an interfase that exists when plants and pathogens interact.

For breeding purposes, characterisation of the most active elements of the pathogen interfase (such as of effector-targeted *S*-genes and/or *S*-genes acting as immune co-receptors) might provide additional tools to engineer pathogen-resistant plants (i.e. either resistant or less susceptible in the strict sense of each term). For example, by disrupting effector activity, to avoid undesired effects of complete loss-of-function. Additionally, by understanding the activities of immune co-factors, it could be possible to design immune receptors which survey specific *S*-genes. While it is currently not possible to bring these modifications to the field by genetic modification (in Europe), it is possible to screen segregating or wild-ancestor populations to find the desired mutations through marker-assisted breeding.

Lastly, the need to protect our crops will persist as an on-going process, since host-

pathogen systems are not static and inevitably remain subject to the constant change of evolution. Upon the implementation of specific strategies, progressively, new approaches will need to be deployed in pest management programs. Molecular biology studies, such as the work presented in this thesis, further our understanding of the mechanisms that regulate plant-pathogen interactions and the interactions in the interfase between plants and their pathogens. Additionally, they provide key knowledge and tools to efficiently and safely manipulate a system with such level of intricacy in its regulatory mechanisms.

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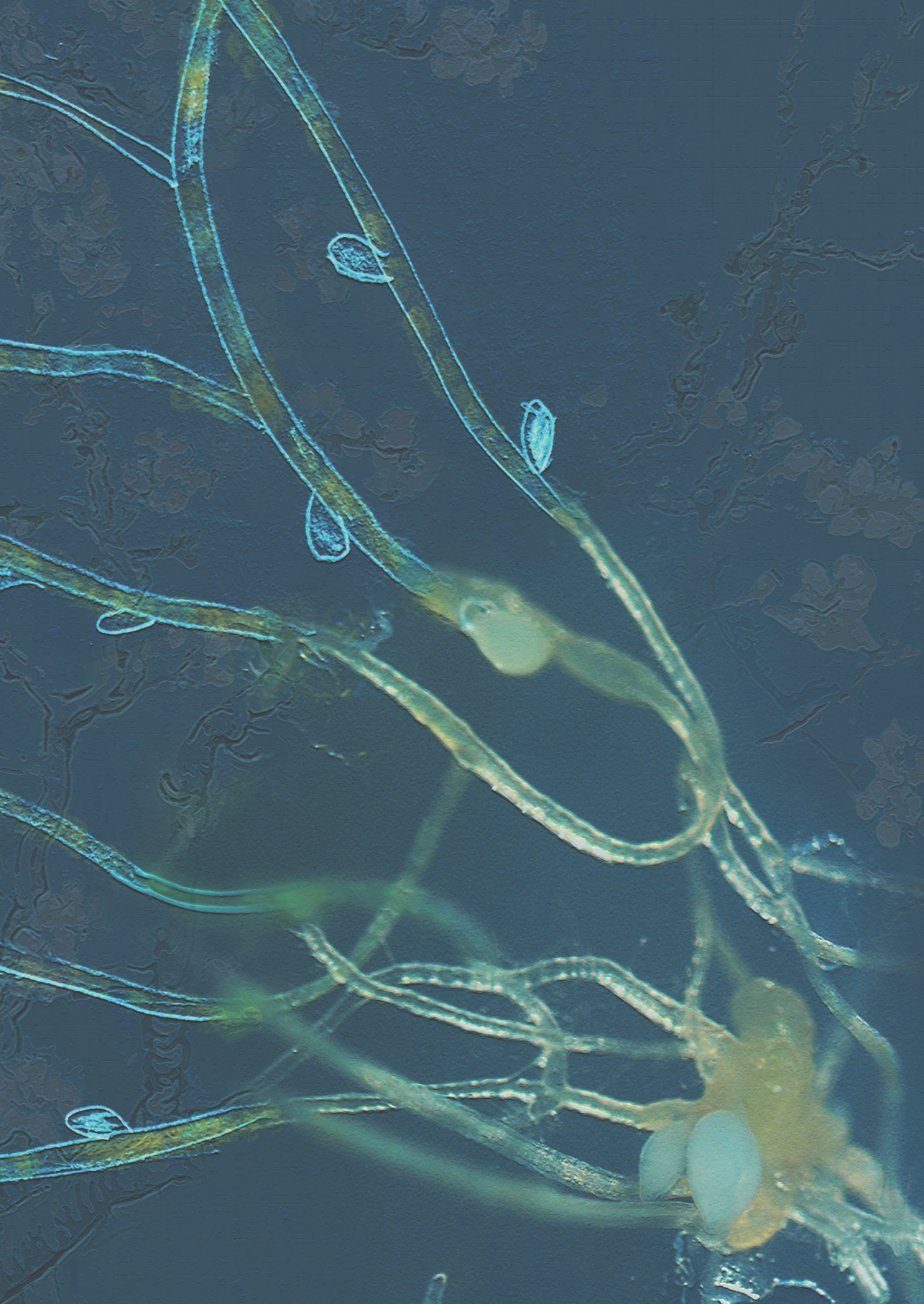

Summary

Plant parasitic nematodes are microscopic animals which have adapted their lifestyle to utilize plants as hosts. Moreover, sedentary nematodes, such as cyst nematodes, inhabit the roots of plants and depend entirely on the host to complete their life cycle. Once inside plant roots, cyst nematodes induce drastic modifications to plant cells to form a syncytium. This permanent feeding site redirects the assimilates in the plant to support the nematode, thereby reducing plant fitness and yield. At the molecular level, effectors secreted by the nematode mediate modifications which allow their establishment inside the plant. In addition, effectors are recognised by plants as distinctive signals of pathogen activity to trigger plant immunity. Therefore, characterisation of effectors and their host targets provides detailed knowledge about the strategies employed by nematodes to parasitize plants, and of plants to halt nematode infections. In turn, this knowledge may be used to design crop-protection strategies to minimize the multi-million economic losses caused by nematode infections in agriculture (**Chapter 1**).

The work presented in this thesis aimed to further understand the roles of SRYSEC effectors in nematode virulence. In particular, a subset of the plant targets of effector GpRbp-1 from the potato cyst nematode *Globodera pallida* were identified and characterized. **Chapter 2** provides an overview of the current knowledge of SPRYSEC effectors, a largely expanded family of effectors from potato cyst nematodes. SPRYSECs have been shown to activate and suppress plant immunity, but their roles in promoting nematode virulence are poorly characterized. The main structural characteristic of SPRYSEC effectors is a SPRY domain, which in organisms from different kingdoms acts as a mediator for protein-protein interactions. In addition, SPRYSECs contain hypervariable regions with large sequence variation between individual members of the family. Therefore, SPRYSECs are proposed to act as a versatile binding platform to modify the roles of their plant interactors and promote virulence.

Chapter 3 describes the identification and characterisation of the interaction of GpRbp-1 with E3 Ubiquitin ligase UPL3 from potato (*Solanum tuberosum*). StUPL3 was first identified as a candidate interactor of GpRbp-1 with a yeast two-hybrid screen. The interaction was validated *in planta* using epitope-based co-immunoprecipitations and bimolecular fluorescence complementation. Furthermore, using confocal laser scanning microscopy, we showed that StUPL3 localises to the nucleus of transiently transformed *Nicotiana benthamiana* leaves, where it also interacts with GpRbp-1. To further understand the activity of GpRbp-1, we evaluated the role of UPL3 during cyst nematode infection of Arabidopsis. The number of nematodes infecting the roots of *upl3* mutants is slightly reduced as compared to the wild-type. However, we found a large modifications to the transcriptional regulation of stress-related genes in *upl3* plants infected with nematodes. Together these results suggest that UPL3 is involved in modulation of stress responses to nematode infection, and that this role may be modified by GpRbp-1 to promote nematode virulence.

Similar approaches were used in **Chapter 4** to identify an interaction of GpRbp-1 with E3 SUMO ligase SIZ1 from potato. GpRbp-1 was shown to interact with StSIZ1 in yeast and *in planta* by yeast two-hybrid, epitope-based co-immunoprecipitation, and bimolecular

fluorescence complementation. In addition, the interaction was shown to take place in the nucleus of transiently transformed *N. benthamiana* leaves, where StSIZ1 localizes, similar to its homologue from Arabidopsis. The role of SIZ1 in cyst nematode infection was also evaluated in Arabidopsis, showing that susceptibility to infection is significantly reduced in *siz1* plants. StSIZ1 is involved in the regulation of several physiological processes of the cell, including immunity mediated by the plant hormone salicylic acid (SA). The findings in this chapter suggest that GpRbp-1 may target SIZ1 to modulate SA-mediated plant immunity to promote nematode virulence.

Chapter 5 describes the identification of Ran GTPase Activating Protein RanGAP2 as a virulence target of GpRbp-1. RanGAP2 is a co-factor required for immune responses triggered by NB-LRR receptor Gpa2 from potato, upon recognition of specific GpRbp-1 variants. First, epitope-based co-immunoprecipitations and analysis of Förster resonance energy transfer by fluorescence lifetime imaging (FRET-FLIM) were used to demonstrate a direct interaction of GpRbp-1 and RanGAP2. Interestingly, Gpa2-eliciting and non-eliciting variants of GpRbp-1 target RanGAP2, indicating that this interaction is not the sole determinant of Gpa2 activation. Moreover, GpRbp-1 variants also target the homologue RanGAP1, suggesting a role of both proteins in cyst nematode infections. Subsequently, a possible role of the role of RanGAP2 and RanGAP1 in plant-nematode interactions, was evaluated in the *Heterodera schachtii* – Arabidopsis model system. Both *rangap2* and *rangap1* plants are infected by less nematodes as compared to the wild-type, with a larger decrease in nematode numbers in *rangap1* plants. These results indicate that RanGAP2 and RanGAP1 may be virulence targets of GpRbp-1. Moreover, GpRbp-1 was shown to target a conserved motif of RanGAP2 (WPP domain), known to be required for localization of RanGAP2 to the nuclear envelope of plant cells. This finding suggest that GpRbp-1 may target and modify the activity or localization of RanGAP2 to promote nematode susceptibility.

In conclusion, this thesis shows that GpRbp-1 targets a number of host proteins involved in post-translational modification and nucleo-cytoplasmic transport. Targeting of these proteins most likely leads to modulation of plant immunity to promote nematode virulence. In **Chapter 6** the implications of the main findings of this thesis are considered, including the possibility that these virulence targets may be recruited by GpRbp-1 to act in concert for the promotion of plant susceptibility to nematodes. The experimental approaches and findings presented in this thesis are also placed in the wider perspective of recent advances in the field of plant-pathogen interactions. Finally, we consider the contributions of unravelling the molecular mechanisms exploited by pathogens to infect plants, to the formulation of novel plant protection strategies.

Resumen

Los nematodos parásitos de plantas son animales microscópicos que a través de la evolución han logrado colonizar las plantas y utilizarlas como hospedantes. En particular los nematodos sedentarios, como los nematodos del quiste, habitan en las raíces de las plantas y dependen completamente de este hospedante para completar su ciclo de vida. Luego de penetrar las raíces, los nematodos del quiste inducen drásticas modificaciones a las células vegetales con el fin de generar un sitio permanente de alimentación, denominado sincitio. Esta estructura especializada redirecciona los nutrientes de la planta para alimentar el nematodo, lo que resulta en una disminución en el vigor y productividad de la planta. Estas modificaciones celulares, que permiten el establecimiento de los nematodos dentro de la planta, son soportadas a nivel molecular por productos secretados por los nematodos, denominados efectores. Por su lado, las plantas reconocen los efectores como indicadores de actividad patogénica, lo que les permite activar su sistema inmune y prevenir o repeler las infecciones por parte de estos parásitos. Es por esto que la caracterización de los efectores y sus objetivos o blancos dentro de la planta permite estudiar en detalle las estrategias usadas por los nematodos para infectar las plantas, al igual que las estrategias empleadas por las plantas para impedir las infecciones con nematodos. Los resultados obtenidos por este tipo de estudio, pueden ser a su vez utilizados para diseñar estrategias de protección de cultivos que permitan minimizar las millonarias pérdidas causadas anualmente en el sector agrícola, por las infecciones con nematodos (**Capítulo 1**).

El trabajo presentado en esta tesis está dirigido a elucidar las funciones del grupo de efectores SPRYSEC (por su sigla en inglés), en la virulencia de los nematodos. En esta investigación, se estudió en particular un subconjunto de los blancos del efector GpRbp-1 secretado por el nematodo del quiste *Globodera pallida*. En el **capítulo 2** se presenta una síntesis del estado del arte sobre los efectores SPRYSEC, una familia de efectores con una gran expansión en los nematodos del quiste de la papa. Aunque se ha demostrado que los efectores SPRYSEC pueden tanto activar como suprimir el sistema inmune de las plantas, la actividad de estos efectores que les permite promover la virulencia de los nematodos aún no se ha caracterizado. Estructuralmente estos efectores se caracterizan por contener un dominio SPRY, que en organismos de diferentes reinos funciona como un intermediario en interacciones proteina-proteina. Adicionalmente, los SPRYSECs contienen regiones hipervariables determinadas por una gran variación en la secuencia primaria de miembros individuales de la familia. Por lo tanto, se cree que los efectores SPRYSEC pueden funcionar como una plataforma versátil con capacidad para modificar los roles de sus interactores en la planta, para promover la virulencia de los nematodos.

En el **capítulo 3** se describe la identificación y caracterización de la interacción entre GpRpb-1 y la E3 ubiquitin ligasa UPL3 (por su sigla en inglés) de papa (*Solanum tuberosum*). Inicialmente se identificó a StUPL3 como un interactor de GpRbp-1 en una librería de papa con el método del doble híbrido de levadura. A continuación, la interacción se validó utilizando co-inmunoprecipitaciones basadas en epítopos y complementación bimolecular de fluorescencia. Adicionalmente, se utilizó microscopía confocal láser de barrido para demostrar

que StUPL3 se localiza en el núcleo de células epidermales de ***Nicotiana benthamiana*** transformadas transitoriamente, donde también interactúa con GpRbp-1. Posteriormente se evaluó la función de UPL3 durante la infección por nematodos del quiste en Arabidopsis, para determinar cuál es la posible acción de GpRbp-1 sobre UPL3. Se encontró que el número de nematodos capaces de establecer una infección, en las raíces de mutantes ***upl3,*** tiene una diferencia mínima comparado con el número presente en Arabidopsis de tipo silvestre. Esto sugiere que UPL3 no tiene una función determinante durante el proceso de infección. Sin embargo, se encontró una modificación significativa en la regulación transcripcional de genes relacionados con respuestas a estrés, en plantas ***upl3*** infectadas con nematodos. En conjunto estos resultados indican que UPL3 está implicada en la modulación de las respuestas contra la infección por nematodos, siendo esta la actividad que puede ser modificada por medio del efector GpRbp-1 para promover la virulencia de los nematodos.

En el **capítulo 4** se utilizaron técnicas similares para identificar la interacción de GpRbp-1 con la E3 SUMO ligasa SIZ1 (por su sigla en inglés) de papa. Aquí se demostró que GpRbp-1 interactúa con StSIZ1 tanto en levadura como ***in planta*** utilizando co-inmunoprecipitaciones y complementación bimolecular de fluorescencia. Adicionalmente, se demostró que la interacción entre estas dos proteínas ocurre en el núcleo de células de ***N. benthamiana*** transformadas transitoriamente, donde se localiza StSIZ1 al igual que su proteína homologa de Arabidopsis. La actividad de SIZ1 durante la infección por nematodos se evaluó en Arabidopsis y se encontró que la susceptibilidad a nematodos es significativamente menor en las plantas mutantes ***siz1-2***, comparadas con Arabidopsis tipo salvaje. Se encontró también que StSIZ1 está implicada en la regulación de varios procesos fisiológicos de la célula, incluyendo la inmunidad mediada por la hormona vegetal ácido salicílico (SA, por su sigla en inglés). Los hallazgos presentados en este capítulo sugieren que GpRbp-1 podría interactuar con SIZ1 para modular la inmunidad mediada por SA para promover la virulencia de los nematodos.

En el **capítulo 5** se describe la identificación de la Proteina Activadora de la RanGTPasa (RanGAP2, por su sigla en inglés) como un blanco de virulencia de GpRbp-1. RanGAP2 es un cofactor necesario para la activación de las respuestas inmunes mediadas por el receptor Gpa2 de papa. Este receptor, perteneciente a la familia NB-LRR activa respuestas inmunes a partir del reconocimiento de variantes específicas del efector GpRbp-1. En primer lugar, se utilizó co-inmunoprecipitación basada en epítopes y medición de la vida media de fluorescencia por transferencia de energía de resonancia (FRET-FLIM por su sigla en inglés), para demostrar la interacción directa entre GpRbp-1 y RanGAP2. Vale la pena mencionar que RanGAP2 es blanco tanto de variantes elicitantes y no elicitantes de Gpa2, lo que indica que la interacción con RanGAP2 no es el determinante único de la activación de Gpa2. Adicionalmente, algunas variantes de GpRbp-1 también interactúan con la proteína homologa RanGAP1, lo que siguiere que tanto RanGAP2 como RanGAP1 tienen una actividad relevante en las infecciones por nematodos del quiste. Por lo tanto, se estudió el posible rol de RanGAP2 y RanGAP1 en estas infecciones utilizando el sistema modelo ***Heterodera schactii – Arabidopsis thaliana***. Con esta evaluación se encontró que tanto las plantas ***rangap2*** como las ***rangap1*** tienen un menor índice de infección que las plantas tipo salvaje, siendo el índice de las plantas ***rangap1*** el más bajo. Estos resultados indican que RanGAP2 y RanGAP1 son blancos de virulencia

del efector GpRbp-1. Por otra parte, se estableció que GpRbp-1 interactúa con un dominio altamente conservado de RanGAP2 (dominio WPP por su sigla en inglés), que está a su vez implicado en la localización de RanGAP en la envoltura nuclear de las células vegetales. Este hallazgo sugiere que GpRbp-1 puede modificar la actividad y la localización de RanGAP2 (y RanGAP1) para promover la susceptibilidad a los nematodos.

Para concluir, en esta tesis se demuestra que GpRbp-1 tiene como blanco varias proteínas de la planta que están a su vez implicadas en los mecanismos de modificación post-transcripcional y de transporte nucleo-citoplasmático. Es probable que la utilización de estas proteínas como blancos de virulencia determine la modulación de la inmunidad de la planta ,para promover la virulencia de los nematodos. Las implicaciones de los resultados más importantes de esta tesis se consideran en el **capítulo 6**, incluyendo la posibilidad de que estos blancos de virulencia sean reclutados como un conjunto para modular la susceptibilidad de las plantas a nematodos. En esta sección también se analizan desde un perspectiva más amplia los resultados y técnicas experimentales mencionados en esta tesis, considerando los avances más recientes en el campo de las interacciones planta-patógeno. Finalmente se considera cómo la disección de los mecanismos moleculares que utilizan los patógenos para infectar las plantas, resulta útil para la formulación de nuevas estrategias para la protección de cultivos.

Samenvatting

Plant parasitaire nematoden zijn microscopisch kleine worpjes die voor hun overleving en voortplanting gebruik maken van planten. Sedentaire nematoden, zoals bijvoorbeeld de cystenaaltjes, zijn voor wat betreft hun ontwikkeling volledig afhankelijk van planten. Hierbij veranderen de cystenaaltjes meerdere plantencellen in de wortels van hun gastheer in een syncytium, dat na inductie functioneert als een permanente bron voor nutriënten. Het voeden van nematoden via een syncytium gaat ten koste van de fitness van geïnfecteerde planten. De vorming van het syncytium is een reactie van de plant op zogenaamde effector eiwitten in het speeksel van de nematoden. Sommige van deze effectoren worden echter ook herkend door het immuunsysteem van planten en kunnen een afweerreactie ingang zetten. Het bestuderen van effector eiwitten in het speeksel van nematoden en de bijbehorende moleculaire targets in waardplanten kan inzicht geven in de wijze waarop parasitisme tot stand komt. Dit soort inzicht kan helpen bij het ontwikkelen van nieuwe gewasbeschermingsstrategieën om de economische impact van plant parasitaire nematoden te beperken (**hoofdstuk 1**).

Het doel van dit proefschrift is het ontrafelen van de rol van SPRYSEC-effectoren in de virulentie van nematoden. Meer in het bijzonder is gekeken naar de moleculaire targets van de effector GpRbp-1 van het aardappelcystenaaltje *Globodera pallida* in waardplanten. GpRbp-1 behoort tot één van de meest uitgebreide genfamilies in het genoom van de aardappelcystenaaltjes (**hoofdstuk 2**). Het was al bekend dat SPRYSEC-effectoren betrokken zijn bij de activatie en onderdrukking van het afweersysteem van planten, maar een mogelijke rol bij virulentie was nog niet goed onderzocht. Kenmerkend voor SPRYSEC-effectoren is het zogenaamde SPRY-domein, waarvan in andere organismen vast staat dat het betrokken is bij eiwit-eiwit interacties. Een ander opvallend kenmerk van de SPRYSEC-effectoren is de aanwezigheid van hypervariabele regio's in het SPRY-domein, waardoor er veel variatie in aminozuur sequentie bestaat tussen verschillende leden van de SPRYSEC-genfamilie. Deze informatie vormde de basis van de hypothese dat cystenaaltjes SPRYSEC-effectoren gebruiken als een multifunctioneel platform om verschillende moleculaire targets in waardplanten te kunnen manipuleren ten behoeve van hun virulentie.

Hoofdstuk 3 betreft de beschrijving van het E3 ubiquitine ligase UPL3 uit aardappel (*Solanum tuberosum*) als mogelijk moleculair target van GpRbp-1. De interactie van StUPL3 met GpRbp-1 is ontdekt met behulp van een *yeast-two-hybrid s*creening en vervolgens gevalideerd met co-immunoprecipitatie en bi-moleculaire fluorescentie complementatie. Met confocale microscopie bleek verder dat de interactie tussen GpRbp-1 en StUPL3 ook optreedt in de kernen van bladeren van *Nicotiana benthamiana* als ze beide tegelijk heteroloog tot expressie worden gebracht. Mutanten van *Arabidopsis thaliana* met verminderde expressie van het homologe *AtUPL3* zijn niet significant meer of minder vatbaar voor cystenaaltjes dan wildtype planten. Niettemin, heeft deze mutatie een groot effect op de transcriptionele regulatie van stress genen, maar verrassend genoeg alleen in nematode geïnfecteerde worteltjes. Dit suggereert dat UPL3 betrokken is bij de modulatie van stress in nematode geïnfecteerde planten, en dat de effector GpRbp-1 hierop aangrijpt ten behoeve van de virulentie van de nematoden.

In een soortgelijke benadering is ook het E3 SUMO ligase SIZ1 van aardappel naar voren gekomen als mogelijke tweede interactor van GpRbp-1 (**hoofdstuk 4**). Deze interactie is ook ontdekt in *yeast-two-hybrid* screening en gevalideerd met co-immunoprecipitatie en bi-moleculaire fluorescentie complementatie. GpRbp-1 en StSIZ1 interacteren ook met elkaar in kernen van bladeren van *N. bethamiana* in een heteroloog co-expressie systeem. De vatbaarheid van een Arabidopsis mutant met verminderde expressie van het homologe AtSIZ1 was wel significant lager dan dat van wildtype Arabidopsis planten. Van AtSIZ1 is bekend dat het betrokken is bij allerlei fysiologische processen in plantencellen, waaronder de regulatie van het immuunsysteem door het hormoon salicylzuur. Deze data suggereren dat de effector GpRbp-1 SIZ1 als doelwit heeft om afweerreacties van planten te moduleren, die afhankelijk zijn van salicylzuur signalering.

Het derde moleculaire target van GpRbp-1, dat in **hoofdstuk 5** van dit proefschrift is beschreven, is het Ran GTPse Activating Protein RanGAP2. RanGAP2 fungeert als co-factor van de NB-LRR immuunreceptor Gpa2 uit aardappel en is nodig voor de herkenning van GpRbp-1 door dit resistentie eiwit. Bij specifieke celdood inducerende varianten van GpRbp-1 zet deze herkenning een afweerreactie in werking. Uit co-immunoprecipitaties en analyses van *Förster resonance energy transfer by fluorescence lifetime imaging* data blijkt dat GpRbp-1 direct bindt aan RanGap2, zelfs in afwezigheid van Gpa2. Opmerkelijk genoeg interacteren varianten van GpRbp-1 die geen celdood induceren toch met RanGAP2. Dit betekent dat naast RanGAP2 nog andere factoren nodig zijn voor de activatie van Gpa2-afhankelijke afweerreacties. Verder is gebleken dat GpRbp1 ook interacteert met RanGAP1, een homoloog van RanGAP2. Arabidopsis knock-out mutanten van zowel RanGAP1 en RanGap2 zijn minder vatbaar voor infecties door cystenaaltjes, wat duidt op het belang van deze eiwitten bij virulentie. GpRbp-1 lijkt specifiek het zogenaamde WPP-motief in RanGAP2 te binden. Van dit motief is bekend dat het de lokalisatie van RanGAP2 in de kernenvelop bepaald. Het is mogelijk dat GpRbp1 dit specifieke motief bindt om de lokalisatie van RanGAP2 te beïnvloeden, om zo de vatbaarheid van planten te kunnen veranderen.

Tot slot, dit proefschrift toont aan dat de effector GpRbp1 vermoedelijk via post-translationele modificaties en nucleo-cytoplasmatische transportmechanismen de vatbaarheid van waardplanten voor cystenaaltjes verandert. Het beïnvloeden van deze processen geschiedt waarschijnlijk door gelijktijdige manipulatie van verschillende moleculaire targets. In **hoofdstuk 6** van dit proefschrift worden de implicaties van deze waarnemingen bediscussieerd en in de bredere context van recente ontwikkelingen binnen het veld van plant pathogeen interacties geplaatst. Verder wordt in dit afsluitende hoofdstuk beschreven hoe deze kennis gebruikt zou kunnen worden voor het ontwerpen van nieuwe gewasbeschermingsstrategieën.

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Isi, you are also one of my oldest friends in Wageningen, and I am so happy you are still here! Thank you for your friendship and for not giving up on inviting me to places even though I have only a third (or less) of your energy. Thank you also to **Leah** and **Lau**, and for always being fun and wise. **Kathi** and **Jimmy** (somehow you always end up being the one guy among a lot of women...) I only see you in the hallways these days, but I've got great memories of our younger years and great appreciation for you.

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And now we turn back to Radix for more great people. **Magda, Chara, Aranka, Valeria, Corien,** it was fantastic having you around to support each other, do sports together and conquer the PhD together. Big hugs to each one of you! **Eleni, William, Ale, Daniel M** thank you for accepting me in your lunch group and for making it fun to be the "elder". Thank you also to everyone from the EPS PhD council, especially **Kim, Jordi P, Magda, Peter, Manos, Kiki, Hana, Jordi B, Chris** and **Tanya**, I really enjoyed our time together and wish you all the best with your postdocs and jobs! A big special thank you to **Iliana**, for patiently helping me to make sure this book looks great. And for your friendship, of course!

Véronique, Daniel, Christophe et **Eliane**. Je veux vous remercier beaucoup de m'avoir accepté et accueilli dans votre famille. Et aussi de m'avoir donné une maison loin de ma maison, avec des plats vraiment délicieux ;) Finalement, merci de nous donner à Bruno et moi un bel exemple de compassion et d'unité.

To my friends from years back, **Cami, July M, Ana, Pascui, July V, Maria, Davis, Juan Pa, Cata, Cris,** you are great foundations, inspiration, and support. I love that every time we meet, we can come back to our younger selves and realize that despite the distance, time does not erode our friendship.

Los tíos **Gloria, Alvaro, Alvaro** and **Beatriz** are one of the most important pillars of my life. You have always loved me, supported me, motivated me, advised me, spoiled me and welcomed me generously into your homes. I am truly lucky to be part of this family! I am especially happy and grateful to have had **Alvaro** as advisor for all the statistics in this thesis. Thank you for your patience and constant availability to help me. También gracias a toda la familia **Diazgranados**, por tenerme en sus pensamientos y brindarme su cariño, a pesar de la distancia.

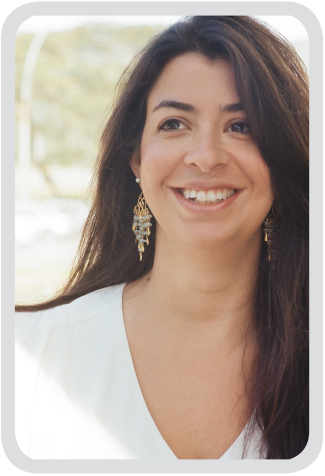
Dear **Bruno**, first as per your request, I publicly acknowledge the DELICIOUSNESS of all the meals you single-handedly prepared so I could work late many many evenings, to finish my thesis (I told you I'd put it in haha). You wrote in your thesis "I hope to be as supportive for you during your PhD as you have been of me", and boy, have you lived up to that promise!!! Not just with tasty food. You've served as cheerleader, sounding board, career strategist, psychologist, and even travel agent. I am sure this journey would have been a LOT harder without your love and dedication. You are one of the most sweet, caring, supportive and bad jokers I know, and all these qualities have been equally important for keeping me happy and sane. I will forever be grateful for the possibility to share these years with such an amazing human being. And last, sorry dearest **Dr Sovran**, soon you will loose your monopoly over the title at home :P.

Finalmente, a mis **papás,** mi más profunda gratitud. Gracias por su compañía permanente a pesar de la distancia y por acomodar sus vidas para que yo pudiera alcanzar esta meta. Sin su amor incondicional, su ejemplo, sus esfuerzos y sacrificios, su ayuda, consejos y apoyo, sencillamente no hubiera logrado dar este paso. La gratitud y felicidad reflejada en los mensajes para esta larga lista de personas, es posible sólo porque ustedes siempre han estado presentes como el motor de mi vida.

About the author

Amalia Diaz Granados Muñoz was born on June 7th, 1986 in Medellín, Colombia. She was passionate about animals and understanding nature from an early age.

After graduating from high school, she studied Biological Engineering at the Universidad Nacional de Colombia in Medellín. Despite being a biologist at heart, she pursued engineering as this combination would allow her a solid mathematical background. It was during this time that she became interested in molecular biology and how it could explain the functioning of life. During her BSc she had the opportunity to do an internship at Johns Hopkins University, (Baltimore, USA) working in the groups of Dr. Chris Zink and Dr. Chloe Thio. This experience shaped her conviction to work in molecular biology, but taught her a career involving interventions on animals was not in her horizon.



Back in Colombia, she pursued a Specialization in Biotechnology, at the Universidad Nacional de Colombia. It was here that she discovered the wonderful world of Plant Sciences, thanks to her literature review of plant immunity against soil-borne pathogens in the group of Prof. Mauricio Marín (Unal, Medellín).

To fulfill a long-time dream to study abroad, she then pursued an MSc in Molecular Life Sciences at Wageningen University, working in research on plant-pathogen interactions in the groups of Dr. Geert Smant (Wageningen) and Dr. Hans Thordal-Christensen (Copenhagen) for her thesis and internship, respectively. She had the privilege to be offered a PhD position in the group of Dr. Geert Smant in the Laboratory of Nematology of Wageningen University, where this thesis was carried out.

Towards the end of her PhD, Amalia had the honor to be awarded a position as postdoctoral researcher in the group of Prof. Richard Immink in the group of Plant Developmental Systems of Wageningen Research. Having entered a new field, she continues to learn about plants and how they interact with their environment, and hopes to make a worthy contribution to the “Climate-proof plant reproduction” project.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to:	**Amalia Diaz Granados Muñoz**
Date:	**24 October 2019**
Group:	**Laboratory of Nematology**
University:	**Wageningen University and Research**

1) Start-Up Phase	*date*	*cp*
▶ **First presentation of your project**		
SPRYSEC effectors from potato cyst nematodes: manipulators of host protein ubiquitination for suppression of immunity?	30 Oct 2014	1.5
▶ **Writing or rewriting a project proposal**		
▶ **Writing a review or book chapter**		
Diaz-Granados, A., Petrescu, A.-J., Goverse, A. and Smant, G. (2016). SPRYSEC Effectors: A Versatile Protein-Binding Platform to Disrupt Plant Innate Immunity. Frontiers in Plant Science, 7. 10.3389/fpls.2016.01575	20 Oct 2016	1.0
▶ **MSc courses**		
Subtotal Start-Up Phase		2.5

2) Scientific Exposure	*date*	*cp*
▶ **EPS PhD student days**		
Get2gether 2015	29 to 30 Jan 2015	0.6
Get2gether 2016	28 to 29 Jan 2015	0.6
Get2gether 2017	09 to 10 Feb 2017	0.6
▶ **EPS theme symposia**		
Theme 2 symposium 2016	22 Jan 2016	0.3
Theme 2 symposium 2017	23 Jan 2017	0.3
Theme 2 symposium 2018	24 Jan 2018	0.3
▶ **Lunteren Days and other national platforms**		
Annual meeting Experimental Plant Sciences Lunteren 2014	14 to 15 Apr 2014	0.6
Annual meeting Experimental Plant Sciences Lunteren 2015	13 to 14 Apr 2014	0.6
Annual meeting Experimental Plant Sciences Lunteren 2016	11 to 12 Apr 2016	0.6
Annual meeting Experimental Plant Sciences Lunteren 2017	10 to 11 Apr 2017	0.6
Annual meeting Experimental Plant Sciences Lunteren 2019	08 Apr 2019	0.3
▶ **Seminars (series), workshops and symposia**		
Pathogenomics Seminar S. Kamoun	28 May 2014	0.1
EPS flying seminar Jane Parker	21 Jan 2016	0.1
EPS flying seminar Hans Thordal	12 Dec 2016	0.1

	Symposium farewell Ton Bisseling	8 Feb 2017	0.2
	EPS flying seminar Gerben van Ooijen	29 May 2017	0.1
	EPS flying seminar Martin Cann	13 Jul 2017	0.1
	Symposia “Changing and creating life” (100 years WUR)	14 Mar 2018	0.3
	Talk Fangming Xiao (guest Nematology)	20 Mar 2017	0.1
	Talk Nicole van ‘t Wout Hofland (guest Nematology)	5 Sep 2018	0.1
	Nematology Symposium KeyGene	26 Sep 2018	0.1
	Talk Sebastian Eves van der Akker (guest Nematology)	13 Feb 2019	0.1
	Seminars Ronnie de Jonge and Andrea Sanchez Vallet (EPS)	20 Feb 2019	0.2
	Talk Tina Kyndt (guest Nematology)	17 May 2019	0.1
	Talk Jijie Chai (guest Phytopathology)	5 Jun 2019	0.1
►	**Seminar plus**		
	Discussion EPS flying seminar Hans Thordal	12 Dec 2016	0.1
	Discussion EPS flying seminar Gerben van Ooijen	29 May 2017	0.1
	Discussion EPS flying seminar Martin Cann	13 Jul 2017	0.1
►	**International symposia and congresses**		
	International Society for Molecular Plant-Microbe Interactions (IS-MPMI) meeting (2014), Rhodes, Greece	06 to 10 Jul 2014	1.2
	COST Sustain meeting (2014), Zakopane, Poland	15 to 17 Oct 2014	0.9
	International Molecular Plant-Nematode interactions group (IMPNIG) meeting (2015)	17 to 18 Sep 2015	0.6
	COST Sustain meeting (2016), Banyuls sur mer, France	17 to 19 Feb 2016	0.9
	European Society of Nematology Conference (ESN) (2016), Braga, Portugal	29 to 01 Sep 2016	1.2
	European Society of Nematology Conference (2018), Ghent, Belgium	09 to 13 Sep 2018	1.2
►	**Presentations**		
	Poster presentation 2014 (COST Sustain)	15 Oct 2014	1.0
	Poster presentation 2015 (Lunteren Experimental Plant Sciences meeting)	13 Apr 2015	1.0
	Poster 2016 (Lunteren and COST)	11 Apr 2016	1.0
	Poster Lunteren 2019	09 Apr 2019	1.0
	Oral presentation IMPNIG meeting 2015	17 Sep 2015	1.0
	Oral presentation ESN symposium Braga 2016	30 Aug 2016	1.0
	Oral presentation EPS Lunteren meeting 2017	11 Apr 2017	1.0
	Oral presentation ESN symposium Gent 2018	13 Sep 2018	1.0
►	**IAB interview**		
►	**Excursions**		
	Company visit Enza Zaden - EPS PhD council	12 Jun 2015	0.2
		Subtotal Scientific Exposure	21.7

3) In-Depth Studies	*date*	*cp*
► **Advanced scientific courses & workshops**		
Spring school, Host microbe interactomics	02 to 04 Jun 2014	0.9
Genome assembly	28 to 29 Apr 2015	0.6
Basic Statistics	22 Jun to 01 Jul 2016	1.5
Introduction to R	06 to 07 Jun 2017	0.6
Chemometrics	26 to 30 Jun 2017	0.9
The power of RNASeq	11 to 13 Jun 2018	0.9
Data Analysis and Visualizations in R	08 to 09 May 2018	0.6
► **Journal club**		
► **Individual research training**		
Subtotal In-Depth Studies		6.0

4) Personal Development	*date*	*cp*
► **General skill training courses**		
Techniques for writing and presenting a scientific paper	14 to 17 Apr 2015	1.2
Project and Time Management	12 Jan to 23 Feb 2015	1.5
PhD Competence assessment	24 Sep 2014	0.3
EPS Introduction course	22 Sep 2015	0.2
Adobe InDesign Essential Training	29 to 30 Sep 2015	0.6
NWO “Pump your career” 2015	19 Nov 2015	0.3
Scientific Artwork with Photoshop and Illustrator	01 to 02 Mar 2016	0.6
Reviewing a scientific paper	22 Sep 2016	0.1
Career orientation	04 to 25 Oct 2016	1.5
Wageningen Career day 2016	02 Feb 2016	0.3
WGS PhD Workshop Carousel 2017	07 Apr 2017	0.3
Last stretch of PhD and writing propositions	27 Nov 2018	0.0
► **Organisation of meetings, PhD courses or outreach activities**		
Organization Lunteren PhD hour and poster award 2015 (EPS PhD council)	14 Apr 2015	0.1
Volunteer Fascination of Plants Day 2015	16 May 2015	0.1
Organization EPS Get2gether 2017	09 to 10 Feb 2017	0.1
Organization Lunteren PhD hour 2018 (EPS PhD council)	09 to 10 Apr 2018	0.1
Organization Flying seminars: Hans Thordal, Gerben van Ooijen, Martin Cann	(See above)	0.3
► **Membership of EPS PhD Council**		
EPS PhD council member	2015-2017	1.4
Subtotal Personal Development		9.0

TOTAL NUMBER OF CREDIT POINTS*	**39.2**

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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