On the modulation of innate immunity by plant-parasitic cyst nematodes

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“Le sentiment de l'absurdité au détour de n'importe quelle rue

peut frapper à la face de n'importe quel homme”

(At any street corner the feeling of absurdity can strike any man in the face)

Albert Camus – *Le Mythe de Sisyphe* (1942)

Contents

1

General Introduction

1.1 Plant-parasitic nematodes

Nematodes (roundworms, belonging to the phylum Nematoda) are amongst the most abundant animals on this planet. Over 28,000 species have been described to date (Wyss, 2002), which is less than 3% of the total number of nematode species that are estimated to exist (De Deyn et al., 2003). Nematodes live in many and very diverse habitats but the vast majority is found in the soil. The bulk of all nematode species are free-living, others have evolved a parasitic lifestyle on animals, and an estimated 15% are plant parasites. More than 4,100 species of plant-parasitic nematodes have been described in 197 genera (Wyss, 1997). The sheer variety and abundance of plant-parasitic nematodes ensures that almost all plants will encounter them. This makes nematodes a crucial driving factor for plant succession in natural environments (De Deyn et al., 2003). In agriculture, nematodes are recognized as a major threat for food security with annual worldwide crop losses estimated to exceed 118 billion US dollars (McCarter, 2009).

The majority of the plant-parasitic nematodes are root parasites (Kile et al., 2002; Woo et al., 2006a). Depending on their parasitic strategy, plant-parasitic nematodes can be divided into 5 classes (Lambshead, 2004). Ectoparasites feed on root tissue without entering the plant. They feed for short periods of time on a selected site (e.g. migratory ectoparasites, such as *Tylenchorhynchus* and *Longidorus* spp.), or feed for many days on the same cells or root section (e.g. sedentary ectoparasites such as *Paratylenchus* and *Criconemella* spp.). Semi-endoparasites are nematodes that can feed ectoparasitically but often partially enter the root tissue to feed on cortical or outer stelar cells (e.g. *Hoplolaimus* and *Helicotylenchus* spp.). Endoparasitic nematodes feed inside the host root. They may migrate through the root while feeding (e.g. migratory endoparasites such as *Pratylenchus* and *Radopholus* spp.), or spend a prolonged time in association with a host cell inside the root (sedentary endoparasites). Among the latter are the notorious root-knot nematodes (i.e. *Meloidogyne* spp.) and cyst nematodes (i.e. *Heterodera* and *Globodera* spp.). Root-knot nematodes and cyst nematodes are

major pests of several important food crops, including potato and tomato, and they are the most studied and therefore currently the best characterized group of plant parasitic nematodes.

1.2 Sedentary endoparasitic nematodes

Sedentary endoparasitic nematodes maintain a complex relationship with their host. As obligate parasites, the outcome of their relationship with the host plant determines whether their life cycle can be completed. The life cycle starts with the hatching of infective juveniles from eggs upon the perception of chemical cues released by the roots of a host plant into the soil. Next, the infective juvenile nematodes migrate to the roots of the host plant. Root-knot nematodes penetrate the root tip and stealthily move intracellularly towards a vascular cylinder cell without causing significant damage to host tissues. Cyst nematodes, in contrast, reach vascular cylinder cells in a more destructive fashion, as they force their way through cells of the root cortex. Both root-knot and cyst nematodes use a protrusible oral stylet to penetrate a suitable cell close to the vascular cylinder and inject secretions that transform the cell into a feeding structure. This feeding structure nurtures the nematode until it reaches the adult stage (Gheysen and Mitchum, 2009). Root-knot nematodes and cyst nematodes use different strategies for the formation of their feeding structures. Root-knot nematodes induce repeated mitosis without cytokinesis in several cells close to its head, resulting in several discrete giant cells each of which may contain over a hundred enlarged nuclei. The surrounding hyperplastic tissue forms a root-knot or gall around the giant cells (Caillaud et al., 2008). Cyst nematodes induce partial cell wall dissolution and subsequent fusion of the protoplast of the initial feeding cell and an increasing number of adjacent cells, resulting in the formation of a syncytium (Sobczak et al., 2009).

Cyst nematodes feed off the syncytium until maturity. Mature male cyst nematodes exit the roots to fertilize the females. Females lose their locomotory

muscles at the onset of the sedentary phase and remain associated with the syncytium until the end of their life cycle (De Boer et al., 1992). The body cavity of a fertilized female swells and fills with eggs, and after the nematode's death the body wall hardens into a cyst. Protected by the cyst, the eggs can remain dormant for years, making cyst nematode infestations particularly persistent and difficult to eradicate. The golden cyst nematode (*Globodera rostochiensis*) and white cyst nematode (*Globodera pallida*) infect solanaceous plants including the major crop species potato and tomato. Most of the current potato cyst nematode populations originate from the same area in south Peru (Sijmons et al., 1994), and were dispersed by humans along with propagative material to most of the potato growing areas of the world. Potato cyst nematodes increasingly cause problems in crop production, with estimated annual losses in the UK alone of approximately 70 million US dollars, or 9% of the total potato yield (Plantard et al., 2008). Worldwide yield losses are estimated to exceed 10% (Oerke et al., 1994). Because pesticides and crop rotation provide only limited success in the control of potato cyst nematodes, the control of potato cyst nematodes is largely dependent on a few major resistance genes that are crossed into potato cultivars. However, the successive use of such cultivars has selected virulent nematodes that can overcome resistance (Molinari, 2011), and for that reason new sources of natural resistance are highly desired.

1.3 The plant immune system

Unlike animals, plants lack adaptive immunity supported by an efficient circulatory system with mobile effector cells and associated humoral responses. Instead, plants effectively defend themselves against attacks by a myriad of invaders with a limited innate immune receptor repertoire. Plant innate immunity is often portrayed as a multi-layered system that reflects the evolutionary history of plant immune receptors (Jones and Dangl, 2006; Dodds and Rathjen, 2010). As a first line of defense numerous physical and chemical barriers exist, such as a thick epidermis, wax layers on leaves, and detoxifying enzymes, which limit the

possibility of serving as a host for most pathogens. This is known as non-host resistance (Nicol et al., 2011). The operatives in the first line of active defense are pattern recognition receptors (PRRs) that recognize pathogen- or microbe-associated molecular patterns (PAMPs) (Zipfel, 2009). PAMPs are common epitopes of pathogenic microbes that are indispensable and do not naturally occur in healthy plants. This enables plants to recognize an array of microbes with a limited number of PRRs. After detecting these conserved molecular patterns, PRRs can activate a basal level of broad-spectrum resistance that is defined as PAMP-triggered immunity (PTI). However, some successful strains of pathogens circumvent or suppress PTI by delivering effector molecules into the apoplast or cytoplasm of host cells (Jones and Dangl, 2006). Plants equipped with highly specific immune receptors that recognize these effectors or their actions (i.e. resistance or R proteins) can activate immune signaling pathways that result in effector-triggered immunity (ETI). R protein-mediated resistance often provides complete resistance to a specific pathogen species, population, or strain. Some plant pathogens turn the table again by using other or modified effectors that suppress ETI, or by deletion or mutation of the recognized effector (Lipka et al., 2005; Ali and Bakkeren, 2011).

Sedentary endoparasitic nematodes are usually able to enter the roots of both host and non-host plants (Kaplan and Keen, 1980). In the latter, the nematodes subsequently fail to initiate a feeding site. In incompatible plant-nematode interactions, PTI-like responses such as reactive oxygen species (ROS) production (Waetzig et al., 1999b) and callose deposition (Grundler et al., 1997) have been observed. However, PAMPs of plant parasitic nematodes that trigger PTI responses remain to be discovered. A range of resistance loci that confer partial or quantitative resistance to sedentary endoparasitic nematodes has been identified in several plant species, including fourteen loci in potato (Tomczak et al., 2009b). However, only a few *R* genes underlying resistance traits against nematodes have been cloned. Resistant host plants seem to exploit the inherent vulnerability of the nematode's dependence on the feeding structure, since ETI responses against

sedentary endoparasitic nematodes are mostly directed against the giant cell or syncytium. The result appears to be the isolation of the feeding structure from the vascular cylinder, causing stagnation of the development of the nematode. For example, the cells of a layer positioned between the syncytium and the xylem cells of the vascular cylinder undergo programmed cell death in the *Hero*-mediated resistance to *G. rostochiensis* in tomato (Sobczak et al., 2005). In potato, *Gpa2*-mediated resistance to *G. pallida* and *H1*-mediated resistance to *G. rostochiensis* results in defense-related programmed cell death in cells surrounding the syncytium (Rice et al., 1985; Koropacka, 2010). Depending on the timing and amplitude of the resistance response, the result ranges from death of the nematode to a significant reduction of adult females, or a higher ratio of nematodes that develop into males due to a reduction in feeding site size (Tomczak et al., 2009b). The only effector of a plant parasitic nematode currently connected to a nematode resistance response in plants is the *G. pallida* SPRYSEC protein named Rbp1. Co-expression of *Rbp1* and the resistance gene *Gpa2* in *Nicotiana benthamiana* leaves results in Gpa2-mediated programmed cell death (Sacco et al., 2009). To date, a direct physical interaction between Gpa2 and Rbp1 has not been shown. Since the feeding site of the nematode is of existential importance for the nematode, it is assumed (but not shown) that nematodes produce effectors that down-regulate immune responses and defense-related programmed cell death.

1.4 Secretions of plant-parasitic nematodes

Sedentary endoparasitic nematodes produce numerous secreted proteins in three single-celled esophageal glands that are connected to the oral stylet via the lumen of the esophagus. Two of these glands are subventrally localized and are most active during host invasion and during the transition from migratory to sedentary phase. Secretions produced during host invasion consist of cell wall degrading enzymes and possibly many other molecules that aid in migration, protection of the nematode, and protection against plant immune responses (Haegeman et al., 2012). The dorsal gland is most active during feeding site formation and

maintenance in the sedentary phase. Proteins and other molecules produced in these glands are secreted through the stylet into the apoplast and cytoplasm of host cells. Secretions injected through the stylet into the feeding cell are thought to be instrumental to the formation, maintenance and protection of the syncytium, and feeding (Haegeman et al., 2012). In order to successfully parasitize a host plant the nematode probably needs to modulate host immunity. It is likely that the secretions produced by plant parasitic nematodes contain immune modulating effectors, but as reviewed in chapter 6 of this thesis, the current evidence for such effectors is scarce. It is likely that a plethora of effectors is secreted by sedentary endoparasitic nematodes, some of which will be recognized by the plant and may trigger resistance responses, while others will aid infection by modulating host immunity.

1.5 Thesis outline

This thesis is a study on interactions of cyst nematodes with the plant immune system, and the role of effectors secreted by potato cyst nematodes (*Globodera rostochiensis*) in the protection of the nematode and its feeding site. In **chapter 2,** we describe the importance of the plant's immune system in compatible plant-nematode interactions. To do so, we compared the infectivity of beet cyst nematodes (*Heterodera schachtii*) on different immune signaling mutants of *Arabidopsis thaliana* plants. In **chapter 3,** we describe the identification of the SPRYSEC effector family of *G. rostochiensis*. Using molecular techniques, immunodetection, and bioinformatics we characterize several SPRYSEC effectors. We used a yeast-two-hybrid screening to identify host proteins that interact with SPRYSECs. One of the interacting host proteins is a member of the *SW5* resistance gene cluster in tomato. **Chapter 4** describes the functional implications of the interaction between SPRYSEC-19 and the putative R protein SW5F. This work centered on two hypotheses. First, we tested whether the physical interaction between SPRYSEC-19 and SW5F activates programmed cell death and nematode resistance. Second, we tested whether SPRYCSEC-19 interacts with resistance

proteins to suppress the activation of these immune receptors. In **chapter 5,** we describe the identification of the AMP-1 effector of *G. rostochiensis*. Using various *in vitro* assays, we found that AMP-1 exerts antimicrobial activity. We discuss the role of the AMP-1 effector in nematode immunity. **Chapter 6** reviews the current evidence that plant parasitic nematodes produce effectors to modulate host innate immunity. In this chapter, we discuss the experiments and the associated hypotheses currently used to conclude that nematode effectors have immune modulating properties.

2

The cyst nematode *Heterodera schachtii* is restricted by extracellular plant immune receptors in *Arabidopsis thaliana*

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Abstract

Plant-parasitic cyst nematodes maintain an intricate relationship with their host as they induce a feeding structure in host tissue on which they depend for the completion of their life cycle. Little is known about immune signaling pathways necessary for immune responses against nematodes, or about the mechanisms by which successful nematodes avoid or suppress the plant immune system. *Arabidopsis thaliana* immune signaling mutants were infected with the beet cyst nematode *Heterodera schachtii.* Susceptibility was compared to susceptibility of corresponding wildtype plants to investigate the contribution of immune signaling pathways to the compatible interaction between host and cyst nematode. We found a possible role for extracellular immune receptor signaling and jasmonic acid (JA)-mediated signaling in the containment of nematode infection in wildtype plants. Salicylic acid (SA)-signaling impairment did not increase susceptibility, but increased SA-mediated signaling lowered susceptibility. This indicates that in the compatible wildtype interaction, necrotroph-directed defense contains the nematode infection and biotroph-directed defense is not active or suppressed. Ethylene and abscisic acid signaling mutants were less susceptible, possibly reflecting effects on syncytium formation. Only few of several WRKY mutants showed altered susceptibility, possibly due to functional redundancy in WRKY factors.

2.1 Introduction

Cyst nematodes are obligate sedentary endoparasites that infect the roots of a restricted number of host plants. They cause yield losses of over 1 billion US dollars annually in crops such as potato (*Globodera* spp.), beet, soybean, and cereals (*Heterodera* spp.) (Wang et al., 2002; Bird and Kaloshian, 2003; Denti et al., 2004; Xie et al., 2006; Zheng et al., 2006; Hu et al., 2012). Parasitism starts when a second stage juvenile burrows into a root of the host plant. The nematode migrates through cells of the root cortex, until it reaches a suitable cell near the vascular

cylinder (Gheysen and Mitchum, 2009). With its protrusible stylet, the nematode punctures the cell wall of this host cell. Through a small pore in the underlying cell membrane, the nematode delivers secretions that are produced in the pharyngeal glands of the nematode into the cytoplasm of the host cell (Hussey, 1989; Davis et al., 2008). Successive rounds of withdrawal of cellular contents and injecting of secretions into the cytoplasm of the host cell follow. The affected host cell responds with hypertrophy and partial cell wall dissolution, which fuses the initial host cell and neighboring cells into a multinucleate syncytium (Sobczak et al., 2009). The syncytium functions as a nutrient sink in the plant and provides the nematode with all the necessary metabolites to enable its development into the adult reproductive stage (Jones and Northcote, 1972). The cyst nematodes are completely dependent on the syncytium as the sessile feeding juveniles lose their locomotory muscles (De Boer et al., 1992), and abrogation of the syncytium by, for example, plant immune responses leads to a strongly reduced reproductive success or even death of the nematode (Jones and Northcote, 1972; Rice et al., 1985; Sobczak et al., 2005; Williamson and Kumar, 2006; Tomczak et al., 2009a). Successful nematodes are able to feed from the plant for several weeks until the completion of their life cycle, upon which the bodies of egg-filled females turn into protective cysts in which a new generation of nematodes, ready to infect new host plants, may remain dormant in the soil for years.

Incompatibility of pathogen and host, mediated for example by mechanical and chemical barriers, is known as non-host resistance and fends off the majority of potential pathogens, including most nematodes (Nicol et al., 2011). Microbes that overcome non-host resistance and manage to attack the host may be perceived by two types of immune receptors. Extracellular pattern recognition receptors (PRRs) recognize epitopes shared by many pathogens called pathogen-associated molecular patterns (PAMPs). Activation of plant immune receptors by PAMPs results in immune responses that culminate in PAMP-triggered immunity (PTI) (Zipfel, 2009). Pathogens may overcome PTI by deploying effectors that help to avoid or suppress PTI (Jones and Dangl, 2006). A second line of plant immune

receptors consists of resistance proteins (R proteins) that recognize these pathogen effectors or perturbations of host proteins due to effector activity. Activation of R proteins triggers immune responses that lead to race- or pathovar-specific resistance called effector-triggered immunity (ETI) (Jones and Dangl, 2006; Katagiri and Tsuda, 2010; Thomma et al., 2011). Pathogens may overcome ETI by developing novel or modified effectors, to which plants may in turn evolve new R proteins, ensuing in an evolutionary arms race (Lipka et al., 2005; Anderson et al., 2010; Ali and Bakkeren, 2011).

The signaling pathways that lead from activated plant immune receptors to the initiation of defense responses are poorly understood, but generally involve a downstream mitogen-activated protein kinase (MAPK) cascade and subsequent transcriptional changes in so-called defense genes (reviewed in (Tena et al., 2011; Spoel and Dong, 2012)). Defense gene activation in plants is often regulated by WRKY transcription factors (Pandey and Somssich, 2009). The activated host defense responses in plants are seemingly generic and largely overlapping in both PTI and ETI. Defense responses associated with the latter are often faster, amplified and more prolonged (Thomma et al., 2011). Early responses are ion fluxes, rapid production of reactive oxygen species (called oxidative burst), and changes in intracellular redox state, which in the event of ETI may end in a type of programmed cell death called hypersensitive response (Coll et al., 2011). The defense response also involves a potent chemical component using an array of antimicrobial compounds, which are collectively referred to as pathogenesis-related (PR) proteins (Van Loon et al., 2006). The interplay between different plant hormones further fine-tunes the immune response towards the type of attacker that is perceived.

Secretions injected into the host cell by cyst nematodes are thought to be instrumental in the formation and maintenance of the syncytium (Haegeman et al., 2012). Although it is generally assumed that these secretions also include effectors that modulate immunity, the evidence to date is limited (reviewed in (Haegeman et al., 2012), and Chapter 6). Interactions with the plant immune system and plant

hormonal pathways are quite well studied in other plant parasitic nematodes (mainly root-knot nematodes), but little is known about cyst nematode parasitism. In numerous gene expression studies on both compatible and incompatible interactions with cyst nematodes, downregulation as well as upregulation of components of various plant immune pathways have been observed (Hermsmeier et al., 2000; Puthoff et al., 2003; Alkharouf et al., 2004; Khan et al., 2004; Alkharouf et al., 2006; Fuller et al., 2007; Puthoff et al., 2007; Szakasits et al., 2009; Hamamouch et al., 2011). From such complex and often contradictory data it is difficult to extract the actual contribution of an individual immune signaling pathway to resistance or susceptibility to cyst nematodes.

In several studies, the functions of specific plant genes or compounds in nematode parasitism in plants have been studied by means of nematode infection assays. For most of these studies the model system *Arabidopsis thaliana* challenged with the beet cyst nematode *Heterodera schachtii* was used (Sijmons et al., 1991). For instance, the importance of the plant hormones auxin and ET to the formation of syncytia (Goverse et al., 2000; Grunewald et al., 2009a) and to susceptibility (Wubben Ii et al., 2001; Wubben Ii et al., 2004; Wubben et al., 2008) have been proven using known *A. thaliana* mutants. Screening of randomly mutated *A. thaliana* resulted in the identification of novel genes that alter susceptibility to nematodes (Baum et al., 2000). In general, these studies have focused on developmental aspects of feeding cell formation rather than immune signaling and defense. Here, we report on the susceptibility to *H. schachtii* of *A. thaliana* mutants with specific defects in immune signaling pathways and hormonal pathways.

Immune signaling is initiated by immune receptor activation. Knockout mutants of key upstream components of PTI and ETI immune receptor signaling were used to investigate whether PTI and ETI immune receptor signaling play a role in the susceptibility to *H. schachtii*. The plasma membrane-associated receptor-like kinase BAK1 is a cofactor in several PTI and ETI responses activated by extracellular plant immune receptors. BAK1 complexes with the PRRs flagellin sensing 2 (FLS2) as well

as brassinosteroid insensitive 1 (BRI1) and is required for FLS2 and BRI1 signaling in *A. thaliana* (Li et al., 2002; Nam and Li, 2002; Heese et al., 2007). In *Nicotiana benthamiana*, BAK1 is required for immune responses against several unrelated PAMPs and for resistance against bacterial and oomycete infections (Heese et al., 2007). The tomato (*Solanum lycopersicum*) BAK1 homologue SlSERK1 is required for Mi1-mediated resistance to aphids but not to root-knot nematodes (Mantelin et al., 2011). The co-chaperones required for MLA12 resistance (RAR1) and suppressor of G2 allele of SKP1 (SGT1) function together with heat shock protein 90 (HSP90) to stabilize cytoplasmic immune receptors (Rice et al., 1985). Non-race-specific disease resistance1 (NDR1) is an integrin-like protein involved in stress response signaling. NDR1 was originally described as a master regulator of the hypersensitive response associated with activation of R proteins of the CC-NB-LRR type (Century et al., 1997; Aarts et al., 1998; Day et al., 2006). Recently, its functional role was extended to the early activation and amplification of both PTI and ETI responses (Knepper et al., 2011). NDR1 mutants are impaired in SA accumulation following ROS perception (Shapiro and Zhang, 2001).

Following immune receptor signaling, plant hormone signaling pathways regulate the activation and execution of immune responses. Over the years, much evidence has been collected in favor of the hypothesis that SA signals for defense against biotrophs, whereas JA signaling is involved in resistance responses against necrotrophs (Glazebrook, 2005). Although plant parasitic nematodes are biotrophs, JA responses have been associated with resistance to nematodes (Nahar et al., 2011) and JA biosynthesis and responses are downregulated in syncytia (Ithal et al., 2007). The phytohormone ET has been implicated in directing host defense responses in interplay with the JA, SA, and abscisic acid (ABA) signaling pathways. ET generally acts synergistically with JA in defense against necrotrophs and antagonizes SA-dependent immune signaling against biotrophs. However, in some instances ET has also been shown to stimulate defenses against biotrophs in synergy with SA (Adie et al., 2007a). Abscisic acid is mostly known for its role in stress responses but has also been implicated in defense responses against

necrotrophic pathogens, by synergistically acting with JA and by antagonizing SA-mediated defenses (Adie et al., 2007b). The role of ABA in nematode infections is currently unclear, with both up- and downregulation of ABA responsive genes in syncytia being reported (Szakasits et al., 2009; Mazarei et al., 2011). Knockout mutants of key components in hormone signaling were used to investigate the role of hormone signaling in the susceptibility to *H. schachtii*.

Further downstream, defense gene activation is often mediated by a network of WRKY transcription factors. These transcription factors constitute a large family of more than 200 members in *A. thaliana*. Knockout mutants of several key WRKY factors were used to investigate the contribution of these signaling components in susceptibility to *H. schachtii*.

A. thaliana wildtypes are considered susceptible to *H. schachtii*, and no ETI responses to *H. schachtii* have been reported so far. Non-host resistance has been observed to soybean cyst nematodes (*H. glycines*) and potato cyst nematodes (*Globodera* species), but not to *H. schachtii*. *H. schachtii* is thus able to overcome non-host resistance in these plants. Since no plant parasitic nematode-derived PAMPs are known, it is not clear if and how plants perceive the invading nematodes. Possibly, PTI responses to *H. schachtii* are suppressed by the nematodes.

Knockout plants and corresponding wildtype plants were grown *in vitro*, and after infection with *H. schachtii* the number of adult female cysts developing on the mutants plants compared to wildtype was used as a measure of susceptibility. By comparing the susceptibility of various immune signaling mutants to the wildtypes, we investigated the contribution of downstream signaling cascades to defense, pathogen recognition and susceptibility of *A. thaliana* to cyst nematodes.

2.2 Results and discussion

2.2.1 Immune receptor signaling

We have used the BAK1 knockout mutant *bak1-4* to test its requirement for susceptibility to cyst nematodes in *A. thaliana*. Infections with *H. schachtii* on *bak1-4* resulted in significantly higher number of cysts per plant than on the corresponding wild type plants Col-0 (134%±SEM) (Table 2.1). Other studies of this *bak1-4* mutant have revealed no altered susceptibility to *Pseudomonas syringae*, no reduced containment of microbial infection-induced cell death, and no spreading necrosis or enhanced susceptibility to fungal pathogens (Kemmerling et al., 2007). However, the *bak1-4* mutant is more susceptible to Turnip crinkle virus (TCV) (Yang et al., 2010). In microarray data of *H. schachtii*-infected *A. thaliana*, BAK1 was downregulated 5.3-fold in 5-to-15-day-old syncytia compared to non-infected root tissue (Szakasits et al., 2009). The increased susceptibility of *bak1-4* mutants show that BAK1, and thus possibly BAK1-mediated signaling of extracellular immune receptors, plays a role in containing the infection by *H. schachtii*. Hypothetically, this could mean that BAK1-mediated PTI responses against *H. schachtii* are active in wildtype plants, which could be partially suppressed by virulent *H. schachtii*.

Challenging of the *A. thaliana* mutant *sgt1-b3* with *H. schachtii* resulted in a significantly reduced number of cysts per plant (68%±SEM) as compared to the corresponding wildtype Ler-0 plants. By contrast, the *rar1-13* knockout mutant in *A. thaliana* did not show altered susceptibility to *H. schachtii* as compared to wildtype Ler-0 plants (Table 2.1). The only phenotype that has been observed for the *rar1* mutant before is loss of ETI, indicating that other than stabilizing highly specific R proteins, RAR1 may not have additional functions in plant defense (Muskett et al., 2002). The wildtype *A. thaliana* ecotype Ler-0 is considered susceptible to *H. schachtii*, as no programmed cell death in or around the feeding site has been observed (Sijmons et al., 1991). It is likely that no effector-triggered immune signaling takes place in infected wildtype plants, which could explain the unaltered susceptibility of *rar1-13* mutant plants to *H. schachtii*. In contrast to RAR1, SGT1 has many different functions in plant cells, including ubiquitination and activation of the cyclic adenosine mono phosphate (cAMP) pathways (Rice et al.,

1985). Mutants of SGT1 may therefore be impaired in diverse key biological processes, which do not necessarily have a link with host defenses. Some of these processes may affect the formation and maintenance of the syncytium, resulting in a lowered susceptibility of SGT1 mutant plants to *H. schachtii*.

We used the NDR1 knockout mutant *ndr1-1* to investigate whether ROS perception and early immune signaling play a role in the compatible interaction between *H. schachtii* and *A. thaliana*. No significant change in susceptibility compared to wildtype Col-0 plants was found. Because the interaction between *A. thaliana* and *H. schachtii* is a compatible interaction, the role of NDR1 as master regulator of CC-NB-LRR signaling is unlikely to restrict *H. schachtii*, which fits the observed result. However, plant parasitic cyst nematodes cause ROS production during the migratory phase, and possibly trigger other PTI responses. The observation that plants with impaired ROS perception did not become more susceptible suggests that ROS-related immune signaling is not hindering the nematodes in the susceptible wildtype interaction.

2.2.2 SA signaling

To investigate the role of SA in the interaction between *H. schachtii* and *A. thaliana*, we used several mutant lines and the transgenic *NahG* line. The latter ectopically expresses NahG, which converts SA to catechol resulting in SA deficiency. We found that the transgenic line *NahG* and the mutant lines *ndr1-1*, *npr1-1*, and *npr1-2*, which are all impaired in SA signaling, were equally susceptible to *H. schachtii* as wildtype *col-0* plants (Table 2.1). The SA-impaired mutant *npr1-3* and the double knockout *ndr1-1/npr1-2* were less susceptible (76% and 70% ±SEM, respectively). The constitutive expression of PR-1 (*cep1*) mutant, which in addition to overexpression of PR-1, 2, and 5 has elevated levels of SA (Silva et al., 1999) was one of the most resistant lines in our study, with only 40% of the number of cysts per plant as compared to the Col-0 wildtype plants. The susceptibility of *A. thaliana* to *H. schachtii* was not affected in several mutants impaired in SA–dependent immune signaling. Our data suggest that the activation of these signaling pathways

is not a limiting factor in the compatible interaction between the wildtype *A. thaliana* plants and *H. schachtii*. In contrast to the weaker *npr1-1* and *npr1-2* mutations, the *ndr1-3* mutant is considered a complete null mutation of NPR1 (Cao et al., 1997). The observed lower infection rates in this mutant suggest that an intact SA signaling pathway is required by the nematode to successfully infect *A. thaliana*. These findings contrast with earlier findings of (Wubben et al., 2008), who found that *NahG*, *npr1-2*, and *npr1-3* were more susceptible to *H. schachtii*. The double knockout mutant *ndr1-1/npr1-2* is impaired in ROS perception and negative feedback on ROS production. Possibly, this results in elevated levels of ROS that negatively impact the nematode, leading to reduced infection numbers. We found that the *cep1* mutant is significantly less susceptible than the corresponding wild type plants. Mutations in two genes with unknown function cause the phenotype of constitutive PR-1 expression and increased levels of SA (Silva et al., 1999). High levels of SA thus decrease susceptibility of *A. thaliana* to *H. schachtii*. (Wubben et al., 2008) observed a similar decrease in susceptibility in SA-treated wildtype plants. Also, they found an inverse correlation between PR-1 expression and susceptibility, which is in agreement with the decreased susceptibility of *cep1*, but not with the NPR1 mutants. Our results suggest that on the one hand SA signaling may be required by the nematode for infection, but on the other hand high levels of SA and PR-1 expression negatively impact the nematodes. Possibly, an intact SA signaling pathway benefits nematodes by antagonizing JA-mediated defense responses, while elevated SA levels induce PR-1-related defense responses which increase resistance to cyst nematodes.

2.2.3 JA signaling

We used the JA insensitive mutants, *coi1-16*, *jin*, and *jar*, and the JA deficient mutant *aos* of *A. thaliana* to investigate the effect of JA on susceptibility to *H. schachtii*. The JA deficient *aos* mutant was significantly more susceptible to *H. schachtii* than wildtype plants (115%) (Table 2.1). The JA insensitive *jin* mutant seemed slightly, but not significantly, more susceptible to *H. schachtii*. The JA

insensitive *jar* mutant was more susceptible (119%). The *coi1-16* mutant was less susceptible to *H. schachtii* (67%) as compared to wildtype Col-0 plants. Next to the mutation causing JA insensitivity, *coi1-16* carries a mutation in *Penetration2* (*PEN2*) (Westphal et al., 2008). PEN2 encodes a glycoside hydrolase required for non-host resistance to powdery mildew (Lipka et al., 2005). Possibly, the *pen2* mutation underlies the decreased susceptibility to *H. schachtii* in these plants by an unknown mechanism. Our findings indicate that in *A. thaliana*, JA-dependent immune signaling is a limiting factor in the susceptibility to *H. schachtii*. This suggests that in a compatible interaction, JA-mediated defenses are active which negatively impact the nematode. Although *H. schachtii* and other sedentary plant parasitic nematodes are biotrophs, they do considerable damage during the initial migratory phase at the onset of parasitism, leaving behind a trail of necrotic host cells (Sobczak et al., 2009). This damage may be perceived by the plant as caused by a necrotrophic attacker, which could trigger JA-mediated host defenses in the early stages of infection by *H. schachtii*. These JA-mediated defenses might negatively impact the success of the nematode.

2.2.4 ET signaling

We used the ET insensitive mutants *etr1-1, ein2*, *ein3*, and *eir1-1* to investigate the role of ET in *H. schachtii* infection of *A. thaliana*, and found significantly altered infection rates of 45%, 51%, 88%, and 112% compared to wildtype plants, respectively (Table 2.1). The ethylene receptor ETR1 negatively regulates ethylene signaling and requires the downstream positioned EIN2 for signal transduction. Loss of ETR1 or EIN2 results in complete ET insensitivity (reviewed in (Robert-Seilaniantz et al., 2011)). EIN3 is a transcriptional activator downstream of EIN2 with five functional homologs termed EIN3-Like elements (EILs). Loss of EIN3 results in reduced ET sensitivity but not complete insensitivity (Chao et al., 1997), which may explain why compared to the *etr1* and *ein2* mutants, *ein3* shows less reduction in *H. schachtii* infection. The *eir1-1* mutant displays root-specific ET insensitivity (Roman et al., 1995) which is probably due to defective polar auxin

transport (Luschnig et al., 1998). The higher infection rates in the latter mutant might be explained by altered auxin transport, since the nematodes need to redirect auxin transport for syncytium formation (Grunewald et al., 2009b).

ET plays a critical role in the formation of syncytia of cyst nematodes in *A. thaliana*. (Goverse et al., 2000; Wubben Ii et al., 2001) found that the ET overproducing mutants *eto1*, *eto2*, and *eto3* were not only hypersusceptible to *H. schachtii* but also facilitated accelerated nematode development and growth. Syncytia in these plants were larger and expanded faster through enhanced cell wall degradation. (Wubben Ii et al., 2001) confirmed hypersusceptibility of the same mutants and in addition showed that the ET insensitive mutants *axr2*, *etr1-1, ein2*, *ein3*, and *eir1-1* were much less susceptible to *H. schachtii*. Our findings show that ethylene insensitivity strongly affects *H. schachtii* parasitism. Whether the reduced susceptibility of the ET mutants used here reflect effects on defenses against *H. schachtii* or whether it merely indicates the necessity of an intact ET pathway for feeding site formation is not clear.

2.2.5 ABA signaling

In the ABA-deficient mutant *aba2-1*, ABA levels in unstressed leaves are 75-80% lower than in wildtype plants (González-Guzmán et al., 2002). Challenging the *aba2-1* plants with *H. schachtii* resulted in an infection rate of 71% as compared to the corresponding *Col-0* wild type plants (Table 2.1). An intact ABA signaling pathway thus favors nematode development in *A. thaliana*. Given the diversity of functions assigned to ABA in plant development, it is conceivable that nematodes require ABA signaling for the formation and maintenance of the syncytium. However, ABA also has been shown to negatively regulate host defenses against the soil-borne fungus *Fusarium oxysporum*, and promotes virulence of several other pathogens (Van Loon et al., 2006). Regulation of developmental pathways in syncytia and negative host defense regulation by ABA could thus both explain the decrease of susceptibility to *H. schachtii* in the *aba2-1* mutants.

2.2.6 WRKY transcription factors

We used *A. thaliana* mutants of WRKY22, 33, 53, 60, 70, and 72 to investigate the role of these transcription factors in defense against *H. schachtii*. We found that in most WRKY knockout mutants we tested, *H. schachtii* infection rates were unaltered compared to wildtype plants (Table 2.1). The *WRKY22* mutant was less susceptible (77%) and the WRKY60 mutant was slightly more susceptible (118%). WRKY22 is thought to be an early defense marker promoting PTI responses (Asai et al., 2002; Zhang et al., 2007). It is therefore surprising that knockout of *WRKY22* renders plants less susceptible to *H. schachtii*. Possibly, unknown functions of WKRY22 such as repression of other immune responses underlie this phenotype. WRKY60 is a negative regulator of ABA signaling (Shang et al., 2010). The possible benefit of elevated levels of ABA to *H. schachtii* is in agreement with our findings with the *aba2-1* mutant. That elevated levels of ABA could be beneficial to *H. schachtii* is supported by our findings with the *aba2-1* mutant. Mutants of WRKY33, 53, 70 and 72 showed no altered susceptibility. WRKY33 is important for JA-mediated resistance against necrotrophic pathogens mediated by JA (Zheng et al., 2006). The increased susceptibility of the JA insensitive mutants showed that JA responses are probably containing *H. schachtii* infection in wildtype interactions. The observation that the susceptibility of ATWRKY33 is not significantly altered is similar to the observations of (Zheng et al., 2006), who found no effect of *WKRY33* knockout on susceptibility of *A. thaliana* to the virulent biotrophic bacterial pathogen *Pseudomonas syringae*. Possibly, knockout of WRKY33 does not sufficiently tip the balance of defenses against necrotrophs and defenses against biotrophs to have an effect on susceptibility to *H. schachtii*. WRKY53 and WRKY70 are involved in the SA-signaling pathway and have overlapping roles in mediating basal resistance against biotrophs (Murray et al., 2007; Van Eck et al., 2010). Functional redundancy in this system could explain why *WRKY53* plants are equally susceptible to wildtype plants. WRKY72 is required for *Mi*-mediated resistance to

root–knot nematodes in tomato, and basal defense against root-knot nematodes in *A. thaliana* (Bhattarai et al., 2010). The unaltered susceptibility to *H. schachtii* of *WRKY72* knockout plants suggests that WRKY72 may not be required for basal defense against cyst nematodes.

Table 2.1: Susceptibility of *A. thaliana* immune signaling mutants to *H. schachtii* (SEM, standard error of mean; N, number of plants inoculated in at least three independent experiments), green is more susceptible, red is less susceptible.

Mutant	Average relative susceptibility compared to wildtype	SEM	N inoculations	Wildtype
bak 1-4	134%	3%	24	*Col-0*
sgt1-b3	68%	9%	42	*Ler-0*
rar1-13	95%	13%	36	*Ler-0*
ndr1-1	100%	9%	45	*Col-0*
NahG	106%	11%	41	*Col-0*
npr1-1	90%	10%	44	*Col-0*
npr1-2	90%	10%	29	*Col-0*
ndr1-1 npr1-2	70%	9%	24	*Col-2*
npr1-3	76%	9%	42	*Col-0*
cep1	40%	8%	23	*Ws*
coi1-16	67%	13%	28	*Col-0*
jin	106%	11%	45	*Col-0*
jar	119%	11%	43	*Col-0*
aos	115%	9%	24	*Col-6*
etr1-1	45%	6%	31	*Col-0*
ein2	51%	5%	26	*Col-0*
ein3	88%	9%	48	*Col-0*
eir1-1	112%	7%	23	*Col-0*
aba2-1	71%	6%	12	*Col-0*
WRKY22	77%	7%	23	*Col-0*
WRKY33	91%	11%	22	*Ler-0*
WRKY53	96%	11%	29	*Col-0*
WRKY60	118%	9%	23	*Ler-0*
WRKY70-1	91%	19%	26	*Col-0*
WRKY72	90%	13%	25	*Col-0*

In a microarray experiment of (Szakasits et al., 2009) 5-15-day-old syncytia of *H. schachtii* were compared to uninfected *A. thaliana* roots. WRKY33, 60, and 72 were downregulated 10.7, 4.5, and 4.2 fold, respectively, while the expression of WRKY22, 53, and 70 was not significantly different. Our data show that knockout of WRKY33, which is also strongly downregulated in syncytia, does not give an additional advantage to nematodes. Knockout of WRKY60, which is less strongly downregulated in syncytia, slightly enhances nematode success. WRKY22 might be required for nematode success, as it is not downregulated in syncytia and knockout renders plants less susceptible. Functional redundancy or lack of involvement in basal resistance against *H. schachtii* could explain the unaltered susceptibility of the other WRKY knockout plants.

2.2.7 General discussion

Plant host defenses are triggered after direct or indirect detection of the invading pathogen by extracellular or intracellular plant immune receptors. Although the *A. thaliana* ecotypes Col-0 and Landsberg used in this study are generally considered susceptible to *H. schachtii*, it is not known if PAMP-triggered immunity to *H. schachtii* occurs in these ecotypes.

Because no ETI responses against *H. schachtii* have been described in *A. thaliana* wildtypes in the past, it is unlikely that R protein signaling and ETI responses are active during *H. schachtii* infection. Our findings with the *rar1-13*, *sgt-1* and *ndr1-1* mutants did not support infection-limiting cytoplasmic immune receptor signaling in the interaction between *A. thaliana* and *H. schachtii*. The increased susceptibility of *bak1-4* plants demonstrates that extracellular immune receptor signaling limits nematode success in the compatible wildtype interaction. The results with the JA signaling mutants show that containment of nematode infection is likely mediated by active JA-mediated immune signaling in wildtype plants. Decreased SA-dependent signaling did not increase susceptibility to *H. schachtii* in our experiments, but increased SA levels and subsequent PR-1 expression lowered susceptibility. SA-mediated immune signaling, which is generally considered to

mediate defense against biotrophs, is thus not limiting nematodes in a compatible interaction, but, once activated, provides a level of resistance. JA-mediated immune signaling limited nematode development in *A. thaliana*, as was shown by the increased susceptibility of JA insensitive mutants *jar* and *aos*. Basal defense against *H. schachtii* thus seems to be mainly a defense against a necrotrophic attacker. The nematodes are possibly detected indirectly by the physical damage they inflict to plant tissues or by unknown PAMPs, and the lack of SA mediated defense responses indicates that the plant appears to fail to recognize the nematodes as biotrophs.

ET-mediated immune signaling may increase susceptibility to cyst nematodes by antagonizing SA-mediated immune responses. Additionally, ET signaling is well established as beneficial to syncytium formation and increasing susceptibility to *H. schachtii*. ABA may regulate developmental pathways in syncytia and probably regulates host defense in favor of the nematode. The latter has been shown for the rice migratory nematode *Hirschmanniella oryzae* (Nahar et al., 2012) and it will be interesting to see more research done on the contribution of this hitherto rather ignored plant hormone on cyst nematode parasitism.

Bioassays with *A. thaliana* mutants such as performed in this study provide a fast and relatively easy way to gain insight in plant-nematode interactions. There are, however, limitations to this experimental approach and the interpretation of the acquired data. Hormonal pathways do not only function in defense, but fulfill key roles in plant development and other processes. Cyst nematodes skillfully manipulate a plant cell into a feeding structure, which goes accompanied by huge transcriptional, metabolic and developmental changes in the affected cells. At least some of these changes are induced through hormonal pathways. Auxin, for example, is required for the successful development of syncytia. Many of the mutants we used here were impaired in hormone signaling. Some of the observed changes in nematode virulence on these mutants could be a consequence of disturbed syncytium formation rather than effects on immune signaling and immune responses.

We have looked mostly into single mutations that have large effects on one or several defense or hormonal pathways. Often the affected genes have homologs that may compensate a mutation in one of the genes. The functional redundancy among homologous genes may in future experiments be addressed by using multi-gene knockout mutants, in which entire pathways are knocked out. Such a setup would also provide possibilities for network analyses (Tsuda et al., 2009), which may provide insights into the relative contribution of single genes to defense and their interplay with other signaling components. Recent findings point at a role for small RNAs in defense against plant parasitic nematodes, and for the utilization by nematodes of small RNAs to manipulate host cells (Hewezi et al., 2008a). Mutants would provide an interesting way to gain more insight in the significance of these findings to nematode susceptibility.

Activation of extracellular immune signaling by cyst nematodes has rarely been described. Only recently effectors of *H. schachtii* and *G. rostochiensis* have been shown to interact with extracellular receptors. In *H. schachtii*, the effector Hs30C02 was shown to interact with an extracellular β-1,3-glucanase of *A. thaliana* (Hamamouch et al., 2012). Knocking down the effector reduced nematode virulence and ectopic overexpression in *A. thaliana* conversely increased host susceptibility. In *G. rostochiensis*, effector-induced changes to an apoplastic cysteine protease are recognized by the extracellular resistance protein Cf-2, which leads to resistance (Lozano-Torres et al., 2012). Extracellular immune receptors thus play a role in both compatible and incompatible host-nematode interactions. Detection of nematode-derived molecules in the apoplast by extracellular immune receptors, whether they are PAMPs or effectors, offers a new field of research for the development of nematode resistance in important food crops.

2.3 Materials and methods

2.3.1 Nematodes

A *H. schachtii* field population from IRS was maintained under greenhouse conditions on cabbage plants. Cysts were isolated and parasitic second stage juveniles were hatched by incubating the cysts in 3 mM $ZnCl_2$. After sucrose purification, the nematodes were sterilized by subsequent incubation for 20 min in 0.5% (w/v) streptomycin-penicillin, 20 min in 0.1% (v/v) ampicillin gentamycin, 5 minutes in sterile tap water, 3 minutes in 0.1% (v/v) chlorhexidine and washing three times with sterile tap water. Nematodes were resuspended 0.7 % sterile Gelrite prior to infection.

2.3.2 Plants and nematode infections

A. thaliana mutants were obtained from ABRC or NASC or kindly provided by Emilie Fradin (Phytopathology, Wageningen University). *Aba2-1*, *Clv1* and *Clv2* were kindly provided by Maarten Koornneef (Genetics, Wageningen University). Seeds were vapor-sterilized according to (Clough and Bent, 1998). For each test 10-15 seeds per genotype were sown in a randomized block design in 12-well tissue culture plates, with each well containing one seed on 1.5 ml modified KNOP medium with 1.0% sucrose and 0.8% Daishin agar (Duchefa, Haarlem, The Netherlands). After 48h stratification at 4°C, the plates with seeds were incubated at 24°C under a 12h light/dark regime. Plants (12-14 days old) were inoculated with approximately 200 sterile *H. schachtii* J2 juveniles, after which the plates were transferred to the dark at 18°C. After 4 weeks the numbers of adult female cysts per plant were counted. Infection rates were calculated relative to wildtypes and significance was calculated by two-tailed Student's t-test assuming unequal variance, with $P<0.05$ considered as the threshold for significance.

3

A secreted SPRY domain-containing protein (SPRYSEC) from the plant-parasitic nematode *Globodera rostochiensis* interacts with a CC-NB-LRR protein in susceptible tomato

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Abstract

Esophageal gland secretions from nematodes are believed to include effectors that play important roles in plant parasitism. We have identified a novel gene family encoding secreted proteins specifically expressed in the dorsal esophageal gland of *Globodera rostochiensis* early in the parasitic cycle, and which contain the B30.2/SPRY domain. The secondary structure of these proteins, named the secreted SPRY domain-containing proteins (SPRYSEC), includes highly conserved regions folding into β-strands interspersed with loops varying in sequence and in length. Mapping sequence diversity onto a three-dimensional structure model of the SPRYSEC indicated that most of the variability is in the extended loops that shape the so-called surface A in the SPRY domains. Seven of nine amino acid sites subjected to diversifying selection in the SPRYSEC are also at this surface. In both yeast-two-hybrid screening using a library from a susceptible tomato and in an in vitro pull-down assay, one of the SPRYSEC interacted with the leucine-rich repeat (LRR) region of a novel coiled-coil nucleotide-binding LRR protein, which is highly similar to members of the SW5 resistance gene cluster. Given that the tomato cultivar used is susceptible to nematode infection, this SPRYSEC could be an evolutionary intermediate that binds to a classical immune receptor but does not yet, or no longer, triggers a resistance response. Alternatively, this SPRYSEC may bind to the immune receptor to downregulate its activity.

3.1 Introduction

Parasitism of the obligate biotrophic cyst nematodes (e.g. *Globodera* spp and *Heterodera* spp.) essentially progresses through two stages (Hussey and Grundler, 1998). In the first stage, infective juveniles hatch from eggs in the soil to invade the roots of a nearby host plant. The infective juveniles preferentially penetrate the root close to the root apex. After breaching the epidermal cell layer they destructively migrate in the cortex. Shortly after penetration the juveniles settle down and subtly start probing host cells with their oral stylet, which marks the

beginning of the second phase. One of the probed host cells will respond to secretions injected into the host cell cytoplasm through the stylet of the nematode. In the hours that follow the responsive host cell transforms into a transfer cell, on which the nematode fully depends for its development. The feeding site of cyst nematode ultimately expands into a large conglomerate of hundreds of cells by highly directed local cell wall degradation and subsequent protoplast fusion, hence its name syncytium.

Cyst nematode esophageal gland secretions are believed to produce important effectors in plant parasitism (Davis et al., 2008). Despite significant progress in the identification of genes coding for stylet secretions in nematodes, little is known about the molecular targets of most of these effectors in host cells. Nor is it clear what the effects of most of the components in nematode secretions are on the constitution of the recipient host cells. Nonetheless, nematode effectors are likely key players in host invasion, feeding structure formation and maintenance, and suppression or evasion of innate immunity in host plants.

In this paper, we report the identification of a novel gene family in *G. rostochiensis* whose members code for secretory proteins consisting of a single B30.2/SPRY domain. The SPRY domain (~ 120 amino acids) was first identified in SPlA and in RYanodine receptors in *Dictyostelium discoideum* (reviewed in (Woo et al., 2006b)). At about the same time, the term B30.2 (~170 amino acids) was coined for a domain encoded by an exon in the human class I major histocompatibility complex region. The B30.2 domain comprises a conserved C-terminal SPRY domain, preceded by a more variable PRY subdomain. The SPRY domain has some structural resemblance to the immunoglobulin fold and provides an extremely versatile scaffold to facilitate intermolecular protein-protein interactions for its carrier (reviewed in (Rhodes et al., 2005)). We therefore named this novel gene family the *SPRYSEC*s in *G. rostochiensis* and show that one of its members interacts with a CC-NB-LRR type of disease resistance protein (Van Ooijen et al., 2008) in the susceptible host plant tomato. Despite its interaction with a classical immune

receptor protein and the discovery of diversifying selection in one its surfaces, we have found no evidence that SPRYSEC-19 activates effector-triggered immunity in host plants to date. In the discussion section we propose two models that could explain this phenomenon in the context of susceptible host plant. First, the SPRYSEC could be evolutionary intermediate that binds to the immune receptor-like protein, but does yet trigger a response of the plant's innate immune system. Alternatively, the nematode may co-secrete other effectors that suppress a SPRYSEC-triggered response rendering the plant susceptible. In the second model, the SPRYSEC could interact with this receptor-like protein to down-regulate its activity.

3.2 Results

3.2.1 Identification of the SPRYSEC gene family

Gene expression patterns in five distinct developmental stages of *G. rostochiensis* using cDNA-AFLP resulted in the display of 16,500 transcript-derived fragments (TDFs) of which 216 were solely or predominantly expressed in potato root diffusate-exposed pre-parasitic second stage juveniles (J2s) and water re-hydrated J2s (Qin et al., 2000). The sequences of two of these TDFs (A18 and A41) showed significant similarity to human RAN-binding proteins (RanBPM [BAB62525], RanBP9 [AAH19886], and RanBP10 [AAI21178]). To resolve the full-length transcripts from which the TDFs originated and to find homologous sequences, we first mined approximately 11,851 ESTs from *G. rostochiensis* using the TDF sequences as queries in the BLASTXN algorithm. A total of forty-two matching ESTs were found with varying degrees of similarity to the TDFs. The cDNA library clones from which the matching ESTs had been generated were re-sequenced from both ends to further obtain the full insert sequences. For some of the sequences gene specific primers were designed to extend the sequences further at the 5'- and/or 3'-ends by RACE. Altogether, the TDFs, the RACE fragments, and the completed library insert

sequences were assembled into thirty-five contigs of which eight contained full-length transcripts (Supplemental table 3.1)

3.2.2 Sequence characterization of the SPRYSEC gene family

The eight full-length transcripts included large open reading frames ranging from 208 to 274 amino acids coding for proteins with molecular masses ranging from 22.7 to 30.4 kiloDaltons (see Table 3.1 for an overview). For each of these eight protein sequences an N-terminal signal peptide for secretion was predicted, while no trans-membrane helices were found in any of the sequences. Thus, the eight transcripts code for proteins that are likely secreted in the nematode.

A comparison of the eight protein sequences with the non-redundant protein databases resulted in significant matches with human RAN-binding proteins (Genbank accessions EAW55354 and AAI21177 respectively; E-values < e^{-16} in BLASTP). The human RAN-binding proteins 9 and 10 are multi-domain proteins of 729 and 620 amino acids respectively, including an SPla/RYanodine receptor (SPRY) domain (pf00622), a Lissencephaly type-1-like homology motif (LisH motif; smart00667), a 'C-Terminal to LisH' motif (CTLH; smart00668), and a C-terminal CT11-RanBPM domain (CRA; smart00757). The best matching putative RAN-binding protein from the free-living nematode *Caenorhabditis elegans* (CAA21656; E-value ~e^{-10}) has the same architecture as human RAN-binding proteins. The significant similarities between the eight predicted proteins from *G. rostochiensis* and these RAN-binding proteins are restricted to the SPRY domain of approximately 120 amino acids. A further search in the conserved domain databases using the eight protein sequences indicated that the SPRY domain is part of a larger structural domain, referred to either in the literature as B30.2, B30.2/SPRY, or PRYSPRY, and consisting of about 210 amino acids (IPR001870). Thus, the eight transcripts from *G. rostchiensis*, hereafter named the SPRYSECs, code for single domain secretory proteins with strong similarity to B30.2/SPRY domains.

Table 3.1: Summary of SPRYSEC family members with features of the coding region.

SPRYSEC	cDNA (in bp)[1]	ORF (in aa)[2]	SP[3]	B30.2[4]	SPRY[5]
4	885	232	1-24	18-228	93-227
5	959	250	1-23	19-232	103-231
8	806	208	1-18	9-200	71-199
9	848	224	1-24	30-214	92-213
15	920	274	1-24	41-238	105-237
16	825	>216*	1-15	21-210	83-209
18	809	224	1-24	30-214	92-213
19	845	216	1-17	25-216	87-215

[1] transcript length in base pairs
[2] size of largest open reading frame in amino acids
[3] position of the signal peptide for secretion according to SignalP
[4] position of B30.2 domain (IPR001870) according to INTERPRO Scan
[5] position of SPRY domain (IPR003877 and PF00622) according to INTERPRO Scan
*Start codon and a few following codons on the 5'-end of the open reading frame are still missing.

3.2.3 The SPRYSECs are expressed in the dorsal esophageal gland

An important criterion in our strategy to identify genes involved in nematode-plant interactions is specific expression in the esophageal glands, the main source of effector proteins in plant-parasitic nematodes. To this purpose, specific antisense cDNA probes were designed on the sequences of six SPRYSECs (-4, -5, -8, -15, -18, and -19) for in situ hybridization microscopy on pre-parasitic second stage juveniles (ppJ2s). The similarities of SPRYSEC-9 and -16 with SPRYSEC-18 and -19 respectively, were too high to make specific probes. In addition, we also designed an antisense probe on the most conserved part at the C-terminus of the SPRY domain in the SPRYSECs. All anti-sense probes, including the probe matching the conserved region, specifically hybridized to the dorsal esophageal gland cell (Fig. 3.1A-G). Also, for each of the anti-sense probes we tested the corresponding sense probes none of which resulted in a specific hybridization of tissues in whole mount nematode sections (e.g. Fig. 3.1H).

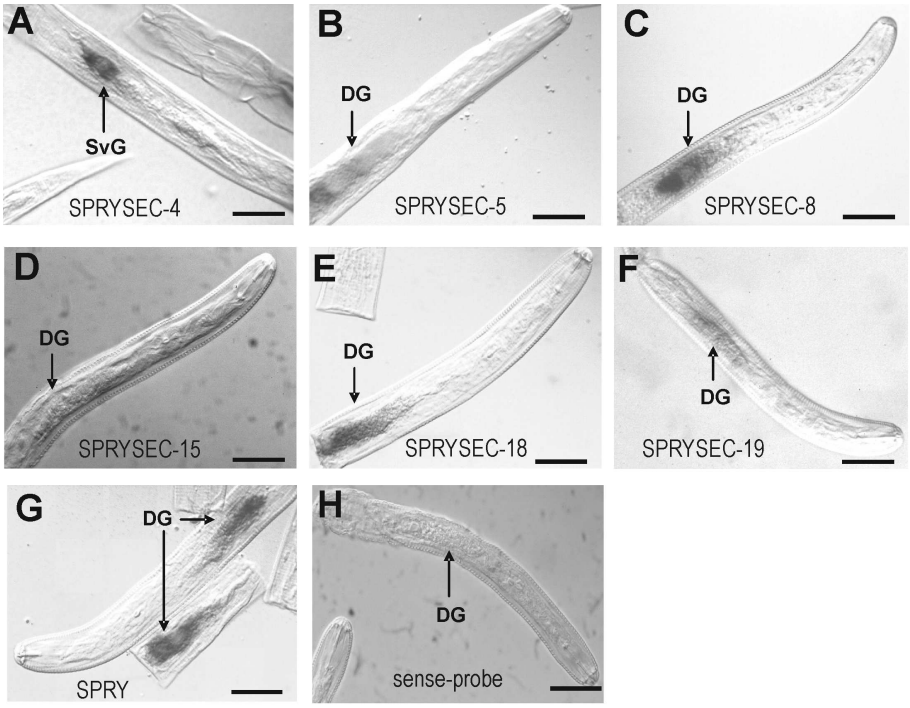

Fig. 3.1. Whole mount in situ hybridization with anti-sense probed specifically designed on six SPRYSECs in anterior sections of pre-parasitic second juveniles of *G. rostochiensis* (**A-F**). A specific hybridization of an anti-sense probe designed on the most conserved region of the SPRY domain in the SPRYSECs in the dorsal esophageal gland cell (DG) is shown in **G**. The sense probes corresponding to each of the anti-sense probes did no show a specific hybridization (e.g. **H**).

3.2.4 The SPRYSECs are expressed in the early stages of the parasitic cycle of the nematode

The SPRYSECs were identified in and cloned from the pre-parasitic second juvenile stage of *G. rostochiensis*, which were exposed to potato root diffusate (Qin et al., 2000). We conducted a semi-quantitative RT-PCR to investigate the expression of the SPRYSECs in successive parasitic stages isolated from root tissues of nematode-infected host plants. SPRYSEC-4 and -5 appeared to be up-regulated in pre-parasitic and parasitic J2 stage only, while SPRYSEC-8, -15, -18, and -19 are also up-regulated in these stage but still showed some expression in later parasitic stages, albeit at lower levels than in J2s (Fig. 3.2). SPRYSEC-9 and -16 were not sufficiently different

to design gene specific primers with similar amplification parameters as the control gene for semi-quantitative RT-PCR. Reactions with uninfected root tissue and reactions without the reverse transcriptase enzyme were included as controls. The *cAMP-dependent protein kinase* gene from *G. rostochiensis* was used as an indicator for constitutive expression throughout the development of the nematode.

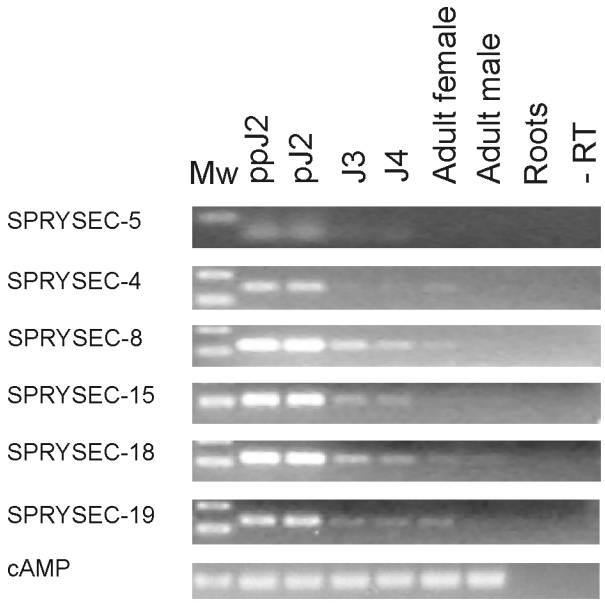

Fig. 3.2. Semi-quantitative RT-PCRs with primers specific for the SPRYSECs on pre-parasitic J2s (ppJ2) and five successive parasitic stages isolated from infected potato roots (pJ2, J3, J4, adult females, and adult males). The first lane includes the molecular weight marker, while a sample from non-infected roots (roots) and a ppJ2 sample without reverse transcriptase were included as negative controls. The cAMP dependent protein kinase (cAMP) from *G. rostochiensis* was used as an indicator for constitutive expression.

3.2.5 The SPRYSECs are a component of stylet secretions

We have raised specific polyclonal antiserum to SPRYSEC-family members to test if the encoded proteins are a component in the stylet secretions of the nematode. The polyclonal antiserum raised to the peptides recognized three bands on western blots (~25, 32 and 37 kDa respectively; Fig. 3.3A) suggesting that at least three

family members were detected in the protein extracts of nematodes. Western blots of recombinantly produced SPRYSEC-15 were also probed with the antiserum to test that it indeed specifically recognized members of the gene family (data not shown). The stylet secretions of about 3 million ppJ2s exposed to potato root diffusates were tested on a native dot blot for reactivity with the anti-SPRYSEC polyclonal antiserum (Fig. 3.3B). This antiserum indeed detected the presence of SPRYSEC proteins in the nematode stylet secretions, whereas the pre-immune serum of the rabbit did not bind to the stylet secretions. A monoclonal antibody to a cellulase in the stylet secretions of ppJ2s was used a positive control. Similarly, an antibody to muscle proteins of the nematodes was used to test if contaminations from nematodes, which may have been accidentally lysed during the procedure, were present in the sample.

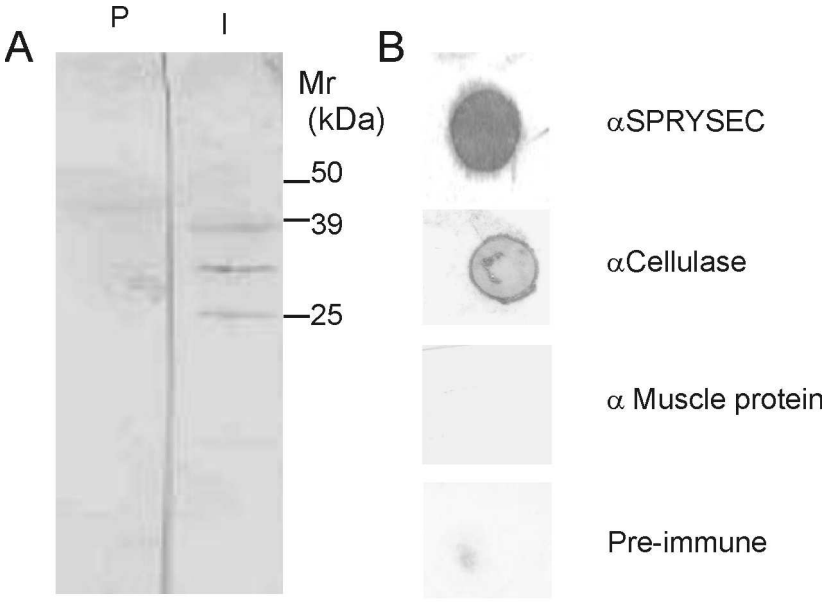

Fig. 3.3. Immunoblots of proteins extracts from homogenized ppJ2s (A) and collected stylet secretions (B) of *G. rostochiensis* with a SPRYSEC specific polyclonal antiserum. **A**, A western blot of a nematode homogenate probed with anti-SPRYSEC polyclonal antiserum raised in rabbits (I) and pre-immuneserum from rabbits (P). **B**, A dot blot of collected stylet secretions from 3 million ppJ2s exposed to potato root diffusate probed with anti-SPRYSEC antiserum raised in rabbits, anti-cellulase antibody MGR048, ant-muscle antibody MGR007, and a pre-immune serum from rabbits.

3.2.6 The diversity in the SPRYSEC gene family mainly localizes to two protein surfaces

A protein sequence alignment of the six most related SPRYSEC family members for protein structure modeling shows an uneven distribution of the sequence similarities between family members (Fig. 3.4). Regions with nearly perfect sequence conservation are interspersed with regions that are highly diverged. Recently, the protein structures of three homologous of B30.2/SPRY have been resolved (SSB-2 from *Mus musculus* with PDB accession 2AFJ (Masters et al., 2006), GUSTAVUS from *Drosophila melanogaster* with PDB accession 2FNJ (Woo et al., 2006b), and PRYSPRY from *Homo sapiens* with PDB accession 2FBE (Grutter et al., 2006)). The overall fold of these proteins is a distorted compact β-sandwich core formed by two anti-parallel β sheets connected by variable loops, with two short α-helices at the N-terminus. Our objective was to investigate if the sequence variability among the SPRYSEC proteins localizes to specific elements in protein folds as predicted by structure homology modeling using the resolved B30.2/SPRY structures as template. SPRYSEC-19 showed the highest level of similarity with any of the three possible templates (~12.1% identity and ~37.1% similarity). From the three structures, GUSTAVUS showed the highest similarity with SPRYSEC-19, and the lowest level of insertions or deletions along the SPRY region and it was therefore used as a template to model SPRYSEC-19 using remote homology modeling. The protein structure model of SPRYSEC-19 was subsequently used to build a consensus structure model of the SPRYSEC family members (Fig. 3.5). Most of the insertion/deletions within SPRYSECs occur in the loops between the β-sheets that form the core of the fold of the protein. As can be readily seen, only two regions of the surface show exceptionally high variability. One highly variable area is located in the so-called surface-A region, and a second region of moderate variability is located at the BC box, corresponding to the α-3 structure (following the annotation of (Woo et al., 2006b)).

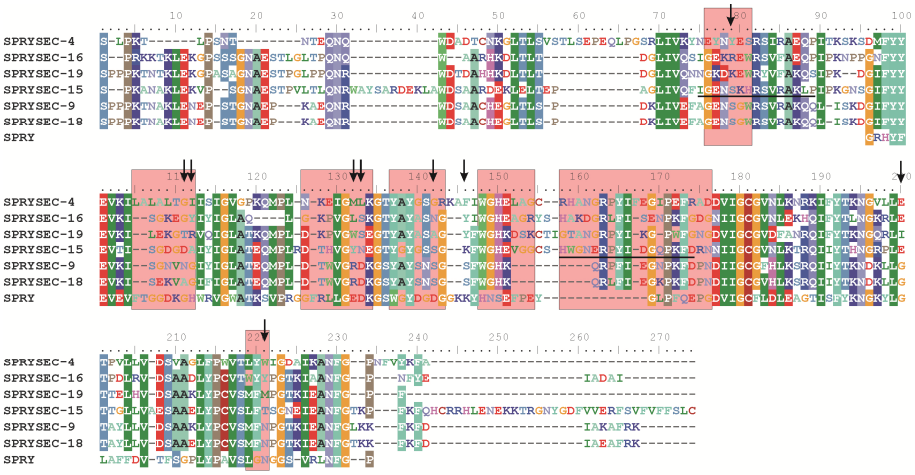

Fig. 3.4. Multiple protein sequence alignment of the SPRYSECs most similar to SPRYSEC-19 and the consensus sequence of the SPRY domains in PFAM accession (PF00622) made for the three-dimensional structure modeling (see Fig. 3.5). The residues are shaded when the conservation level in a column is 80 percent or higher. The residues are color coded according to BLOSUM62 table. The boxes filled pink indicate the extended loops in the protein structure that are part of the surface A in SPRY domains (Woo et al., 2006a). Nine arrows heads indicate positions in the aligned sequences with a probability higher than 0.99 to have been subjected to diversifying selection. Underlines are the sequences of the synthetic peptides used to raise a polyclonal antiserum to the SPRYSECs. The signal peptides were trimmed off prior to the aligning the sequences.

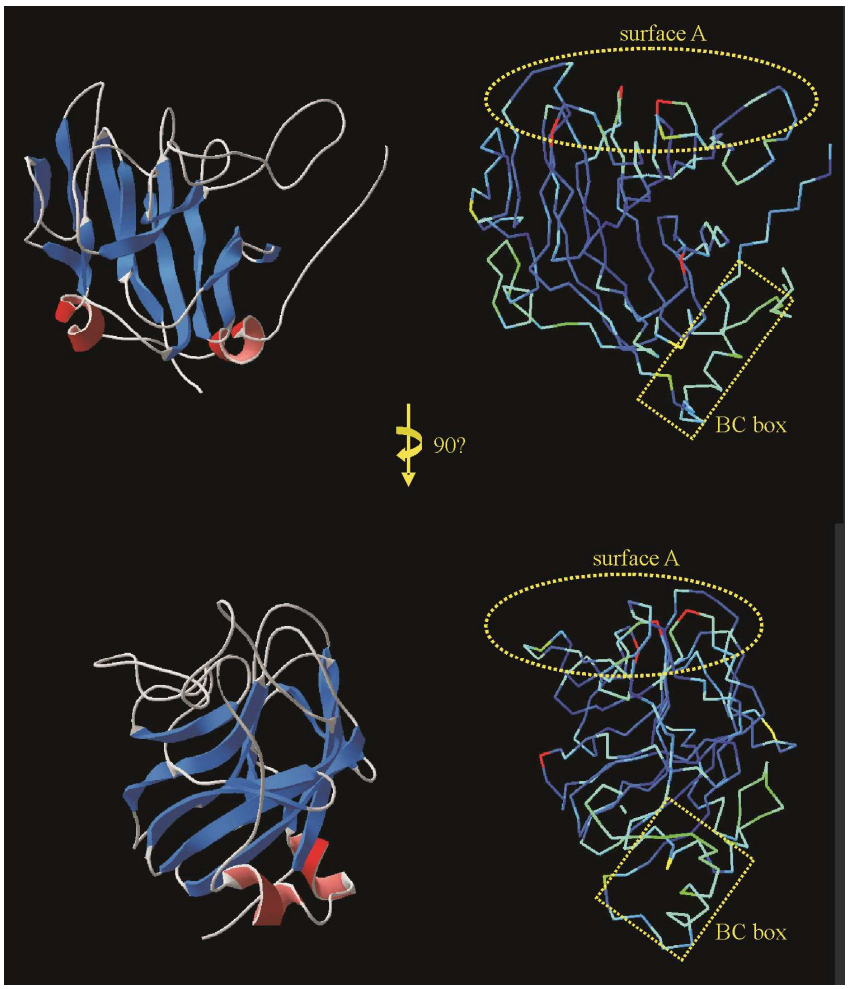

Fig. 3.5. A consensus model of three-dimensional protein structure based on the sequences of SPRYSEC-4, -5, -8, -9, -15, -16, -18, and -19 using PDB accession 2FNJ (GUSTAVUS) as template. The degree of sequence variation in the alignment in Fig. 3.4 is mapped onto the protein structure as a color-coded heat map ranging from high similarity in blue to low similarity in red. Encircled is surface A of the SPRY domains, and boxed is the C-terminal region with structural similarity to the BC box (when following the annotation of (Woo et al., 2006a)).

3.2.7 Most of the positively selected sites in the SPRYSECs localize to surface A

Codon alignments built from cDNA and amino acid sequences of the gene family were analyzed in PAML to test if footprints of diversifying selection are detectable at specific sites in the SPRYSECs (Yang and Bielawski, 2000). We statistically assessed the significance of the ω (dN/dS) > 1 per site under four different evolutionary models (M0, M3, M7, and M8). For nine sites in the alignment of the SPRY domain we found positively selected codons, with probabilities of 0.99 and higher for ω>1 when comparing models M3 with M0, and M8 with M7 (indicated with arrow heads in Fig. 3.4). Seven out of nine sites under diversifying selection localize in the loops that form surface A in the structure model of the SPRYSECs.

3.2.8 SPRYSEC-19 interacts with an LRR domain of a CC-NB-LRR protein

SPRYSEC-15, -18, and -19 were used as bait in yeast-two-hybrid (YTH) screening of a tomato root cDNA library to identify interacting host proteins. After screening of $2x10^5$ independent clones, five cDNA fragments in the library activated the selection markers (AHLW+X-alpha-gal) in yeast in a SPRYSEC-dependent manner for SPRYSEC-19. For SPRYSEC-15 and -18 we did not find specific interactors while using these four selective nutritional markers and various other validations steps. Two of these cDNAs potentially interacting with SPRYSEC-19, named *Int-1* and *Int-2*, included an identical sequence of 894 bp. The interaction between SPRYSEC-19 and *Int-1/2* could be abolished by disrupting the open reading frame (Fig. 3.6A). The interaction between SPRYSEC-19 and *Int1/2* was further validated *in vivo* by yeast-mating (data not shown), and by reciprocal swapping of the BD- and the AD-plasmids for the bait and preys (Fig. 3.6A). Furthermore, SPRYSEC-19 in AD conformation (SPRYSEC-19-AD) did not show autoactivation of auxotrophic yeast strain (AH109) on selective media (without the amino acids AHTL) when co-transformed either with empty vector (BD-E) or with BD-lamin. Similarly, *Int-1/2* did not show autoactivation when co-transformed either with empty vector (BD-E) or with BD-lamin. Thus, in yeast the interaction between SPRYSEC-19 and *Int-1/2* was specific.

Fig. 6A

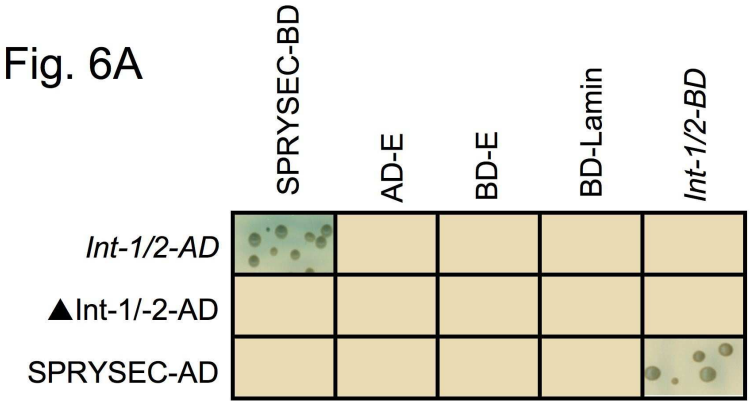

Fig. 6B

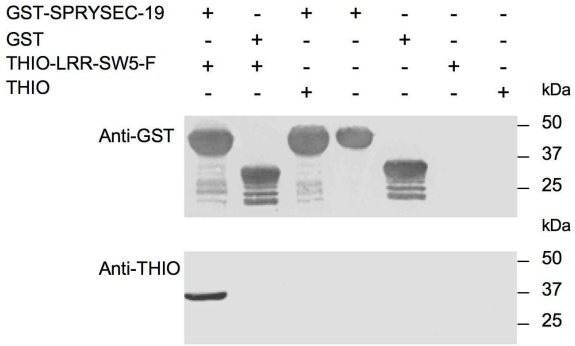

Fig. 6C

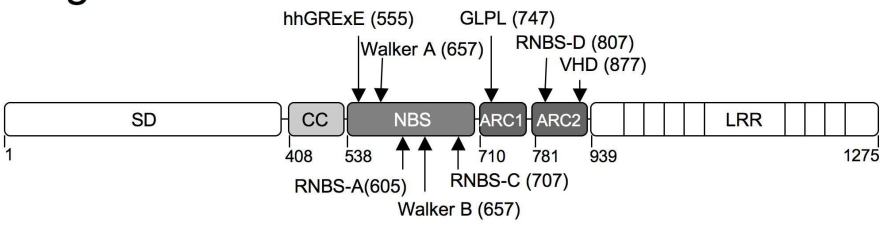

Fig. 3.6A (opposite page). A yeast-two hybrid screening of tomato root cDNA library with SPRYSEC-19. INT1/2-AD is the LRR fragment of a CC-NB-LRR protein fused to the activator domain of GAL4 transcription factor in yeast, which specifically interacts with SPRYSEC-19 fused to the DNA binding domain of GAL4 (SPRYSEC-19-BD) as is shown by the galactosidase activity in yeast cells. The reciprocal constructs with LRR-SW5F fused to BD (Int1/2-BD), and SPRYSEC-19 fused to AD (SPRYSEC-19-AD) also interact in yeast. The LRR-SW5F and the SPRYSEC-19 constructs do not lead to auto-activation of GAL4 when combined with empty vector controls (AD-E and BD-E), and with lamin fused to BD of GAL4. Disrupting the open reading frame of LRR-SW5F (ΔInt1/2) also eliminates the interaction with SPRYSEC-19, which further exclude auto-active GAL4. **Fig. 3.6B.** A GST pull-down assays of recombinant LRR-SW5F fused to thioredoxin (THIO-LRR-SW5F) by the SPRYSEC-19 fused to GST and immobilized on sepharose beads (GST-SPRYSE-19). As controls a pull-down of THIO-LRR-SW5F was also attempted with GST immobilized alone on sepharose beads (GST), and a pull-down of thioredoxin alone (THIO) was also attempted with GST-SPRYSEC-19 immobilized on sepharose beads. The western blot probed with antiserum to GST (A) shows the presence of GST-SPRYSEC-19 or GST alone on the beads. Western blot shows the presence of the LRR-SW5F fused to thioredoxin (THIO-LRR-SW5F) bound to beads carrying GST-SPRY-19 only. **Fig. 3.6C**. The architecture of SW5F showing SD, CC, NBS, ARC1, and ARC2 domains, and the LRR regions. The numbers indicate the positions of the various motifs (as described in the Results section) in the SW5F protein.

The AD-plasmid carrying *Int-1*/2 was rescued from yeast for sequencing of the insert. BLASTX analysis of the insert sequences on the non-redundant database revealed highly significant similarity with the so-called leucine-rich-repeat (LRR)-region of several tospovirus (TSWV) resistance gene homologues from the SW5 resistance gene cluster (AAG31013 through 17), a nematode resistance protein Mi-1.2 (AAC32252), a *Pseudomonas syringae* resistance gene PRF (AAC49408), the *G. rostochiensis* resistance genes Hero (CAD29729) and Gpa-2 (AAF0403). Because *Int-1/2* was highly similar to members of the SW5 cluster from tomato (i.e. SW5A through E) we named it LRR-SW5-F.

LRR-SW5-F matched the C-terminal 194 amino acids of the LRR domain in members of the SW5 resistance gene cluster in tomato. The members of the SW5 cluster

belong to the CC-NB-LRR class of resistance proteins (Spassova et al., 2001), and it was therefore likely that LRR-SW5-F represented only the C-terminus of a larger protein. In order to clone the missing 5`-end of LRR-SW5-F, a degenerate primer (SW5CD in Supplementary Table 3.2) designed on the 5'-end of the members of the SW5 cluster and a gene specific reverse primer (Int-1/2-R) designed on the 3`-untranslated region of LRR-SW5-F were used to PCR-amplify a product of 3992 bp from tomato cDNA. This transcript, named SW5-F, codes for a protein of 1275 amino acids with a predicted molecular mass of 147 kDa (Supplementary Fig. 3.1). The 3`-untranslated region consists of 282 bp and a poly-adenylation signal is located 251 nucleotides downstream of the stop codon. BLASTP searches in non-redundant-protein database using the full SW5-F sequence as query revealed again an extremely high similarity (E-values of 0) with tospovirus resistance gene homologues from the SW5 cluster in tomato (Supplemental Table 3.3).

The architecture of SW5-F displays the typical features of the coiled-coiled, nucleotide binding (NB-ARC), and leucine-rich-repeat family of resistance genes (Fig. 6C; for details Supplementary Fig. 3.1). SW5-F is of approximately the same size as Mi-1.2, SW5-A, SW5-B, SW-5C, SW5-D, and SW5E, while PRF includes a long N-terminal extension of about 300 amino acids (Supplemental Table 3.3). The N-terminal region of SW5-F (amino acids 1-407) showed weak homology with Solanaceae domain (SD; (Mucyn et al., 2006)). Next to the SD of SW5-F (amino acids 435 to 449) a coiled-coil (CC) is predicted by the COILS-program (window 14 with probability of 0.925 and higher), which was also reported for SW5A and SW5B (Spassova et al., 2001). The central NB-ARC1-ARC2 domain contains all the conserved motifs such as the hhGRExE, RNBS, Walker A and B, GLPL, and MHD (VHD in SW5F) motifs as reported for all other disease resistance genes of NB-LRR family (Van Ooijen et al., 2008). In addition, the C-terminal region of SW5-F encodes a leucine-rich-repeat, very similar in size with SW5-A, SW5-B, SW5-C, SW5-D, SW5-E, Mi-1.2, and PRF (Supplemental Fig. 3.1). SW5-F is predicted to have 13 repeats in the LRR domain largely following the consensus sequence xxLxLxx

(starting from amino acid position 939). In conclusion, SW5F has the same CC-NB-LRR architecture as classical receptors of the plant's innate immune system.

A pull-down assay was used to independently confirm the interaction between SPRYSEC-19 and LRR-region of SW5-F *in vitro* (Fig. 6B). To this purpose, SPRYSEC-19 was expressed as an N-terminal gluthathion-S-transferase fusion protein (GST-SPRYSEC-19, ~48kD) immobilized on sepharose beads. *In vitro* translated LRR-SW5-F fused to thioredoxin (THIO-LRR-SW5-F, ~40kD) was incubated with either GST-SPRYSEC-19 immobilized to the beads or GST immobilized to the beads alone (~35kD). After extensive washings, bound proteins were resolved by SDS-PAGE and western blotting with antiserum recognizing GST and THIO. The THIO-LRR-SW5-F bound specifically to GST-SPRYSEC but not to GST alone. Similarly, thioredoxin alone (THIO, 19.5kD) did not bind to GST-SPRYSEC-19 on sepharose beads or to sepharose beads alone.

3.3 Discussion

The plant parasitic nematode *Globodera rostochiensis* delivers the products of its parasitism genes (effectors) directly into host tissues through an oral stylet. These effectors are crucial for host invasion, feeding, and modulation of the host defense responses. Here we present a novel gene family in *G. rostochiensis* coding for a B30.2/SPRY domain in the nematode's stylet secretions. The SPRY is an extremely versatile domain, which so far has been found in 53 different architectures with a variety of other domains (see PFAM accession PF00622). SPRYSECs only consist of a single B30.2/SPRY domain and a signal peptide for secretion. Other proteins with exactly the same architecture are found in the venom glands of venomous snakes and lizards. These proteins, named the *vespryn* (the name derives from Venom PRY-SPRY domain containing proteins with a signal-peptide), includes four members so far (i.e. ohanin/pro-ohanin (Pung et al., 2006)], Lizard venom (Fry et al., 2006), thaicobrin (Junqueira-de-Azevedo et al., 2006), and ohanin-like protein (Li et al., 2004)). Another group of secreted B30.2/SPRY proteins are the

stonustoxins from the stonefish (*Synanceja horrida*, (Ghadessy et al., 1996) and the neoverrucontoxins from the stonefish (*Synanceia verrocusa*; (Ueda et al., 2006)). These stonefish toxins form multimeric complexes of large subunits (70-85 kDa) with the B30.2 at the C-terminus of each subunit. Despite having similar domain architectures, none of the proteins mentioned above have significant similarity to primary amino acid sequences of the SPRYSECs. Therefore, the use of the B30.2/SPRY domain in plant-microbe interactions seems to be an evolutionary innovation in nematodes.

The best matching sequences in the database for the SPRYSECs were the SPRY domains of metazoan RAN-binding proteins. However, the SPRYSECs are much smaller than RAN-binding proteins (about 27 kDa versus 65-70 kDa), and lack the typical LisH and the CTLH domains (see for review (Murrin and Talbot, 2007)). The physical interaction between *ran* and RANBPM is likely mediated through its SPRY domain. It could therefore be argued that the SPRYSECs exert their activity in a manner still similar to that of RAN-binding proteins. In a yeast-two-hybrid analysis with two *ran* genes from tomato and SPRYSEC-19, we have not found a physical interaction (data not shown). Ectopically expressed metazoan RanBPM interferes with the dynamic stability of microtubuli, which leads to uncoordinated aster formation in the recipient cells (Nakamura et al., 1998). Overexpression of SPRYSEC-15 and -19 in a transgenic tobacco cell line with microtubuli-associated protein MAP4 fused to GFP did not show abnormal microtubule organization (data not shown). We therefore conclude that the SPRYSEC gene family and RAN-binding proteins in nematodes may have a common evolutionary history, but that the differences in protein architecture and the experimental data point at different roles.

In addition to its binding to *ran* the SPRY domain in RAN-binding proteins has been shown to interact with a variety of other proteins mostly involved in signaling, including several receptor protein-tyrosine kinases. For example, the SPRY domain of RanBP9 interacts with MET, a receptor protein kinase for the hepatocyte growth factor, which is a multifunctional cytokine controlling cell growth, morphogenesis,

and motility (Wang et al., 2002. RanBPM has also been shown to interact through its SPRY domain with intracellular domain of neurotrophin receptor Trk {Yuan, 2006 #1881). Similarly, the SPRY domain of RanBPM mediates the interaction between RanBPM and human dectin-1 trans-membrane receptor isoform hDectin-1E (Xie et al., 2006). It has also been demonstrated that RanBPM interacts with integrin LFA-1 and is phosphorylated within the SPRY domain at residue Thr320 both constitutively and in response to stress by p58 kinase (Denti et al., 2004). Apparently the SPRY domain in RAN-binding proteins is capable of interacting with a diverse range of receptor-like proteins, which mostly seems to modulate down-stream signal-transduction pathways.

Typically, alignments of SPRY domains show conserved blocks interspersed with highly variable stretches of varying length and sequence in the loops at the surface of the protein (Rhodes et al., 2005). We found that most of the amino acid sites under diversifying selection in the SPRYSECs are also in these loops suggesting that this hypervariable surface likely interacts with host targets. It should be noted that because of a lack of sufficient alignment between members of the SPRYSEC gene family within the loops, which impedes further analysis in PAML in these regions, the number of sites under diversifying selection may even be an underestimation. Seto at al (Seto et al., 1999) noted that the core structure of the SPRY domain is reminiscent of the classical immunoglobulin fold. More recent work suggest, however, that the actual topology of the B30.2/SPRY domain represents a novel fold distinct from the immunoglobulin fold (Masters et al., 2006). Nonetheless, the concept of a stable scaffold with hypervariable complementarity-determining regions of immunoglobulins, which are located in the extended loops at one side of the beta-sandwich, seems applicable to the SPRY domain as well. The wide range of binding specificities in immunoglobulins is brought about by variations in length and in amino acid sequence in the hypervariable loops (reviewed in (Wilson and Stanfield, 1993). A preliminary study of the genetic and allelic variation in SPRYSECs in *G. rostochiensis* suggests that this gene family includes many more members (>50) with sequence and length variations in the extended loops (T. Tytgat,

personal communication). We conclude that the SPRY domain is also used in SPRYSECs in *G. rostochiensis* as a versatile scaffold with a hypervariable surface capable of targeting many different host proteins.

We used a combination of yeast-two-hybrid analysis and *in vitro* GST pull-down assays to find a specific physical interaction of the nematode effector SPRYSEC-19 with the LRR-region of the tospovirus resistance gene homolog SW5-F from tomato. The CC-NB-LRR architecture of SW5-F is typical for the NB-LRR class of disease resistance genes. Furthermore, SW5F is highly similar to the other members of the SW5 disease resistance cluster in tomato (E-value of 0 with 69 – 83% identity and 79 – 87% similarity over 1275 amino acids). In the gene-for-gene model for disease recognition specificity, R proteins activate a resistance response, often a hypersensitive response, only when they detect the presence of specific pathogen effectors (Jones and Dangl, 2006). Detection can either be directly, or indirectly via changes in another guarded host protein. The tomato cultivar harboring the SW5-F gene is susceptible to potato cyst nematodes expressing the SPRYSEC-19. Nonetheless, we tested if SPRYSEC-19-activates a SW5-F-dependent hypersensitive response in plants. We found that transient co-expression of SW5-F and its interacting nematode effector SPRYSEC-19 does not evoke a hypersensitive response in *N. benthamiana* (data not shown). Consequently, we have no evidence that the interaction between SW5-F and SPRYSEC-19 conditions resistance to the nematodes in tomato.

Our findings thus lead us to two alternative models for the role of SPRYSEC-19 and SW5F in nematode-plant interactions which are not mutually exclusive. In our first model, the physical interaction between SPRYSEC-19 and SW5F is an evolutionary intermediate. There is evident binding between a nematode protein and a host plant protein, but this does not lead to the activation of disease resistance signaling (anymore). Other R gene homologs of SW5F may exist in various (resistant) host plants that both bind and elicit a resistance response following the interaction with SPRYSEC-19. Alternatively, other homologs of SPRYSEC-19 that also bind to SW5F and do elicit a resistance response may exist in other (avirulent) *G. rostochiensis*

populations. Although the outcome of our co-expression experiment in *N. benthamiana* does not point in that direction, in principle SPRYSEC-19 could be able to trigger an immune response via its interaction with SW5F, but other co-secreted nematode effectors may suppress this response rendering the plant full susceptible to infections.

In our second model, SPRYSEC-19 binds to SW5-F to promote the virulence of the nematode by modulating host defense responses. Interestingly, a parallel may be found in the protein that was used as the best modeling template for the SPRYECs, the SOCS-box containing protein GUSTAVUS (Woo et al., 2006b). GASTAVUS binds VASA via its SPRY domain, and the heterodimer elongin B and C through its BC box. The BC box is an α-helical element in the SOCS box. It is thought that the elongin BC connects the SOCS-box containing proteins to the so-called elongin C-cullin-SOCs-box (ECS)-type of E3 ubiquitin ligase. SOCS-containing proteins such as GUSTAVUS and VHL may target proteins bound to the SPRY domain to the proteosomal degradation pathway, and thereby regulate their turnover rate (Kile et al., 2002). At the C-terminus of the protein structure model of the SPRYSECs is α-helical structure that could be functionally homologous to the BC box in GUSTAVUS. We therefore hypothesize that some of the SPRYSEC family members may act as adapters to confer diverse recognition specificities to the host's E3 Ubiquitin ligase complexes. The SPRY domain in the SPRYSECs appears to provide a hypervariable binding surface within a stable scaffold structure, which is likely capable of interaction with many different host proteins. Modifying their rate of turnover may thus regulate these molecular targets of the SPRYSECs in host cells, for instance components in the innate immunity in plants. Further research is required to identify more molecular targets of the SPRYSECs in host cells, and to study the possible involvement of the host's ubiquitination machinery in SPRYSEC-mediated regulation of these targets.

SPRYSEC	EST (Genbank accession no)	Clone No[2]	TDF[3]
4	BE607310	cGE2075	
5	BM343869 EE266730 EE266731 BM356075	rr43h11.y1 GRAA-aaa46a03.b1 GRAA-aaa46a03.g1 rr33c05.y1	
8	BM344321 BM344784 EE266329 BM345924 EE266866	rr49d12.y1 rr59c02.y1 GRAA-aaa43f11.g1 rr09g10.y1 GRAA-aaa46g05.g1	
9	BM343244 EE266583 EE266584	rr36b03.y2 GRAA-aaa45b09.b1 GRAA-aaa45b09.g1	
15	BM344614	rr57a05.y1	A41
16	BM343590	rr40d09.y1	
18	BM343498 BM344069 BM344199 BM354706 BM355689 GE1519	rr39c05.y1 rr46c12.y1 rr48a04.y1 rr15h02.y1 rr28c10.y1	E19
19	AJ251757	-	A18

Supplementary Table 3.1: Expressed sequence tags with corresponding clone numbers and transcript-derived fragments assembled into eight contigs coding for full-length SPRYSECs

[2] Nematode Net clone number (http://www.nematode.net/index.php)

[3] cDNA-AFLP Transcript Derived Fragment (TDF) number (Qin et al, 2000)

SPRYSEC	Forward primer (5'→ 3')	Reverse primer (5'→'3)
	In situ hybridization	
4	ATGAAAAGCCCGGACAGAAATG	AAATAAAGGATCGTCTGTTCCTTCC
5	AGAAACTGAAAAACTTGAAAC	CTACTCGGTTTTCAGTTTCTC
8	CTAACCAGTGAAAATCAATGGG	TAGCGAACACAGTGCAAAATCC
15	CATATTCCGCACGTGACGAG	TTTCTCGTCACGTGCAGCGG
18	CTTCCACTCTGCTGGAAACGGATGC	CAATTTGTCGGGCGAGAGTGTCAG
19	Qin et al. (2000)	Qin et al. (2000)
	Semi-quantitative RT-PCR	
4	AAGAATGGAGTGCTTTTAGAAAC	CTGTCCGGGCTTTTCATGCG
5	AGAAACTGAAAAACTTGAAAC	CTACTCGGTTTTCAGTTTCTC
8	GCACTGTGTTCGCTAAAGAG	TGGAAACATAACCTGATTCTGAAG
15	AATTTGACCGTAACAACATCATC	GACTCGGCGACTAACAGC
18	CAAAAGACGGCATTTTCTACTAC	GCGTAAGAGCCTTTGTCAC
19	TTGTACCCGTGCGTTTCG	ACCTCCACAGCAAATTCCTAC
cAMP	ATCAGCCCATTCAAATCTACG	TTCTTCAGCAAGTCCTTCAAC
	PCR based cloning	
YA18F	GACCTG**CATATG**CCGCCGCCAAAAACAAAC	
YA18R	GTCGAC**GGATCC**AATTCAAAATGGGCCAAAG	
SPGF	CTG**GGATCC**CCGCCGCCAAAAACAAAC	
SPGR	CCG**CTCGAG**AATTCAAAATGGGCCAAAGTTC	
SW5CDF	ATGGCTCAAAATGAAATTGA	
Int1/2-R	GCACAGCAAAAGTATCATGTCA	

Supplementary Table 3.2: PCR primers used in situ hybridization, semi-quantitative RT-PCR, and PCR-based cloning

Supplementary Table 3.3: Top-10 of best matching sequences of SW5F in the non-redundant protein databases. Note the high similarity to members of the SW5 tospovirus resistance cluster in tomato.

Gene (accession)	Resistance	Source	Length (AA)	Identity %	Similarity %
SW5C (AA31015)	TSWV	S. lycopersicum	1275	83	87
SW5A (AA31013)	TSWV	S. lycopersicum	1245	69	79
SW5B (AA31014)	TSWV	S. lycopersicum	1246	69	79
SW5D (AA31016)	TSWV	S. lycopersicum	1263	68	77
SW5E (AA31017)	TSWV	S. lycopersicum	1241	69	79
Rpi-blb2 (AAZ95005)	Phytophthora infestans	S. bulbocastanum	1267	31	49
CaMi (ABE688835)	Meloidogyne incognita	Capsicum annuum	1257	32	50
PRF (AAF76308)	Pto-resistance cluster	S. pimpinellifolium	1824	32	52
Mi-1.2 (AAC32252)	Meloidogyne incognita	S. lycopersicum	1206	35	50
R1B-14 (AAT38776)	Phytophthora infestans	S. demissum	1317	34	52

3.4 Materials and methods

3.4.1 Nematodes

Dried cysts of *G. rostochiensis* pathotype Ro1 Mierenbos were soaked on a 100 µm sieve in potato root diffusate (PRD) to collect hatched preparasitic second-stage juveniles (J2s; (De Boer et al., 1992)). Freshly hatched J2s in suspension were mixed with an equal volume of 70% (w/v) sucrose in a centrifuge tube and covered with a layer of sterile tap water. Following centrifugation for 5 minutes at 1,000 *g* juveniles were collected from the sucrose-water interface using a Pasteur pipette and washed 3 times with sterile tap water. Parasitic stages were isolated from roots of potato cultivar Bintje at 13, 19, 23, 27, and 34 days post inoculation to yield samples of second, third, and fourth stage juveniles, adult males and females respectively. To this purpose infected roots were cut into small pieces with a blender, and nematodes were separated from root debris on sieves with a mesh of 250, 175, 100, 22, and 10 µm. The isolated the nematodes were either used for experiments directly or stored at -80°C until further use.

3.4.2 Cloning, sequencing, and analysis of SPRYSECs

The DNA sequences of TDFs were used to mine the 10,000 expressed sequence tags from pre-parasitic and parasitic J2s of *G. rostochiensis* (Supplemental Table 3.1). The library clones from which the ESTs were subsequently sequenced using the T7 and the SP6 primer site of the pCDNAII library plasmid (Baseclear, Leiden, the Netherlands). The sequences were assembled into contigs and checked for likely complete open reading frames as well as the presence of a polyA-tail. For contigs that were suspected to include partial reading frames, primers were designed to amplify flanking regions up- and downstream with the rapid amplification of cDNA ends (Supplemental Table 3.2; Invitrogen, San Diego, USA). The contig assembly was done in Contig Express of the VectorNTI software package (Invitrogen). The assembly criterion was set at 100 percent identity in a minimal overlap of 100 nucleotides.

3.4.3 Semi-quantitative RT-PCR

Messenger RNA was extracted from five developmental stages of *G. rostochiensis* pathotype Ro1-Mierenbos essentially as described previously (Qin et al., 2000). RT-PCR was performed using Superscript III essentially according to the manufacturer's protocols (Invitrogen, San Diego, USA; (Kudla et al., 2007)). Total RNA isolated with TRIzol (Invitrogen) was treated with Turbo DNA-free (Ambion, Austin, USA) to degrade contaminating genomic DNA. Messenger RNA was subsequently isolated from total RNA samples using Dynabead mRNA purification system (Invitrogen). First strand cDNA synthesis was done with a mix of random hexamer and oligo-dT primers in a reaction with 0.5mM dNTPs, 0.1 units RNase-out, 10 units Superscript III for 60 min at 50°C and 15 min at 70°C. Prior to the PCR the samples were incubated with 2 units of RNase-H for 20 min at 37°C. Fragments of SPRYSECs were PCR amplified in 26 cycles with gene primers (Supplemental table 3.2), whereas a forward and reverse primer designed on cAMP were used to amplify a 91 bp fragment of the constitutively expressed cAMP dependent protein kinase (*Gr*-

cAMP; Genbank accession BM343563). Reactions without reverse transcriptase we included to test for possible amplification of the target genes from contaminating genomic DNA. A sample made from non-infected roots was used to check for non-specific amplification from host plant tissues.

3.4.4 In situ hybridization microscopy

DNA probes were amplified from the SPRYSECs by using specific oligonucleotide primers (Supplemental Table 3.2) and digoxigen-11-dUTP. Pre-parasitic J2s were fixed overnight in 2% paraformaldehyde, cut into sections, and permeabilized as described (De Boer et al., 1998; Smant et al., 1998). Fixed sections were then incubated at 50□C with sense or anti-sense DNA-probes followed by digestion with RNAse A and stringency washes. Hybridized DNA-probe was detected using an anti-digoxigenin antibody and alkaline phosphatase staining (Genius kid, Boehringer Mannheim). Stained J2 were examined with differential interference contrast microscopy (Leica, Dfeerfield).

3.4.5 Antiserum and immunodetection

Polyclonal antisera was raised to synthetic peptides designed on the SPRYSEC gene family members(Eurogentec, Ghent, Belgium). To this purpose, two antigenic peptides (IGENSKHRSVRAKLPC [present in SPRYSEC-9, -15, and -18] and HWGNERPYIDGQPKFD [present in all SPRYSECs] were used for the immunization of rabbits.

Western blots of homogenates of pre-parasitic J2s were performed as described by (De Boer et al., 1996). Proteins were separated on 12.5% denaturing polyacrylamide gels by SDS-PAGE and transferred subsequently on 0.2µm nitrocellulose membrane (Schleicher and Schuell) by semi-dry blotter with dry blot buffer (48mM Tris, 150mM Glycine, 10% methanol, pH 8.3). The blots were probed with different primary antibodies, including anti-GST (Amersham), anti-thioredoxin (Invitrogen), and anti-SPRYSEC, followed by their detection with alkaline-phosphatase-conjugated rabbit anti-goat, rabbit-anti-chicken IgY, and rat-anti-

mouse (Jackson) respectively. The blots were developed in substrate buffer supplemented with nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sambrook et al., 1989).

Dot blots of collected stylet secretions were made as was described previously (Smant et al., 1997). The dot blots were probes with anti-SPRYSEC serum, anti-cellulase monoclonal antibody MGR048 (Smant et al., 1998), and a monoclonal antibody to nematode muscle protein MGR007 (De Boer et al., 1996).

3.4.6 PAML

The six sequences most similar to SPRYSEC19 were tested for positive selection, including SPRYSEC-4, -9, -15, -16, and -18. The ratio ω was estimated with the codeml program of PAML (phylogenetic analysis by maximum likelihood) (Yang, 1997; Yang and Bielawski, 2000). Two models of fitting codon substitution were used to calculate likelihood ratio statistics (LR), twice the log-likelihood between models is compared with the value of a χ^2 distribution with branches-1 degrees of freedom. Model M7 (β distributed variable selection pressure) has an ω for each site drawn from a β distribution with parameters *p* and *q*. Model M8 (β plus ω > 1) uses the M7 recipe for a fraction p_0 of the sites and assigns another ω to the remaining fraction. When M8 fits the data significantly better than M7 and the ω ratio estimated under model M8 is greater than 1, we assume evidence of positive selection. To check whether it is significantly greater than 1 the log-likelihood value in M8 is recalculated fixing ω to be 1 (model M8A from (Wong et al., 2004)) and compared to the change in likelihood with a χ^2 distribution with 1 degree of freedom. Likewise the less complicated models M0 (uniform selective pressure among sites) with M3 (variable selective pressure among sites) were calculated and the results were found to give less conservative estimates than M7/M8. The amino acid sequence alignment was generated in ClustalX (v1.83) (Chenna et al., 2003) and pal2nal (v11; (Suyama et al., 2006)) was used to relate the sequences back to a nucleotide alignment.

3.4.7 3-D structure modeling

For the secondary structure prediction, we used the programs GOR IV Jpred, HNN, and PROF. Homology modeling was performed with Insight II software package (Accelrys, Cambridge, UK). The Homology module was used for coordinate transfer and loop generation. Local simulated annealing and energy minimization during modelling steps were performed via the Discovery Studio module with Class II Force Field (CFF). The structures of three SPRY domains are in Protein Data Bank, including SSB-2 from *Mus musculus* (PDB accession 2AFJ; (Masters et al., 2006)), GUSTAVUS from *Drosophila melanogaster* (PDB accession 2FNJ; (Woo et al., 2006b)), and PRYSPRY from *Homo sapiens* (PDB accession 2FBE; (Grutter et al., 2006)). The fold of these proteins is a β-sandwich core formed by two antiparallel β sheets connected by variable loops (Woo et al, 2006). Because of the low overall sequence similarity remote homology modeling had to be used. The approach was to transfer the coordinates along the stretches that form the β-sandwich core and to generate ab initio the loops between the β structures. GUSTAVUS shows the highest similarity with SPRYSEC19 and the lowest level of insertions or deletions along the SPRY region and was therefore used as a template. The variability at a given position was defined as the average of the Blosum62 substitution matrix values between every sequence and the consensus.

$\sum_i \frac{M(S_{ij}, C_j)}{i}$, where S_i - sequence *i*, *C* - consensus sequence, *j* - position

3.4.8 Yeast two hybrid screening

The prey tomato root cDNA library used in present study has been described in Van Bentem et al. (#2005#). The library consisted of 2-million independent clones with average insert size of library 1.1kb. The MATCHMAKER Two-hybrid System 3 (Invitrogen) was used to construct all the bait and prey constructs in this study. For SPRYSEC-19 bait construction, coding region of SPRYSEC-19 was amplified with primers YA18-F and YA18R by PCR, underlined sequence represents these

restriction sites. The amplification products were cloned by restriction digestion in frame with Gal4 DNA-binding domain of vector pGBKT7 (Invitrogen) by using NdeI and BamHI restriction sites. For the construction of ▲Int-1/-2-AD, the reading frame was disturbed by cloning Int-1/2 insert from pACT2 to pGADKT7 by EcoRI and XhoI restriction digestion. For the swap analysis, SPRYSEC-19 was cloned in pGADKT7 by using restriction digestion with NdeI and BamH1 from SPRYSEC-19BD (SPRY-pGBKT7). Similarly, *Int-1/2-BD* was constructed by recloning *Int-1/2* from *Int-1/2-AD* into pGBKT7 from pACT-2 by using EcroR1 and Xho1 restriction sites.

In order to identify SPRYSEC-19 interactors, AH109-yeast cells were simultaneously co-transformed with pACT2-tomato cDNA library (prey) and bait (pGBKT7-SPRYSEC-19) according to manufacturer's protocol. The transformants were plated on minimal SD agar base medium lacking four essential amino acids (–AHLW) but including 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-gal) followed by incubation at 30^{o}C for 10-days. Blue colonies were selected as positive candidates followed by rescuing of AD-plasmid following manufacturer's instructions. The prey and bait plasmids did not auto activate marker genes when co-transformed with empty bait and prey plasmids respectively. For yeast mating, pGBKT7-constructs were introduced into yeast strain Y187 and AD-plasmids (pACT2 and pGADKT7) were introduced into AH109 yeast strain following manufacturer's protocol. For each of Y187 and AH109 transformants, a 2-3 mm colony was vortexed in 200 µl of YPDA and mixed together followed by incubation of the mixture at 30 ^{o}C for 16-hours with shacking. The transformants were spread on plates with minimal SD/-AHLW+ X-α-gal medium followed by incubation at 30 ^{o}C for 3-5 days.

3.4.9 Expression and purification of recombinant protein from *Escherichia coli*

Coding region of SPRYSEC-19 was PCR amplified from cDNA library (Smant et al., 1998) using primers SpGF and SpGR (Supplemental Table 3.2) with BamH1 and Xho1 overhangs at 5`end and directionally cloned in pGEX-KG (Guan and Dixon,

1991). For GST-LRR-SW5-F construct, LRR region of SW5-F from AD-Int1/2 plasmid was isolated by restriction digestion (Nco1 and Xho1) and cloned in pET-42b (Novagen). The same *Int-1/2* fragment was also cloned in pET-32b (Novagen) by restriction digestion to produce THIO-LRR-SW5-F. The BL21 (DE3) cells were transformed with GST, GST-LRR-SW5-F, GST-SPRYSEC-19, THIO, and THIO-LRR-SW5-F followed by induction with 1 mM-IPTG at 30^{o}C for 5-hours. The cells were lysed by sonication in 1 times strength PBS (containing a complete protease inhibitor cocktail) and supernatant was kept in -20C till further use.

3.4.10 *In vitro* GST pull down assay

The protocol for the GST-pull down assay has been adopted from Nguyen and Goodrich (#2006#). In the first strategy, soluble fraction of bacterial lysate containing either GST or GST-SPRYSEC-19 was incubated with glutathione sepharose 4B beads (Amersham) for 2-hours at 4^{o}C followed by removal of lysate by centrifugation. To bound GST or GST-SPRYSEC-19, THIO-LRR-SW5-F, or THIO alone were added and incubated at 4^{o}C for 4-hours. As negative control, sepharose beads were incubated with THIO and THIO-LRR-SW5-F respectively.

3.4.11 Cloning of SW5-F

To amplify full-length SW5-F from tomato cultivar GCR-161, 100 mg of leaf tissue from tomato cultivar GCR-161 (#Kroon and Elgersma, 1993#) was grinded in liquid nitrogen with mortar and pastel and total RNA was isolated using Invisorb spin plant RNA mini kit (Invitek). First strand cDNA was prepared from 5 µg of total RNA using Superscript first-strand cDNA synthesis kit (Invitrogen) following manufacturer's protocol. The cDNA was used for amplification using BD Advantage™ 2 PCR Kit (BD life sciences) with primers SW5CD in combination with Int1/2-R (Supplemental Table 3.2). The amplification product of about 4 kb was cloned in pCR4 TOPO (Invitrogen) and sequenced.

4

The effector SPRYSEC-19 of *Globodera rostochiensis* suppresses CC-NB-LRR-mediated disease resistance in plants

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Abstract

The potato cyst nematode *Globodera rostochiensis* invades roots of host plants where it transforms cells near the vascular cylinder into a permanent feeding site. The host cell modifications are most likely induced by a complex mixture of proteins in the stylet secretions of the nematodes. Resistance to nematodes conferred by nucleotide-binding-leucine-rich repeat (NB-LRR) proteins usually results in a programmed cell death in and around the feeding site, and is most likely triggered by the recognition of effectors in stylet secretions. However, the actual role of these secretions in the activation and suppression of effector-triggered immunity is largely unknown. Here we demonstrate that the effector SPRYSEC-19 of *G. rostochiensis* physically associates in planta with the LRR domain of a member of the SW5 resistance gene cluster in tomato (*Lycopersicon esculentum*). Unexpectedly, this interaction did not trigger defense-related programmed cell death and resistance to *G. rostochiensis*. By contrast, agroinfiltration assays showed that the coexpression of SPRYSEC-19 in leaves of *Nicotiana benthamiana* suppresses programmed cell death mediated by several coiled-coil (CC)-NB-LRR immune receptors. Furthermore, SPRYSEC-19 abrogated resistance to Potato virus X mediated by the CC-NB-LRR resistance protein Rx1, and resistance to *Verticillium dahliae* mediated by an unidentified resistance in potato (*Solanum tuberosum*). The suppression of cell death and disease resistance did not require a physical association of SPRYSEC-19 and the LRR domains of the CC-NB-LRR resistance proteins. Altogether, our data demonstrated that potato cyst nematodes secrete effectors that enable the suppression of programmed cell death and disease resistance mediated by several CC-NB-LRR proteins in plants.

4.1 Introduction

The survival and reproduction of the potato cyst nematode *Globodera rostochiensis* relies on the successful establishment and maintenance of a feeding site inside the root of a host plant. Secretions produced by sedentary plant-parasitic nematodes

such as *G. rostochiensis* are thought to be instrumental in the formation of the feeding site (Haegeman et al., 2012). The nematodes use an oral stylet to deliver these secretions into the apoplast and the cytoplasm of host cells (Hussey, 1989; Davis et al., 2008). In a susceptible host plant, a recipient host cell may respond by increasing its metabolic activity and by progressing through several cycles of endoreduplication. The concomitant local cell wall degradation and subsequent fusion with neighboring protoplasts transform the infected host cells into a multinucleate syncytium (Sobczak et al., 2009). Freshly-hatched infective juveniles of *G. rostochiensis* are mobile, but as soon as feeding on the syncytium commences they lose their body wall muscles and adopt a sedentary lifestyle (De Boer et al., 1992). The syncytium functions as a metabolic sink that transfers plant assimilates from the conductive tissues in the vascular cylinder to the sedentary nematode (Jones and Northcote, 1972). A failure in syncytium formation caused, for example, by host defense responses prevents development of the feeding nematode into its reproductive stage (Sobczak et al., 2009).

The majority of plant resistance proteins are members of the NB-LRR receptor family, which consist of a central nucleotide-binding (NB) domain and a leucine-rich repeat (LRR) domain at the carboxyl terminus (Eitas and Dangl, 2010). At their amino-termini, the NB-LRR plant immune receptors either carry a coiled-coiled (CC) domain, or a Toll/Interleukin-1 receptor like (TIR) domain. The NB domain, which is also referred to as the NB-ARC (nucleotide-binding adaptor shared by APAF-1, certain Resistance proteins, and CED-4) domain, most likely changes from a closed ADP bound state to an open ATP bound state when the resistance protein detects a pathogen (Lukasik and Takken, 2009). The LRR domain is thought to act as the sensor in NB-LRR receptors, which in the absence of the cognate effector keeps the resistance protein in an autoinhibited “off” state. In this model, the recognition of a pathogen effector induces a conformational change in the LRR domain that lifts the inhibition of the NB domain in the core of the resistance protein. Artificially induced mutations in NB-LRR immune receptors suggest that the two functions of the LRR domain, pathogen recognition and negative regulation of the NB domain,

reside in different parts of the domain. Several sequence exchanges and deletions at the N-terminus of the LRR domain switch NB-LRR immune receptors into a permanent effector-independent autoactive state (Rairdan and Moffett, 2006). By contrast, mutations in repeats at the C-terminus of the LRR domain do not lift the autoinhibition, but instead change the recognition specificity of NB-LRR immune receptors (Farnham and Baulcombe, 2006).

The molecular mechanisms underlying effector recognition by plant immune receptors are not well understood. NB-LRR immune receptors may activate signaling pathways that lead to effector-triggered immunity when they physically associate with their cognate effectors (Krasileva et al., 2010). However, the fact that such direct interactions seem to be exceptional inspired the formulation of the 'guard' model in which immune receptors activate host defenses by detecting effector-induced perturbations in other plant proteins (Ghassemian et al., 2000). Plant immune receptors may thus efficiently expand the spectrum of disease resistances of a plant by guarding common virulence targets of multiple effectors (Chung et al., 2011). In the recently proposed intermediate 'bait-and-switch' model a pathogen effector may still directly interact with NB-LRR immune receptors but only after binding to an accessory protein that functions as co-factor for the receptor (Collier and Moffett, 2009).

There are only few examples of plant immune receptors that directly interact with their cognate pathogen effector (Jia et al., 2000; Deslandes et al., 2003; Ellis et al., 2008; Krasileva et al., 2010; Tasset et al., 2010; Chen et al., 2012). For only three of these resistance proteins a physical association with the effector was demonstrated *in planta*. The TIR-NB-LRR resistance protein RPP1 of *Arabidopsis thaliana* associates via its LRR domain with the effector ATR1 of *Peronospora parasitica* (Krasileva et al., 2010). This interaction results in a defense-related programmed cell death in leaves of *Nicotiana tabacum*. Also in *Arabidopsis*, the association of the TIR-NB-LRR resistance protein RRS1-R with the PopP2 effector of *Ralstonia solanacearum* results in immunity (Tasset et al., 2010). Similarly, the

physical association of the CC domain of the resistance protein RB from potato with the IPI-O1 effector of *P. infestans* triggers a programmed cell death in *N. benthamiana* (Chen et al., 2012). Recently, we found that the effector SPRYSEC-19 of *G. rostochiensis* interacts in yeast with the seven C-terminal repeats of the LRR domain of the CC-NB-LRR protein SW5F of tomato (Rehman et al., 2009b). The SW5 resistance gene cluster in tomato confers resistance to a broad range of tospoviruses (Zhao and Guo, 2011). Five other SW5 resistance gene homologs have been identified in tomato. The homolog SW5B confers resistance to tomato spotted wilt virus (TSWV), whereas the functions of SW5A and SW5C-F are currently unknown (Spassova et al., 2001).

SPRYSEC effectors are produced as secretory proteins in the dorsal esophageal gland of *G. rostochiensis* that is connected via the lumen of the esophagus to the oral stylet (Rehman et al., 2009b). They only consist of a SPRY/B30.2 domain, which in many different eukaryotic proteins is involved in intermolecular interactions (Rhodes et al., 2005; Tae et al., 2009). The expression of the SPRYSEC effectors in *G. rostochiensis* is highly upregulated in infective juveniles and during the first few days post invasion. The function of the SPRYSEC effectors in plant parasitism is not well understood. It has been shown that the coexpression of the SPRYSEC GpRBP1 from *G. pallida* and the CC-NB-LRR resistance protein Gpa2 from potato induces a programmed cell death in leaves of *N. benthamiana* (Sacco et al., 2009). This finding suggests that GpRBP1 triggers Gpa2-mediated nematode resistance. However, since both virulent and avirulent *G. pallida* populations harbor GpRBP1, its role in nematode resistance remains to be shown. Furthermore, it is also not clear if the Gpa2-mediated programmed cell death requires a physical association between Gpa2 and GpRBP1.

In this paper we report the functional characterization of the effector SPRYSEC-19 of *G. rostochiensis,* and its interaction with SW5F, in plants. We first tested the hypothesis that SPRYSEC-19 activates SW5F-dependent programmed cell death and nematode resistance. However, co-expression of SPRYSEC-19 and SW5F by agroinfiltration in leaves of *N. benthamiana* and in tomato did not trigger a

defense-related programmed cell death. Moreover, nematode infection assays on tomato plants harboring SW5F showed no resistance to *G. rostochiensis*. Next, we tested the alternative hypothesis that SPRYSEC-19 modulates host defense responses in plants. Our data demonstrated that SPRYSEC-19 selectively suppresses CC-NB-LRR-mediated programmed cell death and disease resistance.

4.2 Results

4.2.1 SPRYSEC-19 does not trigger an SW5F-mediated programmed cell death

Previously, we showed that the effector SPRYSEC-19 of *G. rostochiensis* interacts with a C-terminal fragment of the LRR domain of SW5F (SW5F-LRR7-13) in a yeast-two-hybrid screen on tomato root cDNA (Rehman et al., 2009b). An *in vitro* pull-down assay confirmed that SPRYSEC-19 and SW5F-LRR can interact without cofactors (Rehman et al., 2009b). This specific association of SPRYSEC-19 and SW5F was confirmed *in planta* by bimolecular fluorescence complementation (BiFC) and co-immunoprecipitations (CoIP) (Supplemental Fig. S1). The only other known physical association of a pathogen effector and the LRR domain of a resistance protein *in planta* triggers a defense-related programmed cell death in *N. tabacum* leaves (Krasileva et al., 2010). We expected that co-expression of SPRYSEC-19 and SW5F would also trigger a cell death response in agroinfiltrated leaves of *N. benthamiana*. However, no local cell death was observed within 10 days after transient overexpression of SW5F with either 4MYC–tagged SPRYSEC-19 or untagged SPRYSEC-19 (Supplemental Fig. S2). The fragment of SW5F (SW5F-LRR7-13) that interacted with SPRYSEC-19 in the yeast-two-hybrid screen derived from the near-isogenic line CGR161 of *S. lycopersicum* cultivar MoneyMaker. We reasoned that other close homologs of SW5F either in CGR161 or in the parent cultivar MoneyMaker might be able to mediate a SPRYSEC-19-triggered cell death in *N. benthamiana*. A PCR using SW5F specific primers resulted in the identification of three SW5F homologues (Supplemental Fig. S3). Transient co-expression of

none of the SW5F homologues with either SPRYSEC-19 (Fig. 1A) or 4MYC-SPRYSEC-19 resulted in a local programmed cell death in agroinfiltrated areas of *N. benthamiana* leaves. The three SW5F variants are polymorphic at nine amino acid positions in the LRR region (Supplemental Fig. S3). Despite these differences, 4MYC-SPRYSEC-19 captured on anti-MYC beads pulled-down the transiently expressed LRR domain of all three SW5F variants (Fig. 1B). This demonstrated that the absence of programmed cell death is not caused by lack of a physical interaction between SPRYSEC-19 and the LRR domains of the SW5F homologs.

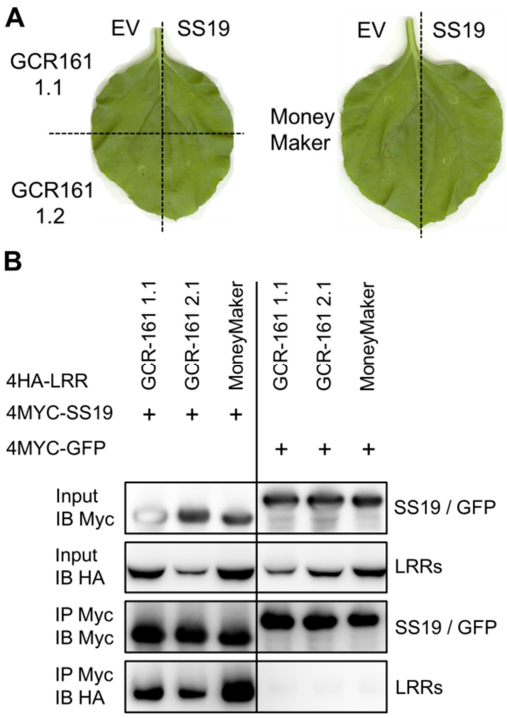

Fig. 4.1. The physical association of SPRYSEC-19 and three SW5F variants in planta does not trigger a programmed cell death. A, Transient expression of SW5F variants (GCR161-1.1 and -1.2, and Moneymaker) by agroinfiltration in *N. benthamiana* leaves together with empty expression vector (EV, left side of leaves) or SPRYSEC-19 (SS19; right side of leaves). Pictures were taken at 10 days post infiltration. B, Co-immunoprecipitation of HA-tagged LRR domains of the SW5F variants by 4MYC-SPRYSEC-19 or 4-MYC-GFP. SPRYSEC-19 and GFP were captured anti-MYC agarose beads (IP MYC/IB MYC) in total protein extracts of agroinfiltrated leaves of *N. benthamiana* transiently co-expressing the proteins. LRR domains pulled-down by either 4-MYC-SPRYSEC-19 or MYC-GFP (IP MYC/ IB HA) were detected on western blots with anti-HA serum.

4.2.2 SW5F does not confer resistance to *G. rostochiensis*

SW5F from tomato might not be able to mediate programmed-cell death in *N. benthamiana*, because it requires accessory proteins that are absent in *N. benthamiana*. However, transient expression of SPRYSEC-19 by agroinfiltration in leaves of the *S. lycopersicum* cultivar MoneyMaker harboring the SW5F gene did not result in a local cell death either (Fig. 4.2). Not all functional disease resistance proteins trigger a local cell death at the infection site of avirulent pathogens (Bendahmane et al., 1999; Bulgarelli et al., 2010), and SW5F might therefore still confer resistance to *G. rostochiensis* in tomato. To test whether SW5F mediates resistance to the population of *G. rostochiensis* from which SPRYSEC-19 was isolated (Ro1 line 19), we inoculated 7 days old seedlings of the tomato cultivar from which SW5F was cloned (i.e. MoneyMaker) with infective second juveniles. Three weeks post-inoculation on average 29 (S.E.M ±1.1) juveniles per tomato plant developed into the adult female stage, which is consistent with a normal susceptibility to *G. rostochiensis* in tomato (Sobczak et al., 2005).

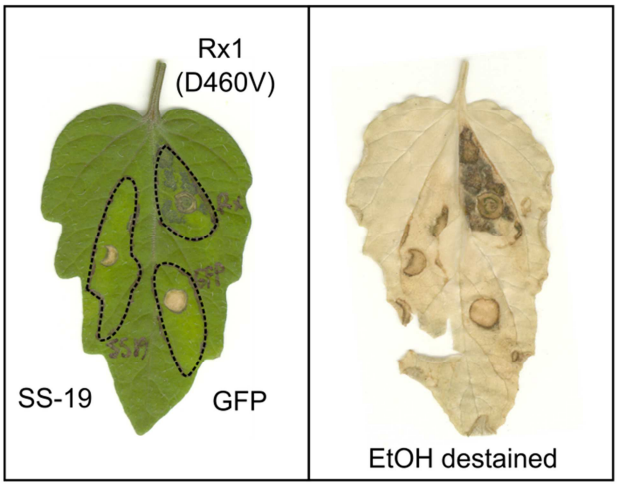

Fig. 4.2. Transient expression of SPRYSEC-19 in tomato cv. MoneyMaker harboring the SW5F gene does not result in local cell death. SPRYSEC-19, GFP, and autoactive mutant of the resistance gene Rx1 (i.e. Rx1[D460V]) were transiently expressed by agroinfiltration in leaves of tomato plants. Photograph was taken seven days post infiltration.

4.2.3 SPRYSEC-19 suppresses programmed cell death mediated by an SW5 homolog in *N. benthamiana* leaves

Next, we reasoned that SPRYSEC-19 interacts with SW5F to suppress effector-triggered activation of SW5F-mediated immune signaling. The SW5F gene has not been linked to a particular disease resistance trait, and by consequence the elicitor of the pathogen that might activate SW5F-mediated signaling is also not known. The TSWV resistance mediated by SW5B is currently the only phenotype linked to the SW5 cluster in tomato. However, the elicitor of the virus that activates SW5B has not been identified either. To be able to test if SPRYSEC-19 suppresses SW5-mediated programmed cell death, we introduced a D-to-V mutation at position 879 in SW5F and at position 857 in SW5B to make the proteins autoactive (Bendahmane et al., 2002; De La Fuente Van Bentem et al., 2005; Tameling et al., 2006; Van Ooijen et al., 2008). Only the expression of SW5B-D857V resulted in an effector-independent cell death response following agroinfiltration of *N. benthamiana* leaves (Fig. 4.3A). Co-expression of 4MYC-SPRYSEC-19 suppressed the effector-independent cell death response mediated by the SW5B-D857V mutant protein in agroinfiltrated leaves of *N. benthamiana* (Fig. 4.3B). This outcome suggested that SPRYSEC-19 suppresses SW5B-mediated activation of effector-triggered immunity.

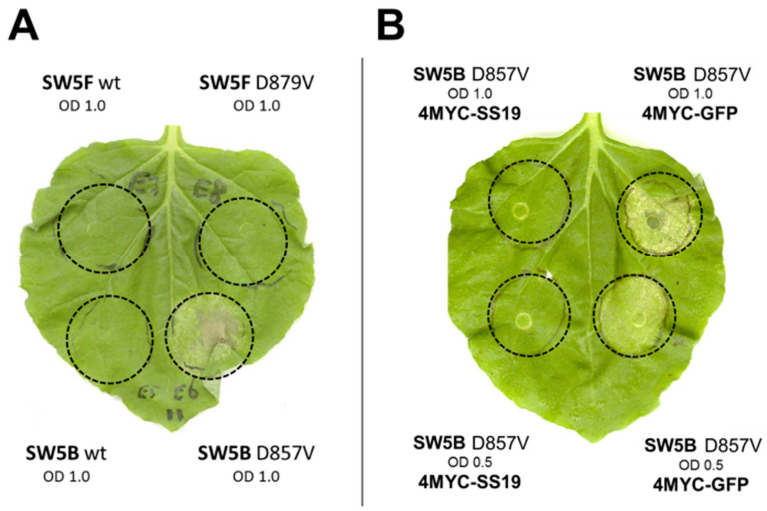

Fig. 4.3. SPRYSEC-19 suppresses SW5B activated programmed cell death. Leaves of *N. benthamiana* were agroinfiltrated with wildtype and autoactive mutant SW5 genes under 35S CaMV promoter and monitored for the initiation of cell death over 10 days. The suspensions of the bacteria carrying the constructs were infiltrated in a 1:1 ratio with a combined OD600 as indicated. Photographs were taken five days post infiltration. A, Wildtype (wt) SW5B and –F genes overexpressed next to SW5 mutants with a D-V mutation. B, Autoactive mutant SW5B(D857V) co-infiltrated with equal amounts of 4MYC-SPRYSEC-19 (left side of leaf) or 4MYC-GFP (right side of leaf).

4.2.4 SPRYSEC-19 selectively suppresses CC-NB-LRR-mediated programmed cell death in *N. benthamiana* leaves

Next, we investigated whether SPRYSEC-19 also suppresses the programmed cell death mediated by other CC-NB-LRR resistance proteins. The SPRYSEC effector GpRBP-1 of the white potato cyst nematode *G. pallida* triggers a Gpa2-mediated cell death in *N. benthamiana* (Sacco et al., 2009). To investigate a possible SPRYSEC-19 controlled suppression of Gpa2-mediated programmed cell death, we co-expressed 4MYC-SPRYSEC-19 together with GpRBP-1 and Gpa2 by agroinfiltration in leaves of *N. benthamiana*. GpRBP-1 transiently expressed with Gpa2 and 4MYC-GFP triggered a strong cell death response in the infiltrated leaf areas within 4-7 days post infiltration. By contrast, no local cell death was observed

following the co-expression of GpRBP-1, Gpa2, and 4MYC-SPRYSEC-19 in *N. benthamiana*. We therefore concluded that SPRYSEC-19 suppressed elicitor dependent programmed cell death mediated by Gpa2. Gpa2 is highly similar to the virus resistance protein Rx1 that recognizes the coat protein of the avirulent PVX strain UK106 (Cp106) (Bendahmane et al., 1995). Cp106 shares no sequence similarity with GpRBP-1 or with other SPRYSEC effectors. We used the Rx1-mediated cell death response in *N. benthamiana* to investigate whether SPRYSEC-19 suppresses the action of a homologous CC-NB-LRR protein that is not triggered by a SPRYSEC. As expected, co-expression of Rx1, Cp106, and 4MYC-GFP resulted in a local cell death response in agroinfiltrated leaf areas of *N. benthamiana* (Fig. 4.4). By contrast, replacing 4MYC-GFP with 4MYC-SPRYSEC-19 completely abrogated the Rx1/Cp106-triggered cell death response in *N. benthamiana* leaves. SPRYSEC-19 of *G. rostochiensis* thus also suppresses programmed cell death mediated by the CC-NB-LRR resistance proteins Gpa2 and Rx1.

To investigate whether the SPRYSEC-19-induced suppression of CC-NB-LRR mediated programmed cell death involves a disturbed effector recognition, we co-expressed SPRYSEC-19 in *N. benthamiana* leaves with an autoactive Gpa2-Rx1 chimera (GG-GRR; (Rairdan and Moffett, 2006)), Mi-1.2 mutant (Mi-1.2(T557S); (Gabriels et al., 2007)), and natural resistance gene homolog 10 from the H1 locus in potato (RGH10; (Finkers-Tomczak et al., 2011)). The transient co-expression of these proteins with 4MYC-GFP led to a local cell death response in agroinfiltrated leaf areas. However, replacing 4MYC-GFP with 4MYC-SPRYSEC-19 abrogated the effector-independent cell death response mediated by GG-GRR and RGH10, but not by Mi-1.2(T557S) (Fig. 4.4). We therefore concluded that SPRYSEC-19 selectively suppresses cell death signaling of a subset of CC-NB-LRR resistance proteins.

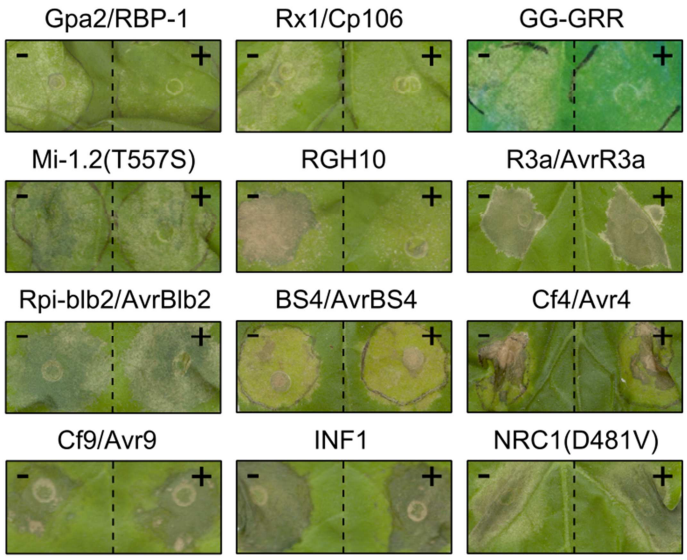

Fig. 4.4. SPRYSEC-19 suppresses programmed cell death mediated by a subset of CC-NB-LRR proteins. Co-expression of 4MYC-SPRYSEC-19 (+) or 4MYC-GFP (-) with several programmed cell death inducing pairs of resistance proteins and their cognate effectors or autoactive resistance proteins (see Results section for details) after agroinfiltration in *N. benthamiana* leaves. Pictures were taken 3-5 days post infiltration.

We also co-expressed SPRYSEC-19 with R3a (Huang et al., 2005b) and Rpi-blb2 (Van Der Vossen et al., 2005; Oh et al., 2009) from potato and their cognate elicitors from *Phytophthora infestans* in *N. benthamiana* leaves to test whether SPRYSEC-19 also modulates the cell death responses mediated by more distantly related CC-NB-LRR resistance proteins (see Supplemental Fig. S4 for an identity matrix). The co-expression of R3a and Rpi-blb-2 and their cognate elicitors resulted in a local cell death response in *N. benthamiana*, which was not suppressed in the presence of SPRYSEC-19 (Fig. 4.4). Similarly, the effector-triggered cell death response mediated by a resistance protein of the TIR-NB-LRR class (i.e. BS4) and the extracellular LRR class (i.e. Cf-4 and Cf-9) was also not affected by co-expression of SPRYSEC-19 either (Fig. 4.4). The *P. infestans* secreted elicitin INF1 has features of pathogen-associated molecular patterns and autonomously elicits a strong cell

death response in leaves of *N. benthamiana* (Heese et al., 2007). The expression of SPRYSEC-19 did not suppress INF1-induced cell death in agroinfiltrated leaves of *N. benthamiana* (Fig. 4.4). The CC-NB-LRR protein NRC1 likely operates in signaling pathways downstream of different types of resistance proteins (e.g. Rx1, Mi-1.2, Cf4, and Cf-9) (Gabriels et al., 2007). To investigate whether SPRYSEC-19 modulates immune signaling downstream of resistance proteins, we co-expressed SPRYSEC-19 with an autoactive mutant of NRC1(D481V) by agroinfiltration in leaves of *N. benthamiana*. Expression of NRC1(D481V) caused a strong cell death response within 24 hours after agroinfiltration in *N. benthamiana* leaves, which was not suppressed by SPRYSEC-19 (Fig. 4.4). Altogether, our data demonstrated that SPRYSEC-19 suppresses the programmed cell death mediated by a group of closely related CC-NB-LRR resistance proteins.

4.2.5 SPRYSEC-19 suppresses disease resistance mediated by Rx1

The local cell death mediated by resistance proteins may be a consequence rather than a prerequisite of disease resistance in plants (Coll et al., 2011). To determine if SPRYSEC-19 also suppresses disease resistance mediated by a CC-NB-LRR protein, we assessed the replication of the avirulent PVX strain UK106 in the presence of both the resistance protein Rx1 and SPRYSEC-19, and in the presence of Rx1 alone. To this purpose, PVX was introduced into *N. benthamiana* leaves by agroinfiltrating the complete viral amplicon including GFP (PVX::GFP). Virus replication was first deduced from the accumulation of GFP in mesophyll cells in infiltrated leaf areas (Fig. 4.5A). As expected, the co-expression of Rx1, PVX::GFP, and GUS resulted in poor accumulation of GFP in agroinfiltrated areas. However, replacing GUS with 4MYC-SPRYSEC-19 in the agroinfiltration mix led to a strong GFP signal. We also co-expressed PVX::GFP and 4MYC-SPRYSEC-19 alone in *N. benthamiana* mesophyll cells to demonstrate that 4MYC-SPRYSEC-19 targeted the action of Rx1 and not the replication of PVX directly (Fig. 4.5A). To confirm that the accumulation of GFP reflects PVX replication in mesophyll cells, we also quantified the accumulation of

PVX coat protein by using a specific antibody in an ELISA on total protein extracts isolated from agroinfiltrated leaf areas (Fig. 4.5B). We concluded that the suppression of Rx1-mediated immune signaling by SPRYSEC-19 also results in loss of disease resistance.

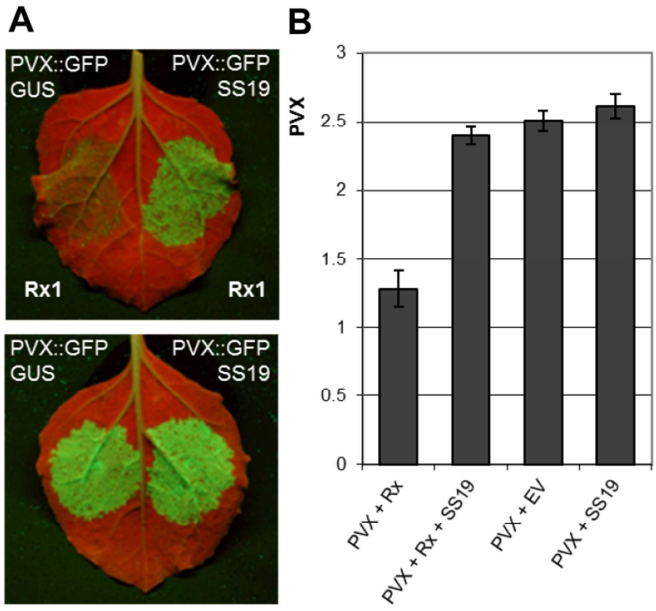

Fig. 4.5. SPRYSEC-19 suppresses resistance to Potato virus X mediated by the CC-NB-LRR protein Rx1. A, Transient expression of GFP-labeled PVX (PVX:GFP) in leaves of *N. benthamiana* after agroinfiltration together with the β-glucuronidase gene (GUS) or SPRYSEC-19 (SS19), with (top panel) or without (lower panel) the resistance gene Rx1 under control of a leaky scan 35S CaMV promoter. GFP expression was visualized under a UV lamp four days post infiltration. Agroinfiltrations with the empty binary expression vector (EV) were included as control. B, Quantification of PVX replication by ELISA directed against the PVX coat protein. Bars represent ELISA signal intensity; error bars represent standard error of mean.

4.2.6 SPRYSEC-19 overexpression renders a fungal resistant potato genotype susceptible to *Verticillium dahliae*

To investigate whether stable overexpression of SPRYSEC-19 enhances the susceptibility of plants to plant pathogens, we first inoculated transgenic potato plants (line V) overexpressing untagged or 4MYC-tagged SPRYSEC-19 with *G. rostochiensis*. Four weeks post inoculation the number of adult females per plant was not significantly higher in at least twelve independent transgenic potato lines overexpressing SPRYSEC-19 as compared to transgenic plants harboring the corresponding empty binary expression vector (Supplemental Fig. S5). The draft genome sequence of the sister species *G. pallida* suggests that potato cyst nematodes carry over 200 different SPRYSEC genes (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/g_pallida). We therefore reasoned that the overexpression of one specific SPRYSEC gene family member in a host plant might have little impact on the virulence of *G. rostochiensis*. However, the potato line V is resistant to *Verticillium dahliae*, and the *V. dahliae* genome does not harbor homologues of nematode SPRYSEC effectors. We therefore challenged the transgenic potato lines overexpressing either SPRYSEC-19 or 4MYC-SPRYSEC-19 with *V. dahlia*e strain 5361, to test whether SPRYSEC-19 alters the resistance of potato plants to this fungus. Four weeks post inoculation with *V. dahliae* the SPRYSEC-19 overexpressing plants showed a strong reduction in shoot growth as compared mock-inoculated plants, and as compared to the empty vector plants inoculated with *V. dahliae* (Fig. 4.6A). To further quantify the level of resistance to *V. dahliae* in the transgenic potato lines, we measured the accumulation of fungal biomass in plants harboring either SPRYSEC-19, 4MYC-SPRYSEC-19, or the empty expression vector by specifically amplifying the internal transcribed spacer (ITS) region of *V. dahliae* with PCR (Fradin et al., 2011). The ITS region of *V. dahliae* was amplified from plants overexpressing either SPRYSEC-19 or 4MYC-SPRYSEC-19 three weeks post inoculation with fungal spores (Fig. 4.6B). As expected, no amplification product of the ITS region in *V. dahliae* was observed in the empty vector plants three weeks post inoculation with fungal spores. These data suggest that SPRYSEC-19 suppresses a yet unidentified fungal resistance in potato, rendering these plants susceptible to an otherwise avirulent strain of *V. dahliae*.

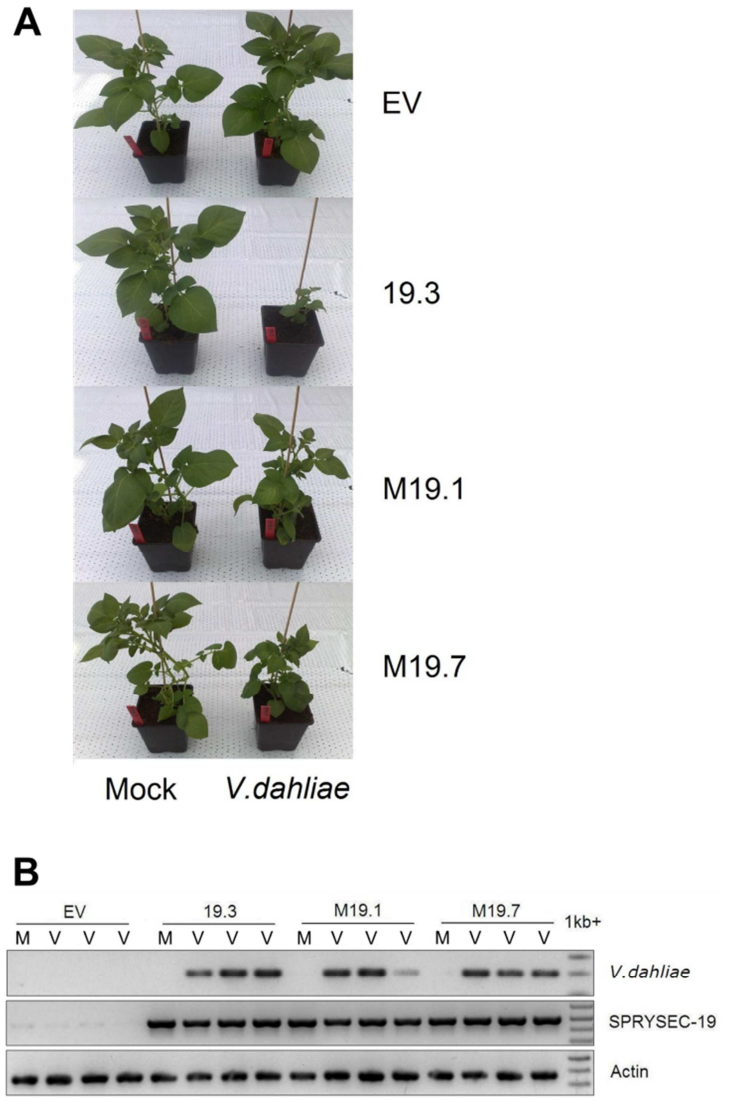

Fig. 4.6. SPRYSEC-19 overexpression renders a resistant potato genotype susceptible to *Verticillium dahliae*. Stable transgenic potato (*S. tuberosum* line V) overexpressing SPRYSEC-19 (19.3) or 4MYC-tagged SPRYSEC-19 (M19.1 and M19.7) under control of the 35S CaMV promoter infected with *V. dahliae* strain 5361. A, Shoot growth of *V. dahliae* (V) and mock (M) -inoculated transgenic potato lines four weeks post inoculation. EV is a transgenic potato line harboring the corresponding empty binary expression vector. B, Quantification of *V. dahliae* biomass by PCR-amplification of the internal transcribed spacer region of *V. dahliae* in total DNA extracts of *V. dahliae* and mock-inoculated transgenic potato lines (top panel). SPRYSEC-19 and actin genes were PCR amplified as internal controls (middle and bottom panels). 1kb+: DNA size marker.

4.2.7 Suppression of disease resistance responses by SPRYSEC-19 does not require a direct interaction with R proteins

To investigate whether the suppression of Gpa2, Rx1, and autoactive SW5B requires a physical interaction with SPRYSEC-19, we co-expressed 4MYC-SPRYSEC-19 and the LRR domains of these proteins fused to a 4HA tag in leaves of *N. benthamiana* for co-immunoprecipitation. Capturing 4MYC-SPRYSEC-19 in total protein extracts of agroinfiltrated leaf areas with anti-MYC beads did not result in the co-immunoprecipitation of the LRR domains of Sw5B, Rx1, and Gpa2 (Fig. 4.7). We therefore concluded that SPRYSEC-19-mediated suppression of CC-NB-LRR-mediated programmed cell death and resistance does not require a physical interaction of SPRYSEC-19 with the LRR domains of these resistance proteins.

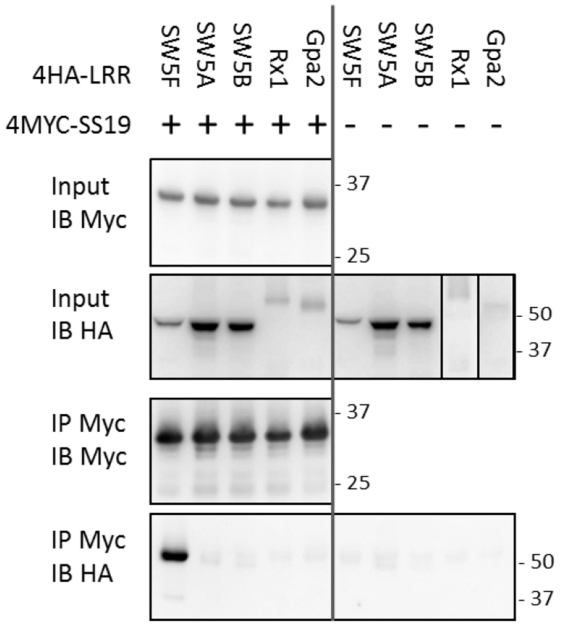

Fig. 4.7. SPRYSEC-19 does not bind the LRR domains of suppressed R proteins. Co-immunoprecipitation of different 4HA-tagged LRR domains of SW5F, -A, -B, Rx1, and Gpa2 with 4MYC-SPRYSEC-19 (SS19) transiently co-expressed in *N. benthamiana*. The LRR domains pulled-down (IP MYC/IB HA) by 4MYC-SPRYSEC-19 on anti-MYC agarose beads (IP MYC/ IB MYC) were detected on western blots with anti-HA serum.

4.3 Discussion

We have shown that the resistance protein SW5F of tomato interacts specifically with the effector SPRYSEC-19 of *G. rostochiensis in planta*. Surprisingly, this interaction did not lead to the effector-triggered activation of SW5F-mediated programmed cell death and nematode resistance. Instead, SPRYSEC-19 is the first nematode effector to demonstrate suppression of defense-related programmed cell death by some, but not all, CC-NB-LRR resistance proteins (i.e. SW5B, Rx1, Gpa2, and RGH10). The suppression of CC-NB-LRR-mediated signaling does not require a physical association between SPRYSEC-19 and these resistance proteins. Furthermore, the suppression of programmed cell death mediated by autoactive mutant CC-NB-LRR proteins suggested that SPRYSEC-19 most likely disturbs receptor-mediated immune signaling rather than effector recognition. In addition to abrogating the programmed cell death mediated by Rx1, the nematode effector SPRYSEC-19 also repressed virus resistance mediated by this CC-NB-LRR protein. Altogether, our data demonstrates that SPRYSEC-19 of *G. rostochiensis* functions as a suppressor of CC-NB-LRR-mediated programmed cell death and disease resistance.

SPRYSEC-19 physically associates with SW5F *in planta* through its interaction with seven C-terminal leucine-rich repeats of the LRR domain of SW5F. There are only a few other plant resistance proteins for which a physical interaction with a pathogen effector *in planta* has been demonstrated. These interactions agree with the model of effector-triggered immunity following direct recognition of effectors by plant immune receptors. Like ATR1/PPR1 and IPI-O1/RB, we expected that the physical association of SPRYSEC-19 and SW5F would also activate effector-triggered immunity to *G. rostochiensis*. However, the absence of SPRYSEC-19-dependent SW5F-mediated programmed cell death *N. benthamiana* and SW5F-mediated resistance to *G. rostochiensis* in tomato and potato led us to reject this hypothesis.

We have demonstrated with four different experimental designs that the physical association between SPRYSEC19 and the LRR domain of SW5F is robust. That this association does not activate effector-triggered programmed cell death and resistance may indicate that SW5F is an inactive gene duplicate of a paralogous functional CC-NB-LRR resistance protein to *G. rostochiensis*. In this scenario the lack of functional constraints on the *SW5F* gene may have rendered its activation domains (i.e. CC-NB) dysfunctional, while binding to the sensor (i.e. LRR) domain is still intact (Van Eck et al., 2010). We tried to make SW5F, along with SW5B, constitutively active by introducing mutations at positions that switch several other CC-NB-LRR resistance proteins into a permanent "on"-state. However, these mutations only induced autoactivity in SW5B, which is thus far the only member of the SW5 cluster linked to a known resistance (Spassova et al., 2001). The lack of autoactivity in SW5F mutants therefore favors the hypothesis that SW5F is a dysfunctional paralogue of a functional nematode resistance gene.

As SPRYSEC-19 lacked any evident avirulence activity on the three SW5F homologs isolated in this study, we also reasoned that SPRYSEC-19 might interact with the LRR domain of SW5F to suppress the activation of the CC-NB-LRR-mediated immune signaling. Using agroinfiltration assays, we have demonstrated that SPRYSEC-19 suppresses programmed cell death mediated by some, but not all, CC-NB-LRR resistance protein in *N. benthamiana*. Moreover, SPRYSEC-19 suppressed none of the members of the TIR-NB-LRR and extracellular LRR classes of resistance proteins tested in this study. We found no evidence in our co-immunoprecipitations that suppression of CC-NB-LRR-mediated programmed cell death requires the binding of SPRYSEC-19 to these receptor proteins. However, it should be noted that mostly high affinity interactions between proteins can be demonstrated with co-immunoprecipitations. We therefore cannot exclude the possibility that SPRYSEC-19 more transiently interacts with the LRR domains of the resistance proteins it suppresses.

As the suppression of autoactive mutant CC-NB-LRR proteins demonstrated, SPRYSEC19 most likely does not disturb the recognition of specific cognate

pathogen effectors that activates these resistance proteins. It is nonetheless conceivable that SPRYSEC-19 is able to outcompete other SPRYSEC effectors of *G. rostochiensis* that trigger the activation of a functional homolog of SW5F. Such a mechanism seems to determine the virulence of *P. infestans* strains on potato plants harboring the RB resistance protein (Chen et al., 2012). Alternatively, as discussed earlier SPRYSEC-19 may also suppress CC-NB-LRR resistance proteins by targeting the immune receptors to the proteasome for degradation (Rehman et al., 2009b). However, western blots of total protein extracts of agroinfiltrated leaf areas revealed no enhanced breakdown of CC-NB-LRR proteins or parts thereof in the presence of SPRYSEC-19. We therefore conclude that our current data does not support a model in which SPRYSEC-19 interacts with CC-NB-LRR resistance proteins to alter their turnover rate.

Programmed cell death in the site of pathogen infections is often associated with effector-triggered immunity in plants, but may not be required for disease resistance (Coll et al., 2011). It could therefore be argued that the suppression of programmed cell death by SPRYSEC-19 in agroinfiltration assays bears little biological significance with regard to disease resistance. Using an avirulent PVX strain that was modified to express GFP but that was still recognized and restrained by the resistance protein Rx1, we have demonstrated that SPRYSEC-19 also suppresses CC-NB-LRR-mediated disease resistance. Furthermore, our observation that the overexpression of SPRYSEC-19 in potato plants abrogated the resistance of this potato genotype to *V. dahliae* further supports that this effector functions as a suppressor of disease resistance.

Next to the ability to induce and maintain feeding cells, the survival and reproduction of sedentary plant-parasitic nematodes is most likely determined by their ability to suppress host defenses. The molecular mechanisms underlying the suppression of host defense responses by plant-parasitic nematodes are not known. All known plant immune receptors conferring resistance to *G. rostochiensis* belong to the CC-NB-LRR class of resistance proteins (Molinari, 2011). Here we

showed that *G. rostochiensis* has evolved several SPRYSEC effectors that selectively suppress CC-NB-LRR mediated programmed cell death and disease resistance. The SPRYSECs in the potato cyst nematodes *G. rostochiensis* and *G. pallida* constitute the largest effector family found in a plant parasitic nematode to date. If the SPRYSEC effector family functions as suppressors of effector-triggered immunity, the expansion of this effector family may reflect adaptations to functional diversifications in plant immune receptors. As the SPRYSEC effector GpRBP1 of *G. pallida* suggests, on their turn plants may have evolved novel NB-LRR plant immune receptors (e.g. Gpa2) that recognize and neutralize SPRYSEC effectors again. It will be highly interesting to investigate if GpRBP1 also suppresses CC-NB-LRR resistance proteins, and if the activation of Gpa2-mediated resistance also involves a physical association between the LRR domain of Gpa2 and GpRBP1.

4.4 Materials and methods

4.4.1 Plant material

For nematode infection assays explants of *in vitro* cultured tomato (*L. esculentum* cv. GCR-161; (Kroon and Elgersma, 1993)) or potato (*S. tuberosum,* line V, genotype 6487-9; (Schouten et al., 1997)) were grown on B5 medium (3.29 g/L Gamborg B5, 20 g/L sucrose, 15 g/L bacto agar, pH 6.2) at 24 °C and 16/8 h photoperiod for 3 weeks prior to inoculation. All other experiments were performed year-round on 3-week-old tomato (*L. esculentum* cv. MoneyMaker) or *N. benthamiana* plants that were grown in a greenhouse in 15cm diameter pots with potting soil.

4.4.2 Cloning and plasmid construction

SPRYSEC-19 was subcloned from pGBKT7-A18-2 (Rehman et al., 2009b) as a BspMI-BamHI fragment an inserted jointly with the complementary oligo pair A18For + A18Rev (Supplemental Table S1) into pRAP digested with NheI-BglII. The coding regions of the mature peptides of other SPRYSECs without their native signal peptides for secretion were PCR-amplified from *G. rostochiensis* cDNA. The full-

length SW5F genes of tomato cv. GCR161 were PCR-amplified as described before (Rehman et al., 2009b). The regions of *R* genes coding for the LRR domain were subcloned from existing plasmids: SW5A and –B (Spassova et al., 2001), SW5F (Rehman et al., 2009b), Gpa2 (Rairdan and Moffett, 2006) and Rx1 (Slootweg et al., 2010). PCR-amplification products were cloned into vector pRAP using specific restriction sites and confirmed by DNA sequencing. The fragments cloned into the pRAP vector were cloned in frame with the CaMV 35S promoter and N- or C-terminal 4HA or 4MYC affinity tags, or no additional tags. Primers and restriction sites used for the cloning of novel genes are listed in Supplemental Table S2. Expression cassettes of pRAP, including promoter, affinity tags and the gene of interest, were subcloned into binary vector pBINPLUS (Van Engelen et al., 1995) using *AscI* and *PacI* restriction sites. All SW5F genes were cloned with the 3' UTR (polyadenylation signal and terminator) of the SW5F gene from isolated from MoneyMaker (Rehman et al., 2009b). Autoactive SW5 mutants were made by inserting the annealed oligo pair D879V-1 and D879V-2 (Supplemental Table S3) between the BspHI and XbaI restriction sites of the SW5 genes in pRAP. All the above described constructs were mobilized to *A. tumefaciens* strain MOG101 (Hood et al., 1993), which was selectively grown on 50 mg/L kanamycin and 20 mg/L rifampicin. For the expression of SPRYSEC-19 in tomato, the coding region for the mature peptide of SPRYSEC-19 without its signal peptide was PCR-amplified from *G. rostochiensis* cDNA using primers listed in Supplemental Table S1 and cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA). After confirmation of the sequence by DNA sequencing, SPRYSEC-19 was subcloned to the expression vector SOL2085 (kindly provided by Patrick Smit) using LR clonase (Invitrogen, Carlsbad, CA, USA), resulting in vector SOL2085:SS19. For agroinfiltrations in tomato leaves the constructs were mobilized to *A. tumefaciens* strain 1D1249 (Wroblewski et al., 2005) which was selectively grown on 100 mg/L kanamycin, 100 mg/L spectinomycin, and 1 mg/L tetracyclin.

4.4.3 Agroinfiltrations

Agrobacterium tumefaciens harboring the individual binary vectors was grown at 28°C in YEP medium (per liter: 10 g peptone, 10 g yeast extract, 5 g NaCl) with appropriate antibiotics. The bacteria were spun down and resuspended in MMA infiltration medium (per liter: 5 g Murashige and Skoog salts, 1.95 g MES, 20 g sucrose). The bacterial solution was diluted to an OD600 of 0.5 (for infiltration in *N. benthamiana*) or 0.1 (for infiltration in tomato) in MMA and infiltrated in the abaxial side of the leaves using a syringe. Coinfiltration of different constructs was performed by mixing equal volumes of the bacterial suspensions to a final OD600 as described above.

4.4.4 Suppression of programmed cell death

The suppression of programmed cell death in leaves of *N. benthamiana* was assessed using the pBINPLUS with MYC tagged SPRYSEC-19 construct described above. The 4MYC:GFP construct was used as a negative control for suppression. The following pairs of resistance genes and cognate elicitors were used to induce programmed cell death in leaves: Gpa2 / RBP1 (Sacco et al., 2009), Rx1 / cp106 (Slootweg et al., 2010), Cf4 / Avr4 (Thomas et al., 2000), Cf9 / Avr9 (Thomas et al., 2000), R3a / AvrR3a (Huang et al., 2005b), Rpi-blb2 / AvrBlb2 (Van Der Vossen et al., 2005), BS4 (Schornack et al., 2005) / AvrBS4 (Ballvora et al., 2001). The following constructs of mutant CC-NB-LRR proteins were used to trigger an elicitor-independent programmed cell death: GG-GRR (Rairdan and Moffett, 2006), Mi-1.2(T557S) (Gabriels et al., 2007), RGH10 (Finkers-Tomczak, 2011), NRC1(D481V) (Gabriels et al., 2007), and INF1 (Kamoun et al., 2003). Agroinfiltrated leaves were monitored up to 10 days for visual assessment of cell death.

4.4.5 SPRYSEC-19 in tomato

SOL2085:GFP (kindly provided by Patrick Smit), SOL2085:SS19 (see above), pBIN61:Rx(D460V) (Bendahmane et al., 2002) in *A. tumefaciens* strain 1D1249 were

agroinfiltrated in leaves of *L. esculentum* cv. MoneyMaker. Agroinfiltrated leaves were monitored up to 10 days for visual assessment of cell death.

4.4.6 Bimolecular fluorescence complementation

The coding regions of SPRYSEC-18 and -19 without signal peptide and the coding regions of LRR7-13 of SW5B and SW5F were PCR-amplified from the pRAP vectors described above using the primers listed in Supplemental Table S4. The amplification products were cloned into vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and verified by DNA sequencing. Using the Gateway LR clonase reaction (Invitrogen, Carlsbad, CA, USA) the amplification products were subcloned into vectors pGREENII:35S:YFPc and pGREENII:35S:YFPn (Zhong et al., 2008), and confirmed by restriction digestion. pGREEN vectors were mobilized to *A. tumefaciens* strain GV3101 (Holsters et al., 1980), which was selectively grown on 50 mg/L kanamycin, 20 mg/L rifampicin and 50 mg/L carbenicillin. Two days after agroinfiltration in leaves of *N. benthamiana* fluorescence analysis was performed on a Zeiss 510 confocal laser scanning microscope setup. YFP fluorescence was assessed at 514nm (excitation) using an argon laser with an emission band of 535-590 nm and 650 nm (chlorophyll autofluorescence).

4.4.7 Co-immunoprecipitation

Total protein extracts of transient transformed *N. benthamiana* leaves were made by grinding leaf material in protein extraction buffer (50 mM Tris-HCl pH7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 2% polyclar-AT PVPP, 0.4 mg/ml Pefabloc SC plus (Roche, Basel, Switzerland), 5 mM DTT) on ice. For co-immunoprecipitation the total protein extract was first passed over a sephadex G-25 column (GE Healthcare, Little Chalfront, UK). The protein extract was treated with rabbit-IgG agarose (40 µl slurry per mL protein extract). After preclearing the protein extract was mixed with 25 µl anti-Myc agarose beads (Sigma, MO, USA) or anti-HA agarose beads (Roche) and incubated for 2 hours at 4°C. After washing six times with washing buffer

(protein extraction buffer with 0.15% Igepal CA-630) the beads were resuspended in Laemmli buffer (Sambrook, 1989) and the bound protein was separated by SDS-PAGE and blotted on PVDF membrane. For immuno detection we used antibodies goat anti-Myc (Abcam, Cambridge, UK) and HRP-conjugated donkey anti-goat (Jackson, West Grove, PA, USA) or HRP conjugated rat anti-HA (Roche). Peroxidase activity was visualized using Thermoscientific Supersignal West Femto or Dura substrate and imaging the luminesence with G:BOX gel documentation system (Syngene, Cambridge, UK).

4.4.8 Plant transformation

Potato *S. tuberosum* line V (genotype 6487-9) was transformed as described by (Van Engelen et al., 1994) using *A. tumefaciens* strain MOG101 with vector pBINPLUS containing SPRYSEC-19, 4MYC:SPRYSEC-19, SW5F, or 4HA:SW5F under the control of a 35S promoter (described above). Genomic DNA was extracted from plant leaves by grinding tissues in liquid nitrogen and purifying DNA with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For every construct at least four independent transformation lines were tested and for each line ten biological replicates were used.

4.4.9 Nematode resistance assay

Dried cysts of *G. rostochiensis* pathotype Ro1 Mierenbos were soaked on a 100-µm sieve in potato root diffusate to collect hatched ppJ2s (De Boer et al., 1992). Freshly hatched preparasitic second stage juveniles in suspension were mixed with an equal volume of 70% (w/v) sucrose in a centrifuge tube and covered with a layer of sterile tap water. Following centrifugation for 5 min at 1,000 × *g*, juveniles were collected from the sucrose–water interface using a Pasteur pipette and washed three times with sterile tap water. The nematodes were surface sterilized by incubation for 20 min in 0.5 % (w/v) streptomycin/penicillin solution, for 20 min in 0.1% (w/v) ampicillin/gentamycin solution, for 5 min in sterile tap water, and for 3 min in 0.1% (v/v) chlorhexidine solution. The nematodes were subsequently

washed three times in sterile tap water, resuspended in sterile 0.7% solution of Gelrite, and pipetted along the roots of 3-week-old *in vitro* grown plants. Routinely, we used between 150 and 200 pre-J2s per plate containing one plant. Adult females per plate were counted six to eight weeks after inoculation. For each transformant tested at least 15 independent lines were used.

4.4.10 PVX resistance assay

A. tumefaciens strain MOG101 carrying vector pBINPLUS with 35S:4MYC:SPRYSEC-19 (described above), $35S_{LS}$:Rx1:GFP (Slootweg et al., 2010), 35S:GFP:PVX (Peart et al., 2002), or GPA2:GUS (Koropacka, 2010) were used for agroinfiltration of *N. benthamiana* leaves. Three days post infiltration GFP expression of GFP-tagged PVX was visualized under UV light. Virus concentration was determined using DAS-ELISA (Mäki-Valkama et al., 2000). Plates were coated with a 1:1000 dilution of a polyclonal antibody against PVX to bind the antigen, and a second polyclonal antibody against PVX conjugated with alkaline phosphatase was used for detection via the phosphatase substrate p-nitrophenyl phosphate.

4.4.11 *Verticillium dahliae* resistance assay

Verticillium dahliae isolate 5361 (kindly provided by Richard Cooper) was grown on 4% potato dextrose media (Duchefa, Haarlem, The Netherlands) at 28°C for 2 weeks. Fungal spores were transferred to sterile de-ionized water to a concentration of 1 x 10^6 spores/ml. The roots of three-week old *in vitro* grown transgenic potato plants were soaked in spore suspension for five minutes and transferred to pots with soil in a greenhouse. For each transformant at least 10 independent lines were used with 4 biological controls. At 20 days post inoculation pictures were taken and to determine the fungal biomass in infected plants, stem pieces were cut from the potato plants just above ground level and flash frozen in liquid nitrogen. Total DNA was extracted from plant tissues using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). A 200-bp fragment of the ITS gene of *V. dahliae* was PCR-amplified using primers ITS1-F (Gardes and Bruns, 1993) and ST-VE1

(Lievens et al., 2006) on DNA samples using FirePol polymerase (Solis BioDyne, Tartu, Estonia). As an internal control potato actin was amplified from the same templates using primers StActinF and StActinR (Nicot et al., 2005).

5

Gr-AMP1: a novel secreted γ-core peptide with antimicrobial activity from the potato cyst nematode *Globodera rostochiensis*

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Abstract

Secretions released by plant-parasitic nematodes are thought to be important for host invasion, transformation of host cells into feeding structures, and suppression of host innate immunity. Many invertebrates also secrete peptides with antimicrobial activity as part of their own innate immunity against hyperparasitism by pathogenic bacteria and fungi. At present, it is not known if plant-parasitic nematodes secrete antimicrobial factors for self-protection against soil-borne pathogenic microbes. Here, we demonstrate that a small, highly charged, cationic polypeptide (Gr-AMP1) produced in the dorsal esophageal gland of the potato cyst nematode *Globodera rostochiensis* functions an antimicrobial peptide. The *Gr-AMP1* gene is highly expressed in second juveniles freshly hatched from eggs prior to host invasion, while it is down-regulated during the onset of parasitism inside host roots. Three-dimensional protein structure modeling of Gr-AMP1 suggests that this protein includes a γ-core fold that is typical for many antimicrobial peptides across different kingdoms. Synthetically produced Gr-AMP1 exhibits antimicrobial activity in a radial diffusion assay, on a standardized set of bacteria and yeast known to be sensitive to antimicrobial peptides in vitro. Furthermore, treatment with synthetic Gr-AMP1 extends the lifespan of the free-living nematode *Caenorhabditis elegans* exposed to a pathogenic strain of *Pseudomonas aeruginosa*. Altogether, our data suggest that esophageal gland secretions of *G. rostochiensis* may have a role in protecting the nematode against infections by pathogenic bacteria and fungi.

5.1 Introduction

Dormant preparasitic second stage juveniles (pre-J2s) of the potato cyst nematode *Globodera rostochiensis* can survive in the soil for many years (Perry, 1989). However, as soon as a Solanaceous plant grows in the vicinity of a dormant nematode, root exudates of this plant may activate the juvenile and trigger it to hatch. A freshly-hatched juvenile then has a limited amount of time to find a root

of the host plant, while living on the lipid reserves stored in its intestinal cells and pseudocoelome. The pre-J2s of *G. rostochiensis* penetrate the epidermis of the roots of a host plant with brute force by using their protrusible oral stylet. Next, the juveniles burrow some distance through the underlying cortex, while causing considerable damage to the plant tissue. Within 24 h after penetration the juveniles select a cortex cell close to the pericycle to transform it into a feeding site initial (Sobczak et al., 2005). The induction of the feeding site coincides with a remarkable change in behavior of the nematode, from a highly destructive migratory behavior to a subtle benign sedentary lifestyle. Over the course of weeks, the feeding site of *G. rostochiensis* expands by local cell wall degradation and subsequent fusion of neighboring protoplasts. The feeding site, which is called syncytium, is the sole source of nutrients for the endoparasitic biotrophic *G. rostochiensis*.

The anatomical hallmarks of plant-parasitic nematodes are an oral stylet and several large single-celled esophageal glands (Davis et al., 2004). The hollow oral stylet not only functions as a device to penetrate plant cell walls, it also delivers esophageal gland secretions into the apoplast and the cytoplasm of host cells and facilitates the uptake of plant nutrients from the syncytial cytoplasm. The esophageal glands are indirectly connected to the stylet via the lumen of the esophagus. The secretions produced in the esophageal glands flow forward through the stylet to the exterior of the nematode. However, these secretions may also pass along with plant nutrients into the intestine of the feeding nematode. In the past decade a flurry of papers on plant parasitic nematodes have described many esophageal gland-specific transcripts encoding putative secreted proteins (reviewed in (Haegeman et al., 2012)). However, only a small number of these candidate nematode effectors have been traced back in nematode secretions either outside or inside plants. Similarly, only a few of the candidate effectors of plant parasitic nematodes have been functionally characterized. Nonetheless, it is thought that the molecular components in nematode secretions are instrumental in host invasion, feeding site formation, and suppression of host defenses.

Most animals secrete host defense effector polypeptides as part of their own innate immune system against bacterial and fungal pathogens (Yeaman and Yount, 2007). Such antimicrobial factors have also been found in the free-living nematode *Caenorhabditis elegans* and the animal-parasitic nematode *Ascaris suum* (Kato, 2007), but it is unknown if plant-parasitic nematodes secrete antibacterial factors for self-protection. Antimicrobial factors are small peptides (<50 amino acids), have a net positive charge, and exhibit an amphipathic structure that enables them to interact with bacterial cell walls (Auvynet and Rosenstein, 2009). The antimicrobial factors are subject to positive Darwinian selection that renders them hypervariable in primary amino acid sequence (Nicolas et al., 2003). Other than an even number of cysteine residues and conserved secondary structure motifs, antimicrobial peptides have little in common. Even the sequence similarity among the highly diverse antimicrobial peptides in closely related species is often too weak to support a robust phylogeny. However, despite a lack of direct sequence similarity most antimicrobial factors across different kingdoms display a so-called γ-core signature, which consists of two antiparallel β sheets that contain a strong polarization of charges (Yount and Yeaman, 2004).

Here we report the identification of a small secretory polypeptide in the dorsal esophageal gland of *G. rostochiensis* by using a differential display of transcripts expressed during the transition of pre-J2s through dormancy. At the end of dormancy *G. rostochiensis* specifically fills both its esophageal glands. The secondary structure of the novel secretory polypeptide showed features of an antimicrobial peptide (AMP) and was therefore named Gr-AMP1. By using an in vitro antimicrobial activity assay on a panel of bacteria and yeast we demonstrate that Gr-AMP1 has antibacterial and antifungal activity. Finally, we demonstrate that Gr-AMP1 can extent the survival of the *C. elegans* suffering from an infection with a pathogenic strain of *Pseudomonas aeruginosa*.

5.2 Results

To identify novel effectors of *G. rostochiensis* we analyzed differential gene expression in second stage juveniles during their transition through dormancy (Qin et al., 2000). One of the transcript-derived fragments that was specifically upregulated in freshly hatched pre-J2s of *G. rostochiensis* (hereafter named A42) was excised from the gel (Fig. 5.1), and subjected to DNA sequencing. The DNA sequence of A42 included a fragment of 339 base pairs that was flanked by the EcoRI and TaqI restriction sites used in the cDNA-AFLP protocol.

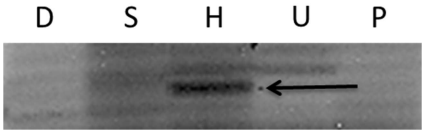

Fig. 5.1: Differential display of gene expression in second stage juveniles (J2) of *G. rostochiensis* during transition through diapause. D = J2 in diapause; S = J2 exited diapause, but still in eggs; H = J2 hatched in potato root exudates; U = embryonic stage inside females; P = J2 prior entering diapause.

To localize messenger RNA matching the A42 sequence in pre-J2s of *G. rostochiensis*, we conducted whole mount situ hybridization microscopy using an antisense cDNA probe designed on A42. The A42 antisense probe uniquely hybridized to the dorsal esophageal gland cell in pre-J2s of *G. rostochiensis* (Fig. 5.2). By contrast, a corresponding sense probe designed on the A42 sequence did not hybridize to any tissue in the nematodes. We therefore concluded that the transcript from which A42 derives is specifically expressed in the dorsal esophageal gland of *G. rostochiensis*.

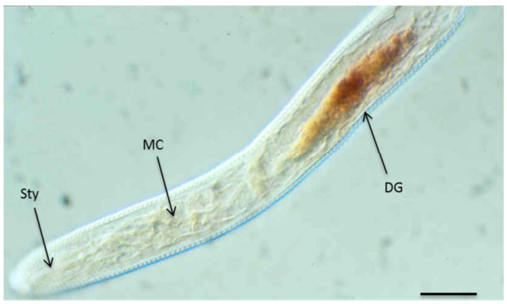

Fig. 5.2: Whole mount in situ hybridization of a cDNA probe corresponding to Gr-AMP1 transcripts in the dorsal esophageal gland of preparasitic second stage juveniles of *G. rostochiensis*. Dark labeling marks the localization of mRNA of Gr-AMP1 (DG, dorsal esophageal gland; MC, metacorpus; Sty, stylet; bar = 20 µm).

To clone the full-length transcript from which A42 is derived, we first searched the non-redundant sequence database at NCBI for matching expressed-sequence tags. Two expressed-sequence tags isolated from parasitic J2s of *G. rostochiensis* matched the A42 sequence (GenBank accessions EE2659321 and EE265932). The inserts of the original clones from which the matching expressed-sequence tags were derived were resequenced to resolve additional sequence information up- and down-stream of A42. Furthermore, rapid amplification of cDNA ends (RACE) on mRNA isolated from pre-J2s of *G. rostochiensis* was used to clone the full-length cDNA sequence of 427 base pairs including A42 (GenBank accession AJ536823). Further database searches with this full-length sequence in the non-redundant nucleotide and protein databases did not reveal any significant matches with sequences from other nematodes or more distantly related organisms. Similarly, searching the genome of the sister potato cyst nematode *Globodera pallida* (version April 2012) with the sequence of A42 did not result in significantly matching sequences either. To independently confirm that the A42 transcript originates from the nematode we used primers matching the 5'-end and the putative polyadenylation site of the transcript to amplify the corresponding region of 644 base pairs from genomic DNA of *G. rostochiensis*. Close inspection of the

genomic sequence showed that besides an intron of 243 base pairs it perfectly matched the sequence of transcript A42 (Fig. 5.3).

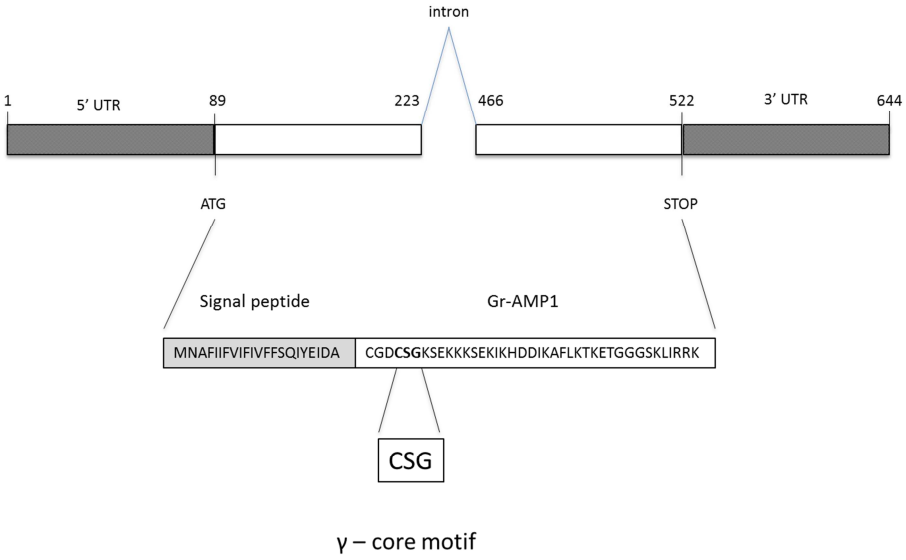

Fig. 5.3: The genomic organization of Gr-AMP1 including an intron, the predicted open reading frame, and γ-core motif.

The largest open reading frame in the full-length A42 sequence includes 186 base pairs corresponding to a polypeptide of 62 amino acids (Fig. 5.3). The first 22 amino acids at the amino-terminus of this polypeptide are predicted to function as a classical eukaryotic signal peptide for secretion (Signal P v3; probability = 0.951). The predicted molecular mass of the remaining mature A42 polypeptide is 4.44 kDa. The isoelectric point of A42 is 9.8, with a net positive charge at pH 7.0 of +7.0. Altogether, our data suggest that A42 encodes a highly charged cationic polypeptide that is most likely secreted by the dorsal esophageal gland of *G. rostochiensis*.

Many multicellular eukaryotes secrete highly charged cationic polypeptides that function as antimicrobial peptides in host defense against pathogenic bacteria and

fungi. This observation inspired us to investigate if A42 shares other features with classical antimicrobial peptides. At its amino terminus mature A42 harbors a set of two closely spaced cysteine residues. The second cysteine at position 4 of A42 is part of a predicted levomeric C-X-G motif (Yount and Yeaman, 2004), which is thought to stabilize a gamma-core fold. Initial structure modeling of A42 using the antimicrobial peptide 1KRH (GenBank accession 1KRH_A) as template suggested that A42 likely folds into a γ-core (Fig. 5.4).

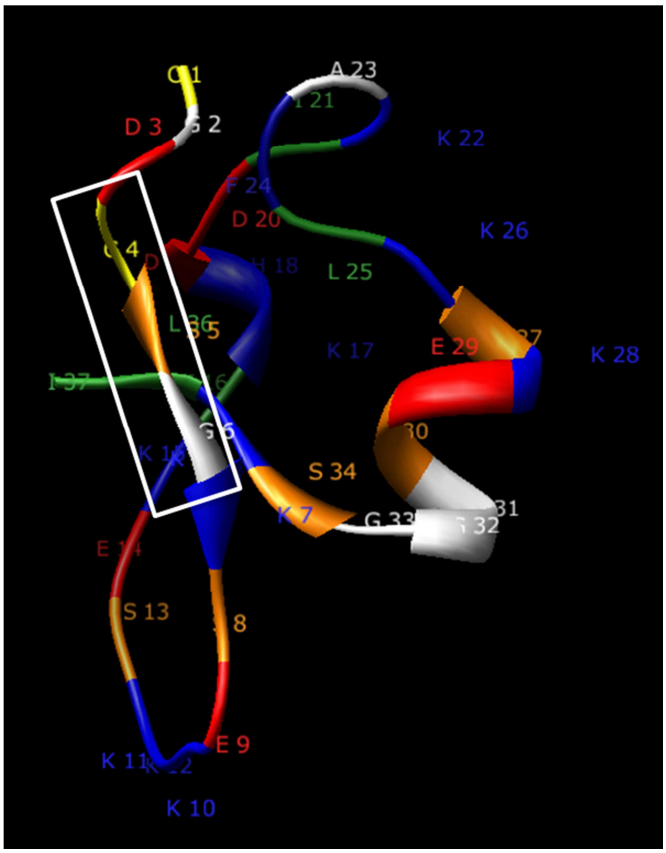

Fig. 5.4: Predicted three-dimensional structure of Gr-AMP1. Amino acids are indicated in ASMOL colorations and their position is included. Box indicates the levomeric G-X-C γ-core motif.

To investigate if A42 can function as an antimicrobial peptide in vitro, we tested synthetic A42 in a radial diffusion assay for antimicrobial activity on a standardized panel of strains of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Candida albicans* with known sensitivity to antimicrobial peptides (Tang et al., 2002). The microbial activity of synthetic A42 was deduced from the zone-of-inhibition of bacterial and fungal growth on solid agar, and compared to that of the synthetic antimicrobial peptide RP.1 (Bourbigot et al., 2009) and buffer alone at two different pHs (Fig. 5.5). At pH 7.5, synthetic A42 reduced the growth of *S. aureus*, *E. coli*, *P. aeruginosa*, *S. typhimurium*, but not of *C. albicans*. At pH 5.5, however, synthetic A42 did reduce growth of *C. albicans*, while its antimicrobial activity on bacteria was significantly less or even absent. These data suggest that synthetic A42 exerts antimicrobial activity on bacteria and a yeast in vitro.

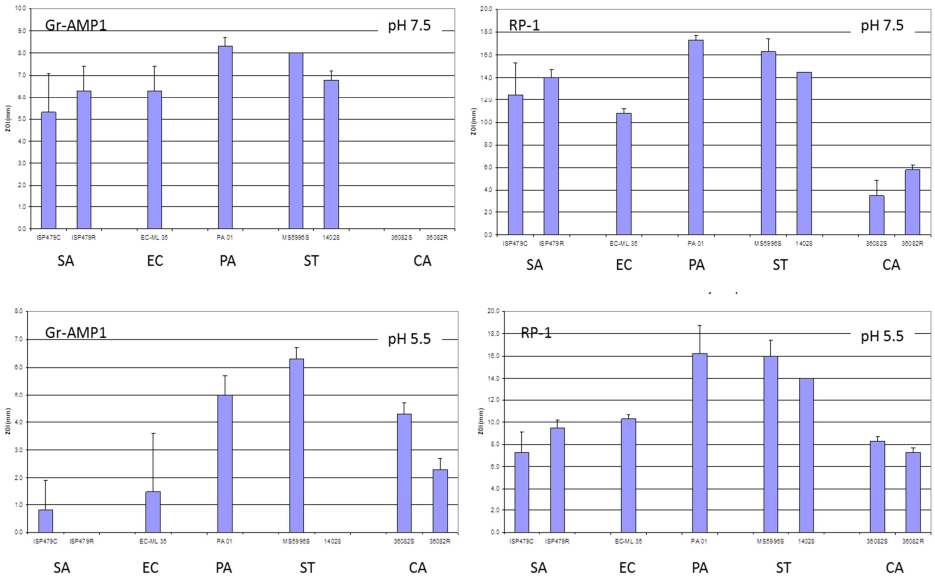

Fig. 5.5: Zone of inhibition (Y-axis) of bacterial and fungal growth following the application of synthetic Gr-AMP1 and RP-1 to wells in agar plates at pH 7.5 and pH 5.5 (SA, two strains *of Staphylococcus aureus*; EC, *Escherichia coli*; PA, *Pseudomonas aeruginosa*; ST, two strains of *Salmonella typhimurium*; CA, *Candida albicans;* Error bars, standard error of mean).

Because *G. rostochiensis* is an obligate endoparasite of plants with a lifecycle of two months, it is not feasible to test if synthetic A42 can improve the survival of this nematode following an infection with a bacterial or fungal pathogen. However, *C. elegans* is routinely used in high-throughput screens to test for antimicrobial activity of highly diverse compounds (Moy et al., 2006). To investigate if synthetic A42 can improve the survival of *C. elegans* following a bacterial infection, we exposed synchronized cultures of *C. elegans* to *P. aeruginosa* and treated the worms with synthetic A42, gentamycin, or buffer alone. Albeit at a lower level than gentamycin, synthetic A42 significantly reduced at two different concentrations the percentage of dead worms at 120h and at 144h post treatment (Fig. 5.6). Repeating the same experiment while replacing *P. aeruginosa* by *E. coli* demonstrated that synthetic A42 alone does not affect the survival rate of *C. elegans*.

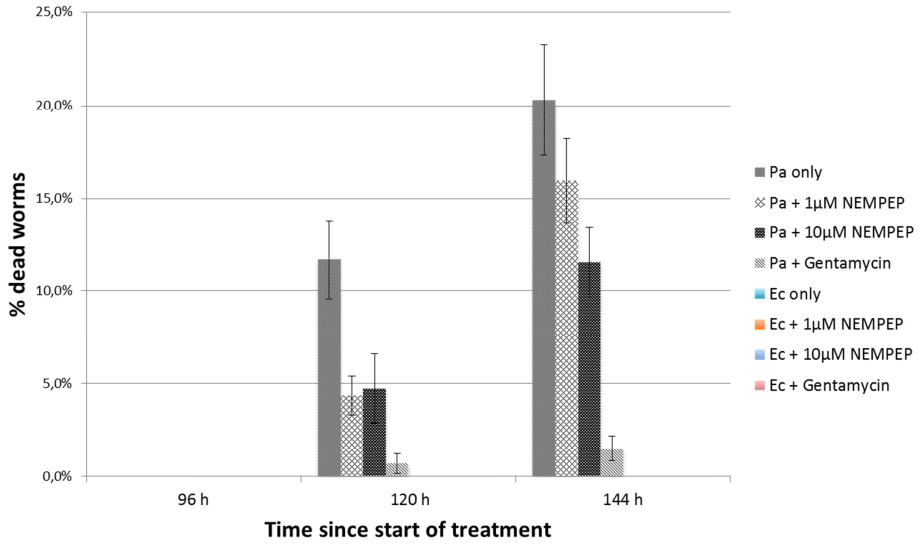

Fig. 5.6: Percentage of dead *C. elegans* infected either with pathogenic *P. aeruginosa* or *E. coli* at three time points after treatment with synthetic Gr-AMP1 and Gentamycin as control (EC, *Escherichia coli*; PA, *Pseudomonas aeruginosa*; Error bars, standard error of mean).

5.3 Discussion

Secretions produced by plant parasitic nematodes have been linked to host invasion, establishment of the feeding site in the host, and suppression of host innate immunity (Haegeman et al., 2012). These secretions consist of a highly complex mixture of proteins and peptides most of which are produced in the three single-celled esophageal glands of the nematodes. Small polypeptides synthesized in the dorsal esophageal gland of cyst nematodes have been shown to activate receptor-mediated signaling of developmental processes in host plants (Replogle et al., 2011). Here we demonstrate that the potato cyst nematode *G. rostochiensis* also produces a highly charged cationic polypeptide with antimicrobial activity (Gr-AMP1) that may protect the nematode against microbial infections.

Remarkably, Gr-AMP1 has no sequence similarity with other (predicted) proteins in the non-redundant sequence databases and in the genome of the sister potato cyst nematode species *G. pallida*. This may not be so surprising as antimicrobial peptides are rarely identified by direct sequence similarity with polypeptides that have demonstrated antimicrobial activity. The extraordinary diversity and thus lack of sequence conservation among antimicrobial peptides even among closely related species is most likely caused by positive Darwinian selection, which favors the accelerated evolution of the novel peptide variants (Nicolas et al., 2003). The evolutionary plasticity in antimicrobial peptides is, for instance, demonstrated by hypervariable peptides on the skin of the frog (Duda Jr et al., 2002). However, the overall physiochemical characteristics of antimicrobial peptides seem to be more conserved than their primary amino acid sequences. Many antimicrobial peptides, including Gr-AMP1, are smaller than 50 amino acids and have a net cationic charge with a propensity for amphipatic surfaces. Recent attempts to further classify antimicrobial peptides based on common structural features have focused on the gamma-core motif, which is defined by a hallmark Gly-X-Cys motif (or Cys-X-Gly in levomeric forms; (Yount and Yeaman, 2004)). Gr-AMP1 harbors a γ-core motif at its amino-terminus, and initial structure modeling using 1KRH as a template suggests

that this motif in Gr-AMP1 may indeed adopt a γ-core fold. Our initial structural model of Gr-AMP1 further suggests that seven residues close to the carboxyl terminus likely fold into an alpha helical structure. We therefore concluded that the overall structure of Gr-AMP1 resembles a γ-α topology as is, for instance, found in invertebrate defensins (Yeaman and Yount, 2007).

Gr-AMP1 is the first secretory polypeptide of a plant parasitic nematode to demonstrate antimicrobial activity on a standardized panel of bacteria and a fungus. Polypeptides with antimicrobial activity, so-called antibacterial factors (ABFs), have been reported for the animal parasitic nematode *A. suum* and the free-living nematode *C. elegans (Kato, 2007)*. Unlike Gr-AMP1, ABFs are small proteins characterized by an array consisting of an even number (>6) of cysteine residues. Searching DNA sequences from a representative set of members of the phylum Nematoda with the cysteine array as query revealed that plant-parasitic nematodes might also use ABFs (Tarr, 2012). Future studies will have to show if these ABF homologs in plant parasitic nematodes encode functional antibacterial and antifungal activity. A second class of antimicrobial peptides that have thus far only been found in animal parasitic nematodes and that lack a cysteine array are the cecropins (Pillai et al., 2005; Tarr, 2012). Cecropins are also small highly charged cationic polypeptides (about 30 amino acids), but do not have any significant sequence or structural similarity with Gr-AMP1.

Typical for ABFs, and for most antimicrobial peptides, is that they are synthesized as pre-pro-proteins, with at the amino terminus a classical eukaryotic signal peptide for secretion and either at the carboxyl or amino terminus of the mature protein a pro-peptide (Tarr, 2012). It is thought that the pro-peptide may inhibit the activity of an antimicrobial peptide until it is post-translationally processed. We have no evidence that Gr-AMP1 is post-translationally cleaved into a mature protein, except for the removal of the signal sequence for secretion. Prediction software trained to recognize pro-peptide cleavage sites (PropP v1) did not detect any likely cleavage sites in Gr-AMP1. Second, the synthetic peptide that we designed on the Gr-AMP1 protein sequence and that showed antimicrobial activity

in our assays included the complete open reading frame down-stream of the signal peptide for secretion.

We have shown that Gr-AMP1 improves the survival of *C. elegans* suffering from infections by the bacterial pathogen *P. aeruginosa*. These data suggest that Gr-AMP1 may function as a host defense peptide to protect *G. rostochiensis* from infection by pathogenic microbes. Like *G. rostochiensis*, *C. elegans* produces antimicrobial peptides in tissues close to the esophagus (Kato, 2007). It is thought that these peptides in *C. elegans* function as an effector component of the worm's innate immune system in the digestive tract of the nematode. Unfortunately, as *G. rostochiensis* is an obligate endoparasite of plants with an average lifespan of 2 months, we cannot test if Gr-AMP1 is able to extend the lifespan of *G. rostochiensis* suffering from an infection with pathogenic bacteria or fungi. In fact, it is not known if and to what extent plant-parasitic nematodes are vulnerable to intestinal infections by other microbes.

As Gr-AMP1 is highly expressed in freshly hatched pre-J2s, it is conceivable that *G. rostochiensis* releases Gr-AMP1 through its stylet during migration through the soil and during host invasion. As such Gr-AMP1 may exert its antimicrobial activity outside the body of *G. rostochiensis* either to protect the cuticle of the nematode against invading hyperparasites or to protect the host from secondary infections. The invasion of host plants by *G. rostochiensis* typically involves considerable wounding of host tissues, which creates ample opportunities for soil-borne pathogens to infect these plants. It would be detrimental to the biotrophic nematode if the plant succumbs to infections by these opportunistic bacteria and fungi. By releasing antimicrobial peptides inside the plant the nematode may thus increase the fitness of the plant. However, despite extensive proteomic analysis of collected stylet secretions of *G. rostochiensis*, we have found no evidence that Gr-AMP1 is actually being secreted at the onset of parasitism. We can therefore not conclude that Gr-AMP1 functions inside or outside the nematode's body, or both.

5.4 Materials and methods

5.4.1 Cloning of Gr-AMP-1

Preparasitic juveniles of *G. rostochiensis* pathotype Ro1-Mierenbos were collected as described previously. Messenger RNA was extracted from five developmental stages of *G. rostochiensis* and cDNA-AFLP analysis was performed essentially as described previously (Qin et al., 2000). The primary cDNA templates synthesized from each of the five mRNA pools were digested using the restriction enzymes *EcoR*I and *Taq*I. For the specific amplification reactions, oligonucleotide primers annealing to the EcoRI and *Taq*I adapter sequences were used in standard protocols. Specific transcript-derived-fragments (TDFs) were excised from acrylamide gels. Following reamplification using the original primers, TDFs were cloned into the TOPO-pCR4 plasmid (Invitrogen, Leek, The Netherlands).

Five µg of total RNA was used as the starting material for full-length, RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (RLM-RACE) with the GeneRacer Kit (Invitrogen). Super-script III reverse transcriptase (Invitrogen) was used for cDNA synthesis, according to the manufacturer protocol. Full-length cDNA was amplified using gene specific primers (5' CCCAGAACAATCACCACAAGCATC 3' for 5'RACE and 5' TCGATGCTTGTGGTGATTG 3' for 3'RACE) in combination with adaptor specific primers, and was subsequently cloned into the pCR4-TOPO vector (Invitrogen). Transformed *E. coli* colonies were checked for presence of the expected insert by PCR with the same primers as used for the amplification. Plasmids were purified using the Wizard *Plus* Miniprep DNA Purification System (Promega Corporation, Madison, WI, USA) and sequenced at the Sequence Facility Wageningen (Wageningen, the Netherlands) or at BaseClear (Leiden, the Netherlands).

Genomic DNA was extracted from preparasitic J2 as described by (Curran et al., 1985). Genomic DNA fragments corresponding to the *Gr_AMP1* were amplified with the gene specific A42 5' FW (5' GATGCTTGTGGTGATTGTTCTGGG 3') and the

A42 3' RV (5' CATTTTCGTCTTATGAGCTTGCTTCC 3') primer. Amplification products were cloned into the pCR4-TOPO vector (Invitrogen).

5.4.2 *In situ* hybridization microscopy

In situ hybridisation microscopy was done on preparasitic juveniles (J2). The nematode fixation, hybridization and detection steps were essentially as described by De Boer et al (De Boer et al., 1998; Smant et al., 1998), except for the use of single stranded DNA probes, which were synthesized by linear PCR. The sequences of the primers used to generate the probes are *Taq* FW (5'TCGATGCTTGTGGTGATTG 3') and *EcoRI* RV(5' GAATTCTAAAGTTTGTC 3').

5.4.3 Sequence analyses and 3-D structure modeling

DNA and amino-acid sequences were analyzed using the DNASTAR (Lasergene, ###). The computer algorithm SignalP 3.0 was used to predict the presence of a signal peptide for secretion and the corresponding putative cleavage site (Neural Networks). BLAST at NCBI (www.ncbi.nlm.nih.gov), at nemaBLAST (www.nematode.net), or at the Sanger Centre (http://www.sanger.ac.uk/resources/downloads/helminths/globodera-pallida.html) was used to search for matching sequences in databases.

A three-dimensional model of Gr-AMP1 was generated by homology modeling (Phyre; 3D-PSSM folding server). In brief, this method aligns a test sequence to one or more template structures with known structures as determined by crystallization/X-ray diffraction, or NMR spectrometry. This method allows for the identification of homology based on PSI-BLAST alignments in combination with a profile–profile-matching algorithm which adjusts for secondary structure alignments. Non-self identifying (i.e., non-sequence identify) comparator template with greatest homology to the target Gr-AMP1 sequence was ferrodoxin-like domain from benzoate 1,2-dioxygenase reductase in *Acinetobacter* (PDB accession

1KRH). This structures had >95% precision and served as control for homology modeling of Gr-AMP1.

5.4.4 Assay for antimicrobial activity

Antimicrobial assays were performed by using a well-established radial diffusion method modified to pH 5.5 or 7.5 (Tang et al., 2002). Synthetic Gr-AMP1 peptide was obtained from commercial sources. RP-1 was included in each assay as a control. A panel of microorganisms was tested: Gram-positive *Staphylococcus aureus* (ISP479S and R), Gram-negative *Escherichia coli* (strain ML-35), *Pseudomonas aeruginosa* (PA01), and *Salmonella typhimurium* (MS5996S and 14028), and the fungus *Candida albicans* (ATCC 36082S and R). Logarithmic-phase organisms were inoculated (10^6 colony-forming units per ml) into buffered agarose, and poured into plates. Peptides (10 µg) were introduced into wells in the seeded matrix and incubated for 3 h at 37°C. Nutrient overlay medium was applied, and assays were incubated at 37°C for bacteria or 30°C for fungi. After 24 h, zones of inhibition were measured. Independent experiments were repeated a minimum of two times.

5.4.5 *C. elegans* survival assay

C. elegans wildtype Bristol N2 was maintained using standard procedures (Stiernagle, 2006). Synchronized (Emmons et al., 1979) L4 stage to young adult worms were infected for 24 hours on lawns of *P. aeruginosa* strain PAO1 or *E. coli* strain OP50 (control), on nematode growth medium (NGM) plates containing 200µM 5-fluoro-2'-deoxyuridine (FUDR) at 20°C. The worms were washed in M9 buffer (Ghadessy et al., 1996), resuspended in 0.7% Gelrite and transferred into 96-well plates (15-20 worms/well). Single wells contained 100µl S-medium (Ghadessy et al., 1996) with 0.5mg *E.coli* OP50, 1.2mM FUDR, and 1µM or 10µM synthetic Gr-AMP1 peptide, or 100µg/ml gentamicin, or no antimicrobial compound. The plates were sealed with gas-permeable membranes and incubated at 20°C. The numbers of dead worms were counted daily. Significance of survival rates was calculated by

two-tailed Student's t-test assuming unequal variance, with $P<0.05$ considered as the threshold for significance.

6

General discussion

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6.1 Introduction

Most plant-parasitic nematodes are soil dwellers that survive and reproduce inside or on the surface of the roots of host plants. Economically the most damaging, and therefore the best-studied plant-parasitic nematodes, are the sedentary endoparasitic cyst nematodes (*Globodera* and *Heterodera* spp.) and root-knot nematodes (*Meloidogyne* spp.). Sedentary endoparasitic nematodes typically invade a host plant and transform host cells into a permanent feeding structure. Infective juveniles of sedentary endoparasites first penetrate the epidermis of the root and then migrate through the root cortex. Cyst nematodes do so by destructively burrowing their way through root tissues, whereas root-knot nematodes stealthily push host cells aside with their head while causing relatively little damage to host cells. This migratory phase ends when the infective juveniles settle down close to (i.e. cyst nematodes) or inside (i.e. root-knot nematodes) the vascular cylinder to start the formation of a feeding structure (Gheysen and Mitchum, 2009).

The feeding structure of cyst nematodes arises by progressive local cell wall degradation and subsequent fusion of neighboring host cells into an elaborate multinucleate syncytium (Sobczak et al., 2009). Root-knot nematodes have a different strategy, and drive several host cells close to the head through repeated rounds of karyokinesis without cytokinesis, leading to the expansion of several cells into so-called giant cells (Caillaud et al., 2008). For several weeks onwards, the syncytium and the giant cells provide all the nutrients that the feeding nematodes require for their development and growth. It is crucial for these nematodes to successfully initiate, maintain, and protect their feeding structures, as abortion of the feeding structure leads to a strong reduction of reproductive success or even death of the nematodes (Jones and Northcote, 1972; Rice et al., 1985; Sobczak et al., 2005; Williamson and Kumar, 2006; Tomczak et al., 2009a).

Sedentary endoparasitic nematodes release secretory-excretory molecules from several specialized gland cells and the hy

podermis into surrounding host tissues (Vanholme et al., 2004). Secretions produced in the dorsal- and subventral esophageal glands of these nematodes are thought to be instrumental in host invasion and the formation of the feeding site (Haegeman et al., 2012). The nematodes deliver these esophageal gland secretions into the apoplast and cytoplasm of host cells via a protrusible stylet (Hussey, 1989; Davis et al., 2008). The esophageal gland secretions are complex mixtures of highly diverse secretory proteins, most of which have not been functionally characterized. Nonetheless, many of these secretory proteins are thought to act as effectors in the parasitism of the nematodes in host plants. The term effector is used differently in various scientific communities. Here, we use the broader and more inclusive definition of effectors as all molecules of parasites that alter host cell structure and function to promote the virulence of the parasite (Hoogenhout et al 2009). Although it is assumed that sedentary plant-parasitic nematodes also deploy effectors to protect themselves and their feeding structures against host defense responses, relatively little is known of their role in immunomodulation in host plants.

Studies on effectors that activate and suppress plant innate immunity have opened up an exciting and rapidly expanding research field. Most of what is known to date originates from work on bacterial plant pathogens (reviewed in (da Cunha et al., 2007)), and to a lesser extent on plant pathogenic fungi and oomycetes (reviewed in (Ali and Bakkeren, 2011)). In this chapter, we review the current evidence suggesting that plant-parasitic nematodes deliver effectors into the apoplast and cytoplasm of host cells to modulate host defenses. This discussion is organized in three main sections. The first section gives a summary of the type of experimental evidence supporting the idea that plant-parasitic nematodes deliberately secrete proteins to modulate host defenses. The second and third sections respectively focus on nematode effectors in the apoplast and the cytoplasm of host cells.

6.2 Identification of immunomodulators in plant parasitic nematodes

The identification of effectors of plant-parasitic nematodes is less straightforward than it is for fungal and bacterial plant pathogens. Nematodes are metazoans with body plans consisting of multiple highly differentiated cells, most of which have specialized functions in organs and tissues. Most of the secretory proteins in nematodes are therefore important for the maintenance of the nematode's internal structure and physiology. This maintenance also includes the nematode's own innate immune system that provides protection against hyperparasitism by soil-borne fungi and bacteria (see chapter 5). At present, several common features are collectively used to single out effectors from other secretory proteins in plant-parasitic nematodes. It is assumed that most effectors are expressed in organs in the nematode that are in contact with plant tissues either directly (i.e. body wall, amphidial, and rectal glands) or indirectly via the oral stylet (i.e. the dorsal and subventral esophageal glands; see chapters 3 and 5).

As a first step in the identification of nematode effectors, whole mount *in situ* hybridization microscopy and immunofluorescence microscopy are used to localize transcription of candidate effector genes or effector proteins in these organs (Muskett et al., 2002). An experimentally more demanding next step is to show their actual delivery into to apoplast and cytoplasm of host cells using immunofluorescence microscopy. This method, however, relies on raising specific antiserum against synthetic peptides matching the candidate effector sequence, which has been moderately successful so far. As a surrogate for *in situ* localization in host cells, candidate effectors have been shown to be part of chemically-induced stylet secretions collected from pre-parasitic infective juveniles. Although this approach does not show the actual delivery of candidate effectors *in planta*, it can demonstrate that a secretory protein of a nematode most likely functions outside of the nematode body at the interface with the plant. The identification of candidate effectors in collected stylet secretions can be based on a targeted

approach with specific anti-serum raised against a synthetic peptide matching part of the candidate effector sequence ((Smant et al., 1997; Rehman et al., 2009a), and Chapter 3) or on a non-targeted approach using tandem liquid chromatography and microspectroscopic analysis (Bellafiore et al., 2008; Lozano-Torres et al., 2012). Both methods have their limitations including the success rate of raising antiserum specifically recognizing nematode effectors, unintentional leakage of non-effector proteins during the handling of the nematodes, and their restricted applicability to pre-parasitic nematodes only.

After establishing that a secretory protein from a plant-parasitic nematode most likely functions as an effector in host cells, several lines of evidence have been used to support a role for an effector as modulator of host defenses. First of all, some nematode effectors share sequence similarity with enzymes that have been linked to modulating host defenses by other plant pathogens. Being guilty-by-association has merely been used as a starting point for further experimental research on an effector. For instance, biochemical assays have been used both on collected stylet secretions and on recombinantly-produced effector proteins to affirm that a particular enzymatic activity may indeed be important for modulation of host defenses (see for example secreted glutathione peroxidase below).

More direct leads to the function of nematode effectors can be obtained by identifying the molecular components in host cells that function as virulence targets of these effectors. Yeast-two-hybrid screening (Hewezi et al., 2008b; Rehman et al., 2009b; Patel et al., 2010), co-immunoprecipitation (Lozano-Torres et al., 2012; Postma et al., 2012) and bimolecular fluorescence complementation microscopy (Sacco et al., 2009; Postma et al., 2012) have all been used to identify the host targets of nematode effectors. Some of the recently identified virulence targets appeared to be molecular components of immune signaling cascades, suggesting that these effectors may interfere with the activation of innate immunity in plants (see for example Gr-SPRYSEC19 and Gr-VAP1 below).

Stable heterologous overexpression of nematode effectors in transgenic plants has been used to further demonstrate that these effectors suppress host defenses, as the overexpression of effectors can render plants hypersusceptible to nematodes and other plant pathogens (see Hs10A06 below). The interpretation of such data requires some caution as the hypersusceptibility in the transgenic plants might also result from non-specific cell stress induced by the large amounts of recombinant protein in host cells and from an altered constitution of transgenic plants caused by selection on antibiotics. For instance, overexpression of the green-fluorescent protein in plants can enhance the susceptibility of plants to nematodes (own data, unpublished). Using transgenic plants overexpressing non-sense proteins as baseline reference for nematode susceptibility in such assays can largely avoid these caveats. Furthermore, analyzing defense gene expression in transgenic plants with altered susceptibility to nematodes can give greater confidence that these effectors specifically target the immune system of the host (see for example Hs10A06 and Gr-1106 below). Lastly, exposing transgenic plants overexpressing a nematode effector to known elicitors of PTI (e.g. elf18 and flg22) can offer conclusive evidence that the effector alters the immune competence of these plants (see Mi-CRT below).

Further attempts to demonstrate the actions of nematode effectors on plant innate immunity have focused on specific cellular changes associated with innate immune responses in plant cells such as the hypersensitive response type of programmed cell death (HR-PCD). Instead of using transgenic plants stably overexpressing nematode effectors, the suppression of the defense-related programmed cell death in these assays is gauged following transient expression using agroinfiltration in plant leaves (e.g. *Nicotiana benthamiana*). The co-expression of a plant resistance protein and a cognate pathogen effector by agroinfiltration can result in a local programmed-cell death in the infiltrated leaf area. A tripartite co-expression by agroinfiltration of a pathogen effector (e.g. virus coat protein) and a matching resistance protein (e.g. Rx1) with either a nematode effector or a control protein, can demonstrate that a nematode effector suppresses the onset of the

programmed cell death and disease resistance (see Gr-SPRYSEC-19 and Gr-1106). A drawback of this type of assay is that it is hard to quantify the level of suppression by the degree of cell death in the infiltrated leaf area. Furthermore, defense-related programmed cell death is notoriously sensitive to environmental factors such as incoming UV radiation and leaf age. To better quantify the level of suppression of cell death, conductivity measurements on apoplastic fluids isolated from agroinfiltrated leaves undergoing a defense-related programmed cell death can provide an added value. This method makes use of the fact that defense-related programmed cell death is preceded by extensive ion leakage into the apoplast (see chapter 4).

6.3 Nematode effectors targeting the apoplast of host cells

One of the first signs in plant defense responses is the rapid production of reactive oxygen species (ROS), in an event called extracellular oxidative burst. Nematode invasions also trigger an oxidative burst in host plants, especially during the destructive migration of nematodes through the roots (Waetzig et al., 1999a; Melillo et al., 2006). Reactive oxygen species can damage the nematode directly, but they can also activate other signaling pathways leading to defense responses such as cell-wall reinforcement (Smant et al., 2011) and activation of the salicylic acid-dependent defense pathway (Grant and Loake, 2000). To protect themselves against the products of an oxidative burst, nematodes most likely secrete a range of antioxidant proteins into the apoplast of host cells to neutralize reactive oxygen species.

One type of the antioxidant enzymes most likely involved in self-protection are secreted superoxide dismutases that catalyze the dismutation of superoxide radicals into hydrogen peroxide and oxygen (McCord and Fridovich, 1969). Superoxide dismutases have been identified in collected secretions of preparasitic infective juveniles of *Globodera rostochiensis* using a specific antiserum (Robertson

et al., 1999) and in secretions of *Meloidogyne incognita* using microspectroscopy (Bellafiore et al., 2008). As expected, several genes encoding superoxide dismutases are present in the genomes of *G. pallida* (http://www.sanger.ac.uk/resources/downloads/helminths/globodera-pallida.html)*, M. hapla* and *M. incognita* (Abad et al., 2008; Opperman et al., 2008). It has been suggested that these nematodes secrete superoxide dismutases in the early stages of parasitism to scavenge reactive oxygen species released during host invasion (Kaplan and Keen, 1980; Guo et al., 2009). Different isoforms and differences in activity of nematode superoxide dismutases correlated with virulence and avirulence of *M. incognita* (Oerke et al., 1994).

Animal-parasitic nematodes also use glutathione peroxidases and peroxiredoxins to cope with the oxidative stress caused by host-generated hydrogen peroxide (Henkle-Duhrsen and Kampkotter, 2001). Similarly, preparasitic juveniles of *G. rostochiensis* secrete glutathione peroxidases through the hypodermis in all parasitic stages, but the substrates of these enzymes are larger hydroperoxide substrates rather than hydrogen peroxide alone (Jones et al., 2004). Immune labeling studies with specific antiserum also demonstrated the presence of peroxiredoxin on the surface of *G. rostochiensis* prior to and post invasion of the host (Waetzig et al., 1999b). The recombinantly produced peroxiredoxin of *G. rostochiensis* catalyzes the breakdown of hydrogen peroxide. Similarly, the root-knot nematode *M. incognita* expresses several peroxiredoxins in all parasitic stages (Grundler et al., 1997). Knocking down expression of these peroxiredoxins by RNA interference reduces both the viability of preparasitic juveniles after an exposure to hydrogen peroxide *in vitro* and the virulence of *M. incognita* on tomato plants. Like their homologues in *G. rostochiensis*, the recombinant peroxiredoxins of *M. incognita* catalyze the removal of hydrogen peroxide in a concentration-dependent manner. Moreover, these recombinant peroxiredoxins are able to protect *Escherichia coli* against organic peroxides.

In addition to secreting enzymes capable of neutralizing the oxidative burst, the cyst nematode *G. pallida* carries a fatty acid- and retinol-binding protein at its

surface in the early stages of parasitism (Prior et al., 2001). Recombinantly produced fatty acid- and retinol-binding protein of *G. pallida* inhibits lipoxygenase-mediated modification of linolenic and linoleic acid compounds. This modification is an early step in the octadecanoid signaling pathway, which leads to the synthesis of jasmonic acid, and which mediates immune responses against necrotrophic pathogens (Gutjahr and Paszkowski, 2009). Thus, surface-bound fatty acid- and retinol-binding protein of the nematode may interfere with the lipid-based signaling involved in regulating host defenses. Further research such as a knock-down of the expression of the fatty acid- and retinol-binding protein may demonstrate whether this protein acts as a modulator of host innate immunity.

Plant-parasitic nematodes also target the activity of apoplastic host enzymes directly. For instance, the venom allergen-like protein Gr-VAP-1 from *G. rostochiensis* specifically interacts with the apoplastic cysteine protease $Rcr3^{pim}$ of the currant tomato species *Solanum pimpinellifolium* (Lozano-Torres et al., 2012). Although the exact function of $Rcr3^{pim}$ remains to be shown, a range of different plant pathogens produce effectors that target this enzyme (Song et al., 2009). It is thought that $Rcr3^{pim}$ acts as node in an apoplastic defense-related signaling network. Mutant tomato plants lacking Rcr3 or having a different allele of *Rcr3* are significantly less susceptible than plants with $Rcr3^{pim}$ (Lozano-Torres et al., 2012). It is thought that *G. rostochiensis* secretes venom allergen like proteins to inhibit $Rc3^{pim}$ in tomato to modulate host defenses. Remarkably, perturbations of $Rcr3^{pim}$ brought about by GrVAP1 are recognized in some tomato genotypes by the extracellular resistance protein Cf-2, which leads to resistance to the nematodes (Lozano-Torres et al., 2012).

Similarly, the effector Hs30C02 from *H. schachtii* has been shown to interact with an extracellular β-1,3-glucanase of *A. thaliana* (Nicolas et al., 2003). This β-1,3-glucanase is considered to be a pathogenesis-related protein involved in the defense response against fungal plant pathogens in plants (Mahalingam et al., 2003; Doxey et al., 2007; Lashbrook and Cai, 2008). Knocking-down Hs30C02

expression in *H. schachtii* by plant-mediated RNA interference strongly reduces the development of the nematodes in plants. Conversely, the overexpressing Hs30C02 in *A. thaliana* increased susceptibility of these transgenic plants to *H. schachtii*, but did not affect the expression of a representative set of defense-related genes.

Recently, it has been shown that secretions of *M. incognita* also include a calreticulin named Mi-CRT (Jaubert et al., 2002; Jaubert et al., 2005). Calreticulins are calcium-binding proteins that control intracellular calcium homeostasis and protein and glycoprotein folding in the endoplasmic reticulum (Michalak et al., 2009). Calcium ions also function as secondary messengers in the host defense responses in plants (Ma et al., 2008). Immunolabeling using specific antiserum to Mi-CRT show that this effector accumulates in the apoplast along the cell wall of giant cells (Jaubert et al., 2005). Knocking-down the expression of Mi-CRT in *M. incognita* significantly reduces nematode virulence (Arguel et al., 2012). Transgenic *A. thaliana* plants overexpressing Mi-CRT in the apoplast show enhanced susceptibility to *M. incognita* and to the unrelated plant pathogenic oomycete *Phytophthora parasitica*. Furthermore, challenging these transgenic plants with the PTI-inducing peptide elf18 resulted in a reduced defense-related gene expression and callose deposition as compared to wild type plants (Jaouannet et al., 2012). Altogether, these data suggest that apoplastic Mi-CRT modulates the activation of innate immune responses of the host.

6.4 Nematode effectors targeting cytoplasmic host proteins

As explained in the introduction, sedentary plant-parasitic nematodes deliver stylet secretions into the cytoplasm of host cells to initiate the transformation of host cells into a feeding structure, and to subsequently maintain this feeding structure for several weeks. Given the importance of the feeding structure for the survival and reproduction of sedentary plant-parasitic nematodes, it is thought that these stylet secretions include multiple effectors to suppress cytotoxic host defense

responses inside the feeding structure. Failing to do so often results in an HR-PCD inside or in cells around the feeding structure of the nematodes. Below we have summarized the current insights in nematode effectors that are most likely delivered into cytoplasm of host cells to suppress defense responses.

Many sedentary plant-parasitic nematodes produce effectors with significant homology to chorismate mutases from gram-negative bacteria (Lambert et al., 1999; Popeijus et al., 2000; Bekal et al., 2003; Huang et al., 2005a; Long et al., 2006; Opperman et al., 2008; Jones, 2009; Vanholme et al., 2009; Haegeman et al., 2011). In both plants and bacteria, chorismate mutases operate in the Shikimate pathway in which chorismate is converted into different compounds. As animals lack the Shikimate pathway, it is thought that plant-parasitic nematodes have acquired these enzymes from bacteria via horizontal gene transfer to modulate the conversion of chorismate in host plants (Jones et al., 2003). Chorismate-derived compounds play critical roles in growth, development and defense of plants (Schmid and Amrhein, 1995; Weaver and Herrmann, 1997). For example, salicylic acid and several phytoalexins are chorismate derivatives (Dixon et al., 1983; Ryals et al., 1994; Dong, 2001). This led to the hypothesis that nematodes use chorismate mutases to reduce the pool of chorismate available for conversion to salicylic acid in host cells. So far, however, it has not been demonstrated that nematode chorismate mutases lower chorismate-derived defense compounds in plants (Doyle and Lambert, 2003). Furthermore, it is also not clear whether the overexpression of nematode chorismate mutases alters the susceptibility of host plants to nematodes and other plant pathogens, as would be expected. Nor is it clear whether exogenous nematode chorismate mutase affects immune signaling in plants.

There are several lines of evidence suggesting that sedentary endoparasitic nematodes use effectors to modulate the host's ubiquitin-based proteasomal degradation machinery. (Gao et al., 2003) have found transcripts encoding secreted ubiquitins, ubiquitin-extension proteins, and homologues of plant SKP1 proteins in esophageal gland specific cDNA libraries of *H. glycines*. Similarly, (Tytgat et al.,

2004) have detected ubiquitin-extension proteins in the secretory granules of the dorsal esophageal gland of the beet cyst nematode *H. schachtii*, while (Bellafiore et al., 2008) have identified ubiquitin-like proteins in collected stylet secretions of *Meloidogyne incognita*. The ubiquitination system in plants comprises a complex process involving many different proteins that in a coordinated fashion target other proteins to the proteasome for degradation (Sadanandom et al., 2012). The ubiquitination system controls the lifespan of proteins in plant cells. Some bacterial plant pathogens deliver effectors with ubiquitin E3 ligase activity into host cells to modify turnover rates of specific defense-related host proteins, such as the flagellin pattern-recognition receptor FLS2 in *A. thaliana* (Spallek et al., 2009). Similarly, the effector Avr3a of the oomycete plant pathogen *Phytophthora infestans* stabilizes an E3 ligase protein of the host involved in defense responses (Tomczak et al., 2009b). It is not clear if and how the different ubiquitination-related proteins in stylet secretions of sedentary endoparasitic nematodes affect the turnover rate of host proteins involved in immunity.

Recently, an annexin-like effector Hs4F01 has been identified from *H. schachtii* (Patel et al., 2010). Annexins function as cellular Ca^{2+} and phospholipid-binding proteins in most eukaryotes. Plant annexins have been linked to abiotic stress responses (Clark et al., 2001; Cantero et al., 2006). Nematode annexins may thus play a role in immune or stress responses in host cells. Some support for this hypothesis was provided by overexpressing Hs4F01 in plants, which resulted in enhanced susceptibility to *H. schachtii* (Patel et al., 2010). Hs4F01 interacts with a member of the 2OG-Fe(II) oxygenase family of *A. thaliana*. A knockout mutant of a 2OG-Fe(II) oxygenase family member shows enhanced defense-related gene expression (van Damme et al., 2008). Hs4F01 may thus target host oxireductases to modulate host defense, however a direct down-regulation of the plant immune system by nematode annexins remains to be shown.

Such a down-regulation of host defenses was indeed observed for the effector Hs10A06 from *H. schachtii* and its homolog in *H. glycines* (Gao et al., 2003; Hewezi et al., 2010). The overexpression of 10A06 in *A. thaliana* makes the plants

hypersusceptible to nematodes and a variety of other plant pathogens. Furthermore, these plants demonstrated a significant down-regulation of the expression of pathogenesis-related genes PR-1, PR-2, and PR-5, all of which are associated with salicylic acid dependent immune signaling. 10A06 interacts with *A. thaliana* spermidine synthase 2 (SPDS2) in a yeast-two-hybrid analysis and in bimolecular fluorescent molecular complementation in plant cells (Hewezi et al., 2010). Spermidine synthase is a key enzyme in the biosynthesis of polyamines in plants, and plants overexpressing 10A06 show elevated levels of cellular spermidine and increased polyamine oxidase activity. Thus, 10A06 may target spermidine synthase of the host to increase the level of antioxidants in order to protect the feeding structure against host defense responses.

The Gr1106 effectors of the potato cyst nematode *G. rostochiensis* were initially linked to plant innate immunity based on their strongly enhanced evolutionary rates (Finkers-Tomczak, 2011). Positive diversifying selection in effectors of other plant pathogens often signals a direct involvement in an evolutionary arms race with the plant defense system of a host plant (Silva et al., 1999). The Gr1106 effectors are uniquely expressed in the dorsal esophageal glands in *G. rostochiensis*, but their delivery into host cells has not been demonstrated. They have a modular structure consisting of a highly basic, lysine rich N-terminal domain harboring nuclear and nucleolar localization signals and a C-terminal acidic part. *Gr1106* genes are expressed in all parasitic stages, but they are strongly upregulated in sedentary nematodes. Knocking-down of the Gr1106 effectors in *G. rostochiensis* and overexpressing of the effectors in plants show that they are important for nematode virulence. Potato plants overexpressing different Gr1106 variants also have altered susceptibility to the fungal pathogen *Verticillium dahliae*. These transgenic plants further demonstrate a down-regulation of the pathogenesis-related gene PR1, which is a marker of SA-mediated defense. Furthermore, the Gr1106 effectors selectively suppress the HR-PCD mediated by several cytoplasmic plant immune receptors in agroinfiltration assays in leaves of *N. benthamiana*. The suppression was restricted to some, but not all, CC-NB-LRR

type of resistance proteins. These data suggest that the Gr1106 effectors are capable of suppressing effector-triggered immunity in host plants. Given the timing of their expression in *G. rostochiensis*, they are most likely involved in protecting the feeding structure (Finkers-Tomczak, 2011).

The members of another class of positively selected effectors in *G. rostochiensis* are named SPRYSECs. They only consist of one or multiple SPlA and Ryanodine receptor (SPRY) domains and a typical eukaryotic signal peptide for secretion. SPRYSECs are among the largest gene families in cyst nematodes in the genus *Globodera* (*G. rostochiensis* (>200 members) and in *G. pallida* (>300 members)). However, SPRYSECs seem to be absent in other cyst nematodes and in root-knot nematodes (Abad et al., 2008; Opperman et al., 2008). Gr-SPRYSEC-19 is uniquely expressed in the dorsal esophageal gland in *G. rostochiensis* in the early stages of parasitism. It has been identified in collected stylet secretions of pre-parasitic juveniles using specific antisera. Potato plants overexpressing GrSPRYSEC19 do not display an altered susceptibility to *G. rostochiensis*, but do show a reduced resistance to *V. dahliae*. GrSPRYSEC19 and close homologs selectively suppress the defense-related programmed cell death and disease resistance mediated by several cytoplasmic plant immune receptors belonging to the CC-NB-LRR class in agroinfiltration assays in leaves of *N. benthamiana* (see chapter 4). Moreover, GrSPRYSEC19 also suppresses ion leakage associated with a hypersensitive response in agroinfiltrated leaf areas (not published). Altogether, GrSPRYSEC19 is capable of suppressing effector-triggered immunity mediated by several CC-NB-LRR resistance proteins in plants by a yet unknown mechanism. Gr-SPRYSEC-19 physically associates with the LRR domain of the resistance protein SW5F of tomato (Rehman et al., 2009b). However, this interaction does not lead to a resistance response, and there are no indications that the suppression of CC-NB-LRR mediated defense responses require a physical interaction between GrSPRYSEC19 and the LRR domains of these resistance proteins.

6.5 Concluding remarks

In all fairness, we are just beginning to understand how plant-plant parasitic nematodes use effectors to modulate host innate immunity. Despite the fragmented nature of the data currently available in the literature, it seems that sedentary plant-parasitic nematodes target the host's innate immune system at different levels and in different compartments of host cells. Plant-parasitic nematodes most likely secrete enzymes through their hypodermis to detoxify plant-derived reactive oxygen species. These enzymes may function in self-protection of the nematode, but they may also intercept intercellular signaling mediated by radicals in the apoplast of host cells. As the findings with the effectors GrVAP1, Hs30C02, and Mi-CRT further suggest, plant-parasitic nematodes deliver multiple unrelated effectors in the apoplast to modulate the extracellular molecular components of the plant's innate immune system. The host targets of Hs30C02 and GrVAP1 indicate that nematodes perturb the action of apoplastic enzymes, some of which may be important in activating extracellular immune receptors. At present the host target of Mi-CRT is not known, but as calcium influx is an early step in the activation of host defenses it is conceivable that the calcium-binding activity of this calreticulin alters the calcium fluxes and calcium-mediated signaling across the cell membrane.

The variety of effectors targeting molecular components in the host cell cytoplasm further suggests that nematodes have evolved multiple strategies to suppress the activation of host defenses in feeding structures. The phenotypes of different nematode effectors expressed in the cytoplasm of plant cells clearly demonstrate that they can alter the immune competence of host cells, as is shown by reduced expression of defense genes, reduced response of plant cells to PAMPs of bacterial pathogens, enhanced susceptibility to unrelated plant pathogens, and suppression of HR-PCD. These defense-related phenomena essentially reflect the outcome of activated receptor-mediated immunity in plants, rather than the ability of immune receptors and down-stream signaling pathways to respond to nematodes. In fact,

for none of the nematode effectors studied to date there is evidence that they directly modulate the activation of extracellular and cytoplasmic plant immune receptors, or intercept immune signaling downstream of activated plant immune receptors. Showing this would be the ultimate proof that plant-parasitic nematodes, just like animal-parasitic nematodes, specifically target the host's innate immune system to enable persistent infections. As is done already for several nematode effectors, identifying the host targets is the first step to address this challenge. The next step will be to assess which of these host targets regulate the activation of plant immune receptors or down-stream immune signaling. A comprehensive mutant analysis of genes involved in immune receptor activation and immune signaling such as described in chapter 2 may help to identify which of these pathways are required for the action of candidate immunomodulators of plant-parasitic nematodes in plants.

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Summary

Plant parasitic nematodes are major pests of many important food crops and they cause huge yield losses throughout the world. There are numerous species of plant parasitic nematodes, but agriculturally the most important are the obligate sedentary endoparasitic nematodes, which also happen to have the most complex lifestyle. For most of their active lifetime these nematodes reside inside the host plant. During this time, they are immobile and completely dependent on the host for nutrition. Nutrients are acquired through a specialized feeding structure that each nematode induces by modifying several host cells. This thesis focuses mainly on the cyst nematodes *Globodera rostochiensis* (golden potato cyst nematode) and *Heterodera schachtii* (beet cyst nematode).

Plants are not defenseless against microbial invaders, as they possess a complex multilayered innate immune system consisting of different types of extracellular and intracellular immune receptors. All currently known immune receptors with recognition specificities to plant-parasitic nematodes belong to the CC-NB-LRR type of cytoplasmic immune receptors. These immune receptors enable plants to detect most invading nematodes and to launch potent immune responses resulting in resistance. Often this nematode resistance involves a defense-related programmed cell death in and around the feeding sites of nematodes, which deprives the nematodes from their sole food source. Plant pathogenic bacteria and fungi have evolved ways to avoid detection or to suppress immune signaling or defense responses, which is often mediated by pathogen molecules termed effectors. In this thesis different aspects of the modulation of plant innate immunity by plant parasitic cyst nematodes are investigated. A better understanding of the mechanisms used by plant-parasitic nematodes to overcome the plant's immune system will ultimately aid the engineering of much-needed new nematode-resistant crops.

The thesis starts with an analysis of plant immune signaling pathways that are involved in the parasitism of *H. schachtii* in *Arabidopsis thaliana.* We have challenged a range of *A. thaliana* immune signaling mutants with nematodes and took the number of nematodes that reach the adult female stage as a measure of plant susceptibility (Chapter 2). Wild type *A. thaliana* lacks specific resistance genes that confer resistance to *H. schachtii* infections, and this analysis therefore primarily investigated basal defenses to plant-parasitic nematodes. We found an indication that extracellular immune receptor signaling and several hormone-mediated signaling pathways contain the nematode infection in this compatible interaction.

Chapter 3 describes the identification of an exceptionally large family of effectors in *G. rostochiensis*. The members of this effector family only consist of a SPRY domain and a classical signal peptide for secretion in eukaryotes, and are therefore called SPRYSECs. *G. rostochiensis* secrete the SPRYSEC effectors from the dorsal esophageal gland of the infective juveniles during the early stages of parasitism. Using specific software to detect footprints of accelerated evolution (i.e. PAML) several amino acid sites in the SPRYSECs were shown to be under diversifying selection. This suggested that the SPRYSECs are most likely involved the activation or suppression of host innate immunity. To test this hypothesis we used yeast-two-hybrid screening of a tomato root cDNA library to identify host proteins interacting with SPRYSEC family members. In this screening we found that SPRYSEC-19 interacts with the protein encoded by a novel resistance gene homolog, named SW5F, which belongs to the CC-NB-LRR type of cytoplasmic immune receptors.

The objective of the work described in Chapter 4 was to resolve the biological relevance of the interaction between SPRYSEC-19 and SW5F. Our first hypothesis was that this physical interaction triggers SW5F-mediated defense responses. We reasoned that as SPRYSEC-19 interacted with the LRR domain of SW5F, which is associated with recognition specificity in this type of immune receptors, it might function as a classical avirulence factor. Despite using several experimental setups we have not been able to show that SPRYSEC19 activates defense responses

mediated by SW5F or other closely related resistance gene homologs in tomato. In contrast, we discovered that SPRYSEC-19 is an effector that suppresses defense-related programmed cell death and resistance mediated by several CC-NB-LRR type resistance proteins.

Next to secreting effectors that modulate host innate immunity, *G. rostochiensis* also secretes peptides with antimicrobial activity from the dorsal esophageal gland, which is possibly a component of the nematode's own innate immunity against pathogenic bacteria and fungi. In chapter 5, we describe the identification of a small polypeptide (named Gr-AMP1) with a so-called gamma-core fold that is typically found in many antimicrobial peptides. We show that Gr-AMP1 inhibits growth of a standardized set of bacteria and yeast in vitro. To further prove that Gr-AMP1 can protect nematodes against infection, we infected the free-living nematode *Caenorhabditis elegans* with a pathogenic strain of *Pseudomonas aeruginosa* and subsequently rescued the nematodes by administering synthetic Gr-AMP1. Our data suggests that secretions of *G. rostochiensis* may have a role in protecting the nematode against infections by pathogenic microbes. Alternatively, *G. rostochiensis* might also secrete antimicrobial effectors to protect host cells against secondary infections by opportunistic bacteria and fungi.

In the concluding chapter 6 we discuss the current evidence for the existence of immune modulating effectors of plant parasitic nematodes and propose directions for further research.

Samenvatting

Plant-parasitaire nematoden (aaltjes) vormen een grote plaag in veel voedselgewassen en zorgen wereldwijd voor enorme oogstverliezen. Van de vele soorten aaltjes zijn voor de landbouw de obligatoire sedentaire endoparasieten het belangrijkst. Ze hebben tevens de meest complexe levenswijze: het grootste gedeelte van hun levenscyclus bevinden ze zich in de waardplant en zijn ze volledig immobiel en voor voeding afhankelijk van de waardplant. Om nutriënten te bemachtigen vormen de wormen enkele cellen van de waardplant om in een speciale voedingsstructuur. Dit proefschrift behandelt voornamelijk de cyste-alen *Globodera rostochiensis* (aardappel cyste-aal) en *Heterodera schachtii* (bieten cyste-aal).

Planten zijn niet weerloos tegen aanvallers. Ze bezitten van nature een complex gelaagd immuunsysteem dat bestaat uit verschillende typen extracellulaire en intracellulaire immuunreceptoren. Alle immuunreceptoren die tot op heden in verband zijn gebracht met de herkenning van plant-parasitaire nematoden zijn cytoplasmatische receptoren van het CC-NB-LRR type. Deze immuunreceptoren zorgen ervoor dat de plant de meeste binnendringende wormen kan herkennen en krachtige immuunresponsen kan opwekken die resulteren in resistentie. Resistentie tegen nematoden behelst vaak een door het immuunsysteem ingezette geprogrammeerde celdood in en rondom de voedingsstructuur van de nematode, waardoor uiteindelijk de nematode wordt afgesloten van zijn enige voedselbron. Plant-parasitaire bacteriën en schimmels hebben manieren ontwikkeld om herkenning te voorkomen of immuun signalering of reacties te onderdrukken. Vaak gebruiken deze ziekteverwekkers hierbij moleculen die effectoren worden genoemd. In dit proefschrift worden verschillende aspecten van de modulatie van het immuunsysteem van planten door plant-parasitaire nematoden onderzocht. Kennis over de mechanismen die plant-parasitaire nematoden gebruiken om het

immuunsysteem te overwinnen zal kunnen bijdragen aan het ontwikkelen van nieuwe nematode-resistente gewassen, die hard nodig zijn.

We beginnen dit proefschrift met een analyse van immuun-signaalketens die betrokken zijn bij het parasitisme van *H. schachtii* op het plantje *Arabidopsis thaliana* (zandraket). Daartoe hebben we mutanten die onderdelen van immuun-signaalketens missen geïnfecteerd met *H. schachtii*. Het aantal vrouwelijke nematoden dat het volwassen stadium bereikte werd genomen als maat voor vatbaarheid van de planten (Hoofdstuk 2). Wildtype *A. thaliana* bevat geen specifieke resistentiegenen die de plant resistent maken tegen *H. schachtii* en daarom ging deze analyse primair over basale verdediging tegen plant-parasitaire nematoden. De uitkomst van dit onderzoek is een indicatie dat ondanks het ontbreken van zichtbare immuunreacties, signalering via extracellulaire immuunreceptoren en verscheidene hormoon gestuurde signaalketens de nematode-infectie beperken in deze vatbare interactie.

Hoofdstuk 3 beschrijft de identificatie van een bijzonder grote familie van effectoren in *G. rostochiensis*. Leden van deze effectorfamilie bestaan uit een SPRY domein en een klassiek eukaryotisch signaalpeptide voor secretie en worden daarom SPRYSECs genoemd. *G. rostochiensis* scheidt deze effectoren uit vanuit de dorsale oesophagale klier tijdens het infectieve juveniele stadium bij aanvang van het parasitisme. Met speciale software die kenmerken van versnelde evolutie herkent (PAML) kon worden aangetoond dat een aantal aminozuurresiduen in de SPRYSECs onder divergerende selectiedruk stonden. Dit suggereert dat SPRYSECs betrokken zijn in de activatie of onderdrukking van het immuunsysteem van de gastheer. Om deze hypothese te testen gebruikten we een yeast-two-hybrid screening van een tomaten cDNA-library om gastheer eiwitten te kunnen identificeren die interacteren met SPRYSECs. We vonden dat SPRYSEC-19 interacteert met een eiwit dat wordt gecodeerd door een nieuw resistentiegen homoloog. Dit eiwit noemen we SW5F en het behoort tot de CC-NB-LRR type van de cytoplasmatische immuun receptoren.

Het doel van het werk dat is beschreven in hoofdstuk 4 was om de biologische relevantie van de interactie tussen SPRYSEC-19 en SW5F te ontrafelen. Onze eerste hypothese was dat deze fysieke interactie een SW5F-gemedieerde resistentie reactie teweeg brengt. De redenering was dat als SPRYSEC-19 interacteert met het LRR-domein van SW5F, waarvan in vergelijkbare resistentie eiwitten is aangetoond dat het de herkenning van pathogenen regelt, het mogelijk is dat SPRYSEC-19 een klassiek avirulentiegen is. In verschillende experimenten konden we niet aantonen dat SPRYSEC-19 immuunresponsen opwekt in bijzijn van SW5F en andere gerelateerde resistentie eiwit homologen in tomaat. Integendeel ontdekten we dat SPRYSEC-19 een effector is die immuun-gerelateerde celdood en resistentie van verscheidene CC-NB-LRR resistentie eiwitten onderdrukt.

Naast dat *G. rostochiensis* effectoren secreteert die het immuunsysteem van de gastheer beïnvloeden, scheidt de worm ook peptiden uit de dorsale oesophagale klier uit die antimicrobiële activiteit hebben en die mogelijk onderdeel uitmaken van het immuunsysteem van de worm tegen pathogene bacteriën en schimmels. In hoofdstuk 5 beschrijven we de identificatie van een klein polypeptide (Gr-AMP1 genoemd) dat een zogenaamde gamma-kern bevat die kenmerkend is voor antimicrobiële peptiden. We laten zien dat Gr-AMP1 de groei van een standaard set bacteriën en gist onderdrukt in vitro. Om te bewijzen dat Gr-AMP1 wormen kan beschermen tegen infecties hebben we de vrijlevende wormensoort *Caenorhabditis elegans* geïnfecteerd met een pathogene *Pseudomonas aeruginosa* lijn en de wormen vervolgens gered door toediening van synthetisch Gr-AMP1. Onze data suggereert dat secreties van *G. rostochiensis* een rol spelen bij de bescherming van de nematode tegen infecties met pathogene microben. Het is ook mogelijk dat *G. rostochiensis* antimicrobiële effectoren uitscheidt om gastheercellen te beschermen tegen secundaire infecties met opportunistische bacteriën en schimmels.

In het afsluitende hoofdstuk 6 bespreken we het huidige bewijs voor het bestaan van immuun-modulerende effectoren in plant-parasitaire nematoden en geven we handgrepen voor vervolgonderzoek.

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In the Laboratory of Nematology I was warmly welcomed by an amazing group of people. Together we have spent many coffee and lunch breaks, corridor meetings, and chats in the lab, which I have enjoyed greatly. And then there were the barbecues in Jaap's garden, lab outings, movie nights, vrijdagmiddagborrels, game nights, graduation parties, We-days, and conferences in The Netherlands and abroad. Like other people before me I can only conclude that Nematology is like a big family with ties that extend beyond the work domain.

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I already mentioned some of you, but I want to thank my fellow PhD students and my closest friends in the lab. Casper Q and Mark, good luck with your PhDs. You will have a few determining years in Nematology, use them well and enjoy. Lotte, Ruud, Jan, Debbie and DJ: I think you are doing a great job. Together you are a great team and I am sure your careers will prosper. Kasia, we missed you a lot after you left. I hope you are enjoying your current life in Poland and I promise to visit! Jet, great to know such an entertaining and warm person. Good luck with your defense. Miriam, we shared a lot of gossips and I think I got to know you quite well. Take care, and expect the unexpected. Kamila and Tomek, I am honored to count you among my friends. It is a pity you had to leave Arnhem but we will keep in touch. Jose, I will always remember the days we spent with Anna in Slovenia after the Cost meeting. Good luck with all your intercontinental plans. Dear Anna, it was so much fun to work with you. You are amazingly witty and cool, and now also a well-deserved mom. Casper, mate, I cannot write what I would like to write to you because other people will read this too. I will miss working with you a lot but we will be in touch. Now that I mentioned you and Anna, this would also be the moment to thank the Little People. Even though we never saw you, your presence could always be felt.

A number of people of other departments collaborated with me or helped in one or another way. To all those people, many of them of the Laboratory of Phytopathology, I extend my gratitude. Special thanks to Bart Thomma, Koste Yadete, and Emilie Fradin for their support. Bert Essenstam of Unifarm: thank you for being so well organised and for thinking along whenever I had a request. Matthieu Joosten and Douwe Zuidema, thank you for listening to me and giving

excellent advice. The same accounts for Richard Kormelink of the Department of Virology. Many thanks for the experiments we did together.

During my work in Nematology I supervised various students in different stages of their study. I thank them for their contributions to my research, their effort and their company. Kasper van Gelderen, you were my first student and you wrote an excellent master thesis. Your bimolecular fluorescence complementation work was an important contribution to the SPRYSEC paper (chapter 3 of this thesis). Good luck with your PhD. Dominika, moja najlepsza studentka, it was fun working with you and I wish you all the best in your life. Diana, I have never met such a worm enthousiast. You are a pleasant person to work with and I wish you all the best for the future. Henry, it was an honor to work with you. You worked on difficult projects and managed very well, and in the mean time you made me laugh a lot. Dear Christel, you worked super hard. The monotonous work you did was not in vain because it resulted in a publication (chapter 2 of this thesis). Madzia, Hanna, and Jori, also with you I spent pleasant moments in the lab and I hope you learned a thing or two about science. Thanks and good luck with the remainder of your studies.

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have you around. Finally I would like to thank my family and especially my father and brothers. I am happy that we are close and I am proud of who you are.

July 2012

Wiebe Postma

Curriculum vitae

Wiebe Jan Postma was born on the 10th of February, 1983 in Alkmaar, The Netherlands. After graduating high school at the c.s.g. Jan Arentsz in that same city in 2001, he moved to Wageningen to study biology at Wageningen University. During his BSc and MSc studies he specialized in plant biology and immunology and conducted three undergraduate thesis projects. The first thesis was performed at the institute of Plant Research International in Wageningen in collaboration with the Laboratory of Entomology, Wageningen University, where he investigated the role of the secondary metabolite nerolidol on spider mite-induced production of DMNT in cucumber. For the second thesis he spent half a year in the Laboratory of Virology of the Institute for Medical Microbiology and Hygiene of the Albert-Ludwigs-University in Freiburg, Germany. There, he investigated how cells detect viral dsRNA and ssRNA by studying co-localization with cytosolic factors in human cells. The final half year of his MSc was spent in Boston, in the Department of Pediatric Gastroenterology and Nutrition of Harvard Medical School and Massachusetts General Hospital. In the research group of Professor Alan Walker, he investigated how the hormone adiponectin in human milk protects newborns against infections. During this period, he also volunteered in the Boston Museum of Science where he gave public biology demonstrations. After graduating in Wageningen he briefly worked in the Laboratory of Nematology to develop the practical course Basics of Infectious Diseases that was later thought as one of the core courses in the master Human Health. Shortly after, he was employed as a PhD candidate in the same laboratory to conduct the research that culminated in this thesis. Alongside his PhD work he was active as a Private in the Royal Dutch Army Reserves, which he still is today. Currently he is employed as a clinical research associate for Ophtec BV. in Groningen, The Netherlands.

Publications

Postma WJ, Slootweg EJ, Rehman S, Finkers-Tomczak A, Tytgat TOG, et al. (2012) The effector SPRYSEC-19 of Globodera rostochiensis suppresses CC-NB-LRR-mediated disease resistance in plants. *Plant Physiol.* **160:** 944-954

Rehman S, Postma W, Tytgat T, Prins P, Qin L, et al. (2009) A secreted SPRY domain-containing protein (SPRYSEC) from the plant-parasitic nematode *Globodera rostochiensis* interacts with a CC-NB-LRR protein from a susceptible tomato. *Mol. Plant-Microbe Interact.* **22:** 330-340

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Wiebe Postma
Date: 13 June 2013
Group: Nematology, Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Elucidating signal transduction pathways in the plant immune system: Effects of SPRYSECs on PTI.	Jun 12, 2008
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Wageningen University	May 20, 2011
► **EPS theme symposia**	
EPS theme symposium 2 'Interactions between Plants and Biotic Agents, Wageningen University	Feb 10, 2012
EPS theme symposium 3 'Metabolism and Adaptation, University of Amsterdam	Mar 22, 2013
► **NWO Lunteren days and other National Platforms**	
CBSG Summit (Wageningen)	Mar 15-16, 2010
ALW Symposium Experimental Plant Sciences (Lunteren)	Apr 19-20, 2010
CBSG Summit (Wageningen)	Jan 31- Feb 01, 2011
CBSG Summit (Wageningen)	Feb 29- Mar01, 2012
► **Seminars (series), workshops and symposia**	
Seminar: Richard Oliver: Genomics of Stagonospora nodorum (Wageningen)	Oct 16, 2008
Seminar: James Bradeen: Potato Pathology and Genomics (Wageningen)	Oct 16, 2008
The Schilperoort Lectures: Success stories of entrepreneurial scientists (Wageningen)	Nov 05, 2008
NBV Symposium: Green meets White (Wageningen)	Jan 09, 2009
Seminar: Hiro Nonogaki: Seeds, MicroRNA and Darwin (Wageningen)	Sep 17, 2009
Current Themes in Ecology 15: Revolution in Evolution? Epigenetics in Ecology and Evolution (Amsterdam)	Sep 18, 2009
Plant Sciences Group Seminar (Wageningen)	Oct 13, 2009
Seminar: Valerie Williamson: Connecting genetics and genomics of pathogenicity and behavior in rkn (Wageningen)	Oct 23, 2009
Seminars Frontiers in Plant-Microbe Interactions: Christiane Gebhardt & Peter Moffett (Wageningen)	Feb 05, 2010
TTI Green Genetics Network Meeting (Nieuwegein)	Sep 22, 2010
EPS Expectations Career Event (Wageningen)	Nov 19, 2010
CBSG Workshop Open Source Biotech (Wageningen)	Oct 04, 2011
► **Seminar plus**	
► **International symposia and congresses**	
Keystone Symposia on Plant Innate Immunity (Keystone, CO, USA)	Feb 10-15, 2008
COST 872 Workshop (Postojna, Slovenia)	May 26-29, 2008
ISCP / COST 872 Meeting (Ghent, Belgium)	May 24-25, 2011
IMPNIG/SPIT Meeting (Ghent, Belgium)	May 23-24, 2012
► **Presentations**	
Bioexploit summer school 2008 (Wageningen) poster	Jun 19, 2008
CBSG Cluster Meeting (Wageningen) oral presentation	Sep 18, 2009
CBSG Summit (Wageningen) poster	Mar 16, 2010
TTI Green Genetics Network Meeting (Nieuwegein) poster	Sep 22, 2010
TTI Green Genetics Network Meeting (Nieuwegein) oral presentation	Sep 22, 2010
TTI Green Genetics Cluster Meeting (Enkhuizen) oral presentation	Nov 18, 2010
CBSG Summit (Wageningen) poster	Jan 31, 2011
TTI Green Genetics Cluster Meeting (Wageningen) oral presentation	Apr 07, 2011
CBSG Summit (Wageningen) oral presentation	Oct 20, 2011
CBSG Summit (Wageningen) oral presentation	Feb 29, 2012
IMPNIG/SPIT Meeting (Ghent, Belgium) oral presentation	May 23, 2012
► **IAB interview**	Dec 04, 2009
► **Excursions**	
Subtotal Scientific Exposure	*21.5 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Evolution of plant-pathogen interactions, Bioexploit summer school 2008, Wageningen	Jun 18-20, 2008
PhD Course 'Gateway to Gateway Technology', Wageningen	Nov 17-21, 2008
► **Journal club**	
Participant in literature discussion group at laboratory of Nematology	2008-2012
► **Individual research training**	
Subtotal In-Depth Studies	*5.1 credits**

4) Personal development	date
► **Skill training courses**	
Presentation skills PhD (CENTA)	Jan 13, 2009
PhD competence assessment (WGS)	Sep 18, 2008
Teaching and supervising thesis students (DO)	Jan 19, 2009
Management voor Promovendi en Postdocs (NIBI)	Jan 08,14,15, 2010
Career Orientation (WGS)	Feb-Mar, 2010
Career Perspectives (WGS)	Mar-Apr, 2010
Entrepreneurial Bootcamp (DAFNE)	Nov 13-18, 2011
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*9.3 credits**
TOTAL NUMBER OF CREDIT POINTS*	**37,4**

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

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

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