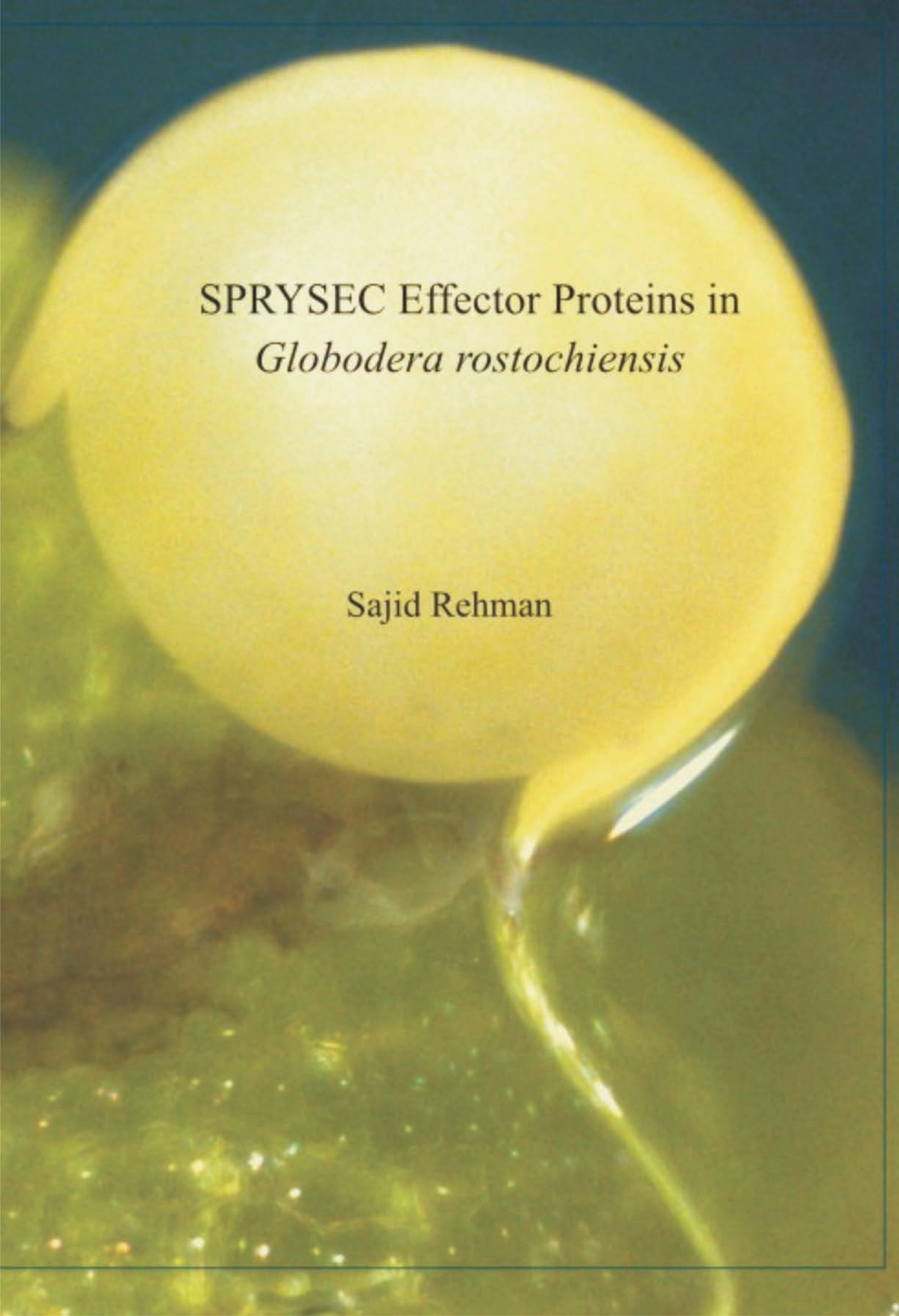
SPRYSEC Effector Proteins in
Globodera rostochiensis
Sajid Rehman

SPRYSEC effector proteins in *Globodera rostochiensis*

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Abstract

Plant pathogens inject so-called effector molecules into the cells of a host plant to promote their growth and reproduction in these hosts. In plant parasitic nematodes, these effector molecules are produced in the salivary glands. The objective of this thesis was to identify and characterize effector molecules produced in the salivary glands of the potato cyst nematode *Globodera rostochiensis*. A combination of cDNA-AFLP and mining of EST-databases resulted in the identification of a large family of effectors named the SPRYSECs. The SPRYSECs essentially consist of a conserved SPRY domain preceded by a signal peptide for secretion. The SPRYSECs are injected into host cells through the oral stylet. A protein structure model of the SPRYSECs indicated that one particular surface of the proteins in the SPRYSEC family was hypervariable and seemed to undergo diversifying selection. This led us to believe that the SPRYSECs are important players in the co-evolution between plant and nematode. Transgenic potato plants overexpressing SPRYSEC-19 appeared to be two- to five-fold more susceptible to infections of nematodes, the fungus *Verticillium dahliae*, and tomato spotted wilt virus (TSWV). This hypersusceptibility to a range of unrelated plant-pathogens suggests that SPRYSECs somehow suppress the basal defense responses that are controlled by the plant's innate immunity. SPRYSEC-19 was found to engage in a specific physical interaction with the Leucine Rich Repeat domain of a protein from the CC-NBS-LRR class of resistance genes. Many immune receptors in the plant's innate immunity belong to the same class of NB-LRR proteins. The host interactor of SPRYSEC19 is most similar to members of SW5 R gene cluster that confers resistance to tospoviruses. Remarkably, plants harboring the CC-NB-LRR interactor of SPRYSEC19 are not resistant to nematodes. Therefore, we hypothesize that the nematode effector SPRYSEC-19 promotes its virulence in susceptible host plants by suppressing basal defense through its interaction with an NB-LRR immune receptor.

Contents

Chapter 1

General introduction

Introduction

In nature plants have to defend themselves against the continuous threat of a wide range of parasites. The infection strategies deployed by plant parasites can be roughly divided into necrotrophic and biotrophic lifestyles. Necrothrophs are ruthless attackers killing the plant on which they thrive. In contrast, biotrophic parasites exploit their hosts in a prudent way, by establishing a long term feeding relationship with their hosts without killing them. Most biotrophs are obligate parasites (e.g. cyst and root-knot nematodes) that need this feeding relationship for their survival and reproduction. This implies that a parasite will aim to avoid the recognition by the host defense systems, while at the same time it will modify plant cells in such a way so that it can feed on plant assimilates.

All plant parasites deliver effector molecules into host plant cells to promote their virulence and to enhance their fitness. These effectors are indispensable for various phases of parasitism, including host penetration, feeding, and reproduction. For example, many plant-parasites use cell wall degrading enzymes to penetrate host plants and to achieve further colonization of plant tissues (Agrios, 2004). Plant-parasitic nematodes transform host cells into feeding sites, and the most plausible explanation to this transformation is likely to be found within nematode effector molecules.

Recent work on the role of microbial effector proteins in plants points at their involvement in the suppression of plant immunity (review Jones and Dangl, 2006). To counteract pathogen ingress plants have evolved a two-layered surveillance system that uses receptor-like proteins, which detect either directly or indirectly specific effector molecules from parasites. The first line of defense in plants is established by extracellular immune receptors that recognize molecular patterns associated with pathogens. A classical example is the recognition of twenty two amino acids in the flagella of bacteria. Recognition by this basal defense system leads to generic defense responses such as cell wall modifications, release of reactive oxygen species, etcetera. Parasites have found ways to breach the basal immunity by suppressing disease signaling with other effectors molecules. These suppressive parasite effectors, however, may induce changes in molecular states of host proteins that are monitored by other immune receptors, so-called R proteins. The probable outcome of pathogen recognition in this second layer of defense is the activation of disease signaling pathways that lead to specific resistances. In many cases effector recognition results in local cell death or a hypersensitive response (HR). HR involves local accumulation of phenolic compounds and cell-wall reinforcements in cells surrounding the area of cell death, thus inhibiting further pathogen infection and colonization. Effector proteins that are being recognized by the products of resistance (R) genes have acquired so-called avirulence (Avr) activity. This gene-for-gene model, which

essentially explains the recognition specificity of disease resistance responses in plants, holds true for most biotrophic plant-pathogen interactions (Jones and Dangl, 2006).

Thus, pathogen effector molecules have three possible roles, including 1) promoting virulence by redirecting metabolic processes to the parasite's benefit, 2) betraying the parasite's presence to the plant immune systems, and 3) suppressing disease signaling in the plant's immune system. This thesis describes the identification and functional characterization of a novel group of microbial effector proteins, encoding by the SPRYSEC-gene family, in the plant-parasitic nematode *Globodera rostochiensis*. The possible roles of these effectors in promoting virulence, suppressing plant defense, and conditioning parasite recognition in plant defense systems are discussed.

Parasitic Nematodes

Nematodes are the most abundant multicellular animals on earth. They can be found from the bottom of the sea to the highest mountains, ranging from Polar Regions to the Tropics (Cobb, 1914; Ditlevesen, 1918). Most of the nematodes are simple, colorless and transparent roundworms with relatively little morphological variation. A vast majority of the nematodes is free living, feeding on fungi, bacteria, organic matter, and other nematodes (predators). Only a small percentage of the phylum Nematoda are parasites of animals and plants. Over 1 billion people are infected by animal parasitic nematodes leading to severe morbidity, blindness, anemia, intestinal infection and respiratory diseases (Hirst and Stapley, 2000).

Plant-parasitic nematodes are classified according to their feeding and reproduction behaviour. The ectoparasites (e.g., *Trichodorus* and *Xiphinema* spp.) mainly feed on epidermal cells, root hairs or on the outer cortical cells beneath the epidermal cell layer using their stylets. The migratory endo-parasites (e.g. *Aphelenchoides* and *Bursaphelenchus* spp.) penetrate plant tissue through several cell layers and feed on cytoplasm of the cells that they come across. Finally, the sedentary endo-parasites (e.g. *Meloidogyne* and *Globodera* spp.) have developed an intimate and long-term feeding relationship with their hosts. They induce a multi-nucleate feeding site close to the vascular bundle in the host and remain sessile for the rest of their life, while extracting nutrients from the feeding site (Wyss, 1997; Dropkin, 1969; Hussey and Grundler, 1998).

The life history of potato cyst nematodes

The potato cyst nematode (PCN), *Globodera rostochiensis*, is an obligate sedentary endoparasite of a small range of Solanaceous plants such as potato, tomato and eggplant. Potato cyst nematodes originate from the Andean region in South America (Evans and

Stone, 1977) and was probably introduced into Western Europe in the second half of the 19th century. Their successful spread over the continent was conditioned by the dissemination of breeding material. Despite their slow propagation rate they have become a major pest and yield limiting factor in potato.

The second stage juveniles (J2) of potato cyst nematodes hatch from the eggs in response to host-plant root exudates. The freshly hatched J2 invade the root just behind the apex, preferably in the differentiation and elongation zone. Plant penetration is achieved by perforating cell walls with the combined effect of physical thrusting of the oral stylet and the enzymatic softening of the cell walls. The infective J2s migrate intracellularly through the cortex in the direction of the vascular cylinder where they select an inner cortical cell as initial syncytial cell (ISC). After careful perforation of the cell wall of the ISC, the stylet of the nematode remains inserted for 6-8 hours (Steinbach, 1973; Wyss and Zunke, 1986; Wyss, 1992).

In this so-called preparation period secretions released from the nematode induce the ISC to redifferentiate into a highly metabolically active cell, which is characterized by small secondary vacuoles, dense cytoplasm, numerous organelles and enlarged amoeboid nucleus (Cole and Howard, 1958; Rice et al., 1985). The developing syncytium extends longitudinally along the vascular cylinder by progressive protoplast fusion with neighboring cells through local cell wall dissolution. Cell wall ingrowths are formed adjacent to xylem elements, facilitating nutrient uptake into the developing syncytium (Jones and Northcote, 1972; Grundler et al., 1998). This multinucleate ‘organ’ acts as nutrient sink, which is continuously replenished by the plant during feeding of the nematode. The syncytium remains the sole source of nourishment for the whole life cycle of this biotroph.

Syncytium formation is accompanied by repeated cycles in the feeding behaviour of the nematode, each cycle consists of three distinct phases:

Phase 1. Nutrient uptake from the syncytium;

Phase 2. Stylet retraction and reinsertion into the syncytium;

Phase 3. Injection of secretions into the syncytium (Steinbach, 1973; Hussey and Mims, 1991).

After successive feeding cycles, the J2s molt into J3 and J4 respectively, finally reaching adulthood within four weeks (Von Mende et al., 1998). The sex ratio in potato cyst nematodes is determined epigenetically during early nematode development (Grundler et al., 1991). A shortage of food leads to formation of more males whereas excess of

nutritional sources will lead to development of more females (Mugniery and Fayet, 1984). Potato cyst nematodes show a high degree of sexual dimorphism resulting in swollen adult females which remain sessile throughout their parasitic life. In contrast, adult males regain motility and become attracted by the females to achieve insemination and fertilization of the eggs. Shortly following the insemination the gravid female dies leaving her remains as a protective container for hundreds of eggs. The first stage juveniles (J1) molt inside the egg and remain dormant for at least one year. These eggs inside the so-called cysts remain viable for many years in the soil (Perry, 1989) (Fig. 1).

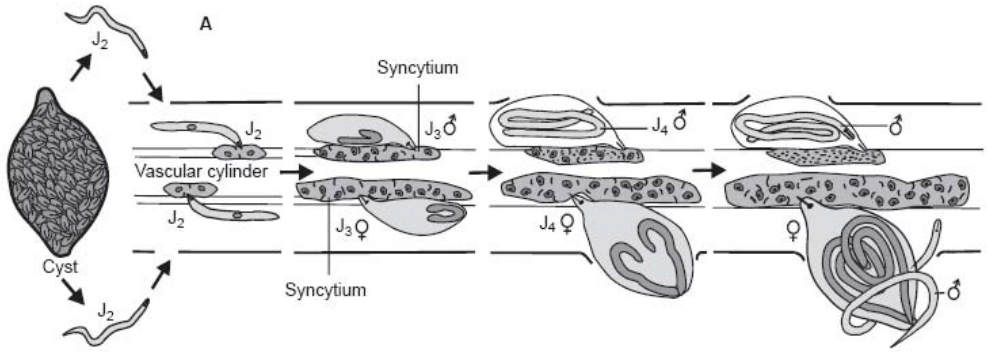

Figure 1. The life cycle of cyst nematodes. J2, J3, J4-juveniles in second, third and fourth developmental stages (Courtesy of Jung et al., 1999)

Nematode Secretions

Nematode secretions are believed to play a key role in the parasitism of plants. These secretions presumably include effector molecules involved in hatching, in self-defense, in movement through plant tissue, and in establishment and maintenance of the feeding site. Nematode secretions are produced in several different organs, including the cuticle, amphids, the excretory/secretory system, the rectal glands and esophageal gland cells (Jones and Robertson, 1997). The oral stylet and the relatively large esophageal glands are thought to be specialized evolutionary adaptations to plant parasitism in nematodes (Hussey, 1989, Hussey and Mims, 1990). As a consequence the esophageal glands are a likely source of most of the nematode effector molecules. Therefore, much of the work done so far on the role of nematode secretions in plant parasitism has been focused on the products of these esophageal glands.

Esophageal Gland Secretions

The stylet is a hollow protrusible structure with a lumen that is connected to the three esophageal gland cells through the esophagus. Potato cyst nematodes have one dorsal and two subventral esophageal glands (Fig. 2). Each gland is a single cell with long cytoplasmic extension that terminates into an ampulla, which serves as a reservoir for secretory granules. The ampulla empties into lumen of esophagus via a valve. The secretions produced in the dorsal esophageal gland are released in the esophagal lumen at the base of stylet, whereas the two subventral glands empty their granules immediately posterior to pump chamber (Hussey et al., 1989).

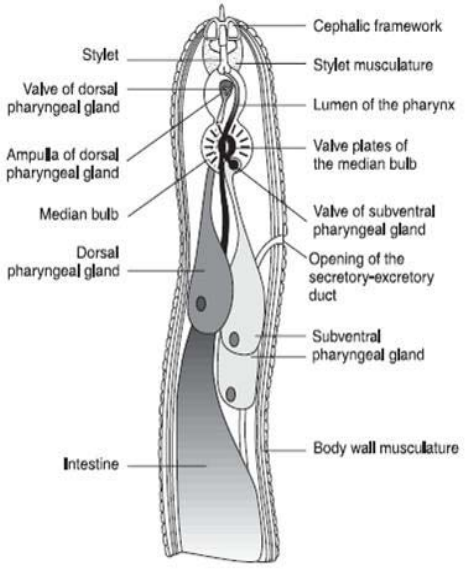

Figure 2. Schematic representation of the anterior region of pre-parasitic J2's of cyst nematodes. The position of the esophageal glands is indicated (Courtesy of Lilley et al., 2005).

The distinct morphological changes of the esophageal glands at specific stages of parasitism hint towards their differential roles. During migration through the plant root, the two subventral glands are large and packed with secretory granules, while shortly after migration ceases they undergo a strong decrease in cell volume. A larger portion of the genes switched on in the subventral esophageal glands during migration code for cell wall modifying proteins, e.g. ß-1,4-endoglucanases (Smant et al., 1998), pectate lyases (Popeijus et al., 2000) and expansins (Qin et al., 2004). In contrast, the dorsal gland shows a remarkable increase in activity during the initiation of the syncytium (Wyss and Zunke, 1986). The nature and the function of dorsal esophageal gland secretions from potato cyst nematodes are not well understood at present.

Identification of genes encoding esophageal gland secretions

Despite considerable efforts, the identity of genes encoding nematode secretions has long been severely hampered because of their microscopic size, long generation time and obligate biotrophic nature. The first attempts to identify the components in nematode secretions used biochemical analysis of fluids in which nematode had been incubated for a certain period. These early analyses indicated the presence of cellulase, pectinase and protease activities in the secretions from *Meloidogyne incognita.* At that time the scientists were not able to establish the origin of these activities (Dropkin, 1963; Myers, 1965).

The direct analysis of the components of nematode secretions is difficult due to the limited amount of material available for analysis. This obstacle was tackled by production of monoclonal antibodies (MAbs) directed against nematode secretions or fractionated homogenate of nematodes. Using MAbs raised against fractionated homogenate of pre-parasitic J2's of *Globodera rostochiensis* Smant et al. (1998) identified several nematode β-1,4-endoglucanases. The success rate of the MAb-based cloning approach was rather limited because of many technical disadvantages associated with it.

A significant technical advance was made by the use of chemical compounds such as the neurotransmitter analogue DMT (5-methoxy-N, N-dimethyl tryptamine) to increase pharyngeal pumping and enhanced release of esophageal gland secretions in cyst nematodes. With these compounds, Goverse et al. (1999) identified a protein fraction in secretions smaller than 3kDa showing mitogenic activity on plant protoplasts and T-cell lymphocytes. Similarly Robertson et al. (1999) demonstrated in-gel activity of proteases and superoxide-dis-mutase in DMT-induced secretions from *G. rostochiensis*. However, the identity of the genes coding for these activities remains elusive to date.

A major leap forward in the identification of parasitism genes was achieved by the work on Expressed Sequence Tags (ESTs). The ESTs are single pass sequences of cDNA clones selected randomly from a cDNA library. cDNA libraries from different developmental stages and different tissues of the nematodes have been used to produce ESTs. These EST are aligned with annotated accessions in reference databases, thus pointing at the possible identity of the corresponding gene (Adams et al., 1991). EST projects so far have resulted in torrents of sequence data from plant-parasitic nematodes, i.e. the number of EST sequences has reached ~150,000 (see for an update www.nematode.net).

A set of criteria based on predicted properties of parasitism genes have been used to identify putative nematode effectors. First, selecting proteins with an N-terminal signal peptide for secretion weeds out approximately 90% of the sequences (Nielsen et al., 1997). The esophageal glands are believed to be important for parasitism, therefore the localization of the transcript within these glands (*in situ* hybridization) is a second important criterion.

To this purpose the digoxygenin labeled anti-sense cDNA strand derived from a putative parasitism gene can be hybridized with mRNA in target tissue. Following an enzymatic reaction, the hybridization signal can be located, thus allowing determination of spatial expression patterns (Vanholme et al., 2002). As a third criterion, the expression of the gene at specific stages of parasitism is being used to further reduce the dataset of potential candidates. Many groups have identified novel parasitism genes such as a pectate lyase (Popeijus et al., 2000), a β-1,4-endoglucanase, xylanase (Dautova et al., 2001) and a polygalacturonase (Jaubert et al., 2002) and an ubiquitin extension protein (Tytgat et al., 2004) by using this approach.

Owing to the technical difficulties associated with collecting sufficient material from parasitic stages, most of the cDNA libraries have been constructed from pre-parasitic stages. Consequently the current database of ESTs are likely biased towards genes involved in the very early stages of parasitism (Lilley et al., 2005). In order to clone the genes involved in later stages of parasitism, Gao et al., (2003) constructed a pharyngeal gland region specific library by micro-aspirating the contents of the gland cells from parasitic stages of the soybean cyst nematode *Heterodera glycines*. A combination of random sequencing of this gland cell specific library with data mining and *in situ* hybridization resulted in the identification of 51 novel *H. glycines* esophageal gland-expressed putative parasitism genes.

An even more stringent selection was achieved by combining gland specific micro-aspirated mRNA with subtractive suppressive hybridization (SSH) of mRNA from the nematode's intestinal region. In SSH, the mRNA isolated from intestinal region of nematodes is used as template to produce first strand driver cDNA. The driver cDNA is immobilized on matrix followed by hybridization with another pool of mRNAs isolated from esophageal glands of various parasitic stages by micro-aspiration. The cDNAs corresponding to mRNA expressed in both tissues will form a DNA:RNA hybrids, which are removed using a column. Therefore, a unique pool of gland specific mRNAs will be produced. The remaining non-hybridized single stranded mRNA is then used for construction of subtracted cDNA library by RT-PCR. Similarly, Lambert et al., (1999) constructed a subtracted cDNA library after differential hybridization of mRNA expressed in posterior and anterior regions of *Meloidogyne javanica* and cloned a esophageal gland specific chorismate mutase (Mj-cm-1) using this method. Homologues of Mj-cm-1 were found in cyst nematode *H. glycines* (Bekal et al., 2003) and *Globodera pallida* (Jones et al., 2003).

Most nematode parasistism genes are not expressed constitutively throughout the nematode life, but in a highly coordinated way at specific events in the nematode-plant interactions. Techniques enabling a global analysis of gene expression between different

developmental stages allow for the identification of novel parasitism genes up-regulated specifically at the onset of parasitism. We have used cDNA-AFLP (complementary DNA-amplified fragment length polymorphism) that allows for a comprehensive analysis of differentially expressed mRNAs isolated from various stages of parasitism (Bachem et al., 1996; see chapter 4). In this technique the cDNA originating from various stages is digested with two restriction enzymes (frequent and rare cutter, respectively) followed by the ligation of oligonculeotide adaptors to generate cDNA for PCR based amplification. PCR primers complementary to the adaptors with additional 1 or 2 nucleotides at 3` end allows selective amplification followed by their visual analysis on gel. We have further used the computer program GenEST to identify for each of the fragments displayed on gel the matching EST in our database. The advantages of cDNA-AFLP are that 1) it requires only minute amounts of RNA because of amplification step involved, 2) due to high annealing temperatures, cDNA-AFLP is stringent and reproducible, 3) it can distinguish between highly homologous genes from individual gene families, 4) it does not need any prior sequence information, and 5) it allows direct cloning of target gene. However, dis-advantages of this technique are that 1) it requires appropriate restriction sites in cDNA, and 2) it will not identify constitutively expressed parasitism genes.

A comprehensive cDNA-AFLP analysis on the expression pattern of various stages of *G. rostchiensis* was done by Qin et al (2000). In total 16,500 transcript-derived fragments were analyzed of which 216 were cloned, sequenced, and used for further analysis. In chapter 4 we used 7 of these fragments, later named SPRYSECs (SPRY: similar to domain found in SPIA and RYanodine receptors, SEC: secreted), to study the role of the corresponding genes in nematode-plant interactions.

Functional characterization of nematode effectors

The list of genes coding for putative parasitism genes from plant-parasitic nematodes has been growing exponentially over the last years. A vast majority of these putative parasitism genes has no match with functionally annotated protein sequences in the non-redundant databases. Earlier it was thought that a fully sequenced genome of *Caenorhabditis elegans*, a free-living bacteriovorous, would aid significantly in the functional characterization of putative parasitism genes. However, many genes identified in plant-parasites do not have a functional counterpart in *C. elegans*, thus making its genome sequence a resource with limited value for our understanding of nematode parasitism (Gao et al., 2003). Therefore, other more sophisticated methods are being deployed to study the features of the novel parasitism genes that may point at a specific role of the encoded protein in nematode-plant interactions. This section gives an overview of the current methodologies used to study pioneering nematode genes including bioinformatics, protein structure modeling, inference

of phylogenies, *in situ* hybridization microscopy, knock-down genes, and protein-protein interaction studies. Each of these methods have been used in the following sections of this thesis.

In silico analysis of candidate effector proteins

Putative parasitism genes are often first identified as gene fragments in ESTs or transcript derived fragments (TDFs) in cDNA-AFLP that require further efforts such as contig building, sequence cluster analysis, and specific amplification of the cDNA ends to end up with the full gene sequence. Once the full-length sequence is resolved the first important feature to look for in the encoded protein is the presence of N-terminal signal peptide for secretion (Nielsen et al., 1997). In eukaryotes, most of the secreted and membrane proteins are exported through the secretory pathway (type II secretion system) via short N-terminal signal peptides. Typically, signal peptides are about 24-amino acid long, including N-terminally positioned charged residues, followed by a hydrophobic core, and a more polar carboxy-terminal region (Von Heijn, 1985; Rapoport, 1992). Several computer algorithms build on the SignalP script, such as in PexFinder and SPIT, have been used to distinguish between genes coding for cytoplasmic and secreted proteins of plant pathogens (Torto et al., 2006; Vanholme et al., 2006). The N-terminal signal peptide conditions the translocation of a protein into the endoplasmatic reticulum (ER). Further separation between proteins that remain in the ER, the Golgi, and/or the cell membrane and proteins that are secreted depends on the presence of transmembrane regions and/or specific retention signal sequences. The next logical step in selecting candidate parasitism genes is therefore to check if the protein includes likely transmembrane regions or retention signals in its sequence. Proteins with an N-terminal signal peptide for secretion but lacking transmembrane regions and other specific retention signal collectively constitute the secretome of the nematode (see Chapter 4).

Resolving the protein structure may be key to understand its biological function, and its role in parasitism and/or disease development. Comparative or homology modeling predicts the three dimensional structure of the target protein sequence based primarily on its alignment to one or more proteins of known structure (template). For example, if members of a protein family share >50% pair-wise amino acid similarity and the structure of one member is determined, it can be used for homology modeling of other family members (Marti-Renom et al., 2000; see Chapter 4). Comparative models can be helpful in designing mutants to test the function of proteins (Boissel et al., 1993), to identify active binding sites (Ring et al., 1993), predicting antigenic epitopes (Sali et al., 1993; see Chapter 5), simulating protein-protein docking (Vakser, 1997), and confirming a remote structural relationship (Miwa et al., 1999). In chapter 4, a three-dimensional structure model of

SPRYSEC-19 was determined by using remote homology modeling, which was later used to construct a consensus structure model for best matching family members. Based on modeling study, antigenic peptides were designed on variable loop regions and antibodies raised were used for immuno-detection of SRPYSEC family members (Chapters 4 and 5).

Localization of candidate effectors in nematodes

The esophageal glands in the plant-parasitic nematode are believed to be an important source for nematode effectors involved in nematode-plant interactions. A second important step in the identification of putative effectors is to assay for a specific expression of the candidate effector gene in the esophageal glands by using *in situ* hybridization microscopy on whole mount nematodes. Further evidence in support of a role as effector in nematode-plant interactions may be found by using specific antisera for immunolocalization of the corresponding protein in stylet secretions and more importantly in plants infected with nematodes; however, raising specific antisera is not a trivial exercise. The heterologous expression of nematode proteins in bacteria and yeast, which is required for antiserum production, has often proven to be difficult. Nematode proteins have to be genetically fused to hydrophilic carrier proteins, such as maltose binding protein *malE* or glutathione-S-transferase (GST; see Chapter 5), which reduces the specificity of the antisera. Synthetic peptides designed on the products of candidate parasitism genes have also been used to raise specific antisera to circumvent the difficulties with expressing nematode proteins in bacteria and yeast. The success rate of this approach is low, which makes it not suitable to be implemented in a high-throughput decision scheme. Consequently, in spite of the superiority of the evidence it may provide, *in planta* immunolocalization of candidate nematode effectors has been done for only two nematode genes to date (Wang et al., 1999; Jaubert et al., 2005).

Functional analysis of candidate effectors by RNA interference

Without further knowledge of the role of a gene in parasitism a knock-out or knock-down may lead to valuable information on its importance in parasitism. For the majority of the genomic loci of *C. elegans* knock-outs (and knock-downs; see below) have been developed to study the associated phenotype. Complete signal transduction pathways have been resolved by systematically making knock-outs and knock-downs in this nematode species. Unlike *C. elegans*, plant-parasitic nematodes have a long generation time, often a sexual mode of reproduction, and an obligate parasitic lifestyle, which have been insurmountable obstacles to achieve knock-outs by genetic transformation. In 1998, Fire and coworkers discovered a phenomenon in *C. elegans* which is now known as gene-silencing by RNA interference (RNAi). RNAi is the ability of double stranded RNA (dsRNA) to direct

sequence specific degradation of homologous RNA. The mechanism of RNAi is thought to be conserved in all eukaryotes. Since its discovery, RNAi has been exploited as a functional genomics tool in insects (Kennerdell and Carthew, 1998), amphibians (Oelgeschlager et al., 2000), mammals and plants (Silva et al., 2004).

When dsRNA is introduced into a cell it is recognized by a protein named Dicer, an RNase III family nuclease (Fig. 3). Dicer cleaves in an ATP dependent manner the dsRNA into 21-23 bp duplexes of small interfering RNAs (siRNAs) with a 2-nucleotide overhang at 3` end. These siRNAs are also called primary small interfering RNAs. siRNAs further associate with an RNA induced silencing complex (RISC) which is activated upon unwinding of the siRNA. The activated RISC, while carrying a single stranded anti-sense strand of the siRNA duplex, scans the whole mRNA population of the cell to find homologous mRNA transcripts which results in the cleavage of target mRNA ~12 nucleotides from 3` end of the hybridized siRNA (Kuznetsov, 2003; Tijsterman et al., 2002).

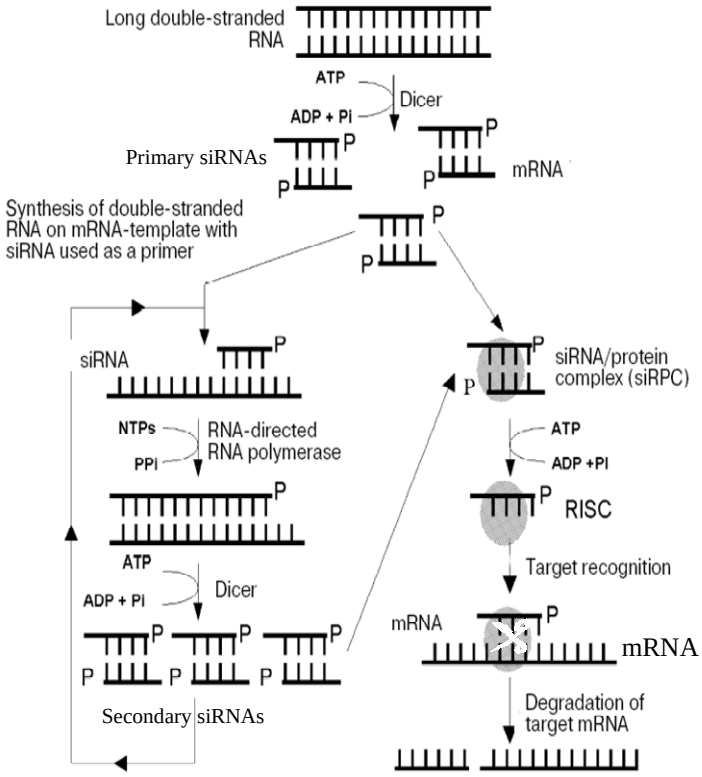

Figure 3. The mechanism of RNAi. Courtesy of V. V. Kuznetsov (2000)

The effect of silencing by RNAi is amplified when the primary siRNAs act as primers for synthesis of longer dsRNA using target mRNA as template. This amplification is mediated by RNA-directed RNA polymerase (RdRP). The long dsRNA is again the substrate of Dicer, resulting in the production of secondary siRNAs, which can lead to target mRNA degradation as well (Forrest et al., 2004).

RNAi functions autonomously in mammalian cells but can be spread systemically to other cells and tissues in nematodes and plants. In *C. elegans* RNAi occurs when bacteria expressing dsRNA are fed to nematodes, by soaking the nematodes in a dsRNA solution, and by microinjection of dsRNA into the nematodes (Kamath et al., 2003). It has been speculated that an alkaline environment in the intestine of *C. elegans* and in other nematodes may avoid degradation of dsRNA. RNAi by feeding nematodes dsRNA may be used effectively to study the importance of parasitism genes in plant-nematode interactions (Bakhetia et al., 2005).

The potato cyst nematode is an obligate biotroph that only feeds from specialized feeding cells in a host plant. This aspect of the biology of the potato cyst nematode raised the question on how to deliver dsRNA into the infective nematode at the onset of parasitism when it has not yet commenced feeding from host cell cytoplasm. A breakthrough in this field came when Urwin et al (2002) published a method to chemically induce feeding behavior in pre-parasitic juveniles. We have invested considerable effort in this method to optimize it for *G. rostochiensis* and used it to study the effect of a nematode cellulase as a model system. The cellulases were chosen because we anticipated an easily observable phenotype associated with successful knock-downs. That is, lack of host penetration and intracellular migration (see Chapters 2 and 3).

Heterologous expression of parasitism genes in plants

Eliminating one specific nematode gene from the molecular interplay of host and parasite as described above is likely to provide insight into the importance, if not the role, of that particular gene. Conversely, constitutive over-expression of a nematode gene in a host plant followed by nematode infections may also shed light on the role of that particular gene in the interaction (see Chapter 4). Nematode effectors induce major morphological and physiological changes in a host such that the host sustains nematode feeding for a long time. The phenotypic changes induced by the over-expression of nematode parasitism genes may result in a direct effect on plant growth and development that can be related to the nematode-induced changes in a host plant. To date, the best characterized example of a profound effect of a nematode gene on plant morphology is the over expression of a nematode chorismate mutase in soybean, which resulted in abnormal morphology of lateral

roots. This observed phenotype was attributed to lower concentrations of auxin which inhibited vascularization of the giant cells (Doyle and Lambert, 2003).

Cellular targets of nematode effectors in host cells

The oral stylet of plant-parasitic nematode delivers effector proteins in and around host cells. The cellular targets of these stylet secretions may reside as extracellular receptors in the plasma membrane of host cells, or alternatively intracellularly in the host cell cytoplasm and nucleus. The heterologous expression of nematode effectors fused to the green fluorescent protein in plant cells may provide information on the sub-cellular compartment that is targeted by these nematode effectors. Using this approach, Tytgat et al (2004) found that an ubiquitin extension protein (Hs-UBI1) in the stylet secretions of *Heterodera schachtii* targets the nucleus of host cells. Gao et al (2003) found that 15 out of 51 candidate effector genes of *H. glycines* include nuclear localization signal suggesting that the host cell nucleus is an major target for nematode effectors. In chapter 4, we have used genetically fused GFP constructs to assess the subcellular targeting of the SPRYSECs in plant cells.

Molecular targets of nematode effectors in host cells

Nematode effectors are likely to interact with host plant molecules in and outside the host cells. The yeast two hybrid method (YTH) has been widely used in plant cells to identify interacting proteins. YTH is based on reconstitution of a functional transcription factor GAL4 consisting of two functional domains, i.e. DNA-binding domain (BD) and activation domain (AD). In our projects, a prey cDNA library that is made from host tissues is fused to the AD-domain of GAL4, whereas the pathogen protein is fused as bait with the BD-domain of GAL4. Upon co-transformation of an auxotrophic yeast strain with the prey and the bait plasmids, a physical interaction of bait and prey may bring the BD- and AD-domain together again which will drive the transcription of nutritional markers downstream of the GAL4 promoter region (Chien et al., 1991; Fields and Sternglanz, 1994). Furthermore, the interaction is often validated and mapped by inducing mutations in bait or prey proteins and reversal of the bait and prey constructs. In chapter 5, we have used YTH to identify interacting host proteins of one of the SPRYSEC family members.

The bait-prey interaction in YTH reconstitutes the GAL4 transcription factor in the nucleoplasm of yeast cells. While some of the parasitism gene products may target the nucleus of host cells, others may interact with host proteins in other subcellular compartments of host cells. Physical interactions that are found in the nucleoplasm of yeast in YTH may not occur in the cytoplasm of host cells. Therefore, in order to assess the

biological relevance of physical interactions found in YTH they need to be confirmed independently by other methods such as co-immuno-precipitation and pull down assays *in vitro* or preferably in plant cells. To achieve a pull down, either bait or prey protein is expressed *in vitro* as GST-fusion protein and immobilized on sepharose beads by affinity chromatography (see Chapter 5). The putative interactor (*in vitro* translated bait or prey) is incubated with the sepharose beads containing immobilized bait or prey followed by their elution together, hence confirming their interaction (Smith and Johnson, 1988; Sambrook and Russell, 2001).

Outline of the thesis

A comprehensive understanding of molecular nematode-plant interactions relies on the identification and functional characterization of effector proteins operating at the interface of nematode and host plant. The overall objective of the thesis was to study the role of one particular family of effector proteins, the SPRYSECs, from the potato cyst nematode *G. rostochiensis*.

To this purpose, we first had to develop a method to test the importance and to assess the role of candidate effectors in nematode-plant interactions. As the potato cyst nematode is not prone to any sort of genetic manipulation, we pursued on a method to knock-down genes in nematode by RNA interference (RNAi). Chapter 2 of this thesis describes a protocol to achieve RNAi in potato cyst nematodes, which enabled us to silence two cell wall degrading enzymes, an amphid secreted protein, and one SPRYSEC gene. Chapter 3 describes two novel and most abundant cellulases in potato cyst nematode secretions, which we used to show that our RNAi knock-down method can provide us insight in the importance of nematode genes in host penetration and intracellular migration.

In the second part of this thesis, we used cDNA-AFLP, ESTs from different cDNA libraries, and whole mount *in situ* hybridization microscopy to identify a novel family of nematode effectors named the SPRYSECs. Overexpression of at least one member of the SPRYSEC gene family in plants suggests that these effectors may be involved in the suppression of basal immunity (Chapter 4). Chapter 5 describes the finding of a physical interaction of SPRYSEC-19 with the LLR domain of a CC-NB-LRR-type immune receptor in tomato roots. This CC-NB-LRR protein is a likely member of the SW5 R gene cluster of which other members condition disease resistance to tospoviruses. We, however, have found no evidence that the interaction between SPRYSEC-19 and the CC-NB-LRR is involved in nematode resistance.

Finally, in chapter 6 our findings are discussed in more detail with a focus on the use of RNAi in the functional analysis of nematode effectors. Furthermore, we propose a model to explain how the physical interaction between a SPRYSEC and a CC-NB-LRR protein may affect disease signaling components in plants.

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Chapter 2

Functional analysis of pathogenicity proteins of the potato cyst nematode *Globodera rostochiensis* using RNAi

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Abstract

RNA interference (RNAi) has been used widely as a tool for examining gene function and a method that allows its use with plant parasitic nematodes has recently been described (Urwin *et al*. (2002), Mol. Plant-Microbe Interact. 15, 747-752). Here we use a modified method to analyse the function of secreted beta-1,4-endoglucanases of the potato cyst nematode, *Globodera rostochiensis,* the first *in vivo* functional analysis of a pathogenicity protein of a plant parasitic nematode. Knockout of the beta- 1,4-endoglucanases reduced the ability of the nematodes to invade roots. We also use RNAi to show that *gr-ams-1,* a secreted protein of the main sense organs (the amphids), is essential for host location.

Additional Keywords: amphid secreted protein, cellulase, gene silencing, invasion.

Introduction

Plant parasitic nematodes are pathogens of almost all crops grown worldwide causing damage valued at over $75 billion per annum (Sasser & Freckmann, 1987). The most economically important species are the biotrophic root knot (*Meloidogyne spp.*) and cyst forming (*Heterodera* and *Globodera* spp.) nematodes. Feeding by these nematodes is not a passive process. Invasive stage juveniles invade roots of potential host plants and migrate to a cell suitable for feeding site induction. In a susceptible interaction the nematode induces the formation of a feeding site, a highly metabolically active multinucleate structure on which the nematode feeds for the next 3-4 weeks. Expression patterns of many plant genes, including those controlling fundamentally important developmental processes such as the cell cycle, are adjusted in the feeding site. The molecular processes underlying feeding site development have been reviewed recently (Gheysen & Fenoll, 2002). Secretions of the subventral and oesophageal gland cells have been the subject of intense study as it is thought that they play important roles in many aspects of the host-parasite interaction including host invasion and induction and maintenance of the feeding site (reviewed by Davis *et al*., 2000). As a result of these studies, most notably the use of expressed sequence tags (*e.g.* Popeijus *et al*., 2000a), genes encoding many secreted proteins have been identified in PCN and other plant parasitic nematodes. Some secreted proteins have functional roles in the host parasite interaction that can be readily tested. Genes that encode secreted plant cell wall degrading enzymes (Smant *et al*., 1998; Popeijus *et al.*, 2000b), antioxidant proteins (*e.g* Robertson *et al.*, 2000) and chorismate mutase (Jones *et al*., 2003) have a predicted biochemical function that can be verified *in vitro*. However, the roles of these proteins *in vivo* remain to be tested and many other genes have been identified that encode secreted proteins and are expressed solely in the oesophageal gland cells, and are therefore candidates for proteins that are important in host parasite interactions, but that have no easily predictable function on the basis of sequence similarity. There is therefore the need for systems that allow gene function to be tested *in vivo* to be developed and tested.

In *C. elegans* and many other organisms RNA interference (RNAi) has been used to investigate gene function in detail. This technique uses the fact that exposure of an organism to double stranded RNA (dsRNA) from a gene of interest causes post transcriptional silencing of the endogenous gene and allows a null phenotype to be mimicked (Fire *et al*., 1998). RNAi has been used for genomic scale studies in *C. elegans* (*e.g.* Maeda *et al*., 2001) but until recently could not be used with plant parasitic nematodes, as the invasive stage juveniles did not take up dsRNA from solution. Recently, Urwin *et al*. (2002) described a method that uses a neurotransmitter, octopamine, to induce feeding in invasive J2 of PCN allowing uptake of dsRNA from solution and used this method to knockout several genes, including one encoding a digestive protease.

Here we describe some modifications to this technique that allow it to be used to perform functional analysis of a variety of PCN proteins, including potential pathogenicity proteins – secreted endoglucanases. A family of genes encoding secreted ß-1,4-endoglucanases has been identified in PCN (Smant *et al*., 1998) and other plant parasitic nematodes (Rosso *et al*., 1999). These genes are expressed in the subventral gland cells and are secreted from the nematode during migration through the host root (De Boer *et al*., 1999; Wang *et al.*, 1999). This, coupled with the observation that the plants own cell wall degrading enzymes are responsible for the degradation of the cell walls within the syncytium (Goellner *et al*., 2001) suggests that the functional role of the nematode ß-1,4-endoglucanases is degradation of plant tissues in order to facilitate invasion and migration. We show that the secreted cellulases of PCN are essential for successful invasion of host tissues. In addition we demonstrate that a secreted protein of the amphids, the main sense organs of nematodes, is critical for host location. Furthermore, we also show silencing of dorsal esophageal gland specific gene SPRYSEC-19. This, however, did not affect the infectivity of the nematodes suggesting that its function may be compensated by other homologs.

Materials and Methods

Biological Material

G. rostochiensis was maintained in a glasshouse on the susceptible potato (*Solanum tuberosum*) cultivar Désirée using standard protocols (*e.g.* Jones *et al*., 1996). Invasive stage juveniles were obtained by soaking dried cysts for 5 days in sterile distilled water (SDW) followed by incubation in tomato root diffusate (TRD) prepared as described by Blair *et al*. (1999). Nematodes harvested for RNAi experiments were used within 24h of hatching. Potato plants (cultivar Désirée) used for invasion studies were maintained in sterile tissue culture using standard protocols (Kumar, 1995).

Generation of dsRNA

Regions of cDNA were selected for silencing by RNAi and PCR primers were designed to amplify the selected fragment. Two PCR products were generated for each gene with the T7 promoter sequence incorporated at the 5' end of either the sense or the antisense strand. For the *Gr-ams-1* gene a region from nucleotides 171 to 415 of the cDNA sequence (Accession number AJ270995) was used. Primers AMSF, AMST7F, AMSR and AMST7R (see below) were used to generate this fragment. For the *Gr-eng* genes a region from nucleotides 299 to 608 of the *Gr-eng-1* cDNA sequence (Accession number AF004523) was used as this showed very high (over 98%) similarity to the *Gr-eng-2* (Accession number AF004716) cDNA sequence and also had high similarity to the *Gr-eng-3/4* sequences (Accession numbers AF408155 and AF408156), including stretches of 20nt with over 95% identity suggesting that it should be possible to reduce expression of all characterised endoglucanase genes with the same dsRNA. Primers ENGF, ENGT7F, ENGR and ENGT7R (see below) were used to generate this fragment. For SPRYSEC-19, a region from nucleotides 179-637bp of the cDNA sequence (Genbank accession AJ251757) was used to generate silencing fragment (458 bp), by using Primers A18F, T7A18F, A18R, and T7A18R. PCR products were purified using a PCR purification kit (Qiagen), quantified by spectrophotometry and used as the templates for *in vitro* transcription reactions using a Megascript RNAi kit (Ambion) following the manufacturers instructions. The single stranded RNAs generated from each of the two strands were then annealed by heating to 75^{o}C for 5 minutes and allowing to cool to room temperature. The dsRNA was then treated with DNAse to remove template, purified using columns supplied in the Megascript kit and precipitated under ethanol. The dsRNA was resuspended in DEPC treated water (Sambrook *et al*., 1989) and quantified by spectrophotometry.

RNAi by soaking

A method was developed based on that described by Urwin *et al*. (2002) but incorporating features of the soaking method used for *C. elegans* studies (Maeda *et al*., 2001). Several thousand J2 were soaked in ¼ X M9 (1X M9 = 43.6mM Na_2HPO_4, 22 mM KH_2PO_4, 18.7mM NH_4Cl, 8.6mM NaCl) containing 50mM octopamine, 3mM spermidine, 0.05% gelatin and between 2 and 5mg/ml dsRNA for at least 24 hours in the dark at room temperature on a rotator. Control samples were incubated in the same solution but without dsRNA. After soaking the nematodes were washed three times in ¼ X M9 to remove dsRNA. An aliquot of the dsRNA was checked on an agarose gel stained in ethidium

bromide to verify that degradation had not occurred during the soaking process. An aliquot of the nematodes was removed for invasion studies and the remaining nematodes were frozen in liquid nitrogen and stored at –80°C before being used for RNA extractions. The FITC in the soaking solution and hand sorting of nematodes showing FITC uptake described by Urwin *et al.* (2002) was found to be unnecessary and was therefore omitted from the protocol. RNAi experiments were repeated on at least three separate occasions and, where possible, in both laboratories participating in this study.

Invasion studies

Internodal cuttings were taken from *S. tuberosum* (cv Désirée) grown in sterile culture and grown in 12 well plates containing MS20 medium. For SPRYSEC-19, the internodal cuttings of *S. tuberosum* Line-V were grown in 9 cm^2 Petri-dishes containing MS20 media. Preliminary studies showed that invasion rates of such cuttings were similar to those in pot experiments (L. Castelli, *in preparation*). Nematodes treated with dsRNA and control nematodes were sterilised by soaking for 30 minutes in 0.1% chlorohexidine digluconate & 0.5µg/µl CTAB followed by two 5 minute washes in 0.01% Tween 20 with a final rinse in SDW. The SPRYSEC-19 dsRNA-treated nematodes were surface sterilized as reported by Goverse et al. (2000). The nematodes were checked under a light microscope after sterilisation to ensure that they were still alive. 100 nematodes were used to infect each plant. Plants were stained in acid fuchsin (Bridge *et al.*, 1982) two weeks later in order to determine the numbers of nematodes that had invaded and set up feeding sites. Replicate experiments were performed and the entire experiment was repeated. Plant root systems were of approximately equal size and mass (not shown).

RT-PCR

RNA was extracted using a Micro Fast Track kit (Invitrogen) following the manufacturers instructions. Each batch of mRNA was checked for contamination with gDNA by performing a PCR reaction with actin primers (below) before use. The entire mRNA extraction for each sample was converted to first strand cDNA using a SMART cDNA synthesis kit (Clontech) following the manufacturers instructions. Aliquots of the resulting cDNA were then used in PCR reactions containing 1X *Taq* buffer (Promega), 1.5mM $MgCl_2$, 200µM dNTPs, 1µM each primer (below) and 2.5 units *Taq DNA* polymerase (Promega). Cycling conditions consisted of one cycle of denaturing at 94°C for 2 minutes followed by 35 cycles of 30s denaturing at 94°C, 30s annealing at 50-55°C (depending on the primer combination) and 30s extension at 72°C. Aliquots of the reaction were removed after 22, 26, 30 and 35 cycles. For each experiment four PCR reactions were set up: two reactions using control primers (ACTF and ACTR) designed to amplify a fragment of the actin cDNA from the control and dsRNA treated cDNA samples and two reactions using primers (XXTESTF and XXTESTR) designed to amplify a fragment of the gene being silenced. In all cases the latter primer set was designed to amplify a different region of the gene than that targeted by the dsRNA fragment. PCR products were visualised on 1.5% agarose gels stained with ethidium bromide using standard protocols (Sambrook *et al.*, 1989).

Table 1. The sequences of the oligonucleotide primers used in the experiments

Primer name	Forward primer (5'-3')	Reverse primer (5'-3')
AMS	CAGGGCCAATGCCGGAATAT	GGAGACTCTCAGTGCTTCAC
AMST7	GTAATACGACTCACTATAGGGCAG	GTAATACGACTCACTATAGGGG
	GGCCAATGCCGGAATAT	GAGACTCTCAGTGCTTCAC
ENG	GTTGCCGTGATTGAGGC	TCTTTGATCGGATTCTGCGA
ENGT7	GTAATACGACTCACTATAGGG	GTAATACGACTCACTATAGGGT
	GTTGCCGTGATTGAGGC	CTTTGATCGGATTCTGCGA
ACT	ATGTGYGAYGARGARGTNGC	ATYTTYTCCATRTCRTCCCA
AMSTESTF	CCGCCTCAATGAACTGCATT	TACTTACTCTCTTTCTCAGAGTATTA
ENG1/2TEST	CACATTGTGTTCTGTAACGC	
ENG1TEST		GTTGAGACAACAGTGACGCT
ENG2TEST		CAGCGGCAGGTTTGGCCGGAGGCG
ENG3TEST	GGCAACCGCTTTTGGGTG	CCAAAGTGCCAACTCTTTGAGA
A18F	GCACATCACAAGGACCTGA	AGGCATGAACATCGAAACG
T7A18	TAATACGACTCACTATAGG	TAATACGACTCACTATAGG
	GCACATCACAAGGACCTGA	AGGCATGAACATCGAAACG
RtA18	AGCCAGTGCTGGCAATGC	GATAATGTTTTGATCGACGAAGAA
GAPDH	GTTCATCGAACTCGACTACAT	GTCATATTTGTCCTCGTTGAC

Results

A modified protocol combining features of that described by Urwin *et al*. (2002), most notably the use of octopamine to induce feeding, and features of the method used for high-throughput screening in *C. elegans* (Maeda *et al.*, 2001) (see Materials and Methods below) allowed reproducible silencing of the genes being tested. The addition of spermidine to the soaking mix and incubation of the nematodes for 24h (compared to the 4 hours described by Urwin *et al*., (2002)) seemed to be important factors. For example, when RT-PCR was used to compare levels of gene expression in nematodes after 4 hours exposure to dsRNA, no reproducible drop in levels of the endogenous mRNA was observed with either the *ams-1* or *eng* genes (not shown).

An amphid secreted protein is essential for sense organ function

The amphids of PCN contain seven nerve processes exposed to the external environment that are bathed in secretions produced by a sheath cell that forms the cavity of the amphid (Jones *et al*., 1994). Although little is known about the role of the secretions produced by the sheath cell, secretions of insect sense organs are important for their function (*e.g.* Vogt *et al.* 1990) and nematicides that target sensory perception in nematodes cause changes in the appearance of these secretions (Trett & Perry, 1985). It is therefore likely that the amphid secretions are important for the function of these sense organs. We therefore attempted to silence a gene (*Gr-ams-1*) that encodes a secreted protein and is expressed solely in the sheath cells of the amphids of PCN (Jones *et al*., 2000) and that is therefore likely to encode a protein forming part of the amphid secretions. RT-PCR experiments comparing expression of the *Gr-ams-1* gene in dsRNA treated and control nematodes showed that the RNAi procedure did indeed result in a down regulation of the *Gr-ams-1* mRNA (Fig. 1).

Expression of a control gene (actin) was, by contrast, similar in both treated and untreated samples (Fig. 1). Infection studies showed that the treated nematodes were almost completely unable to locate and invade host plants as compared to the controls (Fig. 2). All nematodes (control and dsRNA treated) were examined under a microscope after treatment and sterilisation. In all cases the nematodes were alive and moving normally before being used for experiments.

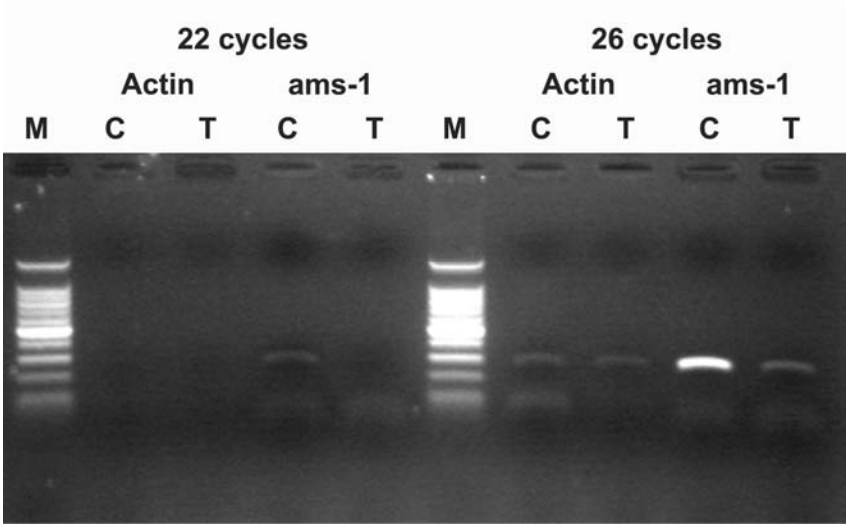

Figure 1. RT-PCR analysis of levels of ams-1 and actin mRNA in control nematodes (C) and nematodes exposed to ams-1 dsRNA. After 22 cycles a band of the expected size is amplified using the ams-1 primers from control nematodes but no band is present in nematodes exposed to ams-1 dsRNA. After 26 cycles the ams-1 band is present in all all samples but is present at far higher levels in control samples. Amplification of a band from actin is similar in control and ams-1 samples. These data indicate a specific reduction in ams-1 transcript in nematodes exposed to ams-1 dsRNA. M – 100bp ladder (Promega).

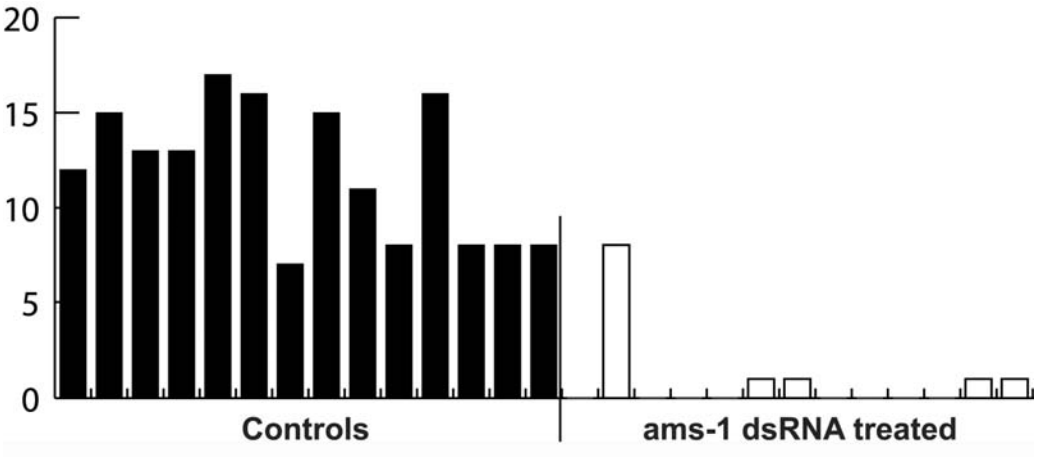

Figure 2. Numbers of *G. rostochiensis* invading roots of potato after exposure to control treatment or ams-1 dsRNA. Each column represents a single plant.

RNAi of nematode cellulases leads to reduced infectivity

A family of genes encoding secreted ß-1,4-endoglucanases has been identified in PCN that have been suggested to degrade plant tissues in order to facilitate invasion and migration. Our RNAi experiments supported this. RT-PCR experiments showed a decrease in the levels of the mRNA of *eng1* (Fig. 3) when compared to control genes. Some decrease in the levels of *eng3/4* was also observed (Fig. 3). Invasion studies showed that treatment of J2 with cellulase dsRNA significantly reduced ($P = <0.002$) the ability of the nematodes to infect plants. Although data was variable, on average almost twice as many untreated nematodes were present in each plant as compared to those treated with dsRNA (an average of 11.9 nematodes per plant compared with an average of 6 nematodes per plant). We compared the developmental fate of those nematodes that were able to infect plants. No differences were found in nematode development (male or female) that could be attributed to treatment with dsRNA. The proportion of feeding nematodes that had a greatly swollen appearance suggesting development to females was 44% in control samples compared with 60% in dsRNA treated nematodes. Far fewer nematodes invaded each plant in dsRNA treated nematodes, which may explain the apparent shift in sex ratio towards female development. Clearly there was no evidence of a trend towards male development in dsRNA treated nematodes. The location of nematodes (but not the feeding sites) within the plant root was also compared. In control samples 40% of the nematodes were lying close to the vascular tissue of the plant roots while the remaining 60% had settled in more peripheral root tissues. In nematodes treated with dsRNA these figures were 37.5% and 62.5% respectively. This suggests that for the cellulase a proportion of the nematodes were affected by the RNAi procedure with other nematodes unaffected.

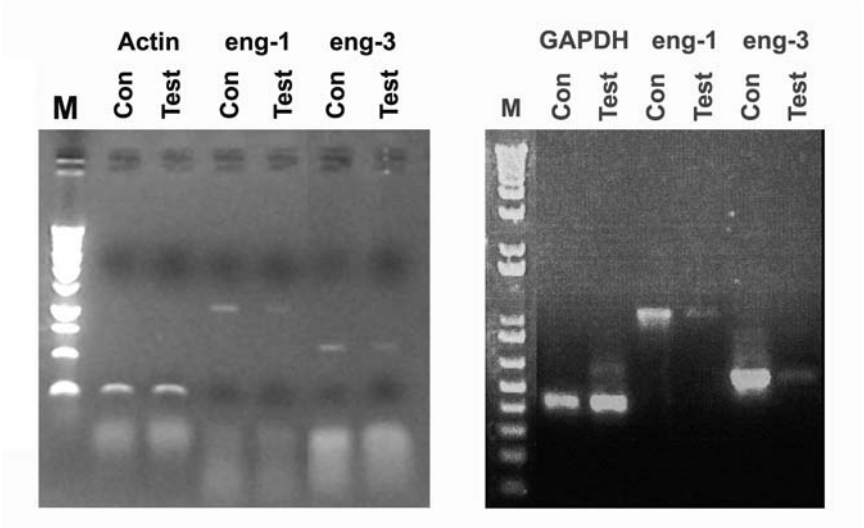

Figure 3. RT-PCR analysis of levels of endoglucanase gene expression compared to two control genes in control nematodes (Con) and nematodes exposed to eng-1/2 dsRNA. Levels of eng-1 and eng-3 mRNA are reduced in samples exposed to eng-1/2 dsRNA. Levels of two control genes, actin (3a) and GAPDH (3b), are similar in control and test samples. M – 100bp DNA ladder (Promega).

RNAi of nematode SPRYSEC does not lead to reduced infectivity

The dorsal esophageal gland specific gene SPRYSEC-19 has been shown to be expressed in pre-parasitic J2s (Qin et al., 2000; see chapter 4). In order to investigate the efficacy of RNAi on a dorsal esophageal gland specific gene, an RT-PCR was done on mRNA isolated from SPRYSEC-19 dsRNA treated and non-treated nematodes (Fig. 4). We observed a significant decrease in SPRYSEC-19 mRNA levels in dsRNA-treated nematodes as compared to the control sample. However, invasion studies showed that treatment of J2 with SPRYSEC-19 dsRNA did not significantly reduce the ability of the nematodes to infect plants (data not shown).

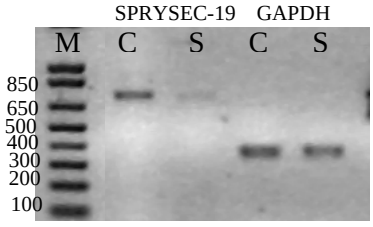

Figure 4. RNAi of dorsal esophageal gland specific gene SPRYSEC-19. RT-PCR analysis of SPRYSEC-19 mRNA in control (C) and SPRYSEC dsRNA-treated (S) nematodes. GAPDH mRNA was uses as a control for off-target effects. M = 1kb DNA ladder.

Discussion

The data presented here show, for the first time, that RNAi can be used to analyse the function of a pathogenicity factor of a plant parasitic nematode. We found that the efficiency of the procedure could be enhanced by combining features of the method described by Urwin *et al.,* (2002) with some of those used for high-throughput analysis of *C. elegans* genes. A 24 hour incubation period, as compared to the 4 hours used by Urwin *et* al., (2002), seemed to be particularly important. A comparison of the data obtained from a gene expressed in sense organs with a gene expressed in the subventral gland cells showed that a certain amount of variability was present, with the *ams-1* gene seemingly far more susceptible to RNAi than the *eng* genes. This variation may be gene specific (possibly associated with levels of expression of the genes) or may relate to the tissues in which the genes are expressed. It is also possible that the fragment targeting *ams-1* contained more regions particularly suited for generation of silencing RNAs than the fragment targeting the endoglucanase genes or that the different sized fragments of dsRNA used to silence the two genes had an effect (although a larger fragment was used for the endoglucanases). Indeed, there are many possible reasons for the observed differences in silencing efficiency and since the mechanisms underlying gene silencing are still in the process of being uncovered it is not possible to state with any confidence the real reasons for the observed effects.

Functional experiments using pathogenicity genes may give phenotypes that require detailed analysis or that are difficult to score. In order to verify that the RNAi method was working and could be used for *in vivo* studies we first sought to disrupt the function of a protein of the main sense organs (the amphids) of PCN. The rationale for this was that disruption of sense organ function should give rise to an easily scored phenotype, an inability to locate host roots. The function of the AMS-1 amphid secreted protein is unknown but the data presented here suggest that it is essential for normal sense organ function. In *C. elegans* at least eight genes encoding proteins similar to AMS-1 are present. The function of these genes has been examined as part of large-scale RNAi studies but these studies have not revealed a role for any of the *C. elegans* genes in sensory perception. However, the scale of these studies mean that few phenotypes are analysed in detail and it is likely that a subtle effect, such as disruption of sensory perception, would be missed in such screens. We have previously demonstrated that at least one other similar protein is present in *G. rostochiensis*. However, the gene encoding this other protein is expressed in the hypodermis (Jones *et al.,* 2000) and it is therefore possible that this group of proteins has a role in the secretion process in a variety of nematode tissues.

Plant parasitic nematodes have been shown to produce a variety of endogenous plant cell wall degrading enzymes. These enzymes are secreted by preparasitic J2 and by parasitic J2 during migration of the nematodes within the root (De Boer *et al.*, 1999). Expression of the plants own endogenous cell wall degrading enzymes is induced in nematode feeding sites in order to achieve the breakdown of the plant cell walls that accompanies development of the syncytium (Goellner *et al.*, 2001). It is therefore thought that the role of the nematode cellulases is to soften and break down plant cell walls in order to assist nematode migration through the root.

The secretions from the dorsal esophageal gland are thought to be involved in feeding site induction and maintenance (Wyss & Zunke, 1986, Smant et al., 1998; Popeijus et al., 2000; Qin et al., 2004). To investigate the efficacy of dsRNA mediated gene silencing in the dorsal esophageal gland we used SPRYSEC-19 as a target gene because of its specific expression in pre-parasitic J2s (Qin et al., 2000). The function of SPRYSEC-19 in parasitism of cyst nematode is unknown at present. RT-PCR did show significant reduction in the transcript level of SPRYSEC-19 following a dsRNA treatment (Fig. 4). However, reduced mRNA levels of SPRYSEC-19 did not result in an easily scored phenotype such as the overall infectivity. The loss of function of one SPRYSEC gene may be compensated by other homologs such that it does not affect the overall infectivity.

Our data support that RNAi can be used as a tool for investigating the role of nematode secreted proteins in the host-parasite interaction. However, experiments using the *eng* treated nematodes suggested that in this case a proportion of the nematodes were not affected by the dsRNA treatment. These nematodes infected plants and developed normally. Although enough nematodes were affected to allow the expected phenotype to be detected, analysing a phenotype where the gene being silenced is of unknown function is likely to be challenging if only a proportion of the nematodes is affected.

Acknowledgements

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Chapter 3

Identification and characterization of the most abundant cellulases in stylet secretions from the potato cyst nematode *Globodera rostochiensis*

Sajid Rehman, Patrick Butterbach, Herman Popeijus, Hein Overmars, Eric L. Davis, John T. Jones, Johannes Helder, Aska Goverse, Jaap Bakker, and Geert Smant

Abstract

Plant-parasitic cyst nematodes secrete cell wall modifying proteins during the invasion of host plants. In this study we used a monoclonal antibody to immunopurify and to sequence the N-terminus of the most abundant cellulases in stylet secretions of pre-parasitic juveniles of *Globodera rostochiensis*. The N-terminal amino acid sequence perfectly matched the sequence of an expressed sequence tag of two nearly identical endoglucanase genes, named *Gr-eng3* and *Gr-eng4*, which show relatively low similarity with the previously identified *Gr-eng1* and *Gr-eng2* (i.e. 62% similarity and 42% identity). The recombinantly produced proteins from *Gr-eng3* and *Gr-eng4* demonstrated specific activity on carboxymethylcellulose indicating that these genes encode active cellulases. Knocking-down *Gr-eng3* and *Gr-eng4* using RNA interference resulted in a reduction of nematode infectivity by 57%. Our observations suggest that the reduced infectivity of the nematodes can be attributed to poor penetration of the host's root system. The cellulases in cyst-nematodes occur in three possible domain structure variants with different types of ancillary domains at the C-terminus of the glycosyl hydrolase family 5 (GHF5) domain. We used Bayesian inference to show that the phylogeny of the GHF5 domain does not support the evolution of cellulases by sequential loss of these ancillary cellulose binding domains and linkers.

Additional keywords: evolution, β-1,4-endoglucanase, plant-nematode interactions

Genbank accession: Gr-eng3, AF408155 (mRNA) and AF408154 (gene); Gr-eng4, AF408156 (mRNA) and AF408157 (gene)

Introduction

Cellulose is made of linear chains of β-1,4 linked D-glucose and constitutes the most abundant natural carbohydrate polymer on earth. In plants, stacked sheets of these parallel β-glucan polymers interconnect along their length with hydrogen bonds to form insoluble microfibrils. The microfibrils assemble into the cellulose network, which represents the main scaffolding structure in plant cell walls. The cellulose scaffold is reinforced by hemicelluloses, such as xyloglucans and glucuronarabinoxylans. This (hemi)cellulose composite is embedded in a matrix of pectic polysaccharides and lignins, which collectively provide plant cells with a strong constitutive protective armor against invading parasites (Carpita 1996; Carpita and Gibeaut 1993).

Sedentary plant-parasitic nematodes penetrate roots of host plants close to the root apex and migrate through the root tissues until they settle and induce the formation of an elaborate feeding site on which they depend for all nutrients required for development to the adult stage. Cyst nematodes migrate intracellularly, breaking down cell walls as they progress from cell to cell, whereas root knot nematodes migrate intercellularly, softening cell walls to aid their migration. During migration of cyst nematodes the cell walls are broken down by strong outward thrusts of the oral stylet of the nematode. Migration is further facilitated by the release of cell wall degrading enzymes (e.g. cellulases, pectate lyases, polygalacturonases, and endoxylanases) from the stylet lumen, which is connected to three esophageal gland cells (Goellner et al. 2000; Jaubert et al. 2002; Mitreva-Dautova et al. 2006; Rosso et al. 1999; Smant et al. 1998; Uehara et al. 2001; Wang et al. 1999; Yan et al. 2001). Recent studies have shown that in addition to the conventional enzymes listed above cyst nematodes also secrete β-expansins (Kudla et al. 2005; Qin et al. 2004) which are believed to disrupt non-covalent interactions between cell wall polymers making them more susceptible to the activity of conventional enzymes.

The first nematode cell wall degrading enzymes - cellulases - were identified in two cyst nematode species (Smant et al. 1998). A specific antibody (MGR48) directed towards at least three proteins in the stylet secretions of the potato cyst nematode *G. rostochiensis* was used to purify two corresponding antigens from nematode homogenates. Following N-terminal amino acid sequencing two cellulase genes, named *Gr-eng1* and *Gr-eng2* and coding for proteins of 49 and 39 kDa respectively, were cloned using degenerate oligonucleotide primers and PCR-based cloning (Smant et al. 1998). The third protein recognized by MGR48 was refractory to amino-acid sequencing, and consequently its corresponding gene could not be characterized. However, in earlier experiments this smaller protein proved to be the most abundant antigen in stylet secretions of the preparasitic juveniles (Smant et al. 1997). Here, we describe the characterization of two genes (*Gr-eng3* and *Gr-eng4*) encoding the 32 kDa antigens recognized by MGR48 using a

modified protocol for N-terminal protein sequencing and 5'-end expressed sequence tags. The genes code for novel cellulases comprising a glycosyl hydrolase family 5 (GHF5) type of catalytic domain.

The group of GHF5 cellulases in plant-parasitic nematodes entails several protein domain structure variants. The largest variant (e.g. *Gr-eng1*) includes a GHF5 catalytic domain, a linker, and a cellulose binding domain, while the novel genes described in this paper only include a GHF5 domain. The third and intermediate variant (e.g. *Gr-eng2*) includes a GHF5 domain and a short ancillary C-terminal domain. Ledger et al. (Ledger et al. 2006) proposed an evolutionary model that explains these extant domain structure variants as a result of a sequential loss of cellulose binding domain and linker sequences. In this paper we test this sequential-loss model using phylogeny inference of the domain structure variants in cyst nematode cellulases.

Materials and Methods

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 (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. Subsequently the J2s were either used for experiments directly or stored at -80°C until further use.

DNA cloning and analysis

An oligo-dT-primed cDNA library from *G. rostochiensis* second stage juveniles was prepared in the vector pcDNAII (Invitrogen, San Diego). Individual colonies plated on Luria-Bertani medium supplemented with ampicillin were randomly picked and grown in 1ml liquid cultures. Plasmid DNA was isolated from the cells following overnight growth at 37°C. The 5'-end expressed sequencing tags were subsequently generated using cycle sequencing with dye terminator chemistry (Popeijus et al. 2000; Smant et al. 1998). The single read sequences were analyzed using the program Basic Local Alignment Search Tool algorithm (TBLASTN, BLASTN and BLASTP) with default settings at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). One expressed sequence tag (named GE1985) encoded a partial open reading that showed similarity to cellulases of various origins. The sequence similarity with other nematode cellulases, however, was only slightly higher than those found in other organisms. The sequence downstream of EST GE1985 was identified using the M13 universal sequencing primer (Table 1). A start codon was missing in GE1985, therefore, a primer (Eng-r1; Table 1) was designed to amplify the region upstream of the tag when combined with a plasmid derived primer (PCDNA5-f1; Table 1) in a PCR using a plasmid prep of the cDNA library. To confirm the integrity of the cDNA that could be deduced from the amplified fragments two primers (Eng-f1 and Eng-r2; Table 1) were combined in a similar PCR that produced a single band of 1194 bp. This band was cloned into the pCR2.1-TOPO (Invitrogen) as subjected to DNA sequencing. A similar PCR with the primers Eng-f1 and Eng-r2 was performed using genomic DNA as template, which was extracted from hatched J2s by alkaline/SDS lysis and phenol/chloroform extraction (Sulston and Hodgkin 1988). The amplified genomic fragment was cloned into pCR2.1-TOPO vector and sequenced as described above.

The program Signal-P was used to predict the presence of a signal peptide consensus sequence (http://www.cbs.dtu.dk/services/SignalP-3.0/). Protein patterns and domains in the predicted open reading frames were identified using the PFAM analysis tool as provided on the world-wide-web (http://pfam.wustl.edu/hmmsearch.shtml).

Table 1. Oligonucleotide primers used for amplification of nematode cDNA (fragments) and genomic DNA. Underlined are the restriction nuclease recognition sites used for subcloning the fragments into the pET expression vector.

Name	Primer sequence (5'→3')
Eng-f	GAATGTGCGCTTTGATTTATG
Eng-r1	GCACAACATTGCTGTTACAGC
Eng-r2	CTAAGCTTTGATTTATTCACCTTTC
Eng-pETf	CACCGTCACAGCCCCTCCCTAT
Eng-pETr	ACCGCGGCAACTTACT
Eng-insitu-f	CCGCGGAATATGCCAAAATGAAG
Eng-insitu-r	CCGGCGTAAAAGGCAATGTGTATG
PCDNA5-f	GGTGACACTATAGAATACTCAAGCTATGCA
PCDNA3-r	GACGGCCAGTGAATTGTAATACGACTCACT
T7ENG3-F	TAATACGACTCACTATAGGCGATGAAATCAATTCTGCTAAA
T7ENG3-R	TAATACGACTCACTATAGGGATTGTTTGCCTCATTTTTG
RT-eng3f	GATCATTCTAAGATCGGCG
RT-eng3r	GGTTGCAAATGTTGTTGATT
RT-eng1f	CTGCACATTGTGTTCTGTAACGC
RT-eng1r	TTGAGACAACAGTGACGCT
RT-eng2r	CAGCGGCAGGTTTGGCCGGAGG
RT-A41f	CAAACTGATGCTTCGCCAA
RT-A41r	TGAATTTGAATGGTTTCGTGC

***In situ* hybridization**

DNA probes were amplified from clone GE1985 using the oligonucleotide primers Eng-insitu-f and Eng-insitu-3 (Table 1) and digoxigen-11-dUTP. 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).

Heterologous Expression and activity assay

The open reading frames of Gr-eng-3 and Gr-eng-4 without the predicted N-terminal signal peptides (nt 77 - 1015) were sub-cloned into the pET101/D-TOPO expression vector using

the oligonucleotide primers Eng-pETf and Eng-pETr (Table 1). The plasmids were introduced into *E. coli* strain TOP10 to sequence the constructs. Recombinant plasmid DNA was introduced into *E. coli* strain BL21Star (DE3) for expression. Expression of the recombinant protein was determined on SDS-PAGE with Coomassie Brilliant Blue staining and western blotting following induction of the transformants with 1 mM isopropyl β-D-thiogalactoside.

Carboxymethylcellulose (CMC) was used as a substrate in a cup plate assay to determine CMC hydrolyse activity of cellulase. CMC (0.2% w/v) was dissolved in a phosphate-citrate-buffer (50 mM K_2HPO_4, adjusted to pH 7.4 with 1 M citric acid) with 0.5% (w/v) agarose. CMC plates were incubated overnight at 30 °C and stained with 0.02% Congo red (Mateos et al. 1992).

N-terminal protein sequencing

Four protein bands (31, 32, 39 and 49 kDa) were detected with the monoclonal antibody MGR048 on western blots of homogenates from *G. rostochiensis*. The protein band of 32 kDa was purified from homogenates of *G. rostochiensis* J2s using continuous flow SDS-PAGE followed by fraction identification and immunoaffinity chromatography with mAb MGR48 (Smant et al. 1998). The purified fraction was rerun on an analytical SDS-PAGE gel utilizing a Tris-tricine buffer system and blotted onto a polyvinylidine fluoride membrane for N-terminal sequencing (ARIAD Pharmaceuticals, Cambridge, MA). The sequence analysis of the protein band showed multiple N-termini including one major sequence. Additional runs using the reagent o-phtalaldehyde at the fourth cycle blocked all N-termini except the major sequence and yielded 26 amino acids.

RNA interference by soaking

For knocking-down expression of *Gr-eng3* and *Gr-eng4* by soaking in gene specific dsRNA, two primers were used T7ENG3-F and T7ENG3-R (Table 1) to generate a dsRNA fragment that targeted the region from nucleotide 403 to 803. This region is identical between *Gr-eng3* and *Gr-eng4*. The T7 promoter sequence was included in the sequence of both primers (underlined). RNA was transcribed *in vitro* using T7 RNA polymerase to prepare dsRNA at 2 mg per milliliter for soaking of the nematodes (Ambion, Austin, USA). A typical soaking reaction included 10,000 nematodes in a dsRNA solution (2 mg/ml dsRNA, 3mMSpermidine, 0.05% gelatin) for 40 hours. Control nematodes were soaked in solutions without dsRNA or with dsRNA from not related genes in *G. rostochiensis*. From each reaction 8,000 J2s were used for RT-PCR, while 2,000 J2s were used for the nematode infection assay. The viability of the nematodes was checked by examination under a microscope after soaking in dsRNA and after sterilization for nematode infection assay. The soaking experiment with dsRNA was repeated three times.

For semi-quantitative RT-PCR, total RNA was extracted from nematodes using TRIzol reagent and transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen). RT-PCR reaction was performed as described by Chen et al. (Chen et al. 2005) using the primers RT-engF and RT-EngR (Table 1) designed outside the region targeted by the dsRNA. Aliquots of reaction were removed after 26 cycles and visualized on 1% agarose gel stained with ethidium bromide (Sambrook et al. 1989).

For the nematode infection assay, internodal cuttings were taken from *Solanum tuberosum* LineV, grown in sterile culture in 12 cm^2 plates containing MS20 media. For

each treatment four plates with 4-internodal cuttings on each plate were inoculated with 175 nematodes. The nematodes were surface sterilized prior to inoculation (Goverse et al. 1999). Nematode penetration was monitored using a binocular microscope. Roots were taken from the plates at 24 hour intervals for acid fuchsin staining (Bird 1983). The number of nematodes (partially) inside the roots were calculated relative to the number of nematodes inoculated. The statistical analysis of the data was done using the Student's *t*-Test for two samples assuming equal variances.

Phylogenetic analysis

The non-redundant protein database at NCBI was queried with the Gr-ENG3/4 sequence using the BLASTP algorithm. All matching cellulase sequences from cyst nematode species were subsequently trimmed in order to include the GHF5 domain in the analysis (i.e. Gr-ENG1, Genbank identification number (gi): 2624931; Gr-ENG2, gi:2654525; Hg-ENG1, gi: 2257849; Hg-ENG2, gi: 2257951; Hg-ENG3, gi: 3513544; Hg-ENG4,gi: 15822650; Hg-ENG5, gi: 33151119; Hg-ENG6, gi: 30348356; Hs-ENG1, gi: 10800865; Hs-ENG2, gi: 10800867; Gts-ENG1, gi: 5923869; Gts-ENG2, gi:5923871). One matching cellulase (Mi-ENG1, gi: 563966) from *Meloidogyne incognita* was used as out-group. A multiple sequence alignment with 15 protein sequences was made in ClustalW version 1.83 at the European Bioinformatics Institute online server using the GONNET250 substitution matrix. The alignment was manually optimized and gaps were removed. In total, 328 informative positions were used for phylogenetic inference using Bayesian analysis (MrBayes version 3.1.2; (Ronquist and Huelsenbeck 2003)). The analysis was run with a mixed model of amino acid evolution to allow model-jumping for fixed-rate models for 4 runs with 4 chains each for 1,000,000 generations. The first 25,000 generations of the run were discarded as burn-in. In the resulting rooted phylogeny the nodes with a posterior probability lower than 0.95 are considered to be unresolved.

Results

Preparative SDS-PAGE was used to isolate a protein fraction ranging from 30 to 35 kDa from homogenates of 5 million preparasitic J2s of *G. rostochiensis*. A 32 kDa protein band was purified from this fraction using monoclonal antibody MGR48. The purified protein was rerun on an analytical SDS-PAGE for western blotting. Five micrograms of blotted protein was subsequently used for N-terminal amino acid sequencing, which resulted in one major sequence of 26 amino acids with two uncertainties because of a weak signal at positions Q_{24} and D_{26} (Fig. 1).

Database searches with the TBASTN algorithm using the N-terminal sequence of the purified 32 kDa antigen of MGR48 yielded one nearly perfect matching nucleotide stretch in one expressed sequence tag (library clone GE1895) from *G. rostochiensis*. All residues that had been resolved reliably in the N-terminus of the antigen of MGR48 were identical to the protein sequence predicted by GE1895. The two residues that were still uncertain in the N-terminal sequence also did not match the predicted sequence in the expressed sequence tag. Further sequencing of the clone from which the tag originated, revealed that a cDNA insert of 1196 bp was present including a region encoding an open reading frame of 337 amino acids. The first 24 amino acids at the N-terminus of the open reading frame were predicted to be part of a signal peptide for secretion using the Signal-P 3.0 software. The sequence of the N-terminus of the purified 32 kDa protein starts exactly where Signal-P software predicts the cleavage site of the signal peptide for secretion in the protein encoded by clone ge1895.

Figure 1. An alignment of the N-terminal amino acid sequences of the 32 kDa protein immunopurified from nematode homogenates using MGR48 (N-terminus), from the predicted open reading frame in *Gr-eng3/4* (N-term. Gr-ENG3/4), and from Gr-ENG1 (N-term. Gr-ENG1) and Gr-ENG2 (N-term. Gr-ENG2). The alignment was generated in ClustalW1.8 using the GONNET matrix to discover identical (*) and similar (.) amino acids.

While sequencing constructs for the recombinant expression of the open reading frame in clone ge1985, we found two highly similar cDNA molecules differing in sequence at eight nucleotide positions. The predicted proteins of these cDNA molecules both have identical matches with the N-terminus of the purified MGR48 antigen. The expression clones were constructed by PCR and the polymorphisms detected in the two cDNAs could therefore reflect errors randomly introduced by DNA polymerases. In order to test this, we amplified and sequenced the corresponding coding sequences from genomic DNA of *G. rostochiensis*. All eight nucleotide differences were also found in the corresponding genomic fragments, making it very unlikely that they are all artifacts caused by PCR errors. Consequently, the epitope of MGR48 is present in two nearly identical proteins, hereafter named Gr-ENG3 and Gr-ENG4. The theoretical molecular masses of Gr-ENG3 and Gr-ENG4 are 35,388 Da and 35,378 Da respectively with predicted isoelectric points of 8.67 and 8.80. Six introns in conserved positions intersperse the coding sequences of the genomic sequences of *Gr-eng3* and *Gr-eng4* (Supplemental Fig. 1). The intron at position I differs in size by 83 nucleotides between the two genes (354 and 271 nucleotides for *Gr-eng*-3 and *Gr-eng-4* respectively). The other introns (at positions II, III, IV, VI, and VIII) in *Gr-eng3* and *Gr-eng4* differ only at a small number of nucleotide positions and are of similar sizes. The nucleotide substitutions at positions 64, 65, 172, 275, and 949 of the coding sequence cause substitution of four amino acids ($K_{21}\rightarrow L_{21}$, $D_{56}\rightarrow Y_{56}$, $N_{91}\rightarrow K_{91}$, and $T_{316}\rightarrow A_{316}$; Supplemental Fig. 1). The three other polymorphisms do not lead to differences in the amino acid composition of the encoded proteins. It is noted that *Gr-eng3* and *Gr-eng4* have one uniquely used intron position (position II) and five intron positions (positions I, III, IV, VI, and VIII) in common with *Gr-eng1* and *Gr-eng2*, with which they share 42% identity and 62% similarity across the coding region.

An antisense digoxigen-11-dUTP labeled cDNA probe was constructed for *in situ* hybridization microscopy to localize the expression of *Gr-eng3* and *Gr-eng4* in nematodes. The high similarity between *Gr-eng3* and *Gr-eng4* did not allow us to design probes specific for each individual gene. The antisense probe specifically hybridized in both subventral esophageal gland cells in preparasitic juveniles of *G. rostochiensis* (Fig. 2a). The sense probe did not hybridize to structures in the nematode (Fig. 2b). Therefore expression of *Gr-eng3* and *Gr-eng4* is restricted to the subventral esophageal gland cells.

Further analysis of the protein sequence using the PFAM HMM search program showed that Gr-ENG3 and Gr-ENG4 comprise a single domain protein (amino acids 37-297) which is recognized as a glycosyl hydrolase family 5 cellulase (GHF 5; *P*-value 10^{-38}). In this domain two conserved regions, IYEIWN**E**$_{163}$P and FVT**E**$_{253}$YGI, including two active site glutamic acid residues were identified in Gr-ENG3 and Gr-ENG4 ((underlined in Supplemental Fig. 1; Jenkins et al. 1995). To test whether the Gr-ENG3 and Gr-ENG4

proteins have cellulase activity, we subcloned the predicted open reading frames into pET101 for recombinant expression in bacterial cells. Cell lysates of transformed *E. coli* containing *Gr-eng3* and *Gr-eng4* constructs showed significant hydrolytic activity in a cup plate assay with 0.2% carboxymethylcellulose (Fig. 3b and c). The lysates of *E. coli* transformants that were not induced to express the protein (data not shown) or that harbored the empty plasmid vector did not show such activity (Fig. 3a). Based on these results we conclude that Gr-ENG3 and Gr-ENG4 encode functional cellulases.

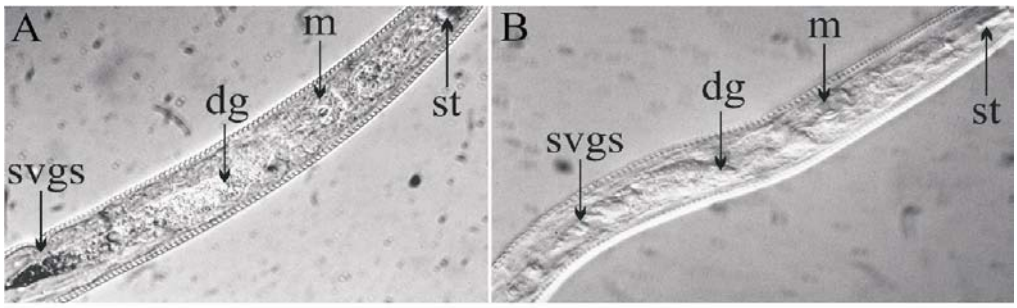


Figure 2. *In situ* hybridization microscopy of *Gr-eng3/4* transcripts using DIG-labeled sense and anti-sense probes amplified from *Gr-eng-3/4* cDNA in sections of second stage pre-parasitic juveniles of *G. rostochiensis*. The anti-sense probe specifically hybridized in the posterior tip of the subventral esophageal gland lobe (svgs) (**A**). No hybridization could be observed with the corresponding sense probe (**B**). In both pictures A and B the dorsal gland (dg), metacorpus (m) and stylet (st) are indicated with arrows.

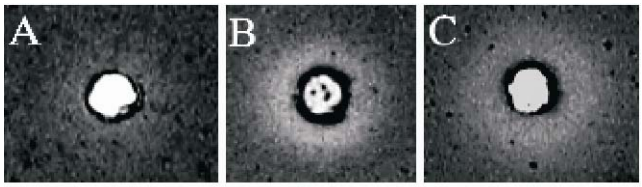


Figure 3. A cup plate enzyme activity assay showing the hydrolytic activity on carboxymethylcellulose of Gr-ENG3 (B) and Gr-ENG4 (C) recombinantly expressed in *E. coli*. The hydrolytic activity in the substrate is visualized as a halo by a Congo Red stain. No halo could be detected while applying lysates of *E. coli* harboring the empty plasmid (A).

The monoclonal antibody MGR48 recognizes at least four proteins encoding cellulase activity of which Gr-ENG3 and Gr-ENG4 were shown to be the most abundant in stylet secretions (Smant et al. 1997). Chen et al. (Chen et al. 2005) found that using RNAi to knock-down expression of *Gr-eng1* and *Gr-eng2* reduced infectivity of the nematodes by approximately 50%. To investigate the relative importance of *Gr-eng3/4* for nematode infectivity we knocked-down these genes in preparasitic juveniles by soaking in specific dsRNA (Fig. 4). Again, the high similarity between *Gr-eng3* and *Gr-eng4* did not allow us to target each gene separately. The nematodes were soaked in dsRNA specifically designed on *Gr-eng3/4*, and off-target effects were not detectable with RT-PCR (Fig. 4).

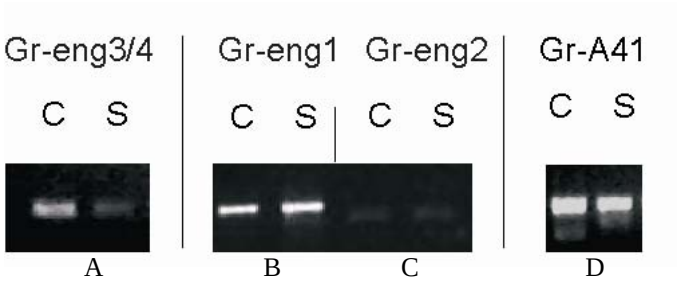


Figure 4. Semi-quantitative RT-PCR showing the expression of *Gr-eng3/4* (**A**), Gr-eng1 (**B**), Gr-eng2 (**C**), and *Gr-A41* (**D**) in preparasitic J2-s of *G. rostochiensis* following a control treatment (*C*) and treatment with *Gr-eng3/4* specific dsRNA (*S*). The samples shown here were taken from the reaction mix after 26 cycles of PCR.

After soaking, the juveniles were transferred to *in vitro* cultured tomato roots, and penetration and intracellular migration was closely monitored for 7 days (experiment was repeated three times). The number of nematodes (partially) inside the roots calculated relative to the number of nematodes transferred to the roots was 10.38% (SD ± 6.01) for silenced nematodes, whereas 23.57% (SD ± 5.79) of the nematodes from the control treatment penetrated the roots (Fig. 5A and D). We further studied the effect of such a significant reduction of infectivity (56.6%; *P*-value in Student's *t*-test of 0.0195) on penetration behavior of the nematodes. Following silencing of *Gr-eng3/4* we observed a high proportion (> 75%) of the penetrating nematodes partially inside the root (Fig. 5A and B). In control nematodes partial penetration of the roots by nematodes was rare (< 1%). We also observed many small brown spots consisting of a number of epidermis cells on roots inoculated with *Gr-eng3/4* dsRNA-treated nematodes. These spots seem to arise from cells in the root epidermis damaged by nematodes attempting to penetrate the root at a specific site. These nematodes fail to enter the root and subsequently move on to another site on the root (Fig. 5B and C). These spots are rarely observed in roots inoculated with nematodes

exposed to the control treatments. None of these specific observations were seen following the knock-down of nematode genes with functions not relating to plant cell wall modification (Gr-GAPDH and Gr-A41; data not shown).

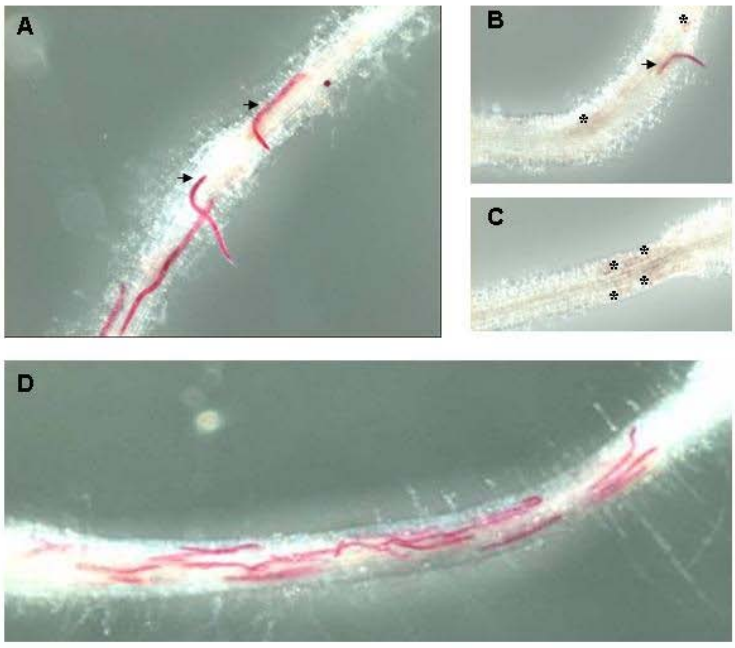


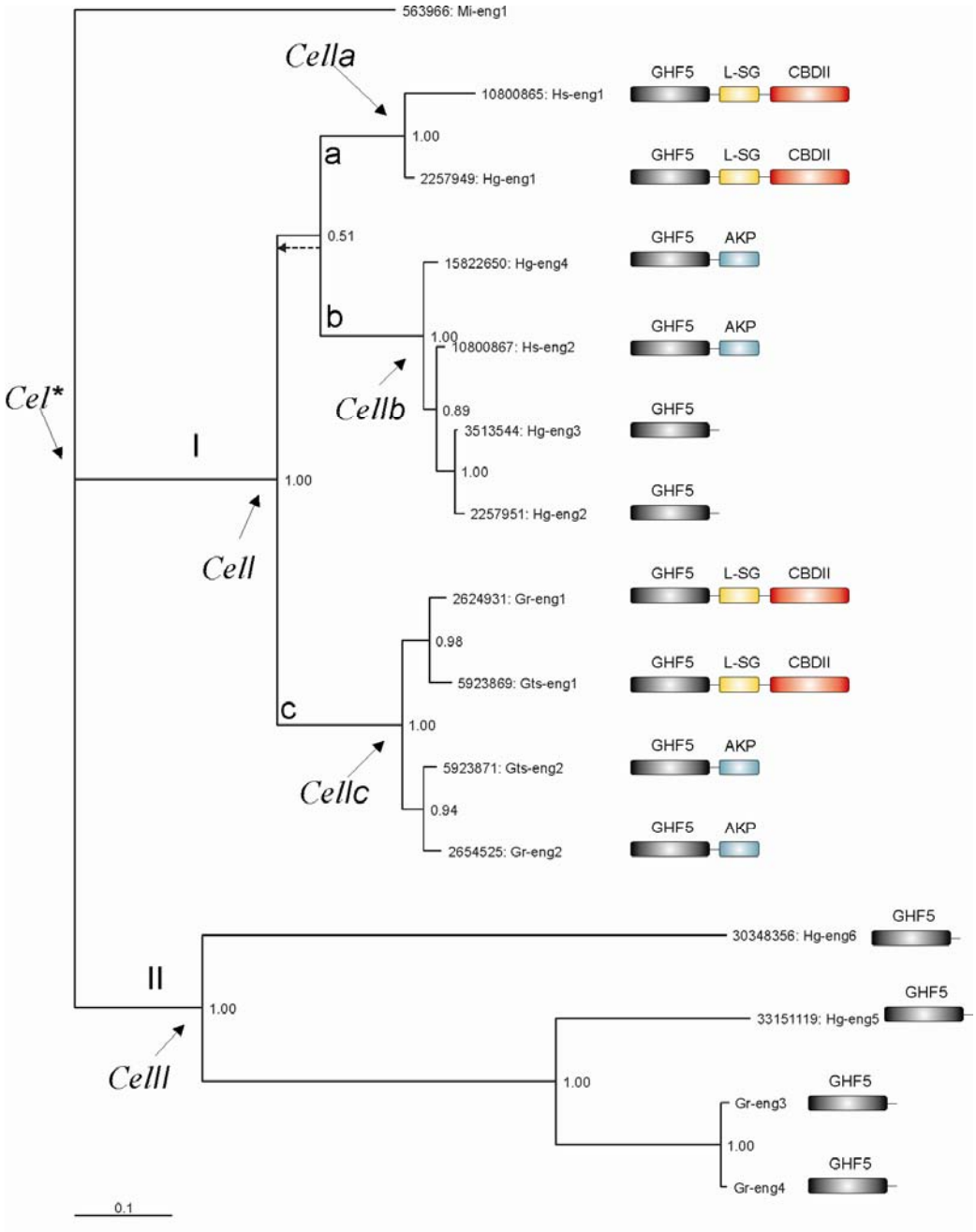
Figure 5. Infections of in vitro cultured potato roots with *G. rostochiensis* J2-s following *Gr-eng3/4* dsRNA treatment (**A**, **B**, and **C**) and control treatment (**D**) at 2 days post inoculation. The roots are stained with acid fuchsin to visualize the nematodes in red. Nematodes that achieved partial penetration are indicated with arrows. Brownish spots of damaged epidermis cells are indicated with an asterisk (*).

Ledger and coworkers (Ledger et al. 2006) proposed an evolutionary scheme for GHF5 cellulases in plant-parasitic nematodes, which suggests that the variants of nematode cellulases found to date arose through sequential loss of the ancillary C-terminal cellulose binding domain and linker domain. To test this 'sequential loss'-hypothesis we used all presently known cellulases from cyst nematodes to infer the evolutionary history of the GHF5 domain with a Bayesian analysis (Fig. 6). In the resulting tree the length of the horizontal branches reflects the accumulated changes in the GHF5 domain in cyst nematode cellulases since the last common ancestor genes (the nodes). Our analysis indicated that there are at least two distinct evolutionary lineages of cellulases in cyst nematodes (clades I

and II) originating from two distinct ancestor GHF5 genes (*CelI* and *CelII*). The sequence of descent of *CelI* and *CelII* from the 'Ur'-cellulase (*Cel**) cannot be reliably resolved with the information captured in currently available sequences. For each cellulase, we indicated in the tree the protein domain structure out of three possible variants found in nematodes so far, i) a GHF5 domain with a glycine/serine rich linker sequence and a type II cellulose binding domain (GHF5+L-SG+CBDII in Gr-ENG1), ii) a GHF5 domain with a so-called highly charged AKP motif (GHF5+AKP in Gr-ENG2) similar to linker histones H1 and H5 (Genbank accession AF107026 and PFAM accession PF00538; (Kasinsky et al. 2001)), and iii) a GHF5 domain alone (GHF5 in Gr-ENG3/4).

The sequential loss-hypothesis is more likely to be true when GHF5+L-SG+CBDII, GHF5+L-SG, and/or GHF5-variants appear in a single monophyletic clade with, ideally, the GHF5+L-SG+CBDII variant being most basal and the GHF5 variant as the most recent speciation. Cellulases in clade II, including Gr-ENG3, Gr-ENG4, Hg-ENG5, and Hg-ENG6, do not have ancillary domains and therefore cannot be used to test the hypothesis. Clade I comprises a polytomy of three groups of cyst nematode cellulases (subclades *a*, *b*, and *c*) for which it is not possible to determine the sequence of descent from *CelI*. But, within each of the subclades *a*, *b*, and *c* the inference of the sequence of descent is robust (posterior probabilities of 0.95 or higher) and can be used to test the hypothesis.

Figure 6. A rooted tree based on a Bayesian inference of phylogeny of fourteen GHF5 cellulases from four cyst nematodes species with significant similarity to Gr-ENG3 and Gr-ENG4. The numbers at the nodes indicate the posterior probabilities as calculated in MrBayes. The root-knot nematode GHF5 cellulase Mi-ENG1 was included in the analysis to root the resulting tree. The protein domain structure out of three possible variants is included for each of the taxa (GHF5, glycosyl hydrolase family 5; L-SG, linker region rich in serine and glycine residues; AKP, short highly charged stretch with similarity to linker histone 1 and 5-like AKP-motifs; CBDII, bacterial type 2 cellulose binding domain). The nematode cellulases are indicated with their protein names (Hs, *Heterodera schachtii*; Hg, *Heterodera glycines*; Gr, *Globodera rostochiensis*; Gts, *Globodera tabacum susp. solanacearum*; Mi, *Meloidogyne incognita*), and their Genbank identification numbers. Robust monophyletic clades (I and II) and inferred ancestor genes coding for GHF5 cellulase (*Cel*) are indicated along the branches and the nodes. The arrow points at the unresolved polytomy of three subclades (a, b, and c) within lineage I.


563966: Mi-eng1
Cella
10800865: Hs-eng1
GHF5 L-SG CBDII
1.00
a
2257949: Hg-eng1
GHF5 L-SG CBDII
0.51
15822650: Hg-eng4
GHF5 AKP
b
1.00
10800867: Hs-eng2
GHF5 AKP
Cellb
Cel*
0.89
3513544: Hg-eng3
GHF5
I
1.00
1.00
2257951: Hg-eng2
GHF5
CelI
2624931: Gr-eng1
GHF5 L-SG CBDII
0.98
5923869: Gts-eng1
GHF5 L-SG CBDII
c
1.00
GHF5 AKP
5923871: Gts-eng2
Cellc
0.94
GHF5 AKP
2654525: Gr-eng2
GHF5
30348356: Hg-eng6
II
1.00
GHF5
33151119: Hg-eng5
CelIII
1.00
GHF5
Gr-eng3
1.00
GHF5
Gr-eng4
0.1

The inferred phylogeny here indicates that none of these three subclades includes all three extant domain structure variants, i.e. the GHF5+L-SG+CBDII and its two possible C-terminal truncations. The GHF5+AKP variant clusters together with the GHF5+L-SG+CBDII variant within subclade *a*, while it also is in monophyly with the GHF5 variant in subclade *b*. The GHF5+LSG+CBDII variant is not found in the same subclade as the GHF5-variant, while it is in monophyly with the GHF5+AKP variant in subclade *c*. The proposed evolutionary scheme that lead to the 'sequential loss'-hypothesis assumes that the L-SG linker and the AKP-motifs are homologous. However, they are not significantly similar in sequence, and thus there is no justification to consider them as homologous traits. It should be further noted that the GHF5+L-SG variant has not been found in nematodes so far. Consequently, there is no support for the sequential loss-hypothesis in currently available data on cyst nematode cellulases.

Discussion

At the onset of parasitism infective nematodes penetrate the host plant to migrate towards the vascular cylinder to establish a permanent feeding site. Plant penetration results from the mechanical impact of stylet thrusts and chemical weakening of the cell wall by nematode proteins secreted through the stylet. The monoclonal antibody MGR048 recognizes at least three proteins in stylet secretions of the potato cyst nematode *G. rostochiensis* of which two were found to be cellulases in a previous study. In this paper, we report on the identification and functional analysis of two novel cellulase genes encoding the most abundant antigens recognized by MGR048 in the stylet secretions of *G. rostochiensis*. Remarkably, the overall identity between the four proteins that evidently have the epitope of MGR048 in common is only 42%.

The cellulases encoded by *Gr-eng1*, *Gr-eng2*, *Gr-eng3*, and *Gr-eng4*, as well as cellulases of other cyst nematodes, show significant similarity to the GHF5 cellulases from the symbiotic parabasalian protists *Spriotrichonympha leidy* (Genbank accession BAD90558 with BLASTP E-value $4*e^{-48}$). In lower termites two separate cellulolytic systems exist, one of endogenous termite origin and one from these eukaryotic protists living in the hindgut (e.g. *S. leidy*, *Holomastigotoides mirabile*; (Inoue et al. 2005)). The cellulases from the symbiotic protists belong to diverse GH families, including GHF5. The endogenous termite cellulases found to date are all classified as members of GHF9.

The four cellulase genes from *G. rostochiensis* (*Gr-eng1*, *Gr-eng2*, *Gr-eng3*, and *Gr-eng4*) are specifically expressed in the subventral esophageal glands of the nematodes. Several studies dating back to the 1980-s reported on the presence of intracellular ricketsia-like bacteria in these esophageal gland cells (Walsh et al. 1983), which suggests that they could contribute to the cellulolytic system of the nematodes in manner analogous to the situation in termites. However, the cellulase genes described here, and all others described from nematodes to date, show features typical of eukaryotic genes (e.g. poly-A tails and introns). To date there is no evidence that intracellular eukaryotic microbes inhabit the esophageal glands of cyst nematodes and we therefore conclude that these cellulases are of nematode origin.

Cyst nematodes are far more damaging to host tissues during migration than root-knot nematodes, suggesting that the celluloytic system in cyst nematodes may be less advanced rendering the nematode more dependent on physical impact of stylet thrusts (Hansen et al. 1996). However, as the cellulolytic system in cyst nematodes unfolds further with each new finding, it appears to be elaborate involving proteins with variety of domain configurations. The configurations known to date comprise a glycosyl hydrolase catalytic domain, a linker rich in serine and glycine, an AKP motif, a bacterial type II cellulose binding domain, and an expansin domain. The glycosyl hydrolase domains exhibit cellulase

activity on a variety of substrates, including cellulose and hemicellulose polymers (Gao et al. 2004). The L-SG linkers appear between GHF5 and CBDII, whereas the AKP motif is present at the C-terminus of GHF5 domains. The AKP motif, whose role remains to be determined experimentally, consists of between 25 and 43 amino acids and is found in Gr-ENG2, Gts-ENG2, Hs-ENG2, and Hg-ENG4, and shows strong similarity with linker histones H1 and H5 (e.g. Genbank accession AF107026). The highly charged AKP helix in linker histones is involved in protein-protein and protein-DNA interactions within aggregated nucleosomes (Kasinsky et al. 2001). It is therefore possible that the AKP-motif in cyst nematode cellulase is an evolutionary remnant that once linked the GHF5 domain to a type II cellulose binding domain. We believe that this C-terminal peptide sequence may serve to aggregate other nematode enzymes and proteins into a cellulolytic complex.

Bayesian inference of the phylogeny of cyst-nematode cellulases combined with the projection of domain structure onto the tree revealed a lack of correlation between the evolutionary history of the GHF5 domain and a sequential loss of the C-terminal CBDII domain and the L-SG linker. Support for the sequential loss-hypothesis would have been provided if the GHF5+L-SG+CBDII variants and the two possible truncates cluster together in a monophyletic clade. However, the theoretical intermediate GHF5+L-SG has not been found in plant-parasitic nematodes to date. The extant cellulase variant that resembles GHF5+L-SG the most includes a GHF5 and a C-terminal AKP-motif (e.g. Gr-ENG2). The linker L-SG and the highly charged C-terminal AKP-motif are not similar in sequence indicating that both domains are not homologous traits. Therefore, the GHF5+AKP variant is not a likely evolutionary intermediate between the GHF5+L-SG+CBD and the GHF5 variants as was suggested by Ledger et al. (Ledger et al. 2006). There is also no support for the proposal that the extant nematode cellulases with only a GHF5 domain originate, as an evolutionary innovation, from an extinct ancestor gene coding for a GHF5 domain and ancillary C-terminal linker (GHF5+L-SG). Nor did we find evidence that this ancestor gene derives from an ancient cellulase that combined a GHF5 domain, a linker and a CBDII (domain). In contrast, we believe that the extant nematode cellulases arose through reshuffling of relatively independent evolutionary units. The fact that highly similar cellulose binding domains occur in proteins with entirely different sequences and activities further supports this model (e.g. the CBDII in Gr-ENG1 and in the β-expansin Gr-EXP1 have 52% identity and 69% similarity).

To study the relative importance of *Gr-eng3* and *Gr-eng4* in the intracellular migration of *G. rostochiensis*, we monitored plant penetration and intracellular migration of infective juveniles in which the genes were knocked-down by RNA interference. Rosso et al. (Rosso et al. 2005) reported for *M. incognita* that RNA interference by soaking in dsRNA is transitory, and may last not longer than a few days. Recent studies, however,

suggest that the effects may last considerably longer in cyst nematodes (Bakhetia et al. 2007). For both cyst and root-knot nematodes soaking in a highly concentrated solution of dsRNA is clearly a valuable method for studying genes involved at the initial stages of parasitism in plants, and we have demonstrated this again in this study. Knocking-down expression of *Gr-eng3* and *Gr-eng4* in pre-parasitic nematodes reduced the penetration rate of plants by the nematodes by more than 50 percent. Chen et al. (Chen et al. 2005) observed some down regulation of *Gr-eng3/4* when they targeted *Gr-eng1* with RNAi, suggesting that in their experiment reduced levels of *Gr-eng3/4* could have affected nematode infectivity. We have seen no such off-target silencing effect on the expression of *Gr-eng1* and *Gr-eng2* in our experiments and therefore the reduced penetration observed here can be solely attributed to removal of *Gr-eng3* and *Gr-eng4*. Previous studies have shown that *Gr-eng1* and *Gr-eng2* (and their close homologs in *H. glycines* and *G. tabacum*) are secreted during the intracellular migration of the nematodes inside host plants. Our observations of the spots of damaged epidermis cells on host plant roots and the inability to achieve full penetration of dsRNA-treated nematode suggest that penetration of plants by cyst nematodes as well as migration within the roots requires activity of cellulases.

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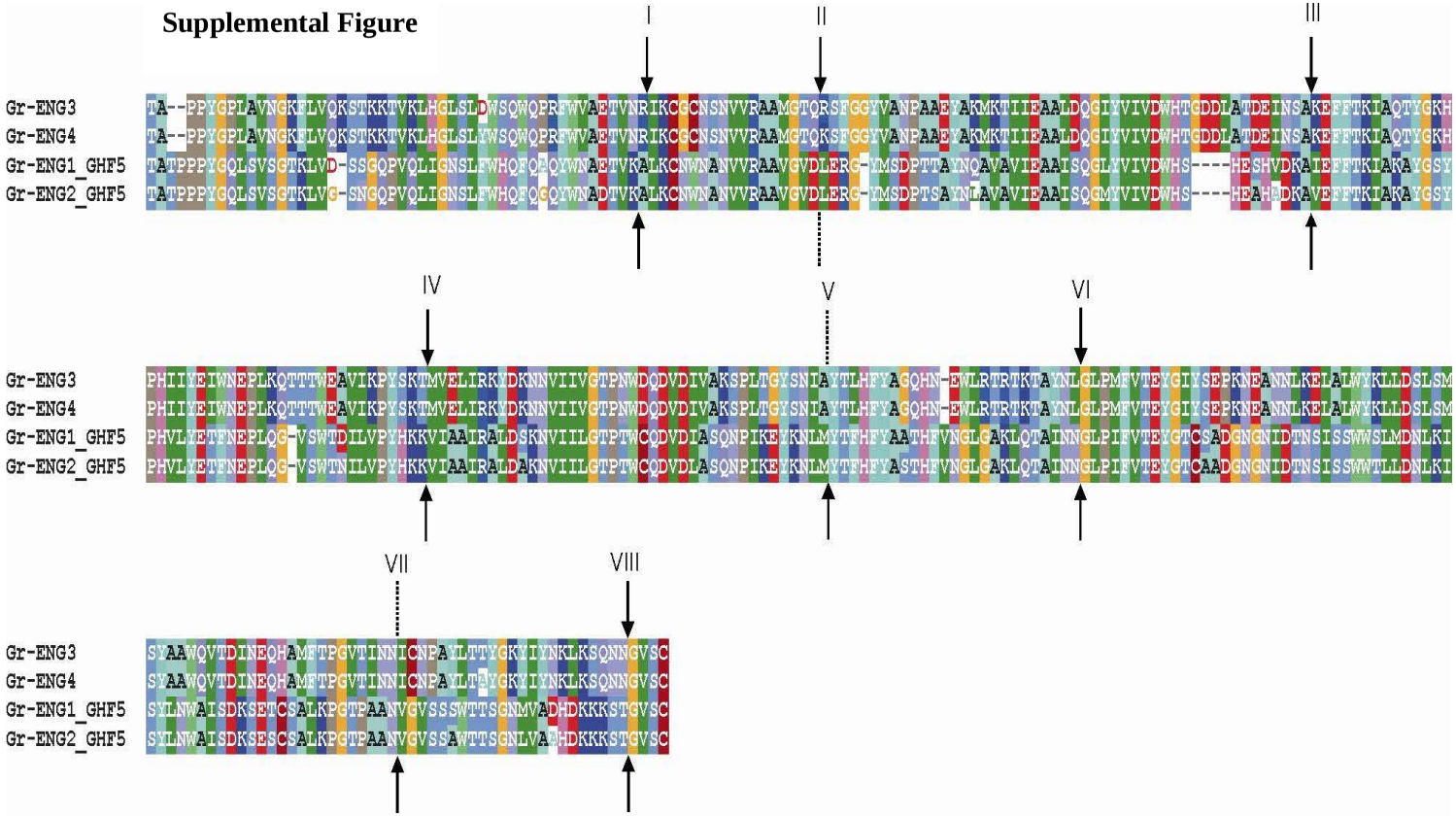

Supplemental Figure 1. A protein sequence alignment of the GHF5 domains in four cellulases of *G. rostochiensis* as generated in ClustalW. The colors indicate similarity between the sequences when using the GONNET matrix. Eight intron positions are indicated by arrows and capital roman-type characters. Unused intron positions are indicated with dotted lines in the alignment. Underlined are residues recognized as part of the signature of glycosyl hydrolase family 5 members.

Chapter 4

Overexpression of a nematode SPRYSEC-gene in potato promotes the virulence of a nematode, a fungus, and a virus

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Abstract

Esophageal gland secretions injected into host cells are believed to be important for plant parasitism of nematodes. We have identified a large family of secretory proteins in *Globodera rostochiensis* consisting only of a B30.2/SPRY domain. The genes coding for this family, named the SPRYSECs, are specifically expressed in the esophageal gland cell of parasitic nematodes. The SPRYSECs are part of the stylet secretions of the nematode suggesting that they may have a role as effector in plant-parasitism. The secondary structure of the SPRYSECs consists of highly conserved regions folding into beta-strands interspersed with stretches varying in sequence and in length. Mapping the sequence diversity among the SPRYSECs onto a three-dimensional structure model of the proteins indicates that most of the variation is in the extended loops that shape the so-called surface A in SPRY-domains. Furthermore, our analysis of the ratio of non-synonymous and synonymous substitutions shows that the majority of amino acids sites that are subjected to diversifying selection are in this same surface. Transgenic plants overexpressing one of the SPRYSECs are three to five times more susceptible to nematodes. A further challenge of these plants with the fungus *Verticillium dahliae* and the *tomato spotted wilt virus* (TSWV) showed a strong increase in susceptibility to these pathogens as well. Our data indicate that this SPRYSEC likely interferes with a host protein, which is essential for basal immunity to *V. dahliae*, TSWV, and *G. rostochiensis*.

Introduction

In the immune system of animals adaptive and innate immunity operate in concert to provide adequate protection against invading micro-organisms. During development of the adaptive immunity the system learns to recognize self-antigens so that it can differentiate in its response between invaders and self-tissue. In contrast to animals, plants only have an innate immune system, which constitutes a multi-layered defense system (Jones and Dangl 2006). The activation of the plant's innate immunity involves consecutive steps of disease recognition by the plant and suppression of disease signaling by the pathogen. In the first line of defense, so-called pathogen associated molecular patterns (PAMPs) are detected by the extracellular pattern recognition receptors. PAMP-triggered immunity (PTI) thus follows on extensive changes in transcription of hundreds of genes leading to cell-wall based defense responses, production of reactive oxygen species, and many other basal defense responses (Nurnberger et al. 2004).

There is an obvious strong selection pressure on the pathogens to jettison the PAMPs, but because of the high fitness costs involved in such innovations, pathogens have evolved an alternative strategy in which they use effector molecules to interfere with PAMP signaling in host cells. Recently it was shown that several bacterial effectors suppress basal innate immunity to promote virulence of the pathogen (De Torres et al. 2006; Hauck et al. 2003; He et al. 2006; Kim et al. 2005; Metz et al. 2005; Oh and Collmer 2005).

A second branch in the innate immunity of plants comprises highly variable NB-LRR proteins that recognize either pathogen effector molecules directly, or the changes induced to self-tissue by these effectors. This, so-called effector-triggered immunity (ETI), often leads to a local hypersensitive response-type of programmed cell death which blocks further pathogen ingress. Pathogens may evolve other effectors capable of suppressing ETI, and plants may on their turn recruit other NB-LRR proteins to guard other components in the ETI signaling pathways.

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 the 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.

Nematode secretions are believed to be important during the host invasion and the feeding stages of plant parasitism (Davis et al. 2000). Despite significant progress in the identification of genes coding for stylet secretions in nematodes during feeding, little is known about the molecular targets of these effectors (> 100) in host cells and the effect individual effectors have on the constitution of the recipient host cells. In this paper, we report the identification of a large gene family whose members code for small secretory proteins including only a B30.2/SPRY domain. We named this gene family the SPRYSECs and found that overexpression of one of its members in host plants promotes virulence of a nematode, a fungus, and a virus. These three pathogens have evolved entirely different styles in their parasitism, which is why we hypothesize that the SPRYSECs may intervene in a generic defense mechanism such as the PAMP-triggered immunity signaling in plants.

Materials and Methods

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.

cDNA-AFLP analysis and RT-PCR

Messenger RNA was extracted from five developmental stages of *G. rostochiensis* pathotype Ro1-Mierenbos, and cDNA-AFLP analysis was performed essentially as described previously (Qin et al. 2000). RNA was isolated from five developmental stages: (D=dormant) dehydrated, unhatched J2s in cysts (in diapause); (S=soaked) rehydrated, unhatched J2 in 1-year old cysts after exposure to tap water for two days; (H=hatched) pre-parasitic J2 (dry cysts were incubated in tap water for 1 week and three days in potato root difusate; (U= undifferentiated into J2) developing nematodes (mostly J1) in gravid females 2 months post inoculation; (P= prediapause) developing nematodes (J2) in gravid females 3 months post inoculation.

The primary cDNA templates synthesized from each of the five mRNA pools were digested using the restriction enzymes with KasI and TaqI, NcoI and AscI, or KasI and TaqI in separate reactions. For the specific amplification reactions, oligonucleotide primers annealing to the KasI and TaqI, NcoI and AscI, or KasI and TaqI adapter sequences were used in standard protocols (Table 1). Differentially expressed transcript-derived-fragments (TDFs) were excised from acrylamide gels. After re-amplification using the original primers, TDFs were cloned into TOPO-pCR4 (Invitrogen, Breda, The Netherlands) and transferred into *E. coli* TOP10 chemically competent cells (Invitrogen). After purification, inserts were sequenced using standard procedures.

Messenger RNA was extracted from five developmental stages of *G. rostochiensis* pathotype Ro1-Mierenbos as described above, and 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 30°C. Fragment of SPRYSECs were PCR amplified in 26 cycles with the primers with specific primers (Table 1), whereas a forward and reverse primer designed on CDPK were used to amplify a 91 bp fragment of the constitutively expressed cAMP dependent protein kinase (*Gr-CDPK*; Genbank accession BM343563). Reactions without reverse transcriptase were 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.

Table 1. The sequences of the oligonucleotide primers used for semi-quantitative RT-PCR (including the predicted amplicon size; see also Fig. 4), and for in situ hybridization microscopy.

SPRYSEC#	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
Semi-quantitative RT-PCR			
1	AACAGAGATTGGAAGAAGTGAAG	AAGATTCCTTAACCTTGTGACC	77
3	TCTATCCCCAAAACTCGCC	GCAAACTCTTCGGCGGGGGGC	132
4	AAGAATGGAGTGCTTTTAGAAAC	CTGTCCGGGCTTTTCATGCG	92
5	AGAAACTGAAAAACTTGAAAC	CTACTCGGTTTTCAGTTTCTC	67
8	GCACTGTGTTCGCTAAAGAG	TGGAAACATAACCTGATTCTGAAG	129
10	GGCAACAAAATTGAAGCGAAC	TCTAAACCCTCTACAGCAAGC	91
12	TGTTACATCGGTGGAAAGC	CAAATAAATTGGCGGTGTCC	124
14	AGCCTCGGTTTGGTGTCC	CGGCATTCATATCCAGAGTTTC	150
15	AATTTGACCGTAACAACATCATC	GACTCGGCGACTAACAGC	112
17	GAGCAGACGACGCCTTGG	CTCTCTCTCCTTTGGTGTTTTG	95
18	CAAAAGACGGCATTTTCTACTAC	GCGTAAGAGCCTTTGTCAC	124
19	TTGTACCCGTGCGTTTCG	ACCTCCACAGCAAATTCCTAC	135
20	TGTATCAGTACCATTCTCATCCG	ACCAGCACGCCTATTAGTTG	80
21	CGAAGGAACCTAGAAAGTG	CCAAAGAAATATGTTAAAACCC	151
22	GTCACGAGGTCAAGGGATG	TTACAATTTCTCCGTCCAGTGT	112
CDPK	ATCAGCCCATTCAAATCTACG	TTCTTCAGCAAGTCCTTCAAC	91
In situ hybridization microscopy			
3	TCTATCCCCAAAACTCGCC	GCAAACTCTTCGGCGGGGGGC	
4	ATGAAAAGCCCGGACAGAAATG	AAATAAAGGATCGTCTGTTCCTTCC	
5	AGAAACTGAAAAACTTGAAAC	CTACTCGGTTTTCAGTTTCTC	
10	CAAGGCTTTATCTATGCGC	TGAATTGGTAAAATGTTTG	
12	CTCGCGTCTGCTGCTGTTGTC	CATTTTTCGTGCAGTTTTTGG	
15	CATATTCCGCACGTGACGAG	TTTCTCGTCACGTGCAGCGG	
17	GAGCAGACGACGCCTTGG	CTCTCTCTCCTTTGGTGTTTTG	
19	GACTGCGTACCAATTCACAT	GATGAGTCCTGACCGACGAA	
20	GACTGCGTACCAATTCTG	GATGAGTCCTGACCGACG	
21	CGAAGGAACCTAGAAAGTG	CCAAAGAAATATGTTAAAACCC	
22	TCGAGCAGTACAGGAAGTAGACC	GGCGCCTATGAGCCCAGA	
SPRY	ACATCATCGGCTGCGGCGTCC	CGTGCCAAAATTCGCTTCAAT	

Cloning, sequencing, and analysis of SPRYSECs

The DNA sequences of TDFs coded A18, A41, E19, A30, A29, and KT12 (Genbank accessions AJ251757, AJ251758, BE607310and AJ536829) were used to search the EST database at the National Centre for Biotechnology Information to identify matching ESTs (Table 2). The library clones from which the EST originated (De Boer et al. 1998; Smant et al. 1998) 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 down stream with the rapid amplification of cDNA ends (RACE; Invitrogen, San Diego, USA). The contig assembly was done in Contig Express of the VectorNTI software package (Invitrogen). The assembly criterion was set at 99% identity in a minimal overlap 100 nucleotides. Initially, the assembly yielded 22 contigs, however, after further manual inspection contigs 2 and 17 were combined into a single contig.

In situ hybridization

DNA probes were amplified from the SPRYSECs by using specific oligonucleotide primers (Table 1) and digoxigen-11-dUTP. 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).

Antiserum and immunodetection

The domain coding for the B30.2/SPRY in SPRYSEC-19 (nt 55-645) was PCR-amplified as described above and cloned in pDEST-17 with Gateway cloning technology (Invitrogen, Carlsbad, CA, USA) to produce 6xHis tagged-protein in BL21-S1 (Invitrogen) cells when induced with 0.3M NaCl at 37 ^{o}C for 16-hours. Recombinant SPRYSEC was purified on Ni-NTA spin columns following the manufacturer's instructions (QIAGEN). Hens were immunized with purified protein and the chicken immunoglobulin IgY was isolated and purified as described by (Kudla et al. 2005). In order to raise specific antibodies to SPRYSEC-family members, two antigenic peptides (IGENSKHRSVRAKLPC [in SPRYSEC-9, -15, and -18] and HWGNERPYIDGQPKFD [in all] were used to immunize rabbits (Eurogentec).

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).

PAML

Six sequences were tested for positive selection, including SPRYSEC-4, -9, -15, -16, -18, and -19. 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. M7 and M8 are nested models, so they can be compared using a likelihood ratio test (LRT) which is generally robust to the assumed distribution of ω over sites. 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.

Next, positive selection was tested for by studying variation among sites identifying amino acids under diversifying selection. This variation is tested with an additional LR test between M7 and M8 (Yang and Nielsen 2000) using the empirical Bayes theorem as implemented in PAML to calculate the posterior probability that a particular amino acid belongs to a particular class (neutral, negative or positive). A particular site that belongs to the class ω>1 with a posterior probability > 95% is most likely under positive selection. This approach makes it possible to detect positive selection and identify sites under positive selection even if the average ω ratio over all sites is less than 1. Meanwhile, for this type of study it is important to note three test characteristics. First, detection of positive selection requires significant differences between M7 and M8 and estimates of ratio that exceed 1. Second, under M8 it is possible to estimate the proportion of sites that are under positive selection, and this proportion is denoted P1. Third, the application of these models requires a topological, or phylogenetic, assumption. For each sequence group, PAML analyses were applied using the M0 generated phylogenetic tree. The amino acid sequence alignment was executed by 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. Pal2nal is a program that converts a multiple sequence alignment of proteins and the corresponding DNA (or mRNA) sequences into a codon-based DNA (nucleotide) alignment. The results were directly used by PAML.

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). Searching the InterPro databases indicated the presence of an SPRY domain within the SPRYSECs sequences (2.9e-18 score with Pfam PF00622 and 1e-21 with SMART SM00449). 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. 2006)), 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). The level of identity of the best matching SPRYSEC-19 with the templates was ~12.1%, while the similarity is ~37.1%. 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

In planta overexpression of SPRYSECs

The coding sequence of SPRYSec without signal peptide (nt 57-745) was amplified as described earlier using primers (A18ns-NcoI: GGCGCG**CCATGG**TGCCGCCAAAAACAA and A18-BglII: GTCGAC**AGATCT** TTGATCGACGAAGAAAAAC) and cloned in pRAP33 using restriction sites (NcoI and BglII) added as overhangs on primer sequences. Expression cassette containing CaMV 35S promoter, SPRYSEC-19 and Tnos was excised from pRAP33 using AscI and PacI followed by cloning in pBINPLUS (Van Engelen et al. 1995) followed by transformation in *Agrobacterium tumefaciens* strain LBA4404 (Shen and Forde 1989). Potato Line V *(Solanum tuberosum)* was transformed by using *A. tumefaciens* mediated plant transformation (Horsch et al. 1986). The explants were maintained on MS20 media supplemented with 100 mg/l kanamycin and 300mg/l of cefatoxime (Duchefa). To determine the expression of SPRYSEC-19 in stable transgenic potato lines, RT-PCR was done. Total RNA was isolated from transgenic lines by using Trizol reagent (Invitrogen) with subsequent cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) following manufacturer's protocol.

Gene silencing by plant delivered dsRNA

Plants expression dsRNA hairpin constructs of SPRYSEC-19 and -15 were generated using the GATEWAY cloning system (Invitrogen) with subsequent cloning into the vectors pDONR207 and pK7GWIWG2 (Karimi et al. 2002). Gene fragments were PCR amplified from SPRYSEC A18 using the primers (B1-A18:5`GGGGACAAGTTTGTACAAAAAAGCAGGCTCC**ATG**CCGCCGCCAAAAACAAA3`; B2-A18:5`GGGGACCACTTTGTACAAGAAAGCTGGGTATGGGCCAAAGTTCGCTTCAAT3`). Each of the primers included the attB sites for the BP reaction in order to create an entry clone in pDONR207 according to the manufacturer's protocol. The entry clones were sequenced and subsequently used for a recombination reaction of the insert with LR Clonase II into the destination vector pK7GWIWG2. Construction into pK7GWIWG2 aimed at a double insertion of the amplicon in reverse orientation such that a dsRNA molecule will arise following expression in the plant. All constructs were made in *E. coli* TOP10. For plant transformation the destination vectors with the SPRYSEC amplicons were introduced into *Agrobacterium tumefaciens* LB4404, which was selected on gentamycin, rifampicin, and spectinomycin medium. Potato Line V *(Solanum tuberosum)* was subsequently transformed by using *A. tumefaciens* (Horsch et al. 1986).

Pathogen infection assays

The pre-parasitic J2s of *Globodera rostochiensis* pathotype Ro1 Mierenbos were hatched and isolated from dried cysts soaked in potato root diffusate (De Boer et al. 1996), and surface sterilized prior to inoculation (Goverse et al. 1999). For the nematode infection assay, inter-nodal cuttings were taken from *Solanum tuberosum* LineV, plants transformed with empty vector, SPRYSEC-19, and SPRYSEC-15 and were grown in sterile culture in 12 cm^2 plates containing MS20 media. For each treatment, 10-plates with 4-internodal cuttings on each plate were inoculated with an average of 172 nematodes. The percentage of well-developed females was determined after 7-weeks post inoculation based on number of developed females relative to number of nematodes inoculated. The statistical analysis of the data was done using the Student's *t*-Test for two samples assuming un-equal variances.

Infection assay with Verticillium dahliae and TSWV

For the infection assay with TSWV, inter-nodal cuttings from the transgenic plants of *Solanum tuberosum* LineV were grown in sterile pots containing MS20 media for two weeks. To acquire the virus inoculum, two fresh leaves of *Nicotiana benthamina,* infected with TSWV isolate BR-01, were ground with sterile mortar and pestle in 3 ml of 1x PBS buffer (de Ávila et al. 1992). Leaves of 2-weeks old *in vitro* grown plantlets were sprinkled with carborundum powder followed by brushing the surface of leaves with a sponge soaked in crude sap extracted from TSWV infected leaves. The plants were monitored for symptoms for 3 weeks. For the fungal infection assay, *V. dahliae* strain 5368 was grown in Petri-dishes containing 4% potato dextrose media (Duchefa) at 28^oC for 2 weeks. The spores were scrapped from agar plates with scapula and transferred into 50 ml of sterile de-ionized water, followed by centrifugation at 4000 rpm at room temperature. The pellet was resuspended in de-ionized water to prepare spore suspension to a concentration of 1 x 10^6/ml. The roots of three-weeks old *in vitro* grown plantlets of transgenic plants of *Solanum tuberosum* LineV were soaked in spore suspension for three minutes and transferred to pots in a green house. The symptoms were scored at 20 day post inoculation.

Results

Identification of the SPRYSEC gene family

A comparison of gene expression patterns in five distinct developmental stages of *G. rostochiensis* using cDNA-AFLP with the enzyme combinations of *Eco*RI and *Taq*I, *Kas*I and *Taq*I, and *Nco*I and *Asc*I resulted in the display of 16,500 TDFs of which 216 were solely or predominantly expressed in potato root diffusate-exposed J2s (H-stage) and water re-hydrated J2s (S-stage). These TDFs, which were strongly and specifically up-regulated in pre-parasitic juveniles exposed to potato root diffusate (Fig. 1), were excised from the gel, cloned and sequenced. Sequence database searches with the sequences of these seven TDFs revealed significant similarity to human *Ran Binding Proteins in the Microtubule Organizing Centre* (RanBPM or RanBP9; Genbank accessions EAW55354 and AAI21177).

To resolve the full-length cDNAs from which the TDFs originated, we first mined approximately 10,000 ESTs generated from the same developmental nematode stages using the TDF sequences as queries in the BLASTN algorithm. A total of 30 matching ESTs were found with varying degrees of similarity to the TDF sequences. The cDNA library clones from which the matching ESTs had been generated were re-sequenced from both ends to resolve the full insert sequences. For some sequences that we still suspected to be partial, gene specific primers were designed to extent the sequences further at the 5'- and/or 3'-ends with RACE. The TDFs, the RACE fragments, and the full library insert sequences were assembled into contigs, thus resulting in 22 sequence contigs (Table 2).

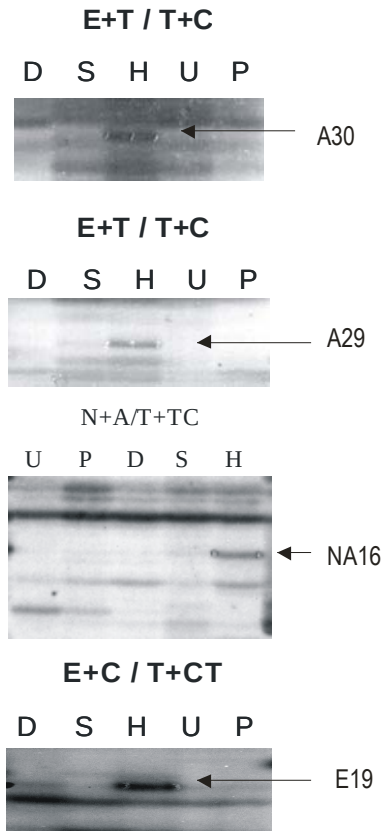


Figure 1. cDNA-AFLP expression profiles of A20, A29, NA16 and E19 from potato cyst nematode used to identify seven members of the SPRYSEC gene family in successive stages of nematode development. The arrow pointed bands were cut out, cloned and sequenced. On the basis of sequence results, the primers E and T were extended with one or two additional selective nucleotides. The letters D, S, H, U and P refer to the different developmental stages. D: unhatched J2 in diapause, S: unhatched J2 after diapause, rehydrated for 2 days on water, H: freshly hatched J2 in potato root diffusate, U: developing nematodes (J1) in gravid females two months post-inoculation, P: developing nematodes (J2) in gravid females three months post-inoculation.

Table 2. ESTs and corresponding library clones matching with the transcript derived fragments A18, A41, E19, A29, A30, NA16, and KT12.

EST (Genbank accession number)	Clone number	Identical to TDF	Assembled SPRYSEC No.
BE607309	rr43h11.y1		6
BE607310	cGE2075		4
BM343244	rr36b03.y2		9
BM343285	rr36f05.y2		12
BM343498	rr39c05.y1	E19	18
BM343589	rr40d08.y1		17
BM343590	rr40d09.y1		16
BM343869	rr43h11.y1		5
BM344069	rr46c12.y1		18
BM344163	rr47e07.y1		1
BM344199	rr48a04.y1		18
BM344321	rr49d12.y1		8
BM344614	rr57a05.y1	A41	15
BM344784	rr59c02.y1		13
BM345554	rr65b10.y1		10
BM345592	rr65f06.y1		14
BM345742	rr07e04.y1		3
BM345924	rr09g10.y1		8
BM354257	rr14d06.y1		14
BM354706	rr15h02.y1		18
BM354784	rr16h04.y1		11
BM355470	rr25d09.y1		17
BM355497	rr25g03.y1	NA16	7
BM355633	rr27f01.y1		5
BM355689	rr28c10.y1		18
BM355763	rr29c02.y1		13
BM356075	rr33c05.y1		5
BM356126	rr33h12.y1		1
GE1519	-		18
GE1156	-		6
AJ251757	-	A18	19
AJ251758	-	A29	20
BE607310	-	A30	21
AJ536829	-	KT12	22

Sequence characterization of the SPRYSEC gene family

Computational translations of 17 consensus sequences revealed large open reading frames ranging from 203 to 280 amino acids coding for proteins with molecular masses ranging from 23.8 to 31.2 kiloDaltons (see Table 3 for an overview). Our analysis indicated that four contigs (SPRYSEC-3, 7, 13, and 21) are still likely representing partial cDNA sequences. The open reading frames were run through the SignalP-prediction software to determine the destiny of the encoded protein in the cellular protein-sorting pathway of the nematode cells. For 13 consensus sequences the software predicted the presence of an N-terminal signal peptide for secretion. To discriminate transmembrane from secreted proteins, the encoded mature proteins were also screened for transmembrane helices with

the TMHMM algorithm. However, no transmembrane helices were predicted for any of the sequences starting with a signal-peptide for secretion. Thus, thirteen contigs code for proteins that are likely to be secreted by the nematode.

Table 3. Summary of the SPRYSEC gene family with features of the coding sequence

Name (SPRYSEC-x)	cDNA (in bp)[1]	ORF (in aa)[2]	SP[3]	B30.2[4]	SPRY[5]
X=1	1016	276	1-25	60-121	124-267
3	1920	-	-	-	-
4	922	232	1-24	18-228	93-227
5	969	250	1-23	19-232	103-231
6	1051	280	1-23	61-267	138-266
7	543	-	-	-	-
8	847	215	1-22	16-207	78-206
9	805	224	1-24	30-214	92-213
10	1014	256	1-25	35-243	98-242
11	880	-	-	-	-
12	818	246	No	41-231	101-230
13	639	177	-	-	-
14	939	226	1-28	8-218	73-217
15	904	261	1-24	41-238	105-237
16	856	218	1-17	23-212	85-211
17	1090	275	-	-	-
18	804	224	1-24	-	-
19	857	216	1-17	25-216	87-216
20	801	267	No	65-260	129-259
21	670	108	No	-	-
22	924	262	1-23	45-251	121-250

[1] transcript length in base pairs
2 size of largest open reading frame in amino acids
3 position of the signal peptide for secretion (if present according to SignalP)
4 position of the B30.2 domain (if present according to INTERPRO Scan: IPR001870)
5 position of the SPRY domain (if present according to INTERPRO Scan: IPR003877)

A comparison of the consensus sequences with other sequences in the non-redundant protein database resulted in significant matches with human RAN binding protein 9 and 10 (E-values < e^{-10} 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 (smart00449), a Lissencephaly type-1-like homology motif (LisH motif; smart00667) and a conserved stretched known as C-Terminal to LisH motif (CTLH; smart00668). Human RAN-binding protein 9 includes a C-terminal to CT11-RanBPM domain, which is absent in the human RAN-binding protein 10 (CRA; smart00757). There is only one matching putative RAN-binding protein from *C. elegans* (Genbank accession CAA21656) including 622 amino acids with the same domain structure as human RAN binding protein 10, and which showed similarity (from 5 to 24 % identity in the SPRY domain) with our contig consensus sequences. This putative RanBPM from *C. elegans* is the best match of nematode origin for the SPRYSECs in the database. The sequence similarities between the predicted proteins from the contigs and the RAN binding proteins are confined to the SPRY domain of approximately 120 amino acids (Fig. 2). A further search in the Interpro-database using the contig consensus sequences indicated that the SPRY domain is part of a larger structural domain named B30.2 (SPRY)-like consisting of 210 amino acids. We thus named the consensus sequences of the contigs SPRYSECs, because of their high similarity with SPRY domains and their predicted secretion.

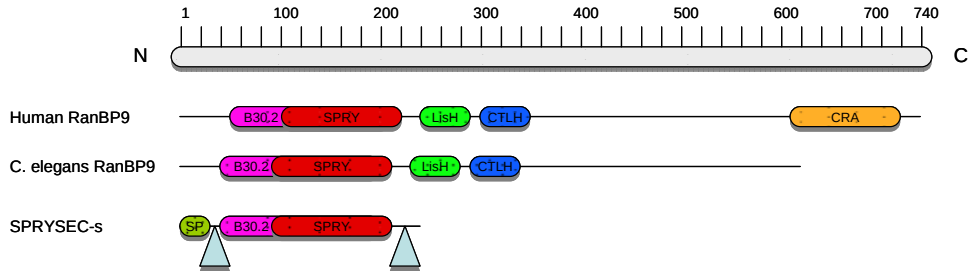
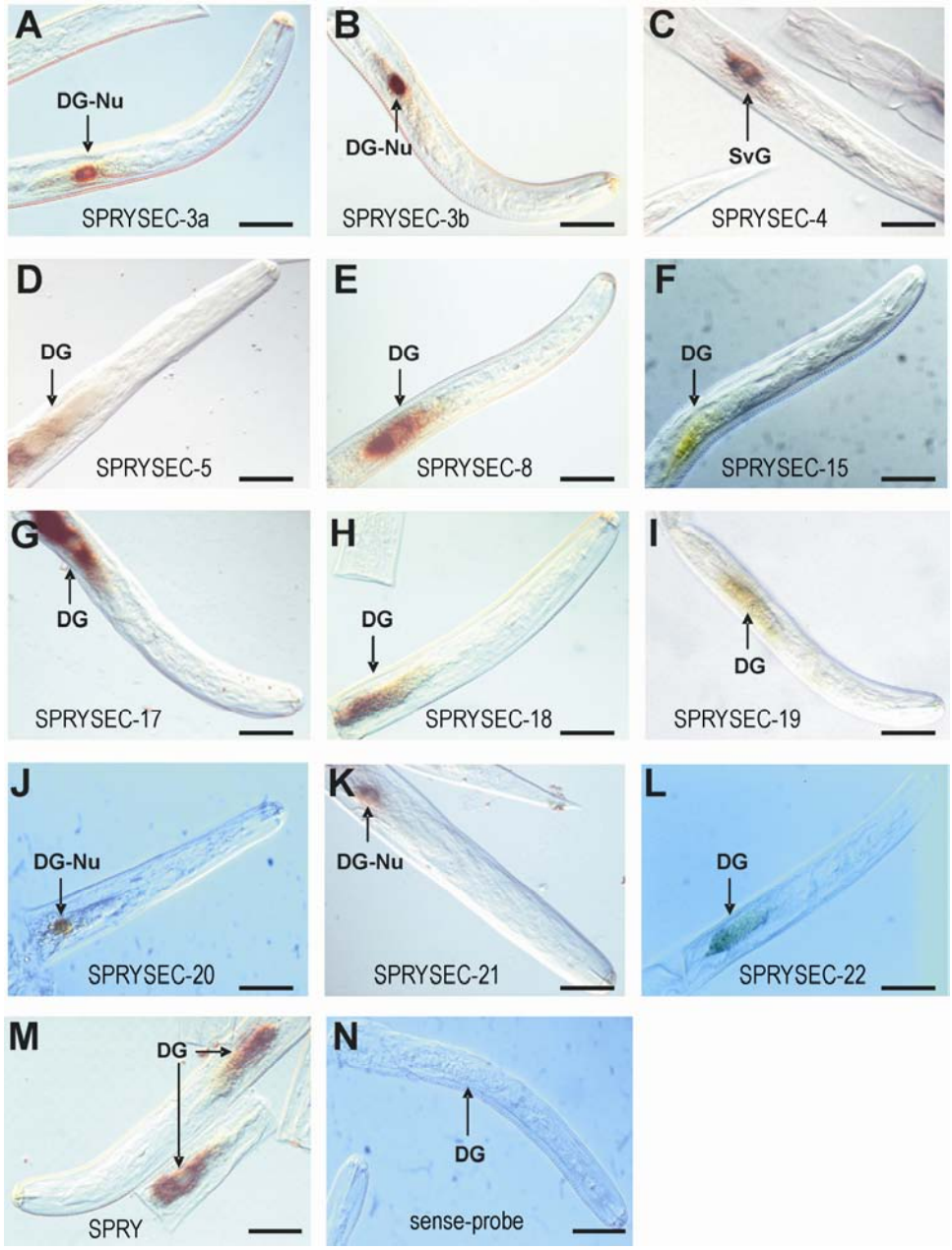


Figure 2. A comparison of the domain structure of the SPRYSEC gene family members and two of the best matching proteins in non-redundant database in a BLASTP search algorithm. The domains are indicated as they are identified in the InterPro database. B30.2 refers to IPR001870 accession that contains accession IPR003877, which is described as SPLa/RYanodine receptor SPRY. LisH (IPR013720) refers to *lis h*omology that contains a Lissencephaly type-1-like homology motif. The C-terminal to LisH (CTLH) motif is a predicted alpha-helical sequence of unknown function that is found adjacent to the LisH motif in a number of proteins. The predicted signal peptide for secretion in the SPRYSEC family members is indicated with SP. The open triangles indicate the regions in the SPRYSEC family with most sequence length variations.

Transcription of SPRYSEC gene family members localizes to the esophageal glands

Secretions produced in the esophageal glands of nematodes have been shown to be important for parasitism on plants. An important criterion in our strategy to identify genes involved in nematode-plant interactions, therefore, is a specific expression in these glands. To this purpose, antisense cDNA probes were designed on the consensus sequences of ten SPRYSECs for in situ hybridization microscopy on preparasitic J2's. For eleven SPRYSECs the similarity with other contigs was too high to make specific probes. Probes designed on nine SPRYSECs specifically hybridized to the dorsal esophageal gland cell. The probe designed on SPRYSEC-4 hybridized to the subventral esophageal glands. The hybridization patterns differ in intensity from a weak (Fig. 3 F, I, and L) to very strong signal (Fig. 3A and G), and in subcellular localization between (peri)nuclear (Fig. 3B, C, J, and K) and whole cell (Fig. 3 D, E, G, H, I, and L). We also designed a probe on a conserved stretch present in most of the SPRYSECs, to test for the expression of homologous transcripts in other tissues in the nematode. However, no hybridization of this conserved-sequence probe was observed in tissues other than in the dorsal esophageal gland cell (Fig. 3M). Also, for each of the antisense probes we tested the corresponding sense probes of which none resulted in a specific hybridization of tissues in whole mount nematode sections (e.g. Fig. 3N).

Figure 3. Whole mount *in situ* hybridization of the SPRY gene family members in infective preparasitic second stage juveniles of *G. rostochiensis*. The sequence divergence within the gene family allowed for the synthesis of twelve discriminative cDNA probes (A to L) and one probe designed on the most conserved part of the SPRY domain (M). Three different hybridization patterns were observed with the family member-specific probes, including a specific hybridization in the nucleus (A, B, J, and K) and a perinuclear hybridization in the lobe (D, E, F, G, H, I, and L) of the dorsal esophageal gland, and a subventral esophageal gland specific hybridization (C). The probe designed on the conserved part of the SPRY domain in the SPRY gene family (M) resulted in a strong dorsal esophageal gland lobe specific hybridization. Hybridization of this latter probe was not observed in any other tissues in the nematode. Similarly, the sense probes for each of the probes above did not result in hybridization in nematode tissues either (e.g. N).


A
DG-Nu
SPRYSEC-3a
B
DG-Nu
SPRYSEC-3b
C
SvG
SPRYSEC-4
D
DG
SPRYSEC-5
E
DG
SPRYSEC-8
F
DG
SPRYSEC-15
G
DG
SPRYSEC-17
H
DG
SPRYSEC-18
I
DG
SPRYSEC-19
J
DG-Nu
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M
DG
SPRY
N
DG
sense-probe

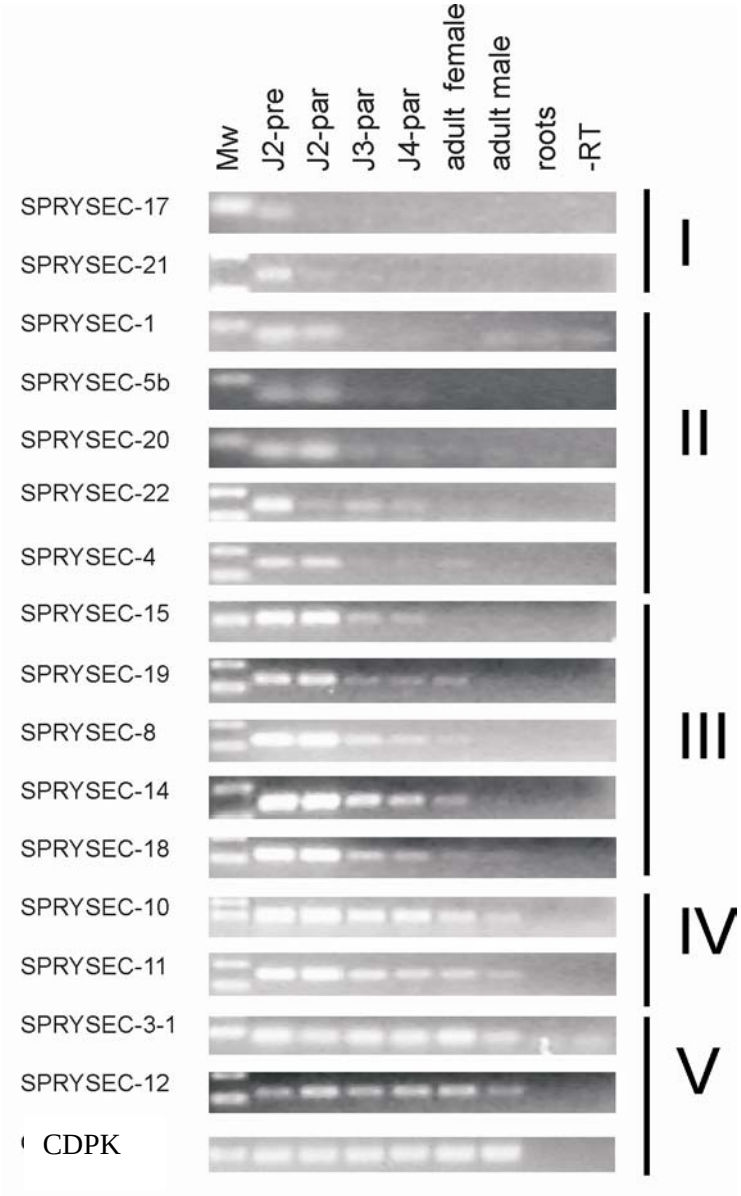

Figure 4. Semi-quantitative reverse transcriptase-PCR of sixteen members of the SPRYSEC gene family revealing five main expression patterns (I, II, III, IV, and V) throughout the parasitic cycle of the nematodes. The expression of the gene family members was assessed relative to the expression of the constitutively expressed CDPK (cAMP dependant protein kinase).

SPRYSECs are differentially expressed throughout the parasitic cycle of the nematode

The contigs were assembled from transcripts expressed in the preparasitic second juvenile stage of *G. rostochiensis*; just prior to penetration of a host plant. Reverse-transcriptase PCR was conducted to investigate the expression of the corresponding genes in successive parasitic stages isolated from root tissues of nematode-infected host plants. Reactions with uninfected root tissue and reactions without the reverse transcriptase enzyme were included as controls. The CDPK (cAMP dependant protein kinase) gene from *G. rostochiensis* (*Gr-CDPK*) was used as an indicator for a constitutive expression throughout the development of the nematode (Fig. 4). The gene expression patterns observed repeatedly in this experiment fall essentially into five distinct profiles. Two genes are expressed in the pre-parasitic J2 stage only (I in Fig. 4). In the second profile expression is high in pre-parasitic and parasitic J2s with a quick decline in the J3 stage. The third expression profile includes strong expression in pre-parasitic and parasitic J2 stage, after which the expression declines to detectable but significantly lower levels in J3, J4 and adult females (profile III in Fig. 4). The fourth observable expression profile also comprises the strongest expression in the early parasitic stages followed by a decline during further development of the nematode, but now including adult males too (IV in Fig. 4). A constitutive expression similar to that of the CDPK gene represents the fifth distinct profile (V in Fig. 4) detected within this gene family.

The SPRYSEC gene family members code for nematode effectors

The computer predictions indicated that a majority of the products of the SPRYSEC gene family members is likely to be secreted by the dorsal esophageal gland. Secretory proteins produced in the dorsal esophageal gland have been shown in the stylet secretions of *M. javanica* (Doyle and Lambert, 2003). We have raised specific antibodies to SPRYSEC-family members to test if the encoded proteins end up in the stylet secretions of the nematode. To this purpose, two antigenic peptides (IGENSKHRSVRAKLPC [in SPRYSEC-9, -15, and -18] and HWGNERPYIDGQPKFD [in all] were used for the immunization of rabbits. The polyclonal antiserum raised to the peptides recognized three bands on western blots (25, 32 and 37 kDa respectively; Fig. 5A) 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 nematodes were subsequently collected in vitro and tested on

a native dot blot (Fig. 5B). The antisera raised to the synthetic peptides detected SPRYSEC-family members in the 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 the nematode was used a positive control, whereas an antibody to muscle proteins of the nematodes was tested as a control for protein contaminations from damaged nematodes.

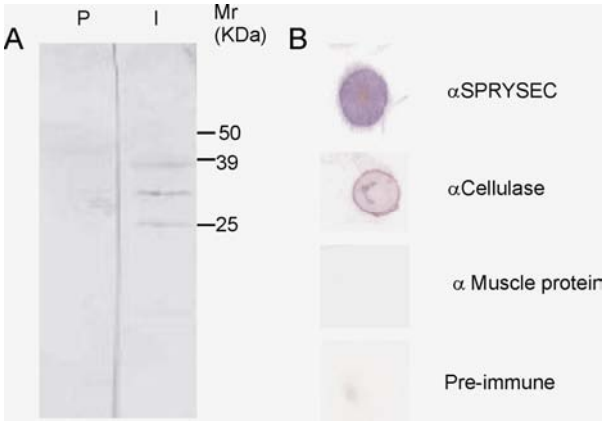

Figure 5. Immunodetection of SPRYSEC family members in nematode homogenates (A) and collected stylet secretions (B) using the antisera raised to two family specific synthetic peptides (see alignment in Fig. 7). The antiserum specifically recognizes three protein bands with molecular masses between 25 and 39 kDa (I), which are not detected in nematode homogenates with the pre-immune serum from the same rabbit (P). The same antiserum shows specific binding to collect stylet secretions of the infective juvenile. Samples probed with MGR048 that recognizes cellulases in stylet secretions also reacted positively, whereas the muscle protein specific antibody MGR007 indicated the absence of potential leakage from damaged nematodes during the procedure.

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

A protein alignment of the SPRY family members shows an uneven distribution of the sequence similarities between family members. Regions with nearly perfect sequence conservation are interspersed with regions that are highly diverged (Fig. 6). 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. 2006), 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 antiparallel 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 gene family members localizes to specific elements in protein folds predicted by structure homology modeling using the resolved B30.2/SPRY structures as template.

Among the gene family members, SPRYSEC-19 shows the highest level of similarity with any of the three possible templates (~12.1% identity and ~37.1% similarity), and consequently remote homology modeling had to be used. The approach was to transfer the coordinates along the stretches that constitute the β-sandwich core and to generate *ab initio* the loops between the β structures by repeated rounds of simulated annealing and minimization. Usually, in a crystal the protein adopts the most favored conformation, so the NMR structure determined for SSB-2 was discarded as a template. From the remaining two crystal structures, GUSTAVUS shows the highest similarity with SPRYSEC-19 and the lowest level of insertions or deletions along the SPRY region and was therefore retained as a modeling template. Even if these levels qualify the templates only for remote homology modeling, the match of the predicted secondary structure of SPRYSEC-19 and the secondary structure patterns of template is very well indicating a high level of the model accuracy. Similarity analysis of SPRYSEC-19 shows that the similarity is clearly clustered within the β stretches that form the core β sandwich while it diminishes drastically along the loops connecting these secondary structure elements.

The 3D protein structure model of SPRYSEC-19 was subsequently used to build a consensus structure model of the best matching family members (Fig. 7). From the original set of fourteen sequences, seven were eliminated in consensus model building due to large gaps/insertions that bias the meaningful information in the 3D representation. The remaining sequences SPRYSEC-4, -9, -18, -15, -16, -19, and -12 were included in the consensus 3D model based on the structural information of SPRYSEC-19. As can be readily seen, only two regions of the surface show exceptionally high variability (Fig. 7). 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 α3 structure (annotation of (Woo et al. 2006).

Positively selected sites in the SPRYSEC gene family partially localize to the protein surfaces

For genes in the secretome of plant-parasitic nematodes the SPRYSEC-gene family is unusually large suggesting that strong selection pressures may act on these genes. Codon alignments build 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. We statistically assessed the significance of the dN/dS ratio > 1 per site under five different evolutionary models (M0, M1, M3, M7, and M8). For twelve sites in the alignment we found significant (P>0.95) dN/dS-ratios > 1 (i.e. positions: 125, 172, 185, 186, 205, 206, 217, 219, 280, 286, 311, and 324; Fig. 7). Eight out of twelve sites that have been subjected to diversifying selection localize to the predicted surface A in the 3D-structure model of the

SPRYSECs. Most of the insertion/deletions within the protein family occur in the loops between the β-sheets that form the core of the fold of the protein. Because of the absence of proper codon alignments in these regions the current number of positively selected sites is likely to be an underestimation.

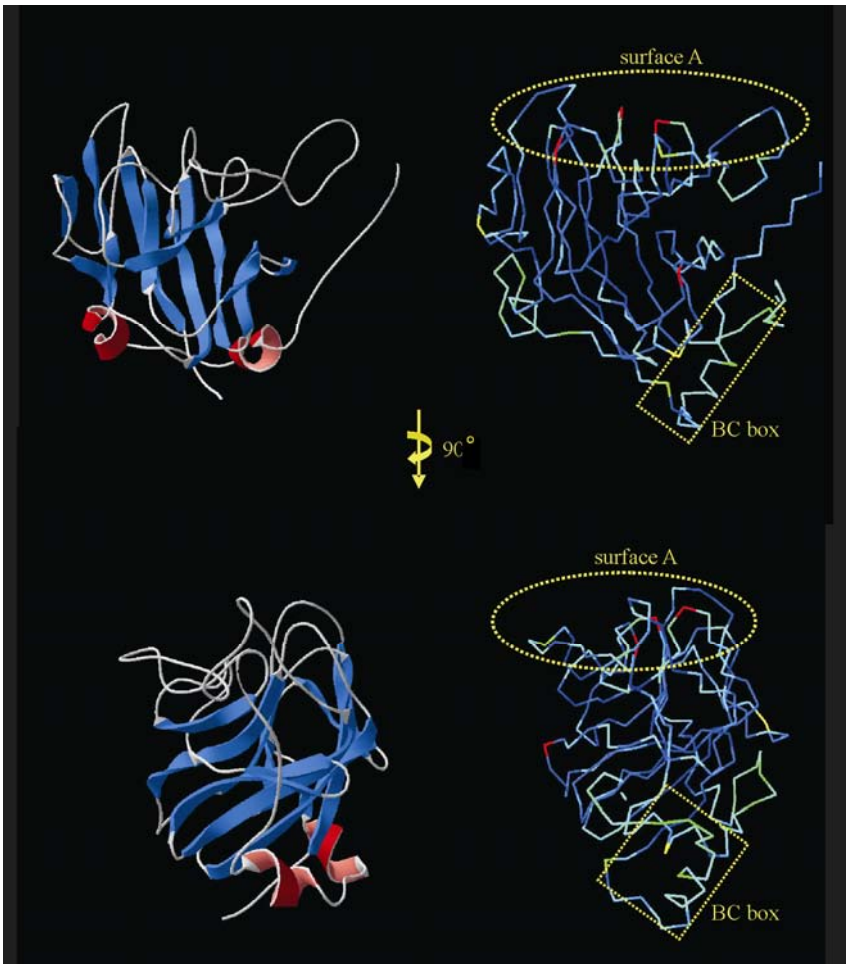

Figure 6. Mapping of the protein sequence diversity onto the consensus 3D structure model built on seven members the SPRYSEC gene family. Based on the consensus sequences of seven most similar family members, a consensus (chimerical) model was built by homology modeling. The variability at a given position was defined as the average of the Blosum62 substitution matrix values between family members and the consensus. The values are mapped onto the 3D model as colors from blue (high similarity) to red (low similarity) in the same manner the b-factor (temperature factor) of a structure are represented. As can be readily seen, only two regions of the surface show increased variability. A highly variable area is located in the surface-A region using the annotation of Woo et al (2006), and a second region of moderate variability is located at the BC box, corresponding to α3 structure using the same annotation.

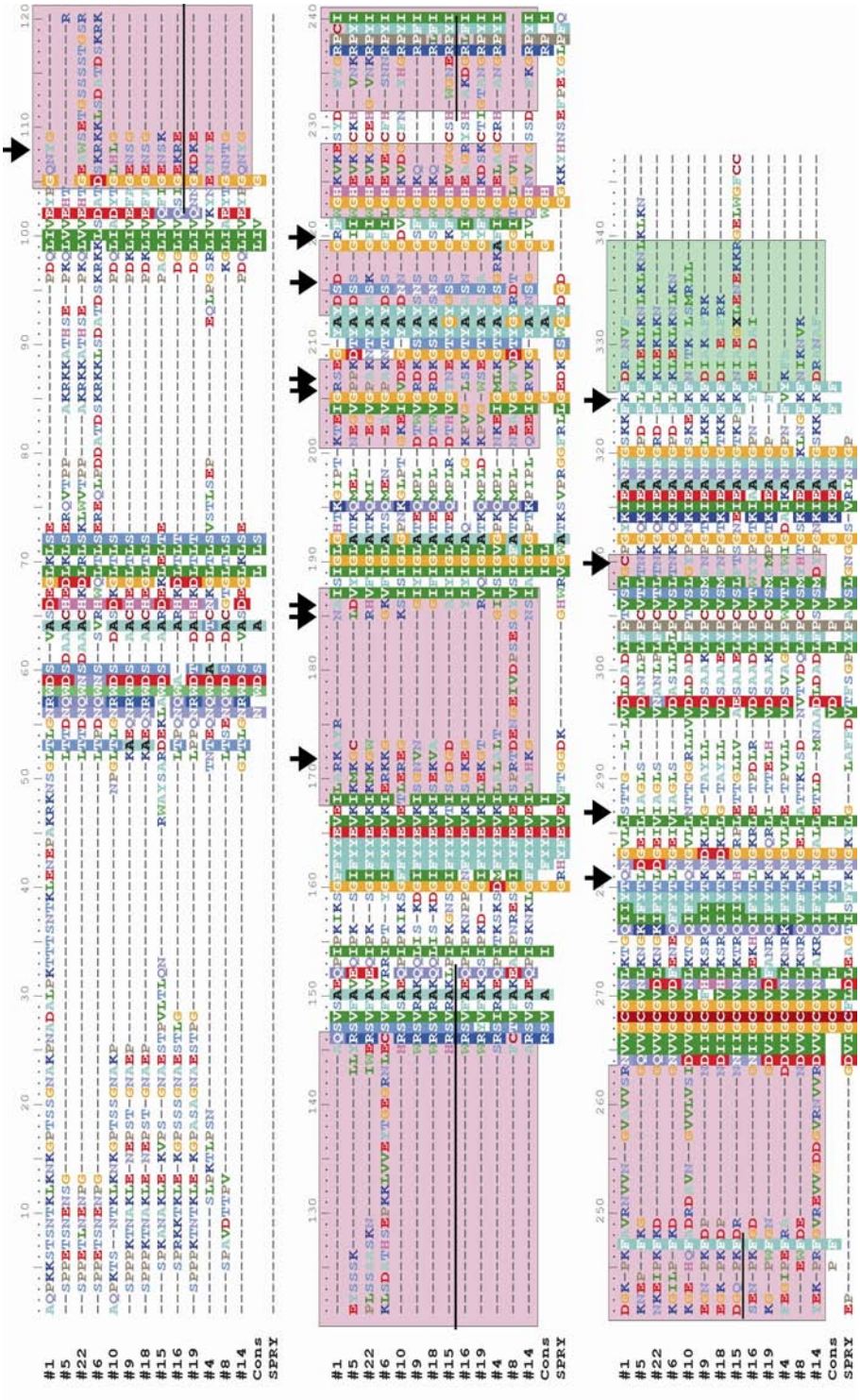

Figure 7. A protein sequence alignment of the SPRYSEC family made in Clustal W using the BLOSUM62 matrix. The boxes filled pink indicate the regions in the alignment that are involved in the construction of surface A. The residues contained in green filled box form the so-called BC box (see also fig. 6). Positively selected amino acids (omega > 1 with probabilities >0.95 in PAML) are indicated with black arrows (black).

Infection assays on plants expressing dsRNA designed on SPRYSECs are inconclusive

Potato plants expressing dsRNA designed on three SPRYSECs to knock-down the corresponding gene in the nematodes were challenged to investigate their importance in nematode-plant interactions. Nematodes on three out of eight transgenic potato lines expressing dsRNA from SPRYSEC-19 showed an arrested or retarded development. Nematodes in plants with the empty vector all developed into cysts, whereas nematodes in plants expressing dsRNA to SPRYSEC-19 were mostly still in the J2 stage with only a few individuals in the J3 stage. For one out of three transgenic lines expressing dsRNA to SPRYSEC15, and four out of ten lines expressing dsRNA to SPRYSEC-18 we observed similar outcomes. However, the lines that tested positively were propagated clonally and retested for nematode development. It appeared that none of the tested lines showed the same phenotype as in the first test. The propagation of the transgenic lines was done by making internodal stem cuttings. For reasons unclear to us the data appeared not to be reproducible in the clonally propagated lines.

Overexpression of SPRYSEC-19 in plants results in super-susceptibility to nematodes, the fungus *Verticillium dahliae*, and tomato spotted wilt virus

To further test the virulence function of the SPRYSECs in parasite-host interactions, we inoculated stable transgenic plants harboring the SPRYSEC-19 gene without the code for the signal peptide with infective *G. rostochiensis*. Nematodes infecting plants overexpressing the SPRYSEC-19 gene exhibit a significantly higher infectivity (Fig. 8A; Student *t*-test p-value < 0.01). In wild type plants, and plants harboring the empty plasmids, only about 5 percent of the nematodes had developed into the adult female stage, while at the same time in two independent overexpression lines (18_3 and 18_4) approximately 18 and 25% of nematodes had reached the adult female stage. The root systems and aerial parts of the wild type plants, the plants with empty vector, and the plants with SPRYSEC-19 overexpression showed no morphological differences.

These same overexpression lines were also inoculated with the fungus *Verticillium dahliae* and *Tomato Spotted Wilt Virus* (TSWV) to monitor the disease symptom development over time. The fresh weight of the plants inoculated with *V. dahliae* spores, and mock-inoculated plants, were assessed four weeks after inoculation as an indicator of disease development (Fig. 8B and 9A). Depending on the transgenic line a four- to tenfold increase in disease development was observed in plants overexpressing SPRYSEC-19 relative to plants harboring the empty vector. Similarly, disease symptom development of tomato spotted wilt virus infections on plants overexpressing SPRYSEC-19 was significantly more severe as compared to plants harboring the empty plasmid (Fig. 9B).

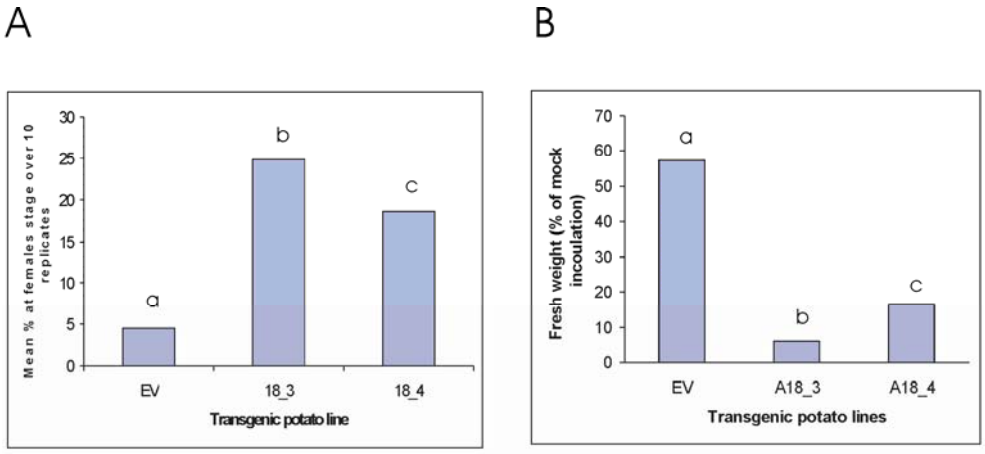

Figure 8. Potato plants overexpressing SPRYSEC-19 are hyper-susceptible to *G. rostochiensis* and *Verticillium dahliae*. Infection assays were done with three lines of transgenic potato plants, including the plants transformed with the empty binary vector pBINPLUS (EV)), and two independent lines overexpressing SPRYSEC-19 (18_3 and 18_4). **A**. Nematode infection was monitored over 35 days, and the infectivity was calculated as the mean percentage individuals developed into adult females inside the root system from the total number of individuals inoculated on the plants over at least 8 independent replicates. **B**. Fresh weight of plants infected with *V. dahliae* strain 5368 expressed as a proportion of the fresh weight of mock inoculated plants 21 days post-inoculation. The characters a, b, and c indicate statistical significances of the observed differences in the plants ($P<0.01$).

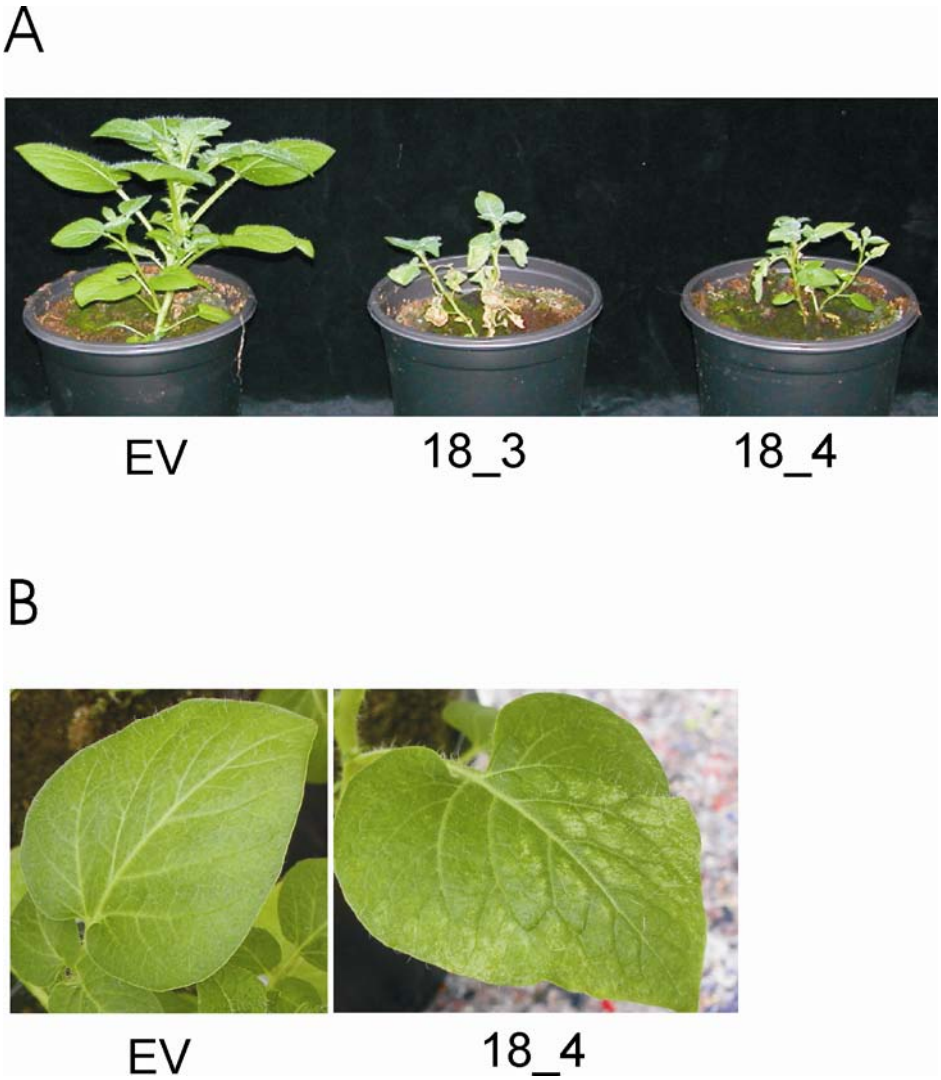

Figure 9. Disease symptoms development in potato plants overexpressing SPRYSEC-19 inoculated with *Verticillium dahliae* (A) and tomato spotted wilt virus (B). Infection assays were done with three lines of transgenic potato plants, including the plants transformed with the empty binary vector pBINPLUS (EV)), and two independent lines overexpressing SPRYSEC-19 (18_3 and 18_4).

Discussion

Over one hundred effector molecules are secreted by sedentary plant-parasitic nematodes to promote their virulence on host plants. To date the functional analyses of these effectors point at a role in host plant invasion and feeding site formation. We found that overexpression of the nematode effector SPRYSEC-19 in potato plants results in two- to threefold increase in susceptibility to the obligate biotroph *G. rostochiensis*. But, more strikingly, the same transgenic lines also showed up to ten-fold increase in susceptibility to a fungus (*V. dahliae*) and an increase in disease symptom development by a virus (TSWV). This effect on the virulence of three pathogens with entirely different modes of host invasion, colonization, and reproduction suggests that SPRYSEC-19 protein suppresses or stimulates a generic process. The morphology of the SPRYSEC overexpression lines of potato does not differ in such a way that could explain the increase in virulence of the three pathogens. We therefore hypothesize that at least some SPRYSECs likely interfere with PAMP-triggered immunity (PTI) signaling in potato that normally restricts the infection of a wide range of pathogens. Recently, a range of bacterial type III effectors with suppressive activity on the basal branch of the innate immune system of *Arabidopsis thaliana* and *Nicotiana benthamiana* have been identified by overexpressing the effectors in these plants (reviewed in (Abramovitch and Martin 2004; Grant et al. 2006; Nomura et al. 2005). There is also evidence that the type III effectors can achieve basal defense suppression by interfering with different PAMP signaling pathways (Zhang et al. 2007). We are currently testing if, and how, the overexpression of SPRYSEC-19 in potato modulates PTI signaling.

The use of the B30.2/SPRY domain as means to modulate disease signaling in a host seems to be an evolutionary innovation of nematodes. The SPRY domain (~ 120 amino acids) was first identified in SPlA and in RYanodine receptors in *Dictyostelium discoideum*. 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 (reviewed in (Woo et al. 2006). The B30.2 domain comprises a C-terminal conserved SPRY domain, preceded by a more variable subdomain named PRY. The SPRY domain occurs in 53 different architectures with a variety of other domains. 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 [Genbank accession P82885], (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 neoverruconotoxins 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 a similar domain architecture, none of the proteins mentioned above has significant similarity to the SPRYSECs.

Primary amino acid sequence of the SPRYSEC members is most similar to the SPRY domain of metazoan Ran-binding proteins, but is much smaller (about 27 kDa versus 65-70 kDa) and lacks the LisH and the CTLH domains (see for review (Murrin and Talbot 2007)). The physical interaction between Ran and RanBPM is 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 microtubules, which leads to uncoordinated aster formation in the recipient cells (Nakamura et al. 1998). Overexpression of two SPRYSECs -19 and -15 in a transgenic tobacco cell line with MAP4 fused to GFP did not show abnormal microtubule organization (data not shown). We therefore conclude that the SPRYSEC gene family and nematode RanBPM may have a common ancestor, but that the differences in protein architecture and the experimental evidence point at different roles.

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 that are under diversifying selection 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 proper analysis in PAML, the current number of sites under diversifying selection may even be an underestimation. The residues in the conserved blocks constitute in total fifteen beta-strands that fold into two beta-sheets of a sandwich shaped core. Seto at al. (1999) noted that the core structure of the SPRY domain is reminiscent of the classical immunoglobulin (Ig) fold. The topology of the Ig-domain consists of beta-strands forming two beta-sheets packed together in a layered structure. The hypervariable complementarity-determining regions (CDR) in immunoglobulins that are held together by framework regions in these variable domains condition antigen-binding, which are located in the extended loops at one side of the beta-sandwich. 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). Recent work on the protein structure of SPRY domains suggests that the core structure is similar to that of the immunoglobulines. However, the actual topology of the B30.2/SPRY domain represents a novel fold distinct from the immunoglobulin fold (Masters et al. 2006).

By using transcript-derived fragments to query a limited database of expressed sequence tags from second stage juveniles of *G. rostochiensis*, we have by no means exhaustively searched the nematode's transcriptome for homologs. For instance, primers designed on a single family member are able to PCR amplify several novel variants per member (data not shown). The sheer size of the SPRYSEC gene family, the hot spots of sequence variation in the SPRY domain, and its structural homology with the complementarity derived regions (CDRs) in immunoglobulines suggest that this framework is capable of displaying extensive sequence diversity. This implicates that strong evolutionary pressure is likely operating on SPRYSECs, and more specifically on the surface A of the SPRY domain. We therefore hypothesize that the SPRYSECs act at the interface of pathogen recognition and evasion of host innate immunity. An interesting similarity to this is found in TRIM5α in the innate immunity of primates to retroviruses. TRIM5α potently restricts infection of HIV-1 replication in rhesus-monkeys, whereas its human ortholog exhibits only a weak restriction (Stremlau et al. 2005). Recently it was shown that the specificity of viral restriction depended on amino-acid differences and length variations in the B30.2/SPRY domain of TRIM5α. A single mutation in surface A of the B30.2/SPRY domain in human TRIM5α can confer the ability to restrict virus replication in a human cell line. Furthermore, a small patch of 13 residues acids on surface A in the SPRY domain of TRIM5α has tested positively for diversifying selection (Sawyer et al. 2005).

The next question then to address is how the SPRYSECs achieve evasion of host innate immunity. We believe that there may be a parallel in the regulatory mechanism of the SPRYSECs and the protein that was actually used as the best modeling template, GUSTAVUS. GUSTAVUS belongs to the SPRY domain-containg SOCS box protein family (Hilton et al. 1998). In the consensus model of the SPRYSECs the C-terminus includes an (partial) alpha helical structure. In SPRY containing SOCS Box (SSB) proteins this alpha helical structure is named a BC box (Woo et al. 2006). The BC box is one of two elements in the SOCS box (~40 amino acids), which were first described in suppressors of cytokine signaling. The LP-rich region – the second element in a SOCS box – is not present in members of the SPRYSEC gene family. Some authors have drawn up a parallel between the role of the F box in the SCF complex and that of the SOCS box in the ESC complex (Kamura et al. 1998; Kile et al. 2002). The SCF complex is assembled from SKP2 (including an F-box), SKP1, Cul-1 (including its associated proteins Roc1, E2, and E1; reviewed in (Willems et al. 2004)). SKP2 is the adapter that determines the binding specificity of the complex to various targets. SKP1 binds to the F-box in SKP2, and bridges between SKP2 and the cul-1 (and its associated proteins) thus forming an E3 ubiquitine ligase complex. In principle SKP2 conditions the recognition specificity to bound substrate (protein), and the turnover of that bound protein will thereafter be determined by the

ubiquination by the assembled Ub ligase complex. The parallel is such that the BC box in SSBs, and possibly the SPRYSECs, binds elongin B (and thereby elongin C). Elongin BC may bridge between the protein that has the BC box and Cul-2 (and its associated proteins) so that an E3 type ubiquitine ligase complex is assembled. The substrate recognition specificity in the protein with the BC box will determine which proteins become regulated by the ESC E3 ubiquitin complex. We hypothesize that some of the SPRYSEC family members may act as adapters to provide diverse recognition specificity to the Ub ligase complex. Modifying their rate of turnover may thus regulate the molecular targets of these SPRYSECs in host cells, for instance components in the PTI signaling pathways. Further research is required to identify these molecular targets of SPRYSEC in host cells, and to study how their activity is regulated.

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Chapter 5

The nematode effector SPRYSEC-19 interacts with the Leucine-Rich-Repeat-domain of a CC-NB-LRR protein from Solanaceae

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Abstract

Parasitism genes expressed in the esophageal gland cells of the potato cyst nematode *Globodera rostochiensis* are believed to be important for nematode-plant interactions. We have previously shown that the nematode effector SPRYSEC-19 is specifically expressed during onset of parasitism and likely suppresses basal immunity in host plants. The objective of this study was to investigate the molecular targets of SPRYSEC-19 in host cells. With yeast-two-hybrid analysis and *in vitro* GST pull-down assays we found a specific physical interaction of the nematode effector SPRYSEC-19 with the LRR-region of a tospovirus resistance gene homolog, named SW5-F, from tomato. The structure of SW5-F resembles that of the CC-NB-LRR class of resistance genes. It shares significant similarity with five other members of the SW5 cluster, the root-knot nematode resistance gene Mi-1.2, and the *Pseudomonas syringae* resistance gene PRF. Furthermore, we found that SPRYSEC-19 bears a nuclear localization signal which targeted a GFP fusion protein of SPRYSEC-19 to the nucleolus of tobacco BY-2 cells. Tomato and potato harboring the SW5-F gene are not resistant to nematode carrying SPRYSEC-19. In addition, co-expression of SPRYSEC-19 and SW5-F in an agroinfiltration assay did not elicit a hypersensitive response. Therefore, we hypothesize that the nematode effector SPRYSEC-19 promotes virulence in susceptible host plants by suppressing basal defense through its interaction with cognate receptor SW5-F.

Introduction

The potato cyst nematode *Globodera rostochiensis* parasitizes a small range of Solanaceous plants such as potato, tomato and eggplant (Evans and Stone, 1970). Freshly hatched from eggs in the soil, the invasive second stage juveniles (J2s) penetrate the roots of a host plant just behind the apex and migrate intracellularly towards the vascular cylinder where they establish a multinucleate feeding site in the inner-cortex. In the feeding site, host cells are transformed into a so-called syncytium, which is marked by protoplast fusion, partial cell wall dissolution, dense cytoplasm, small secondary vacuoles, enlarged nuclei and proliferation of organelles. Developing nematodes remain sessile and complete their development by feeding on these specialized feeding cells (Wyss and Zunke 1986; Wyss, 1992). Nematode effector molecules, secreted from the esophageal glands through the oral stylet into the host cells, are believed to be responsible for induction and maintenance of the syncytium. Recent studies suggest that plant parasitic nematodes secrete a whole suite of effector molecules into the host tissue, probably encoded by more than one hundred different genes (Gao et al., 2003; Qin et al., 2000).

In order to cope with invading pathogens, plants have evolved a multi-facet defense system. Basal immunity is based on recognition of conserved pathogen associated molecular patterns (PAMPs) by extracellular trans-membrane pattern recognition receptors. PAMP recognition may lead to activation of mitogen-activated protein kinase signaling, accumulation of reactive oxygen species, cell wall based defenses, and activation of defense genes to prevent further pathogen ingress (Jones and Dangl, 2006; Ingle et al., 2006; He Ping et al., 2007). In turn pathogens have evolved effector molecules that target signaling components in PAMP-triggered immunity (PTI) to promote virulence by suppressing defense responses. To counteract these insurgents, plants have evolved a second branch in their immune system involving extra-/intracellular immune receptors (R proteins) to survey for pathogen effector molecules. The interaction between pathogen effector molecules and the cognate R proteins may be direct, but more often these effectors induce modifications in host cells that lead to non-self recognition. This so-called effector-triggered immunity (ETI) often leads to a rapid programmed cell death of host cells (a hypersensitive response) which halts the pathogen at its entry site. Recent findings indicate that effector-triggered immunity overlaps with PAMP-triggered immunity but differs quantitatively and kinetically (Chisholm et al., 2006; Kim et al., 2005; Mithofer et al., 1996).

Plant R proteins can be divided into three major classes: receptor like proteins, receptor like kinases, and nucleotide binding site-leucine rich repeat proteins (NB-LRR). Receptor like proteins (e.g. Cf2 and Cf9) have an extracellular LRR-domain connected to a short trans-membrane region, whereas receptor like kinases like Xa21 have an extra-

cellular LRR, a short trans-membrane motif, and an intracellular kinase domain. The vast majority of R proteins belong to the NB-LRR class (Martin et al., 2003; van Ooijen et al., 2007). The nucleotide binding site in NB-LRR proteins actually comprises two subdomains, the NB (nucleotide binding) and ARC (from homologous to human Apoptotic protease-activating factor, R proteins, and CED-4 protein of *C. elegans*). The NB domain is thought to act as a molecular switch following ATP binding (McHale et al., 2006). The LRR domains (LRD) consist of 2 to 42 repeats of which each repeat is formed by 24-28 residues largely, following the consensus sequence LxxLxxLxLxxC/Nxx. LRD have been implicated in protein-protein interactions by forming ligand-binding surfaces (Enkhbayar et al., 2004). Within R-proteins, the LRD is the most variable domain and various studies have demonstrated its role in determining resistance specificity by recognizing different pathogen effector molecules. The NB-LRR domains in R proteins may either be linked to an N-terminal coiled-coiled domain (CC) or a TIR (homologous to *Drosophilla* Toll and human interleukin-1 receptor) domain. A direct interaction between an R-protein and a pathogen effector with avirulence activity is so far found only in three cases, i.e. between Pita/AvrPita, L/AvrL567, and PopP2/RRS-1 (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006). It is believed, however, that most R proteins recognize their cognate pathogen effectors indirectly by the changes in molecular patterns they induce in host cells (Mackey et al., 2002).

Several nematode resistance genes have been isolated from important crop plants (Williamson and Kumar, 2006). $Hs1^{pro-1}$ cloned from sugar beet confers a resistance response to sugar beet cyst nematode (Cai et al., 1997). The tomato R genes, *Mi-1.2* and *HeroA*, condition resistance against root knot nematode species and against different pathotypes of *Globodera rostochiensis* and *Globodera pallida*, respectively (Milligan et al., 1998; Ernst et al., 2002). However, potato plants with the R genes *Gpa2* and *Gro1-4* display resistance to only a few pathotypes of *G. pallida* and *G. rostochiensis*, respectively. The *Mi-1.2*, *Gpa2*, and *HeroA* genes belong to the intracellular NB-LRR class of R-genes with a CC-domain at their N-terminus, whereas *Gro1-4* is a TIR-NB-LRR type of R protein (van der Vossen et al., 2000; Paal et al., 2004). *Rhg1* and *Rhg4* from soybean condition resistance to several pathotypes of the soybean cyst nematode *H. glycines* and likely encode an R protein with an extracellular LRR-domain, a short trans-membrane region and a cytosolic serine-threonine kinase domain. *Rhg4* is structurally related to the rice gene *Xa21*, which displays resistance against the bacterial pathogen *Xanthomonas oryzae* (Song et al., 1995). Similarly, *Gpa-2* conditions nematode resistance while its closest homolog gives resistance to potato virus X. Resistances to nematodes is generally attributed to a failure to form a functional feeding site, which leads to highly skewed male to female ratios. For example, Mi mediated resistance is characterized by a rapid localized cell death near the

anterior side of nematode, whereas *HeroA* mediated resistance leads to atrophy or abnormal development of the feeding site (Branch et al., 2004; Sobczak et al., 2005).

Plant parasitic nematodes inject a suite of effector molecules into host cells. Despite the cloning of several R genes conferring resistance to nematodes there is little known about the corresponding effectors with avirulence activity (Davis et al., 2004). The segregation pattern of virulence in *G. rostochiensis* towards the H1 resistance gene from *Solanum tuberosum* reveals a gene-for-gene relationship (Janssen et al., 1991). However, the (a)virulence factor has not been cloned to date. Likewise, an amphid secreted protein MAP-1 has been found to be expressed only in avirulent near isogenic lines (NILs) of *M. incognita* on tomato with the *Mi1.2* resistance gene, but not in virulent NIL's. Due to the specific expression in avirulent NIL's, MAP-1 is considered as an avirulent gene candidate, but further evidence to support this is currently lacking. Interestingly, the MAP-1 homologs were found in only three root-knot nematode species (*M. incognita, M. arenaria, M. javanica*), which are all controlled by the *Mi-1.2* gene (Semblat et al., 2001). Similarly, a cDNA-AFLP cloning strategy resulted in the identification of twenty-two transcripts specifically expressed in avirulent NILs of *M. incognita*. Most of the differentially expressed genes have no matches in sequence databases so their function still remains to be elucidated (Neveu et al., 2003). So far, chorismate mutase (CM) is the best functionally characterized nematode effector that may play important role in promoting nematode virulence (Lambert et al., 1999). CM is an enzyme secreted from the esophageal glands of many plant-parasitic nematodes, and may manipulate the host plant's shikimate pathway. The shikimate pathway is involved in the synthesis of aromatic amino acids, plant hormones and numerous secondary metabolites with defense activity (Schmid and Amrhein, 1995). Strikingly, in various inbred lines of the soybean cyst nematode the expression of distinct forms of CM correlates with nematode resistance in soybean cultivars. It has been proposed that nematode secreted CM could promote nematode virulence through suppression of plant defense compounds like salicylic acid and phytoalexins (Bekal et al., 2003; Doyle and Lambert, 2003).

We have previously shown that the nematode effector gene SPRYSEC-19, which is specifically expressed during the onset of parasitism of *G. rostochiensis*, likely suppresses basal immunity in host plants. The objective of this study was to investigate the molecular targets of SPRYSEC-19 in host cells. We found a physical interaction of SPRYSEC-19 with the LRR region of a novel CC-NB-LRR protein from tomato by using a combination of yeast two hybrid analysis and *in vitro* GST pull down assays. The CC-NB-LRR protein is a member of the SW5 R gene cluster of which some other members condition disease resistance to tospoviruses. We further show that SPRYSEC-19 targets the nucleus of host plants, which is recently implicated in the disease signaling of some R proteins.

Materials and Methods

Prey cDNA library construction

The prey cDNA library used in present study has been described in Van Bentem et al. (2005). In brief, cDNA prepared from mRNA isolated from tomato plants (*Lycopersicon esculentum* cultivar GCR161; [Kroon and Elgersma, 1993]) infected with *Fusarium oxysporum* forma specialis *lycopersici* race 2 isolate FoI007 was cloned downstream of the Gal4 transcription activation domain (AD) into EcoRI and XhoI sites of Lambda-ACT vector (Elledge et al., 1991). The ligation products were packaged into phage particles using the Gigapack III gold cloning kit (Stratagene). Lambda phages were converted into yeast shuttle vectors using an "*in vivo* mass excision" protocol (Elledge et al., 1991). The library consisted of 2-million independent clones with average size of library 1.1kb.

Bait and Prey plasmid construction

The MATCHMAKER Two-hybrid System 3 (Clontech) 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*: GACCTG**CATATG**CCGCCGCCAAAAACAAAC; *YA18R*: GTCGAC**GGATCC**AATTCAAAATGGGCCAAAG) by polymerase chain reaction (PCR), underlined sequence represents NdeI and BamHI 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. Prey plasmids of LRR-domains of various resistance proteins such as Rx (potato), RPM1 (Arabidopsis), RPS5 (Arabidopsis), I-2S (tomato), and Mi-1.2 (tomato) have been described in Van Bentem et al. (2005). For the LRR-Gpa2-bait, we used a plasmid containing LRR-domain of Gpa2 fused with YFP. The LRR-Gpa2 was digested with NcoI and PstI and cloned in pGBKT7. For Sw5-A and Sw5-B LRR baits, plasmids pBIN61-Sw5a-LRR-HA and pBIN61-Sw5b-LRR-HA were used respectively. LRR of SW5-A and SW5-B were digested with NcoI and Sal1 and cloned in pGBKT7. For 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 by using EcoR1 and Xho1 restriction sites.

Yeast two hybrid analysis

The yeast host strain AH109 harbors three reporter genes (*ADE2*, *HIS3* and *MEL1*) that are tightly regulated by UAS (upstream activated sequence), and requires binding of functional Gal4 transcription factor for the expression of reporter genes. 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°C for 10-days. Blue colonies were selected as positive candidates followed by rescuing of AD-plasmid following manufacturer's instructions. Interaction was scored visually based on growth of transformants on selective plate as negative (-) indicating no growth, or positive (+) indicating growth. Interaction strength was indicated from + (strong) to +++ (very strong) depending upon growth rate. 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 2-3mm colony was vortexed in 200 µl of YPDA and mixed together followed by incubation of the mixture at 30 °C for 16-hours with shaking. The transformants were spread on plates with minimal SD/-AHLW+ X-α-gal medium followed by incubation at 30 °C for 3-5 days.

Expression and purification of recombinant protein from *Escherichia coli*

The coding region of SPRYSEC-19 was PCR amplified from a cDNA library (Smant et al., 1998) using primers (SpG-F: CTG**GGATCC**CCGCCGCCAAAAACAAAC, and SpG-R: CCG**CTCGAG**AATTCAAAATGGGCCAAAGTTC) with BamH1 and Xho1 overhangs at 5`end and directionally cloned in pGEX-KG (Guan and Dixon, 1991). For GST-LRR-SW5-F construct, the 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 1mM-IPTG at 30°C for 5-hours. The cells were lysed by sonication in 1 times strength PBS (containing a complete protease inhibitor cocktail) and the supernatant was kept in -20C till further use.

In vitro GST pull-down assay

The protocol of GST-pull down assay has been adopted from Nguyen and Goodrich (2006) with some modifications. In the first strategy, the 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°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°C for 4-hours. As negative control, sepharose beads were incubated with THIO and THIO-LRR-SW5-F respectively. In the second strategy, the soluble fraction of bacterial lysate containing either GST or GST-LRR-SW5-F was incubated with sepharose beads followed by removal of the lysate by centrifugation. To bound GST or GST-LRR-SW5-F, purified SPRYSEC-19 was added and incubated for 4-hours at 4 °C. Immobilized proteins on sepharose beads were washed 5 times with PBS+0.2% Tween. To determine protein interaction, sepharose beads from all samples were re-suspended in SDS-PAGE sample buffer followed by SDS-PAGE and subsequent western blotting.

SDS-PAGE and western blotting

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 : anti-GST 1:3000 (Amersham), anti-Thioredoxin 1:3000 (Invitrogen), and anti-SPRYSEC-19 1:2500, followed by their detection with alkaline-phosphatase-conjugated rabbit anti-goat 1:5000, rabbit-anti-chicken IgY 1:5000, and rat-anti-mouse 1:5000 (Jackson) respectively. The blots were developed in substrate buffer supplemented with nitroblue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sambrook, 1989).

Localization in BY-2 cells of tobacco

A PCR-based cloning strategy was used to isolate SPRYSEC-19 (nt 55-645) from a cDNA library of *G. rostochiensis* (Smant et al., 1998). The resulting fragment was cloned in pK7FWG2 and pK7WGF2 (Karimi et al., 2002) with Gateway cloning technology (Invitrogen, Carlsbad, CA, USA), to produce a N- and C-terminal GFP-fusion construct for SPRYSEC-19. The fusion constructs were introduced into BY2-cells by the biolistic PDS-1000/He particle delivery system (vacuum, 26 inches Hg, helium pressure, 1, 100psi; gold particles, 1um) (Bio-Rad). Following transformation (16h), the transformed BY2-cells were analyzed by using Radiance2000 (Bio-Rad) confocal microscope.

Antibody production and immuno-assay

The SPRYSEC-19 coding region (nt 55-645) was PCR-amplified as described above and cloned in pDEST-17 with the Gateway cloning technology (Invitrogen, Carlsbad, CA, USA) to produce 6xHis tagged-SPRYSEC-19 in BL21-S1 (Invitrogen) cells when induced with 0.3M NaCl at 37 ^{o}C for 16-hours with shaking. Recombinant SPRYSEC-19 was purified on Ni-NTA spin columns following manufacturer's instructions (QIAGEN). Hens were immunized with purified SPRYSEC-19 and the chicken IgY was isolated and purified as described by Kudla et al. (2005). Western blots of homogenates of pre-parasitic J2's were performed as described by de Boer et al. (1996).

Plant transformation

The coding sequence of SPRYSEC-19 without signal peptide (nt 57-745) was amplified as described earlier using primers (A18ns-NcoI: GGCGCG**CCATGG**TGCCGCCAAAAACAA and A18-BglII: GTCGAC**AGATCT** TTGATCGACGAAGAAAAAC) and cloned in pRAP33 using restriction sites (NcoI and BglII) added as overhangs on primer sequences. The expression cassette containing the CaMV 35S promoter, SPRYSEC-19 and Tnos was excised from pRAP33 using AscI and PacI and cloned in pBINPLUS (van Engelen et al., 1995) followed by transformation in *Agrobacterium tumefaciens* strain LBA4404 (Shen and Forde, 1989). Potato Line V *(Solanum tuberosum)* was transformed by using *A. tumefaciens* (Horsch et al., 1984). The explants were maintained on MS20 media supplemented with 100 mg/l kanamycin and 300 mg/l of cefatoxime (Duchefa). To determine the expression of SPRYSEC-19 in stable transgenic potato lines, RT-PCR was done. Total RNA was isolated from transgenic lines by using Trizol reagent (Invitrogen) with subsequent cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) following the manufacturer's protocol.

Cloning of SW5-F and agroinfiltration assays

To amplify full length SW5-F from the tomato cultivar GCR-161 (Kroon and Elgersma, 1993), 100 mg of leaf tissue was grinded in liquid nitrogen with mortar and pestel 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 the manufacturer's protocol. The cDNA was used for amplification of full-length SW5-F using BD Advantage™ 2 PCR Kit (BD life sciences) with primers SW5AB (5`-ATGGCTGAAAATGAAATTGA-3`) or SW5CD (5`. ATGGCTCAAAATGAAATTGA-3`) in combination with Int-1/2-R (5'-GCACAGCAAAAGTATCATGTCA-3`). The amplification product of about 4kb was cloned in pCR4 TOPO (Invitrogen) and sequenced.

To construct a binary vector containing SW5-F, the coding region of SW5-F was PCR amplified from cDNA clone (described above) using primers (Sw-NcoI: 5'-GAGGAG**CCATGG**CTCAAAATGAAATTGAG-3' and SW-PstI: 5'-GAGGAG**CTGCAG**CTACCATCCTTTGATAATGAG-3**'**) and cloned by restriction digestion with NcoI and PstI into the pRAP vector. The expression cassette containing the 35S promoter, SW5-F, and Tnos was excised from pRAP using AscI and PacI, and cloned in pBINPLUS followed by transformation in *Agrobacterium tumefaciens* strain LBA4404 (Shen and Forde, 1989). pBINPLUS-constructs containing SW5A and SW5B have been described in Spassova et al. (2001), whereas Gpa-2 and GFP constructs were supplied by Dr. Jan Roosien (Laboratory of Nematology, Wageningen University). The agroinfiltration assay was performed (Van der Hoorn et al., 2000) on leaves of 3 weeks old *Nicotiana benthamiana* leaves. The infiltrated leaves were monitored for 7 days to score the phenotype.

Bio-informatics

For DNA sequence analysis, open reading frame (ORF) prediction, sequence alignment, and building contigs the VectorNTi 10 program (Invitrogen) was used. Database searches were done using the BLAST programs from NCBI server (Atschul et al., 1997). The Signal P program was used to predict the presence of a signal peptide for secretion (Nielsen et al., 1997). PSORT (psort.nibb.ac.jp) was used to predict the sub-cellular localization of proteins and CDART was used to find conserved domains from NCBI server. The COILS program (www.ch.embnet.org/software/COILS) was used to predict putative coiled-coil regions from resistance gene homologue SW5-F.

Results

SPRYSEC-19 interacts with an LRR domain in a yeast-two-hybrid analysis

Transgenic potatoes overexpressing SPRYSEC-19 are supersusceptible to the nematode *G. rostochiensis*, the fungus *Verticillium dahliae*, and tomato spotted wilt virus (TSWV). To study the mechanism behind this supersusceptibility, we performed a yeast-two-hybrid (YTH) screening of a tomato root cDNA library to identify interacting host proteins of SPRYSEC-19. The coding region of SPRYSEC-19, fused to the DNA-binding domain of GAL4 (BD), was used as a bait to interact with the tomato root cDNA fused to the activator domain of GAL4 (AD) in pACT2. After screening of $2x10^5$ independent clones, five cDNA fragments in the library activated the YTH-selection markers (AHLW+X-alpha-gal) in a SPRYSEC-dependent manner. Two of these cDNAs, named *Int-1* and *Int-2*, coded for an identical sequence of 894 bp. The interaction between SPRYSEC-19 and both *Int1* and *Int2* was abolished by disruption of their open reading frame (Fig. 1). The interaction between SPRYSEC and *Int1* and *Int2* was further validated *in vivo* by yeast-mating (data not shown), and by swapping the BD- and the AD-plasmids for bait and preys (Fig. 1). 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* and *Int-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.

The AD-plasmids carrying *Int1* and *Int2* were rescued from yeast for sequencing of the inserts. BLASTX analysis of the insert sequences on the non-redundant database revealed significant similarity of *Int1* and *Int2* with the so-called Leucine-Rich-Repeat (LRR)-region of several tospovirus (TSWV) resistance gene homologues SW5-A, -B, -C, -D, and -E (E-values ranging from $6e^{-56}$ to $2e^{-44}$), and a nematode resistance protein Mi-1.2, a *Pseudomonas syringae* resistance gene PRF, and the cyst nematode resistance genes Gpa2 and Hero (E-values, $7e^{-05}$ to $2e^{-11}$; Table 1). Given that *Int1* and *Int2* are identical in sequence and that their best match is highly similar to members of the SW5 cluster we named them LRR-SW5-F.

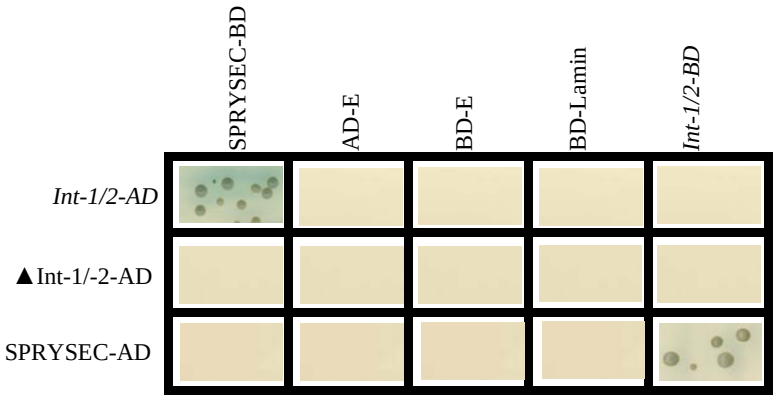


Figure 1. SPRYSEC-19 interacts with LRR domain of tospo virus resistance gene homologue. SPRYSEC-19 fused with DNA-binding domain (SPRYSEC-BD) was used as bait to screen tomato cDNA library fused in frame with activation-domain (AD). Here AD-E is empty vector containing activation domain, BD-E is empty vector with DNA-binding domain, SPRYSEC-AD is SPRYSEC-19 fused with AD-domain, BD-Lamin is BD-vector with in-frame human Lamin gene, *Int-1/2-BD* is *Int-1/2* fused with BD-domain, and ▲Int-1/-2-AD represents Int-1/2 fused with AD-domain where reading frame has been disturbed.

Table 1. Best matching sequences in the database following a BLASTX search with Int1/2 as query.

Accession#	Description	Expect value
AAG31017	Tospo virus resistance protein E from tomato	6e-56
AAG31016	Tospo virus resistance protein D from tomato	8e-56
AAG31015	Tospo virus resistance protein C from tomato	6e-52
AAG31013	Tospo virus resistance protein A from tomato	2e-47
AAG31014	Tospo virus resistance protein B from tomato	2e-44
AAC32252	Disease resistance gene homologue Mi-copy2 from tomato	2e-14
AAC32253	Nematode resistance protein Mi-1.1 from tomato	4e-13
AAC49408	PRF from tomato	4e-07
AAF04603	Disease resistance protein Gpa2 from *Solanum tuberosum*	7e-05
CAD29729	Hero resistance protein from tomato	2e-11

SPRYSEC-19 interacts with an LRR-domain *in vitro*

A pull-down assay was used to independently confirm the interaction between SPRYSEC and LRR-region of SW5-F *in vitro*. 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 or GST alone (~35kD) on sepharose beads. After extensive washings, bound proteins were resolved by SDS-PAGE and western blotting. The THIO-LRR-SW5-F bound specifically to GST-SPRYSEC but not with GST alone. Similarly, thioredoxin alone (THIO, 19.5kD) did not bind to GST-SPRYSEC-19 on sepharose beads or to sepharose beads alone (Fig. 2A).

In a separate experiment, LRR-SW5-F was also fused to GST (GST-LRR-SW5-F), immobilized on sepharose beads, and incubated with bacterially produced SPRYSEC-19. Western blots of this pull-down assay revealed that purified SPRYSEC-19 specifically interacts with GST-LRR-SW5-F but not with GST alone (Fig. 2B). Our findings indicate that the interaction of LRR-SW5-F and SPRYSEC-19 is specific both in yeast and in vitro.

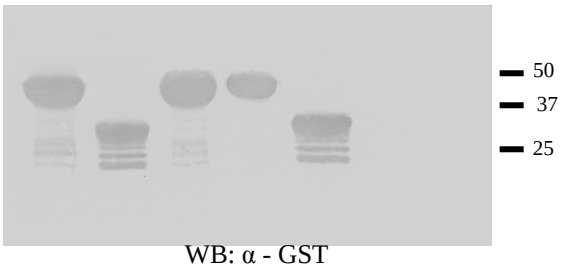
The SPRYSEC-19/LRR-domain interaction is specific for SW5-F

The specificity of the interaction between LRR-SW5-F and SPRYSEC-19 was further tested by yeast-two-hybrid interaction experiments between SPRYSEC-19 and LRR domains of various resistance proteins of the CC-NB-LRR class. The presence of prey and bait plasmids was confirmed by growing the auxotrophic yeast on media lacking leucine and tryptophane (-LW). Interaction was monitored by re-streaking the transformants on medium lacking the amino acids AHLW and allowing them to grow for 10 days. While SPRYSEC-19 interacted specifically with LRR-SW5-F, a similar interaction was not observed with the LRR domain of *Rx* (potato, CAB507886), *Gpa2* (potato, AAF04603), *RPM1* (Arabidopsis, NP187360), *RPS5* (Arabidopsis, NM101094), *I-2* (tomato, AF118127), *Mi-1.2* (tomato, AAC32252), *SW5A* (tomato, AAG31013), and *SW5B* (tomato, AAG31014) (data not shown).

Figure 2. LRR region of SW5-F interacts specifically with SPRYSEC *in vitro*. A) GST-conjugated SPRYSEC-19 (GST-SPRYSEC-19: 48kDa) or GST (35kDa) alone was immobilized on sepharose beads followed by incubation with Thioredoxin-conjugated LRR-region of SW5-F (THIO-LRR-SW5-F: 40kDa) or Thioredoxin alone (THIO: 19.5kDa). After extensive washings, the bound proteins were fractionated on 12.5% SDS-PAGE followed by western blotting. The immunodetection of GST- and Thioredoxin-fusion proteins was done by probing blots with anti-GST followed by alkaline-phosphatase-conjugated rabbit anti-goat or with anti-Thioredoxin followed by alkaline-phosphatase-conjugated rat-anti-mouse, respectively. B) GST-conjugated-LRR region of SW5-F (GST-LRR-SW5-F: 55kDa) or GST (35kDa) alone was immobilized on sepharose beads followed by incubation with purified SPRYSEC-19 (26kDa). The bound proteins were run on 12.5% SDS-PAGE with subsequent transfer on nitrocellulose membrane by western blotting. The bound proteins were detected by probing the blots either with anti-GST and alkaline-phosphatase-conjugated rabbit anti-goat or with anti-SPRYSEC and alkaline-phosphatase-conjugated-rabbit.

A.

GST-SPRYSEC-19	+	-	+	+	-	-	-
THIO-LRR-SW5-F	+	+	-	-	-	+	-
GST	-	+	-	-	+	-	-
THIO	-	-	+	-	-	-	+

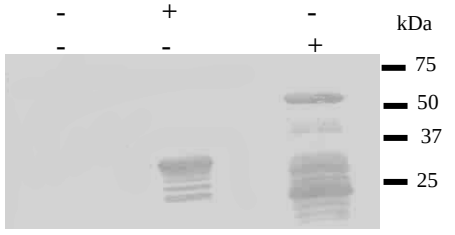


WB: α - GST

WB: α - Thio

B.

SPRYSEC-19	+	+	+
GST	-	+	-
GST-LRR-SW5-F	-	-	+



WB: α - GST

WB: α - SPRYSEC

LRR-SW5-F is part of a CC-NB-LRR protein

LRR-SW5-F shows similarity with 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-F cluster belong to the CC-NB-LRR class of resistance proteins, and it was therefore likely that LRR-SW5-F represented only the C-terminus of a larger protein as well. In order to clone the missing 5`-end of LRR-SW5-F, two degenerate primers (SW5AB and SW5CD) were designed on the 5'-end of the members of the SW5 cluster. A gene specific reverse primer (Int-1/2-R) was designed on the 3`-untranslated region of LRR-SW5-F. Following a polymerase chain reaction (PCR) on tomato cDNA (cultivar GCR-161) with different primer combinations, an amplification product of ~4kb was obtained only with primers SW5CD and Int1/2-R. The amplification product was cloned in pCR-4 vector (Invitrogen) followed by various rounds of DNA-sequencing. Sequences were assembled into a contig of 4110 bp, which was named SW5-F. SW5-F codes for an open reading frame of 1275 amino acids with a predicted molecular mass of 147 kDa and isoelectric point of 5.54. The 3`-untranslated region consists of 282 bp and a poly-adenylation signal is located 251 nucleotides downstream of the stop codon.

Searches in non-redundant-protein database using the full SW5-F sequence as query revealed again a high similarity with tospo virus resistance gene homologues such as Sw5-C (Genbank accession AAG31015; 88% amino acid similarity), Sw5-A (AAG31013; 79% sim.), and Sw5-B (AAG31014; 78% sim.), root-knot nematode resistance gene Mi-1.2 (AAC32252; 50% sim.) and *Pseudomonas syringae* resistance gene PRF (AAC49408, 53% sim.) (Table 1).

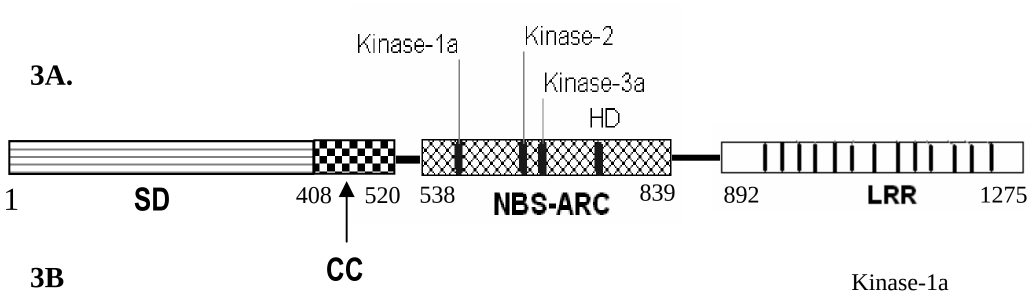
The structure of SW5-F protein displays all features of the coiled-coiled, nucleotide binding (NB-ARC), and leucine-rich-repeat (LRR) family of resistance genes (Pan et al., 2000; Spassova et al., 2001) (Fig. 3A). SW5-F is of approximately the same size as Mi-1.2, SW5-A, SW5-B, SW-5C and SW5-D, while PRF (1824aa) includes a long N-terminal extension of about 300 amino acids (Salmeron et al., 1994). The C-terminal region of SW5-F (amino acids 409-520) is predicted to form coiled-coil by COILS program (Lupas et al., 1991) with window 14 as was also reported for SW5A and SW5B (Spassova et al., 2001). However, the N-terminal region of SW5-F (amino acids 1-408) showed weak homology with solanaceae domain (SD) as described by Mucyn et al (2006). The central NB-ARC domain contains all the conserved motifs such as kinase-1a (P-loop) (583-592), kinase-2 (654-666), kinase-3a (684-691), and HD (hydrophobic domain: 745-759) as reported for all other resistance genes of NB-LRR family (Meyers et al., 1999) (Fig. 3B). In addition, the C-terminal region of SW5-F encodes a leucine-rich-repeat motif (LRR), very similar in size with Sw5-A, Sw5-B, Sw5-C, Mi-1.2, and PRF (Fig. 3C). SW5-F is predicted to have 13 LRR-repeats largely following the consensus sequence LxxLxLxx (starting from

amino acid position 939). The similarity percentage of CC, NB, and LRR domains of Sw5-A, Sw5-B, Sw5-C with SW5-F is very high (61- 92%), where as, Mi-1.2 and PRF show low similarity (21-30%) (Table 2).

Table 2: The sequence amino acid identity between SW5-F and the best matching homologs in the sequence database

	% Identity		
	CC	**NB**	**LRR**
SW5A	68	78	61
SW5B	67	79	60
SW5C	92	89	63
Mi-1.2	21	44	30
PRF	17	45	26

3A.



3B

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                                                                Kinase-1a
Sw5-F    (1) PFEYFPTRHINPVTDEDIVGFGNDIEKMFQYLIRGTNDLDVVPIVGMGGQGKTTVARK  58
Sw5-A    (1) --KHLPDRHSNLVTDEEVVGFEKQAEELIDYLTRGTNELDVVPIVGMGGQGKTTIARK  56
Sw5-B    (1) --KHLPDRHSNLVTDEEVVGFENKAEELIDYLIRGTNELDVVPIVGMGGQGKTTIARK  56
Sw5-C    (1) --------HINPVTDEDIVGFGNDIEKMFQYLIRGTNDLDIVPIVGMGGQGKTTVARK  50
Mi-1.2   (1) –SPKKPVERKSLTTDKIIVGFEEETNLILRKLTSGPADLDVISITGMPGSGKTTLAYK  57
Prf      (1) -YVAPSFSAYTQRANEEMEGFQDTIDELKDKLLGGSPELDVISIVGMPGLGKTTLAKK  57

Sw5-F   (59) VYNSDNIVSHFDVRAWCIVSQTYNRRKLLQDILSQVTGSKDKG-YEDDILADELRKSL  115
Sw5-A   (57) LYNNDIIVSRFDVRAWCIISQTYNRIELLQDIFSQVTGFNDNG-ATVDVLADMLRRKL  113
Sw5-B   (57) LYNNDIIVSRFDVRAWCIISQTYNRRELLQDIFSQVTGSDDNG-ATVDVLADMLRRKL  113
Sw5-C   (51) VYNSDNIVSHFDVRAWCIVSQTYNRRKLLQEILSQVTGSKDKG-YEDDILADELRKSL  107
Mi-1.2  (58) VYNDKSVSRHFDLRAWCTVDQGYDDKKLLDTIFSQVSGSDSNL-SENIDVADKLRKQL  114
Prf     (58) IYNDPEVTSRFDVHAQCVVTQLYSWRELLLTILNDVLEPSDRNEKEDGEIADELRRFL  115

                  Kinase-2                    Kinase-3a
Sw5-F  (116) MGKRYLIVLDDMWDCMAWDDLRLSFPDSGNRSRIVVTTRLEKVGEQVKCHTDPYSLPF  173
Sw5-A  (114) MGKRYLIVLDDMWDCMVWDDLRLSFPDVGIRSRIVVTTRLEEVGKQVKYHTDPYSLPF  171
Sw5-B  (114) MGKRYLIVLDDMWDCMVWDDLRLSFPDDGIRSRIVVTTRLEEVGKQVKYHTDPYSLPF  171
Sw5-C  (108) MGKRYLIILDDMWDCMAWDDLRLSFPDSGNRSRIVVTTRLEKVGEQVKCHTDPYSLPF  165
Mi-1.2 (115) FGKRYLIVLDDVWDTTTLDELTRPFPEAKKGSRIILTTREKEVALHGKLNTDPLDLRL  172
Prf    (116) LTKRFLILIDDVWDYKVWDNLCMCFSDVSNRSRIILTTRLNDVAEYVKCESDPHHLRL  173

                                                              HD
Sw5-F  (174) LTKEESCELLQKKVFQKEDFPPELQDVSRAVAEKCKGLPLVVVLVAGIIKKRKMEESW  231
Sw5-A  (172) LTTEESCQLLQKKVFQKEDCPLELQDVSQAVAEKCKGLPLVVVLVAGIIKKRKMEESW  229
Sw5-B  (172) LTTEESCQLLQKKVFQKEDCPPELQDVSQAVAEKCKGLPLVVVLVAGIIKKRKMEESW  229
Sw5-C  (166) LTKEESCELLQKKVFQKEDFPPELQDVSQAVAEKCKGLPLVIVLVAGIIKKRKMEESW  223
Mi-1.2 (173) LRPDESWELLDKRTFGNESCPDELLDVGKEIAENCKGLPLVADLIAGVIAGREKKRSV  230
Prf    (174) FRDDESWTLLQKEVFQGESCPPELEDVGFEISKSCRGLPLSVVLVAGVLKQKKTLDS   231

Sw5-F  (232) WNEVKDSLFDHLNRESEEYSLSTMQLSYYNLTDCLKPCLLYMGMFQEDAIIRVSELIS  289
Sw5-A  (230) WNEVKDALFDYLDSEFEEYSLATMQLSFDNLPHCLKPCLLYMGMFSEDARIPASTLIS  287
Sw5-B  (230) WNEVKDALFDYLDSEFEEYSLATMQLSFDNLPHCLKPCLLYMGMFSEDARIPASTLIS  287
Sw5-C  (224) WNELKDFLFDYLDCHSEQYSRATMQLSFDNLADCLKPCLLYMGMFPEDASIIVSALLS  281
Mi-1.2 (231) WLEVQSSLSSFI-LNSEVEVYKVIELSYDHLPHHLKPCLLHFASWPKDTPLTIYLFTV  287
Prf    (232) WKVVEQSLSSQR-IGSLEESISIIGFSYKNLPHYLKPCFLYFGGFLQGKDIHVSKMTK  288

Sw5-F  (290) LWIAEGFVQSIES   303
Sw5-A  (288) LWIAEGFVENTES   300
Sw5-B  (288) LWIAEGFVENTES   300
Sw5-C  (282) LWIAEDFVQNIES   294
Mi-1.2 (288) YLGAEGFVEKTEM   300
Prf    (289) LWVAEGFVQANNE   301
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3C.

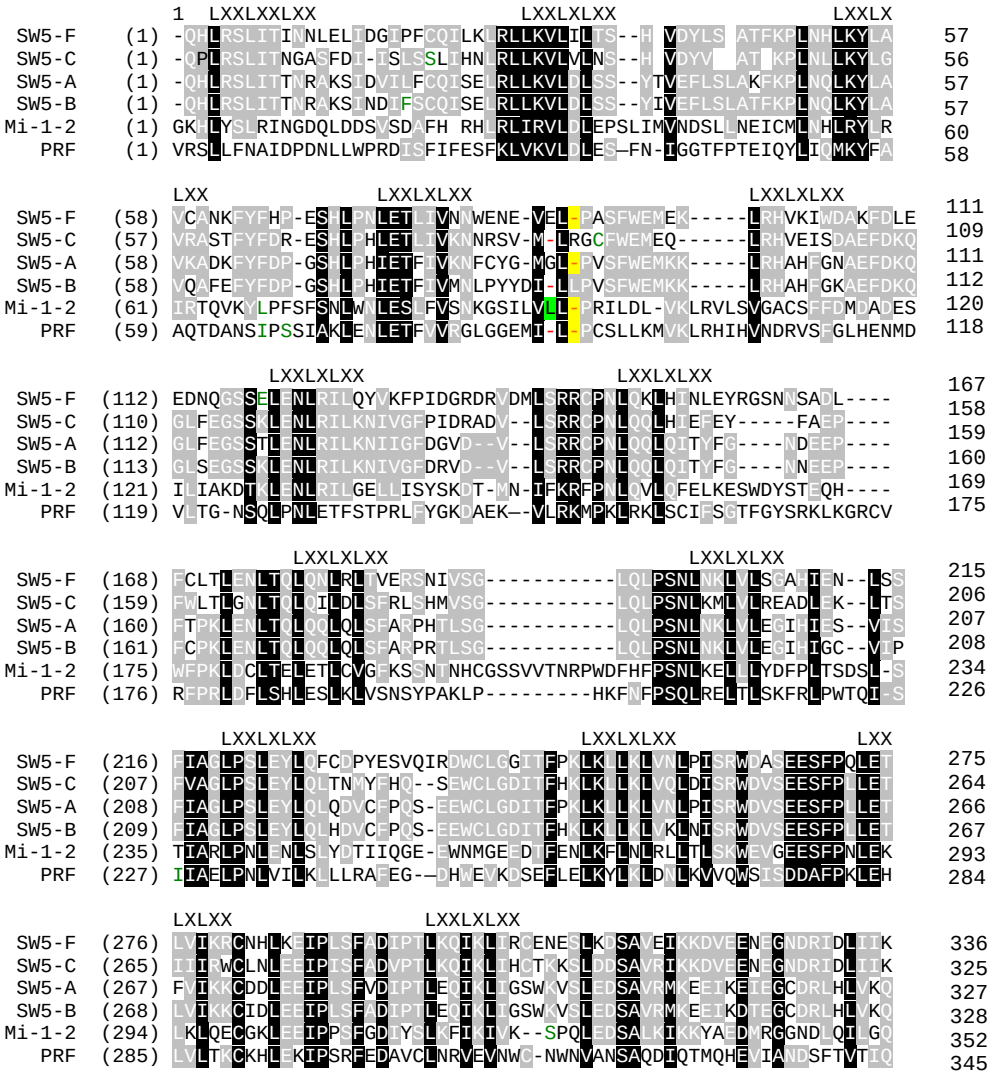

Figure 3. Analysis of SW5-F deduced protein structure. **A**. Schematic domain structure of SW5-F (1275 aa). SD, Solanaceae domain (1-408aa); CC, coiled-coil domain (409--520aa); NB-ARC domain (538-839aa); LRR, Leucine-rich repeat region (892-1275aa), LRR's are represented as (**I**). **B**. Comparison of central conserved NB-ARC domain of SW5-F with Sw5-A (accession no. AAG31013), Sw5-B (AAG31014), Sw5-C (AAG31015), Mi-1.2 (AAC32252), and Prf (AAC49408). Conserved Kinase-1a (p-loop), kinase-2, kinase-3a and Hydophobic domains (HD) are indicated. Displayed are amino acids 538-839 of SW5-F. **C**. Comparison of LRR domain of SW5-F with Sw5-A, Sw5-B, Sw5-C, Mi-1.2, and Prf. The presence of thirteen imperfect Leucine-Rich Repeats in SW5-F is indicated as consensus sequence LxxLxLxx.

SPRYSEC-19 targets the nucleolus of host cells

Due to the presence of an N-terminal signal peptide for secretion, SPRYSEC-19 is likely to be secreted into the cytoplasm of host cells during feeding site initiation and maintenance. Further inspection of the 69 residues between signal peptide and the SPRY domain in SPRYSEC-19 using the algorithm PSORT II predicted (69% probability) that this domain could target the protein to the nucleus of plant cells. To test this, N- and C-terminal GFP fusions were made for SPRYSEC-19 in pK7FWG2 and pK7WGF2, respectively, to assess its subcellular targeting following heterologous expression in tobacco BY2 cells. The predicted signal peptide for secretion was excluded in the constructs to avoid the translocation of the fusion protein to the apoplast. In repeated experiments both the C-terminal and N-terminally GFP fused SPRYSEC-19 localized to the nucleus and nucleolus of BY2 cells (Fig. 4A and B). Similar plasmid harboring GFP alone was used as a reference for the localization of GFP in BY2 cells (Fig. 4C).

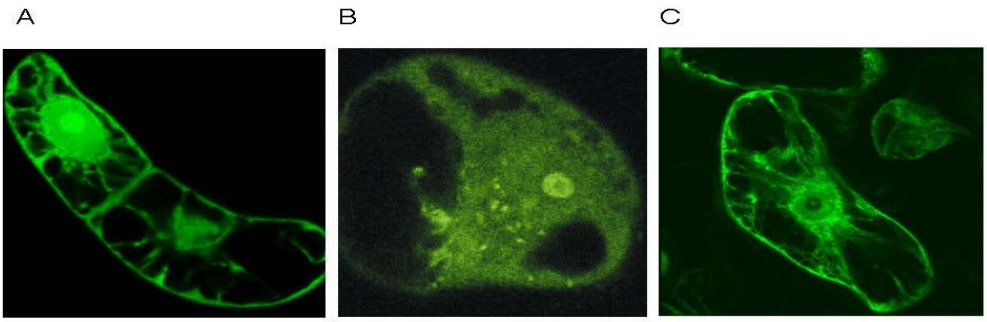

Figure 4A and B. Sub-cellular localization of SPRYSEC-19 in BY-2 cells. C-terminal green fluorescent protein (GFP) fusion of SPRYSEC-19 without signal peptide for secretion localized into nucleus and nucleolus of tobacco BY-2 cells. **C**. Sub-cellular localization of free GFP in BY-2 cells.

Co-expression of SW5-F and SPRYSEC-19 evokes no hypersensitive response

Various R genes of the NB-LRR class mediate a programmed cell death after recognizing pathogen-derived avirulence gene products. The theory predicts that the indirect or direct recognition of pathogen effectors with avirulence activity leads to the activation of disease signaling pathways. We have used agroinfiltration to test if SPRYSEC-19 recognition by SW5-F conditions a hypersensitive response in *N. benthamiana* leaves. Co-expression of SPRYSEC and SW5-F under the control of the cauliflower mosaic virus 35S promoter did not result in a hypersensitive response. In addition, co-infiltration of SPRYSEC-19 with either SW5-A, SW5-B and Gpa2 did not lead to an HR in *N. benthamiana* either. Co-infiltration of PVX coat protein 105 and the R gene Rx1 as a control did produce a strong and specific HR (data not shown).

Discussion

In this study a combination of yeast-two-hybrid analysis and *in vitro* GST pull-down assays showed a specific physical interaction of the nematode effector SPRYSEC-19 with the LRR-region of the tospovirus resistance gene homolog SW5-F from tomato. SW5-F belongs to the CC-NBS-LRR class of resistance genes and shares significant similarity with the tospovirus resistance gene SW5-B (Spassova et al., 2001), the root-knot nematode resistance gene Mi-1.2 (Milligan et al., 1998), and the *Pseudomonas syringae* resistance gene PRF (Salmeron et al., 1994). 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 (Flor, 1971). At the outset of our research, we tested if SPRYSEC-19-activated SW5-F conditions a typical hypersensitive response. 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*. In fact our previous work had already shown that potato plants over-expressing nematode effector SPRYSEC-19 are five times more susceptible to nematodes, fungi, and viruses than wild-type plants. Thus, it seems more likely that nematode-derived effector SPRYSEC-19 promotes the virulence of nematodes in plants by modulating host defense responses possibly through its interaction with SW5-F.

The plant parasitic nematode *Globodera rostochiensis* delivers the products of its parasitism genes (effectors) directly into the host cell cytoplasm through an oral stylet, which essentially is the nematode's equivalent of the bacterial type three secretion system. In the last decade significant effort has been directed to the identification and the functional characterization of a range of nematode effectors but the identity of their molecular targets in plants has largely remained unknown (Qin et al., 2000; Gao et al., 2001; Gao et al., 2003; Huang et al., 2003). Similarly, a number of nematode resistance genes have been identified from host plants but none of the corresponding nematode effectors have been identified at present (Williamson and Kumar, 2006). SPRYSEC-19 is highly expressed and secreted in the early stages of parasitism, and the modulation of SW5-F is likely to take place at the onset of parasitism.

SPRYSEC-19 is a member of large family of proteins consisting of a PRY-SPRY/B30.2 domain and an N-terminal leader peptide to signal for secretion. SPRYSEC-19 is most similar to the SPRY of human RanBP9 and RanBP10 and their functional homologs in other organisms. RanBP9 (RanBPM) was discovered as an interactor of Ran in a yeast-two-hybrid screening. Overexpression of human RanBPM in a metazoan cell line resulted in the distortion of the dynamic stability of microtubules (Nakamura et al., 1998; Nishitani et al., 2001). SPRYSEC-19 does not interact with either of the two Ran protein cloned from tomato (data not shown). Nor does overexpression of SPRYSEC-19 in tobacco

BY-2 cells, harboring the microtubule associated protein 4 (MAP4) fused to GFP, lead to an evident interference in microtubule organization (data not shown).

In addition to its binding to Ran the SPRY domain has been shown to interact with a variety of other proteins mostly involved in signaling pathways, including several receptor protein-tyrosine kinases. For example, the SPRY domain of RanBP9 interacts with MET, a RPTK for hepatocyte growth factor, which is a multifunctional cytokine controlling cell growth, morphogenesis, and motility (Wang et al., 2002). Moreover, constitutive expression of RanBP9 can further activate Ras-Erk-SRE pathway, which is a crucial component of the signaling of many other RPTK (Balkovetz and Lipschutz, 1999). RanBPM also interacts through its SPRY domain with the intracellular domain of the neurotrophin receptor Trk (Yuan et al., 2006), which when activated, initiates several signal transduction pathways such as the MAPK- and the phosphatidylinositol 3-kinase (P13K) pathway (Huang et al., 2003). The SPRY domain of RanBPM mediates the interaction between RanBPM and human dectin-1 trans-membrane receptor isoform hDectin-1E (Xie et al., 2006). The N-terminal part of RanBPM including the SPRY domain is enough to associate with neural cell adhesion molecule L1, which is known to activate extracellular signal regulated kinase pathways (Cheng et al., 2005). Furthermore, Denti et al. (2004) demonstrated that RanBPM is phosphorylated within the SPRY domain at residue Thr320 both constitutively and in response to stress by p58 kinase (Denti et al., 2004). Thr320 is conserved within SPRY domains across species (human, mouse, yeast, *C.elegans, Xenopus*) (Wang et al., 2002), including SPRYSEC-19 (data not shown). It is not clear if SPRYSEC-19 needs to be phosphorylated to be active as an effector in host plants. Further studies are required to investigate if phosphorylation at the conserved threonines in SPRYSEC-19 is required for the activation of its molecular targets in host cells.

Proteins containing SPRY domains have also been implicated in host pathogen interactions in mammals, e.g. the tripartite-motif containing protein TRIM5α. The architecture of TRIM5α consists of ringer finger domain, a B box, a coiled-coiled region, and a C-terminal SPRY domain (Freemont, 2000). TRIM5αrh from rhesus monkeys mediates human immunodeficiency virus-1 restriction at the early post-entry and pre-integration stage of the viral life cycle, whereas the human ortholog of TRIM5αrh does not (Yap et al., 2004). The specificity of the restriction is determined by a small number of residues in the so-called surface A of the SPRY domain of TRIM5α. Even a single amino acid mutation in SPRY (R332P) of human TRIM5α is sufficient to restrict HIV-1 (Yap et al., 2005). Furthermore, a comparative analysis has shown that the residues in surface A of TRIM5α orthologs undergo diversifying selection (Sawyer et al., 2005). The sites that are predicted to constitute the surface A in SPRYSEC-19 are also subject to diversifying selection, and are believed to be pivotal for its interaction with the molecular targets in host cells.

NBS-LRR proteins mediate resistance following direct/indirect molecular detection of pathogen-derived elicitors, during which the LRR domain is involved in determining recognition specificity (McHale et al., 2006). We have shown that a nematode effector, SPRYSEC-19, interacts both *in vivo* and *in vitro* with the seven C-terminal repeats of the LRR-region of the tospovirus resistance gene homologue SW5-F. Our finding supports the hypothesis that the C-terminal part of the LRR domain of CC-NB-LLR type of resistance proteins is involved in pathogen detection. Similar, but indirect, evidence has been found with Rx, which confers resistance against various strains of potato virus X and belongs to NBS-LRR class of R-genes including 15-LRR repeats (Faruham and Baulcombe, 2006). A mutation in 2nd LRR (S516G) enhanced the activation function of Rx whereas the mutation in 12th LRR (N796D) and 14th LRR (N846D) resulted in altered recognition specificity. Other supporting evidence has come from studies on R genes to the rice blast fungus *Magnaporthe grisae*. The R-genes Pi2 and Piz-t, which are functional alleles with only 8 amino acid differences in C-terminal leucine rich repeats (LRR-11,-12, and-13), confer resistance to different isolates of *M. grisae*. A reciprocal exchange of one hydrophilic amino acid within the xxLxLxx motif of LRR-11 between Pi2 and Piz-t did not change the resistance specificity but, rather, abolished the function of both resistance genes (Zhou et al., 2006). Therefore, C-terminal LRR-repeats seem to play a crucial role in pathogen recognition.

In order to further elucidate the role of LRR as a determinant of recognition specificity, we also tested the interaction of SPRYSEC-19 with LRDs of various resistance proteins of the NBS-LRR class in the yeast-two-hybrid system. We found that SPRYSEC-19 interacts with the C-terminal LRR region of SW5F (LRR-SW5-F), but not with LRDs of Rx, Gpa2, RPM1, RPS5, I-2, Mi-1.2, SW5A, and SW5B (data not shown). Similarly, it has been demonstrated that the flax rust R gene allele L6 normally interacts with six variants of Avr567 (a, b, d, f, j, and l), but a chimerical L6 containing 11 amino acids from LRD of L11 (L6L11RV) only abrogated its interaction with Avr567 variants (a, b, d, f, j, l) but instead recognized Bs1-derived Avr567-J (Dodds et al., 2006).

Although many plant R proteins and the corresponding pathogen effectors with avirulence activity have been cloned, a direct interaction has been documented for only a few combinations. Rice expressing the Pi-ta gene develops a resistance response to strains of rice blast fungus *Magnoporthe grisea*, expressing AVR-Pita in a gene-for-gene manner. The LRD of Pita has also been shown to bind directly both *in vivo* and *in vitro* with AVRPita176, which triggers the resistance response (HR; (Jia et al., 2000)). Even a single amino acid substitution either in AVRPita176 or in Pita-LRD not only abolished their physical interaction but also lead to a loss of resistance. A direct binding has also been shown between the products of the tomato bacterial speck resistance gene Pto and the corresponding *Pseudomonas syringae* effector AvrPto. Pto is a protein kinase that requires

the CC-NB-LRR protein Prf to condition disease resistance (Scofield et al., 1996; Tang et al., 1996). Similarly, the *Arabidopsis thaliana* resistance gene RRS1-R binds specifically with effector molecule avrPop2 derived from *Ralstonia solanacearum*, the causal agent of bacterial wilt, and confers broad-spectrum resistance to various bacterial strains (Deslandes et al., 2003). Likewise, the flax (*Linum usitatissimum*) R-genes L5, L6, and L7 interact directly to the products of Avr567 genes derived from various strains of the flax rust fungus (*Melampsora lini*), and confer a resistance response to the fungus (Dodds et al., 2006). All above mentioned examples of direct interactions were identified by a yeast-two-hybrid system. Interestingly, the reciprocal combinations of avr-Pita176 fused to the DNA binding domain of GAL4 and LRD-Pita fused to the activator domain of GAL4 failed to interact in yeast (Jia et al., 2000). Similarly, RRS1-R and avrPop2 also failed to interact in a reciprocal configuration in a yeast-two-hybrid analysis (Deslandes et al., 2003). We found interaction between SPRYSEC-19 and LRR-SW5F in both reciprocal combinations, which may be indicative for the strength of the interaction. It should be noted that full-length SW5-F did not interact with SPRYSEC-19 in a yeast-two-hybrid experiment. Others have had similar observations with full-length R proteins. For example, full-length Pita failed to interact with avrPita176, and full-length MLA6 also failed to interact with HvWRKY-1/2 (Jia et al., 2000; Shen et al., 2007). The loss of binding with the complete proteins may be explained by steric constraints, the lack of specific inter- and intra molecular interactions, or a size limitation of the system.

The structure of the cloned SW5-F shows a high amino acid sequence identity (78-88%) with five members from the SW5 R gene cluster in tomato, of which only SW5-B has been implicated in TSWV resistance (Spassova et al., 2001). We tested if SW5-F confers resistance to TSWV using agroinfiltration assays. Resistance gene homologs from a single cluster can be directed against entirely different pathogens e.g., Rx and Gpa2 belong to same gene cluster (sharing 86% amino acid identity) but display resistance against potato virus x and the parasitic nematode *G. pallida*, respectively (van der Vossen et al., 2000). However, transient expression of SW5-F in *N. benthamiana* did not restrict TSWV suggesting that SW5-F does not induce resistance against tospovirus (data not shown). One could argue that the members of the SW5 R gene cluster from tomato may not be functional in *N. benthamiana*. However, in a recent paper Margaria et al. (2007) have shown that SW5-B is capable of inducing a HR in *N. benthamiana* when co-expressed with its viral avirulence gene, which makes this argument less likely.

Most R-genes of the NB-LRR class trigger a hypersensitive response after recognizing the corresponding avirulence gene product of the pathogen. However, co-expression of SPRYSEC-19 and SW5F in *N. benthamiana* leaves did not result in a hypersensitive response. Potato plants harboring the SW5-F gene are not resistant to potato cyst nematodes, including nematodes expressing the SPRYSEC-19. Given further that the

tomato cultivar used for the yeast-two-hybrid analysis is susceptible to *G. rostochiensis*, we have no evidence that the interaction between SW5-F and SPRYSEC-19 conditions resistance to the nematodes. This data leads us to two alternative models for the role of SPRYSEC-19 and SW5F in nematode-plant interactions. In the first model, the physical interaction between SPRYSEC-19 and SW5F is an evolutionary intermediate. There is evident binding between the two proteins but this does not lead to the activation of disease resistance signaling (anymore). Other R gene homologs of SW5F may exist that both bind and elicit a resistance response following the interaction with SPRYSEC-19. Alternatively, other homologs of SPRYSEC-19 that also bind to SWF and do elicit a resistance response may exist in *G. rostochiensis*. In our second model, SPRYSEC-19 binds to SW5-F to promote the virulence of the nematode by suppressing host defense responses.

In order to test the first model, SPRYSEC-19 was also co-expressed with SW5-A and SW5-B in *N. benthamiana*. Although we cannot exclude that other genotypes do have SW5 homologs capable of triggering an HR when exposed to SPRYSEC-19, none of the combinations tested so far resulted in a hypersensitive response. We have also co-expressed other homologs of SPRYSEC-19 with SW5F, but this did not result in an HR either. In contrast, we found that potato plants overexpressing SPRYSEC-19 in the background of SW5F are supersusceptible to infective juveniles of the potato cyst nematode. In our second model, SPRYSEC-19 modulates or suppresses innate immunity in host plants through its interaction with the CC-NBS-LRR protein SW5-F. Further transcriptome analysis of plants co-expressing SW5-F and SPRYSEC-19 will shed light on the molecular mechanism underlying our observations, and may further establish a causal relationship between the physical interaction of SW5-F and SPRYSEC-19 and suppression of innate immunity in host plants.

Recent studies have shown that bacterial effectors promote virulence by suppressing PAMP-triggered immunity and that this effect is prevented by CC-NB-LRR type R-proteins. Shen et al. (2007) showed that the avirulence gene A10 from *Blumeria graminis* induces the CC-NBS-LRR R protein MLA to localize to the nucleus to interact with WRKY1/2 transcription factors. This nuclear interaction results in de-repression of PAMP-triggered basal defense. Over-expression of HvWRKY1/2 in leaf epidermal cells resulted in hypersusceptibility to *B. graminis*, whereas, silencing of HvWRKY1/2 heightened resistance to various isolates of *B.graminis* (Shen et al., 2007). That the nucleus in host cells is part of the battlefield in molecular plant-pathogen interactions is becoming more evident with the findings of other groups as well. Pop2 is a bacterial effector from *Ralstonia solanacearum* and possesses a full NLS on its N-terminus, and the R-protein RRS1-R localizes to nucleus in a Pop2-dependent manner (Deslandes et al., 2003). We also found that SPRYSEC-19 possesses an N-terminal nuclear localization signal, and that GFP-fused SPRYSEC-19 indeed localizes to the nucleus and nucleolus when expressed in

tobacco BY2 cells. Further work is required to investigate if the SW5-F protein has a nuclear localization, if such a nuclear localization is dependent on SPRYSEC-19, and if a co-localization in the nucleus leads to transcriptional regulation of factors involved in basal defense responses.

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Chapter 6

General discussion

Introduction

Plant-parasitic nematodes have evolved complex relationship with their host plants, which involves the re-differentiation of root cells into specialized feeding structures. Nematodes secrete a whole repertoire of effector molecules to bring about these changes in the host cells. The overall objective of this thesis was to study members of the SPRYSEC gene family including their role as effectors in plant-parasitism. This thesis essentially breaks down in two parts. The first part is more methodological in which we describe our efforts to develop procedures to knock-down genes in the potato cyst nematode with RNA interference (RNAi) to study their importance in parasitism. Where as, the second part of this thesis describes the discovery of the SPRYSEC gene family and their role as nematode effectors in plant-parasitism.

The evidence from the gene knock-downs is important as the associated phenotypes may provide leads to the disease signaling pathways in plant-parasitism. In chapters 2 and 3, we used nematode cellulases to test our methods anticipating that knock-downs of cellulases would give an easily observable phenotype. Previously, it had been shown that these nematode cellulases are involved in the intracellular migration (Chen et al. 2005). The phenotype we expected to observe was first a reduced infectivity (chapter 2), which could be explained by reduced root penetration (chapter 3). To knock-down cellulases, the nematodes were soaked in a highly-concentrated solution of double stranded RNA, which was designed on the sequence of the target gene. Significantly reduced levels of target transcripts were achieved by this so-called soaking method, as well significant phenotypes. The soaking method was developed so that it could be used to test the importance of the SPRYSEC gene family in nematode parasitism. And, if it proved to be significant, to provide clues on the role of the SPRYSECs by deduction from an informative phenotype. Unfortunately, for the SPRYSECs, and as well as many other genes, the soaking method for knocking-down genes seems not adequate enough to provide a phenotype. In this chapter we will further discuss these findings, and provide a perspective on future use of RNAi in plant-parasitic nematodes.

Functional analysis of nematode genes by RNA interference

Previously the functional analysis of parasitism genes was hindered because the plant-parasitic nematodes were refractory to genetic transformation. As a consequence, and in contrast to the free-living bacterivorus nematode *Caenorhabditis elegans*, it has proven not feasible to acquire gene knock-outs by transformation. For long, it was thought that the information from *C. elegans* genes, and corresponding mutants, could be used to acquire more insight in the function of homologs from plant-parasitic nematodes. However, the vast majority of parasitism genes have no match in the genome of *C. elegans*. And, therefore,

for only a small number of parasitism genes there is an informative phenotype available in *C. elegans* (Gao et al. 2003). For instance, the SPRYSECs described in this thesis have no match in the genome of *C. elegans*.

The discovery of dsRNA mediated transient suppression of endogenous homologous transcripts has been instrumental in functional genomics of many eukaryotes. The mechanism of this phenomenon and the different RNAi pathways are particular well studied in *C. elegans*. In short, in *C. elegans* there is genetic evidence for at least three distinct pathways. First, a ribonuclease Dicer cleaves dsRNA into small interfering RNA (siRNA) which leads to specific degradation of target mRNA. A second pathway facilitates the systemic spread of dsRNA molecules. And last, there is evidence for an amplification pathway in which siRNA separates into two single strand RNA molecules that bind to the target sequence and serves as primer for the RNA dependent RNA Polymerase, which amplifies the target RNA (Plasterk, 2002; Kuznetsov, 2003).

A breakthrough for the field of nematode-plant interactions was the finding that genes in *C. elegans* could be silenced by feeding nematodes on bacteria expressing dsRNA or simply by soaking worms in dsRNA (Fire et al. 1998). For, if feeding dsRNA to free-living nematodes could yield a transient but significant reduction in gene expression, the same may hold true for plant-parasitic nematodes. While *C. elegans* readily takes up food *in vitro* this is not the case for the obligate biotrophic plant-parasitic nematodes, especially the sedentary plant parasites. Thus, feeding behavior in plant-parasitic nematodes either had to be induced chemically or dsRNA needed to be delivered through the host plant on which the nematode naturally feed.

Gene knock-down by soaking in dsRNA

Various chemical compounds are known to affect pharyngeal pumping in nematodes which is associated with the release of esophageal gland secretions and, more importantly, the uptake of fluids (Urwin et al. 200o; Rosso et al. 2005; Chen et al. 2005). In chapter 2 and 3, we have shown that *in vitro* uptake of dsRNA by nematodes can be induced when the nematodes are exposed to highly concentrated solution of dsRNA, including the neurotransmitter octopamine. In this procedure nematodes are essentially soaked in a buffer with milligram amounts of dsRNA and are forced to ingest the buffer including the dsRNA for a few hours. Thereafter, the nematodes are transferred to the roots of host plants to assess the importance of the gene targeted by the dsRNA by the loss of infectivity of the nematodes.

Potato cyst nematodes secrete a whole repertoire of cell wall degrading enzymes to facilitate their intracellular migration in the host (Smant et al. 1998; Popeijus et al. 2000). We selected cell wall degrading enzymes as our first targets for RNAi because of their high expression in pre-parasitic J2s and because a reduced ability to penetrate a root is an easily noticeable phenotype. We found that exposure to dsRNA designed on the sequence of Gr-eng-1 resulted in reduced transcript levels of both Gr-eng-1 and its close homolog Gr-eng-2. As a further off-target effect transcript levels of Gr-eng-3 and Gr-eng-4 were also affected by this dsRNA.

In *C. elegans* dsRNA is chopped up in small interfering RNAs (siRNAs) of 21-23 nucleotides by the endo-nuclease *dicer* and these siRNAs in turn associate with and activate the RISC complex (RNA induced silencing complex). The activated RISC complex then screens and degrades specifically homologous mRNA sharing 100% homology with the sequence of the siRNA (Plasterk, 2002). Despite the lack of any evidence that the full RNAi pathways are present in plant-parasitic nematodes, it is likely that the siRNAs are the causal agents in the transcript suppression in these plant-parasites too. Gr-eng-1 and Gr-eng-2 share 72% nucleic acid identity including many stretches of more than 23 nucleotides long with 100% identity. It was therefore not a surprise that dsRNA designed on Gr-eng-1 has an off-target effect on transcripts of Gr-eng-2. Gr-eng-3 and Gr-eng-4 are only less than 44% identical to Gr-eng-1 and Gr-eng-2 with only one stretch of 14 nucleotides long with 100% identity, which makes it more difficult to explain this off-target knock-down by a homologous siRNA. In a recent paper, Rual et al (2007) concluded that off-target effects may occur in mRNA with >95% identity to the dsRNA over 40 nucleotides.

Off-target effects have significant implications for the use of RNAi, especially in organisms for which there is no full genome sequence available. Any dsRNA believed to be specific for a single gene may result in a reduction of transcripts of related, but yet unknown, genes or of unrelated genes that have by chance a short stretch with sequence identity to the primary target gene. Database searches with the sequence of the dsRNA for potential overlapping sequences in other genes may reduce, but cannot completely eliminate, the risk of off-targets. Some caution should therefore be exercised when interpreting an RNAi phenotype in plant-parasitic nematodes. In chapter 2, we found a reduced infectivity of the potato cyst nematode by knocking-down a cellulase. In chapter 3, we tried to monitor the penetration process to find clues in the behavior of the dsRNA-treated nematodes that could explain the reduced infectivity. We found that the dsRNA-treated nematodes were not able to get into the roots or got stuck after breaking down a few cell layers. Thus, close inspection of nematode-plant interaction with dsRNA-treated nematodes such as for instance studying feeding site morphology might be an anticipated link between a knocked-down gene and a phenotype more trustworthy. Further confidence in dsRNA phenotypes can be achieved by complementing a knocked-down gene in the

nematode by overexpressing that same nematode gene in a host plant. However, this may not be feasible for nematode genes whose overexpression have a profound effect on the constitution of the plant.

At present, the importance of several genes has been analyzed by soaking the plant-parasitic nematodes in dsRNA. However, many laboratories have reported that the soaking method seems to work for certain genes, while other putative parasitism genes seem refractory to RNAi by this method. Extensive data from RNAi in *C. elegans* has identified variables which affect the efficiency of dsRNA mediated RNAi, but such variables are just beginning to become clear for plant-parasitic nematodes. In brief, factors that influence RNAi in plant parasites are the length of target dsRNA fragment, topology of the fragment, incubation time, duration of silencing and the target tissue. At least for Gr-eng-3/eng-4, we found that dsRNA designed on either the 5' or 3'-end of the target sequence did not make a significant difference. Although, long dsRNA molecules (~600 bp) were more effective than shorter fragments (150 bp and 300 bp). However, in the gastrointestinal parasitic nematode *Trichostrongylus colubriformis* a 22 bp siRNA was shown to be far more efficient than the longer dsRNA in inducing RNAi (Issa et al. 2005). The direct application of siRNA to plant-parasitic nematodes has not been reported so far. However, it will be a valuable exercise to test siRNA on plant-parasitic nematodes because it may prove to be more efficient than dsRNA, and it will also show if the dsRNA processing in the nematodes is required to achieve efficient RNAi in plant-parasitic nematodes.

The first report of RNAi in plant-parasitic nematodes suggested that soaking in dsRNA for 4 h would be sufficient to achieve RNAi of genes in the potato cyst nematode *G. pallida* (Urwin et al. 2002). We found that an incubation time in dsRNA for at least 24 h was of particular importance to achieve RNAi in *G. rostostochiensis*. We further discovered that longer incubation in the highly concentrated dsRNA of Gr-eng-3 (~40 hours) was more effective than 24 hours soaking. We have also achieved a knock-down of SRPYSEC-19 only after at least 40 h soaking in dsRNA. Experiments with the root-knot nematode *M. incognita* suggest that for this nematode species and the gene that was being targeted an incubation in dsRNA for 4 h was sufficient too. It seems, therefore, that the species of the nematode, and the gene which is targeted by the dsRNA, may both determine the minimal incubation time required to achieve RNAi.

The longevity of the RNAi in plant-parasitic nematode also seems to depend on various factors. Rosso et al (2005), soaked the root-knot nematode *M. incognita* in dsRNA to calreticulin (Mi-crt) and a polygalacturonase (Mi-pg-1) to find that the knock-down was optimal 20 hours and 44 hours after the soaking treatment respectively. But, for both genes they found that the transcripts regained their normal levels 68 hours after treatment. In

contrast, Urwin et al. (2002) showed reduced transcript levels of the major sperm protein Gp-msp for 14 days post treatment. And, Bakhetia et al (2007) showed that the levels of a cellulase mRNA were back at normal levels beyond 10 days post treatment in dsRNA. It needs further investigation to see if the various longevities observed for RNAi are correlated with the tissue in which the target gene is expressed, its transcript level at the time of treatment, and the turnover rate of the transcripts of the target gene.

A further complicating factor in these studies is the storage capacity for proteins in the nematodes. Esophageal gland secretions are expressed and stored in secretory granules in the gland cells well ahead of the anticipated time of their deployment by the nematode. In spite of a profound effect on the transcript level in dsRNA treated nematodes, this may not translate in reduced levels of proteins. We have repeatedly shown a significant reduction in cellulase transcripts in dsRNA-treated nematodes, but in none of these samples we have been able to show reduced protein levels as well. Rosso et al (2005) have made similar observations for the Mi-pg-1 gene in *M. incognita*. If, therefore, the storage capacity for secretory proteins last long enough such that it approaches the time when the mRNA expression recovers from the dsRNA treatment then the actual window for RNAi to achieve a phenotype may be small.

We conclude that RNAi by soaking in dsRNA is a valuable tool for studying nematode genes that are suspected to be involved in parasitism. However, because of the transitory nature of the RNAi following dsRNA by soaking in these nematodes, its use should be limited to the early stages of parasitism. To study genes throughout the parasitic cycle of the nematode, including later parasitic stages, a continuous exposure to dsRNA to nematodes is more appropriate. In the next section, we will discuss a second approach to achieve RNAi in plant-parasitic nematodes by a continuous exposure to host-generated dsRNA.

Gene knock-down by host generated dsRNA

A short exposure to dsRNA seems to induce a transitory RNAi in plant-parasitic nematodes. This phenomenon makes the RNAi by soaking pre-parasitic juveniles in dsRNA of limited value for genes with constitutive expression and for genes expressed later in the parasitic cycle. In order to achieve a constant delivery of dsRNA to the feeding nematode, we (chapter 4) and others have engineered plants such that they express dsRNA molecules to nematode genes. Even if target mRNA is not expressed in the pre-invasive J2 stage, constitutive expression of dsRNA/siRNAs in these transgenic plants may ensure depletion of target transcripts in later stages. A number of potato plants expressing dsRNA to SPRYSEC-19 and SPRYSEC-15 appeared to show a developmental arrest of infecting

potato cyst nematodes. However, in clonal offspring of these plants made by stem cuttings from the primary transformants we have not been able to reproduce this phenotype.

Since 2006, three groups have reported reduced infectivity of nematodes by expressing dsRNA in host plants. Huang et al. (2006a) showed that transgenic *Arabidopsis thaliana* plants expressing dsRNA to the *M. incognita* gene 16D10 resulted in 69-92% reduction in egg count with an overall suppression of nematode development by 74-81% as compared to control plants. The 16D10 gene encodes a conserved secretory peptide in four root knot nematode species (*M. incognita*, *M. arenaria*, *M. javanica*, and *M. hapla*). Overexpression of this peptide in plants stimulates root growth, and molecular analysis suggests that it acts as ligand for a SCARECROW-like transcription factor (Huang et al. 2006b). Huang was able to show the presence of siRNAs in transgenic plants, and a significant correlation was observed between levels of siRNAs and nematode resistance. Unfortunately, the authors did not show a further correlation with a reduction in target mRNA in the nematodes.

Yadav et al. (2003) followed a somewhat different approach and demonstrated that transgenic tobacco lines expressing dsRNA to housekeeping genes of *M. incognita* provided effective resistance against root knot nematodes. Remarkably, in this paper nematodes recovered from these transgenic plants exhibited a knock-down of both integrase and splicing factor mRNA, which were targeted in this experiment. Similarly, nematodes feeding on transgenic tobacco expressing dsRNA to MjTis11, a zinc finger type transcription factor expressed in eggs and eggs producing females, showed depletion of target transcript in these stages although it did not result in a significant decrease in fecundity or egg hatching rate (Fairbairn et al. 2007).

Most of the reports of successful application of host-delivered dsRNA to achieve RNAi in plant-parasitic nematodes involved root-knot nematodes. While many laboratories working with cyst nematodes have failed to achieve similar outcomes for these parasites. It is possible that elements in the biology of the cyst nematodes preclude uptake of dsRNA or siRNA from host plants. For instance, root-knot nematodes and cyst nematode are different in size exclusion limit of stylet orifice. It has been observed that cyst nematodes like *G. pallida* and *H. schachtii* do not ingest dsRNA efficiently, while *M. incognita* readily took up the molecules (Bakhetia et al. 2005). It is not clear if the RNAi by host-delivered dsRNA is conditioned by the uptake of dsRNA molecules or by the uptake of plant-generated siRNA. Root-knot nematodes and cyst nematodes may differ in the susceptibility to siRNA or may differ in their processing ability of dsRNA. Alternatively, the promoters that have been used to control dsRNA expression may be regulated differently in feeding sites of root-knot nematodes and cyst-nematodes. These issues, along with many more that

could be speculated on, underline the need to prioritize further investigation on the RNAi pathways in root-knot nematodes and cyst nematodes.

In principle, host-delivered RNA interference triggered silencing of genes in plant-parasitic nematodes may prove to be a novel disease resistance strategy with wide biotechnological applications. Bioengineering crops with dsRNA to genes of nematodes can disrupt the parasitic process and therefore holds great promise to develop resistant crops against plant-parasitic nematodes. It has been suggested that multi-component dsRNA targeting of two or more genes can further increase the efficacy of RNAi in a parasite.

SPRYSEC proteins from *G. rostochiensis* act as effectors in plants

The second part of this thesis describes the discovery of the SPRYSEC gene family and their role as nematode effectors in plant parasitism. The SPRYSECs constitute a large family of secreted proteins, consisting only of a B30.2/SPRY domain and a signal peptide for secretion. Their specific expression pattern in the esophageal glands of infective nematodes and their predicted secretion from these glands made them interesting candidates as potential effector molecules. In order to demonstrate that the SPRYSECs indeed have a role as effectors, we challenged transgenic potato plants overexpressing SPRYSEC-19 with nematodes. These transgenic plants did not show an aberrant overall morphology, but appeared to be three-to-five time more susceptible to nematodes than wild-type and empty-vector plants. Remarkably, these same potato lines were also found to be supersusceptible to the fungal pathogen *Verticillium dahliae,* and to tomato spotted wilt virus (TSWV). Based on these finding we concluded that SPRYSEC-19 interferes with disease signaling in plants such that it promotes the virulence of diverse pathogens.

The question that immediately arises from these observations is how a SPRYSEC is able to improve the fitness of such unrelated pathogens. The life histories of each of the pathogens in our experiment are essentially covering a whole spectrum. *G. rostochiensis* is a sedentary biotroph that feeds for weeks from a highly advanced and structured feeding cell complex in the roots. The fungus, *V. dahliae,* is a soil-born vascular pathogen that induces chlorosis, necrosis, and wilt in infected plants (Fradin and Thomma, 2006). Rapid colonization by the fungus leads to complete deterioration of the plants within weeks. The biology of the tomato spotted wilt virus (TSWV), with a complete intracellular lifecycle inside living cells, adds more color to this blend (de Avila et al. 1992). Summarizing, there is no clear similarity between the three pathogens in their invasion, feeding, and reproduction behavior. This implicates that SPRYSEC-19 likely modulates a part of the disease-signaling pathways that controls a generic response affecting many different

pathogens, which leaves only a few options of which PAMP-triggered immunity has become our prime candidate.

We have not been able to investigate the molecular mechanism behind the phenotype of SPRYSEC-19 in plants any further experimentally within the framework of this thesis. However, the phenomenon described above appears not to be unique for the nematode effector SPRYSEC-19, and below we want to explore parallels in the literature to construe testable hypotheses for future research.

Suppression of PAMP-triggered immunity in plants

In principle, PAMP recognition by specific extracellular receptors leads to activation of MAPK-signaling, accumulation of ROS, cell wall-based defenses, and the activation of defense related genes to prevent pathogens insurgence (Jones and Dangl, 2006). The suppression of such basal defense responses has been reported for a range of bacterial effectors. *Pseudomonas syringae* and *Xanthomonas campestris* use a type three secretion system (TTSS) as a molecular syringe to inject effector molecules into host cells. The *hrp*-gene cluster in these bacteria codes for components of the TTSS. Consequently, *hrp* mutants are unable to multiply on susceptible hosts, which demonstrates the importance of TTSS effectors (Brown et al., 1995). Overexpression of the bacterial effector AvrPto in plants represses genes encoding proteins involved in cell wall fortification (e.g. extensins) and defense-related proteins. AvrPto overexpression in plants also restores the virulence of *hrp* mutant bacteria, which are normally blocked by localized callose depositions. The exact mechanism behind this is not entirely understood, but AvrPto interacts with two Ras-related Rab proteins which are involved in vesicular trafficking. Vesicle trafficking is required for callose deposition as well for other proteins involved in extra-cellular defense (Hauck et al., 2003). Callose deposition in nematode-plant interactions takes place at the cell wall of the initial feeding cell around the perforations made by the stylet of the nematodes. It is not known if resistance to nematodes is associated with increased callose depositions, for this has not been studied in great detail. But, since the cell wall-based defenses are crucial elements in PAMP-triggered immunity (PTI) in other pathosystems, it is likely to contribute to immunity to nematodes as well. A comparative analysis of the feeding cell structure, including a callose staining, in wild type plants and plants overexpressing SPRYSEC-19 may shed light on the potential role of cell wall based defenses in the phenotype associated with SPRYSEC-19 in plants.

While cell wall based defenses are also involved in fungal infections, this may not hold true for virus-plant interactions. Given that SPRYSEC-19 overexpression in potato affects virus infections, SPRYSEC-19 likely modulates other processes in the host cells. Bacteria have also evolved effector activities to a range of different processes that are

sometimes not mutually exclusive. AvrPtoB overexpression in plants, for instance, repressed the cell death response initiated by Pto/avrPto, by Cf9/avrCf9, and by the pro apoptopic mouse protein Bax (Abramovitch et al., 2003; de Torres et al., 2006). Likewise, AvrPphEpto, HopPtoE, and HopPtoF suppressed host defenses by suppressing the expression of pathogenicity-related proteins such as PR1 (Jamir et al., 2004). To investigate what other mechanisms may explain the phenotype of SPRYSEC-19 overexpression, the impact of SPRYSEC-19 expression on the hypersensitivity response induced by a range of cognate R and avirulence genes should be studied. Furthermore, it will be informative to analyze PTI signaling pathways in infected plants overexpressing SPRYSEC-19 and wild type plants. Similarly, plants overexpressing SPRYSEC-19 could be challenged with bacterial flagellin, a potent inducer of PAMP triggered immunity, to see if in contrast to wild type plants specific pathways are not being activated (Navarro et al. 2004).

The molecular targets of SPRYSECs

In chapter 5, we used the yeast-two-hybrid method to identify the molecular targets of the SPRYSECs in host cells, for we believed that the identity of these interactors could provide us insight in the signaling pathways involved in the phenotype of the SPRYSECs. To this purpose, SPRYSEC-19 was used as bait to fish for interacting proteins in a tomato root cDNA library under high stringency conditions. The potential interactors from the yeast-two-hybrid screening were subsequently validated with GST-pull down assay. In these experiments, we found that SPRYSEC-19 physically associates with the C-terminal part of the leucine rich repeat domain of a CC-NB-LRR protein named by us SW5F. SW5F most likely belongs to the SW5 resistance gene cluster with significant similarities to a range of other Solanaceous NB-LRR genes. These NB-LRR proteins mediate a resistant response, following direct/indirect recognition of pathogen-derived elicitors, which often leads to rapid programmed cell death (hyper sensitive response). We also tested if SPRYSEC-15 interacts with the C-terminal part of the LRR domain of SW5F, and found no evidence for an interaction. SPRYSEC-15 was also used as bait to screen the cDNA library using the same conditions as for SPRYSEC-19. However, none of the initial interactors in this latter screen passed our selection criteria.

To date, a physical interaction between an R protein and a pathogen effector is found only in three other cases, i.e. between Pita/AvrPita, L/AvrL567, and PopP2/RRS-1 (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006). All three examples mentioned above are classical Avr and R gene interactions involved in effector triggered immunity (ETI). Bacterial flagellin interacts with the leucine rich repeat transmembrane kinase receptor protein FLS2. Flagellin reception results in PAMP-triggered immunity (PTI) to the bacteria in plants (Gomez-Gomez and Boller, 2002). A recent study on the CC-NBS-LRR

protein MLA and the corresponding avirulence gene A10 from *Blumeria graminis* has provided a link between PTI and ETI. Shen et al. (2007) showed nuclear localization of MLA in an A10 dependent manner, where it interacts with WRKY1/2 transcription factors to de-repress the PTI. Thus, R-gene association with pathogen derived elicitors could alter host plant resistance responses by modulating activities of negative regulators of basal immunity.

Does SPRYSEC suppression of basal defenses require the NB-LRR protein SW5F

It is likely that SPRYSEC-19 is somehow able to modulate the defense responses to a diverse panel of pathogens. And, we have provided evidence for a physical interaction between SPRYSEC-19 and the NB-LRR protein SW5F. Future research will have to resolve if both observations are causally related or two functionally independent phenomena. As was discussed in chapter 5, SPRYSEC-19 could be an interactor that has either not yet acquired avirulence activity or lost its avirulence activity. Alleles may exist in nematode populations that both bind to SW5F and induce a resistant response. Similarly, orthologs of SW5F in potato and tomato may have evolved to both bind SPRYSEC-19 and activate a disease-resistance signaling pathway. Either way, SPRYSEC-19 and SW5F can be evolutionary intermediates approaching on or departing from a classical gene-for-gene relationship.

When the latter scenario is not true, then SPRYSEC-19 may develop its phenotype through its interaction with SW5F. In this contrasting scenario, binding of SPRYSEC-19 to SW5F leads to suppression rather than activation of disease-resistance pathways. This would implicate that a NB-LRR protein acts as key regulator in the PAMP-triggered immunity of plants. There is an increasing body of evidence that such key regulators exist, but none of currently known regulators belongs to the NB-LRR class of proteins. For instance, RIN4 is a negative regulator of basal defense and bacterial effectors (AvrRpm1, AvrRpt2) induced perturbations of RIN4 are well documented (Mackey et al., 2002; Axtel and Staskawicz, 2003; Kim et al., 2005). In order to test if SW5F, RIN4 alike, is a key regulator of disease resistance signaling, plants lacking SW5F or in which SW5F is silenced, should be challenged with nematodes, fungi, and viruses to assess its effect on disease development. Then, overexpression of SPRYSEC-19 in these same plants will further resolve if the phenotype of SPRYSEC-19 is SW5F dependent.

A testable model for SPRYSECs modulation of molecular targets in host cells

It is likely that the SPRYSECs from nematodes interact with molecular targets in host cells to modulate their activity. The next question that needs to be addressed is how SPRYSECs modulate the activity of host proteins. Three-dimensional structure modeling revealed the presence of a C-terminal helical structure ancillary to the SPRY domain in some of the SPRYSECs. In GUSTAVUS, the protein that was used as a modeling template for the SPRYSECs, this helical structure is annotated as the BC-box. The BC box in SPRY containing SOCS box protein like GUSTAVUS is a crucial element in the ubiquitin-proteasome system. In chapter 4, we proposed a model that explains SPRYSEC-induced modulation of the host proteins by ubiquitylation. The concept of modifying host protein activity by ubiquitylation seems thus to be used by cyst nematodes in more than one way (Davis et al, 2004).

Protein degradation by the ubiquitylation involves the covalent attachment of ubiquitin (Ub) molecules to substrate proteins by the consecutive activities of the Ub activating enzyme E1, the Ub conjugating enzyme E2, and Ub ligase E3. The degradation of ubiquitylated proteins takes place by proteolysis in the 26S proteasome. The specificity of ubiquitin-proteasome system-mediated proteolysis is determined by the E3 ubiquitin ligases, which harbor various interacting domains serving as docking points for particular substrates (Varshavsky, 1991; Willems et al. 2004). Recently, the effector protein AvrPtoB from *Pseudomonas syringae* was shown to have E3 ubiquitin ligase activity *in vitro*, which was required for suppression of plant immunity (Rosebrock et al. 2007). Rosebrock et al (2007) showed that AvrPtoB E3 ligase actually ubiquitylates Fen, which leads to a proteasome dependent degradation of the Fen protein. In tomato plants expressing Fen this degradation results in disease susceptibility. The SPRYSECs do not have a structural similarity to E3 ubiquitin ligases. However, they may act as an adaptor to provide substrate specificity to other E3 ubiquitin complexes.

To date, several multiprotein complexes with E3 activity have been identified (Reviewed in Schwechheimer, 2004). For instance, Skp1-Cullin-F-box (SCF) E3 ubiquitin ligases belong to the RING ubiquitin ligase family characterized by an invariant core composed of Skp1 linker protein, scaffolding protein Cullin, RING finger protein Roc1 (Rbx1) and E2-enzyme. The C-terminus of F-box proteins is highly variable harboring specific protein-protein interaction domains. Skp1 links the substrate containing F-box adaptor protein to the core catalytic unit containing Roc1-E2 enzyme, which catalyses the transfer of Ub-moieties to bound substrate (Willems et al. 2004). Analogous to this SCF-ligase complex, in ElonginB/C-Cullin-SOCS-box (ECS) E3 ligase complex, the SOCS-box couples via the BC box substrates to core components of the E3-ligase complex (Elongin B/C-Cullin-Roc1)(Fig. 1). Structural analysis of ECS-complexes revealed that Cullin serves

as scaffolding protein and that it positions the SOCS-box containing substrate conjugate and E2 for efficient transfer of ubiquitin moieties to the substrate. The BC box is important component of SOCS-box, which uses the Elongin- B/C heterodimer as a bridge to connect to an E3-complex for ubiquitylation (Kile et al. 2002). Similarly, the BC box in SPRYSECs may be used to bind elongin C (and thereby elongin B) and to link up with an E3 ubiquitin ligase complex. In our model the SPRY domain binds to molecular targets in host cells, while the BC box targets the bound SPRYSEC/host protein to E3 ubiquitin ligase complex for ubiquitylation and subsequent proteolysis by the proteasome.

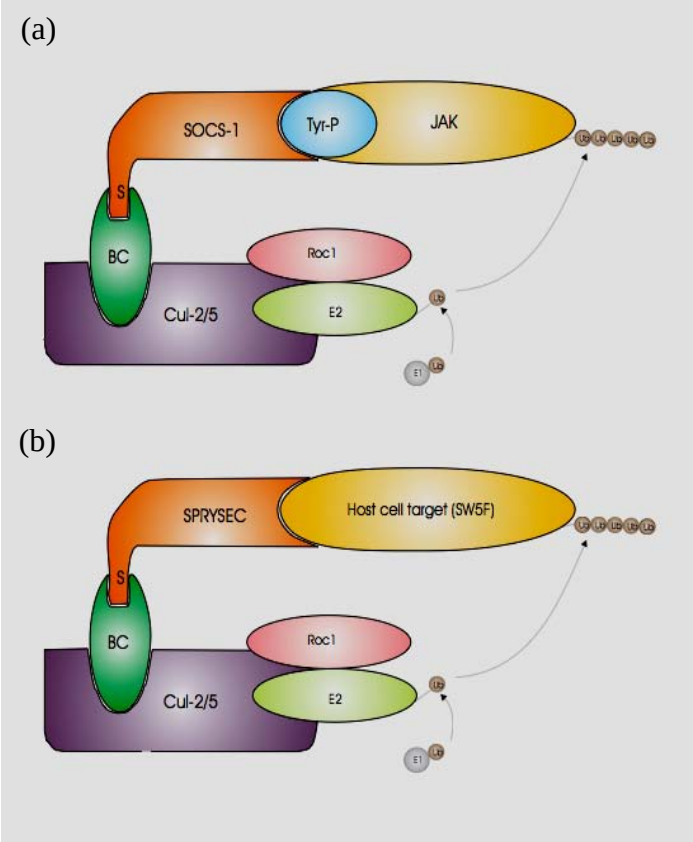

Figure 1.Comparison of the structure of ECS-type E3 ubiquitin ligase with EC-SPRY mediated host proteins ubiquitination model. a) SOCS box binds via its BC box (S) with core components of E3 complex: elongins B and C, the cullin 2/5, and RING-finger protein Roc1 which docks ubiquitin conjugating enzyme E2. Ubiquitin activating enzyme (E1) activates ubiquitin (Ub). b) SPRYSEC binds via its BC box (S) with elonginc BC, which is bound to Cullin-Roc1-E2. Both ECS and EC-SPRY contain substrate recognition motif (orange). SOCS-1 (a) interacts with JAK depending upon post-translational modification, where as, SPRYSEC interacts with host cell target (SW5F). In the next step, the coordinated activities of E1 and E2 mediate transfer of ubiquitin moieties on cysteine residue of target protein (JAK or SW5F), catalyzed by ubiquitin ligase (E3). Abbreviations: ECS, elongin-C-cullin-SOCS-box; JAK, Janus kinase; EC-SPRY, elongin-C-cullin-SPRY; SOCS, suppressor of cytokine signaling.

In chapter 4, we have shown that SPRYSEC is a large gene family comprising of at least 22 members. For each family member we have been able to find several variants suggesting that nematodes inject many different SPRYSECs into host cells. Remote homology modeling revealed that SPRY domains in SPRYSECs adopt a novel fold consisting of a distorted compact ß-sandwich core formed by two anti-parallel ß sheets which are connected with variable loops (Figure 2). Sequence conservations among SPRYSECs are confined to the ß-strands. Most of the variations in sequence and in length are in the loop regions. Some of the amino acid sites in these loop regions are under diversifying selection. The core structure of SPRY domains is reminiscent to that of immunoglobulins, which also comprises a ß-sandwich formed from two anti-parallel ß-sheets (Fig. 2). The antigen binding specificity of immunoglobulins is determined by sequence and length variations in three hyper variable loop regions. These variations in the loop regions provide immunoglobulins the capacity to bind an incredible large number of antigens (Alberts et al. 2004). Similarly, we believe that potato cyst nematodes use the SPRY domain in SPRYSECs as a versatile framework with the capacity to create large binding varieties to various host proteins. With their BC box the SPRYSECs are able to direct a wide range of bound host proteins to an E3 ubiquitin ligase complex for proteolysis by the proteasome.

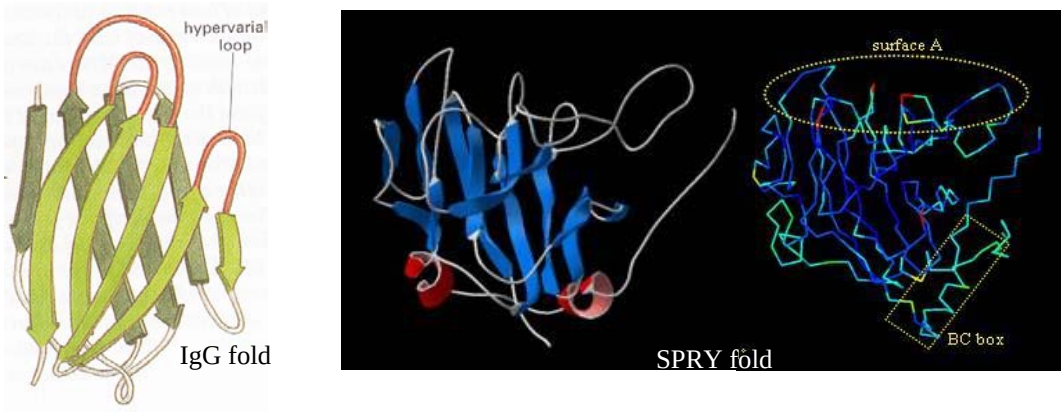

Figure 2. Comparison of IgG and SPRY fold. Hypervariable loops in IgG are shown in red. For SPRY fold loops are indicated as grey color and surface A and BC box are also visible.

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Summary

Plant-parasitic nematodes inject a full repertoire of effector molecules into host cells. These effectors are largely being produced in the esophageal glands of the nematodes. Delivery of the effectors into the host cells occur through the oral stylet of the nematode. For the majority of effectors it is not known what their role in nematode-plant interactions is. However, they are believed to be required for host invasion, feeding cell formation, and evasion and suppression of host innate immunity. This thesis describes the identification and characterization of a novel group of nematode effectors from the potato cyst nematode *Globodera rostochiensis*, which we named the SPRYSECs.

The thesis essentially breaks down into two parts. To better understand the role of the SPRYSECs in nematode-plant interactions, we first developed a method to use RNA interference in the potato cyst nematode *G. rostochiensis*. Chapters 2 and 3 report on our attempts to achieve a transient suppression of mRNA coding for nematode effectors in *G. rostochiensis*. Several papers had described earlier the use of RNA interference in nematodes by soaking the worms in highly concentrated solution of dsRNA. However, these experiments were done with the free-living nematode species *Caenorhabditis elegans*, which naturally feeds *in vitro*. A breakthrough for our research was the finding that dsRNA was taken up by plant-parasitic nematodes when the nematodes were exposed to the neurotransmitter octopamine by a group from the University of Leeds (UK). We have adapted the soaking-method such that it worked on the pre-parasitic juveniles of the potato cyst nematode *G. rostochiensis*. In chapter 2 cellulases, an amphid specific gene (*ams*), and the SPRYSECs were targeted for RNA interference. By using the cellulase and the amphid-specific gene we anticipated to find an easily scored phenotype, for both are likely to be important at the onset of parasitism. Soaking worms in dsRNA with octopamine resulted in reduced mRNA levels for cellulase, *ams*, and SPRYSECs. For the cellulase and *ams* reduced mRNA levels greatly affected the virulence of the nematodes. However, reduced SPRYSEC mRNA did not lead to a reproducible phenotype in nematodes on a plant.

In chapter 3 our method for RNA interference in *G. rostochiensis* was further tested on two novel cellulases, of which it was known that they represented the most abundant cellulases in stylet secretions. Again, we found a strong reduction in mRNA levels and severely compromised pathogenicity in the nematodes. Close inspection of the infection process revealed that the loss of pathogenicity could be explained by poor host penetration capabilities. The nematode appeared to be no longer able to breach the epidermal cell layer, or if they had penetrated the epidermis they got stuck in the subepidermal tissue. These observations matched the expected phenotype of a cell wall degrading enzyme, whose role seems particularly important during invasion of the host.

RNA interference by soaking pre-parasitic nematodes in dsRNA seems a valuable asset to study effectors that are active in the early stages of plant-parasitism. For the SPRYSECs, however, the soaking method did not result in a significant effect on nematode virulence. This could imply that the soaking method was not adequate for all effectors in plant-parasitic nematodes. Alternatively, the SPRYSECs represent a multi-member gene family and reduced levels of a single or a few members does not significantly affect nematode virulence. In order to test the first hypothesis we made transgenic plants that constitutively produce SPRYSEC dsRNA. Nematodes feeding on these plants are likely to ingest host-generated dsRNA too. Unfortunately, these transgenic plants have not resulted in reproducible outcomes.

The SPRYSECs as novel group of effectors in nematode-plant interactions have not been studied before. Our work described in this thesis has revealed some intriguing features of the members of SPRYSEC family. Chapter 4 describes the discovery of the SPRYSECs in cyst nematodes along with their unique structural characteristics. It appears that the SPRYSEC proteins consist of highly conserved stretches that make up the core of the protein interspersed with highly variable segments. Especially the sequence elements that fold into one of the surfaces of the protein is subject to extraordinary evolutionary forces leading to hypervariability. The SPRYSECs are structurally reminiscent to the variable domain in immunoglobulins. The build of these latter variable domains also includes a highly conserved framework with hypervariable complementary determining regions (CDRs).

Overexpression of one of the SPRYSECs in transgenic potato leads to supersusceptibilty of the plants with up to five times increase in infectivity of the nematodes. Strikingly, these same plants appear to be significantly more susceptible to fungi and viruses. We hypothesize that the SPRYSECs modulate disease-signaling pathways in plants, which affects virulence of unrelated pathogens. Given that the biology of nematodes, fungi, and viruses have little in common the supersusceptibility likely involves a highly generic mechanism such as the basal immunity (i.e. PAMP-triggered immunity). In chapter 4, we propose a model that may explain how SPRYSECs regulate their molecular targets inside host cells.

In chapter 5, we describe the finding of a protein segment from the host that interacts with the one of the SPRYSEC family members. This protein segment matches the consensus of the so-called Leucine-Rich Repeat (LRR) from disease resistance genes. Further analysis of the LRR actually showed that it likely originates from a CC-NB-LRR protein from the SW5 R gene cluster in tomato and potato. Other members of the SW5 cluster confer resistance to tospoviruses. Potato and tomato plants harboring this new member of the SW5 cluster (SW5F) are not resistant to *G. rostochiensis*. Transient co-

expression of the SPRYSEC and its interactor SW5F does not lead to hypersensitive response typically associated with disease resistance. Therefore, it seems not likely that SW5F is a functional R gene towards *G. rostochiensis*. Our alternative hypothesis is that the SPRYSEC modulates basal immunity through its interaction with SW5F by a yet unknown mechanism. Further research is required to test if the supersusceptibility following overexpression of the SPRYSEC indeed suppresses basal immunity, and if this suppression is mediated through SW5F.

In the final chapter we further focus on specific aspects of RNA interference in plant-parasitic nematodes, and we elaborate on our immunity modulation model and put it into perspective by studying parallels in the scientific literature.

Samenvatting

Plantparasitaire nematoden injecteren een groot aantal effector moleculen in de cellen van een waardplant. Deze effectors worden geproduceerd in de faryngeale klieren van de nematoden, en worden via een stylet in de mondholte naar buiten gebracht. De functie van veel van deze effectors is nog onbekend, maar het vermoeden bestaat dat ze betrokken zijn bij de invasie van de gastheer, bij de transformatie van gastheercellen tot voedingscellen, en/of bij het onderdrukken of ontwijken van de immuniteit van de gastheer. Dit proefschrift handelt over de identificatie en analyse van een nieuwe groep van effectors uit het aardappelcystenaaltje *Globodera rostochiensis*, die we de SPRYSECs hebben genoemd.

Dit proefschrift bestaat feitelijk uit twee delen. Om de rol van SPRYSECs in nematode-plant interacties beter te kunnen begrijpen, hebben we een manier ontwikkeld om RNA-interferentie toe te kunnen passen in *G. rostochiensis*. In hoofdstuk 2 en 3 beschrijven we onze pogingen om transiente knock-downs te maken van effectors in *G. rostochiensis*. Uit de literatuur bleek dat RNA-interferentie kon optreden in nematoden door ze te induceren in oplossingen met extreem hoge concentraties dsRNA. Deze observaties waren echter gedaan aan de vrij-levende soort *Caenorhabditis elegans*. Uit onderzoek van collega's van de Universiteit van Leeds (UK) was gebleken dat plantparasitaire nematoden ook dsRNA konden opnemen mits de neurotransmitter octopamine was toegevoegd aan de incubatievloeistof. Wij hebben de Leeds-methode aangepast zodanig dat het toepasbaar was op de pre-parasitaire stadia van het aardappelcystenaaltje *G. rostochiensis*. In hoofdstuk 2 zijn een cellulase, een amfiede specifiek gen (*ams*), en de SPRYSECs als target gebruikt. De verwachting was dat een knock-down van de cellulasen en het *ams* duidelijk zichtbaar fenotypen zouden geven, omdat beide genen vroeg in de interactie een rol spelen. Het is ons gelukt om de mRNA niveaus van het cellulase, het *ams*, en de SPRYSECs te reduceren met RNA interferentie door nematode te incuberen in dsRNA. Bovendien bleek dat deze reductie in mRNA voor het cellulase en het *ams*-gen grote invloed had op de virulentie van de nematoden. De reductie van het SPRYSEC mRNA resulteerde niet in een reproduceerbaar fenotype op de plant.

In hoofdstuk 3 is de methode voor RNA interferentie in *G. rostochiensis* uit hoofdstuk 2 verder getest op twee nieuwe cellulasen, waarvan bekend was dat ze de meest abundante cellulasen in de stylet secreties van *G. rostochiensis* zijn. Wederom vond een sterke reductie in mRNA niveau plaats met als gevolg een afname van virulentie van de nematoden. Door het infectieproces nauwgezet te monitoren bleek dat de afname van virulentie verklaard kon worden door een gebrekkig penetratie van de gastheer. De nematoden bleken de epidermis niet meer te kunnen doorbreken, of ze bleven na enkele cellagen steken in het weefsel. Dit beeld past bij de verwachtte activiteit van de cellulasen als celwand afbrekende enzymen, en hun belangrijke rol bij invasie van de gastheer.

RNA interferentie door middel van incubatie van pre-parasitaire nematoden lijkt voor effectors, die actief zijn in de vroege stadia van de interacties met de gastheer, goed te werken. Voor de SPRYSECs bleek de methode niet te leiden tot een duidelijk effect op de virulentie. Dit kon betekenen dat methode op basis van incubatie in dsRNA niet adequaat was voor de SPRYSECs. Een alternatieve verklaring zou zijn de reductie van SPRYSEC mRNA niet een direct meetbaar effect op de virulentie heeft. Als alternatieve methode om dsRNA te voeren aan nematoden hebben we daarom transgene aardappelplanten gemaakt die constitutief dsRNA tegen SPRYSECs produceren. Immers parasitaire nematoden die zich voeden aan deze planten, zouden zo ook voortdurend het dsRNA mee opnemen. Helaas, hebben deze transgene planten geen eenduidig resultaat opgeleverd.

De SPRYSECs als zijn als groep nog nooit eerder bestudeerd. Uit dit proefschrift blijkt dat het een bijzonder boeiende familie van eiwitten is. In hoofdstuk 4 is beschreven hoe de familie is ontdekt, en wat de structurele karakteristieken zijn. Wat opvalt is dat de eiwitten bestaan uit een kern van zeer geconserveerde stukken, die worden afgewisseld met hypervariabele stukken. Vooral de delen van het eiwit aan één van de oppervlaktes lijkt onderhevig aan de evolutionaire krachten die resulteren in extreme diversiteit. Structureel lijken de SPRYSECs op de variabele domeinen in antilichamen, met daarin een hypervariabele complementariteit bepalende regio's (CDRs) die worden bijeengehouden door een stabiel framewerk.

De overexpressie van één van de SPRYSECs in transgene aardappelplanten leid ertoe dat de planten tot vijf keer zo vatbaar worden voor de nematoden. Opvallend is dat deze supervatbaarheid niet beperkt blijft tot de nematoden, maar ook optreedt bij infecties met schimmels en virussen. Onze hypothese is dat de SPRYSECs kennelijk iets in de plant moduleren waardoor het parasitisme van zeer uiteenlopende organismen wordt bevorderd. Gezien het feit dat de infectiecycli van nematoden, schimmels en virussen weinig gemeenschappelijks hebben, lijkt het waarschijnlijk dat de SPRYSECs zoiets generieks als de basale immuniteit (PAMP-geïnduceerde immuniteit) onderdrukken. In hoofdstuk 4 stellen we een model voor hoe de SPRYSECs hun moleculaire targets in een cel van de gastheer zou kunnen beïnvloeden.

Hoofdstuk 5 beschrijven we de vondst van een stuk uit een eiwit uit een waardplant dat fysiek bind aan de één van de leden van de SPRYSEC familie. Dit stuk eiwit bestaat uit zogenaamde *leucine-rich repeats* (LRR) die grote overeenkomst hebben met de LRRs van ziekteresistentie genen. De LRR bleek dan ook afkomstig van een CC-NB-LRR eiwit dat vermoedelijk lid is van het SW5 R gen cluster uit tomaat en aardappel. Andere R genen uit dit cluster geven resistentie tegen tospovirussen. Aardappelplanten en tomaat met het SW5 lid (SW5F) dat bind aan de SPRYSEC zijn niet resistent tegen nematoden. Co-expressie van de SPRYSEC en SW5F resulteert niet in een

overgevoeligheidsreactie. Het lijkt dus niet waarschijnlijk dat het SW5F een functioneel R gen tegen *G. rostochiensis* is. Een alternatieve hypothese is dat de binding van SPRYSEC met SW5F leidt tot modulatie basale immuniteit op een vooralsnog onbekende manier. Toekomstig onderzoek zal moeten uitwijzen of supervatbaarheid na overexpressie van een SPRYSEC inderdaad modulatie van immuniteit is, en of deze modulatie gereguleerd wordt via de interactie met SW5F.

In het laatste hoofdstuk zijn we nog verder ingegaan op de bijzondere aspecten van RNA interferentie in plantparasitaire nematoden, en plaatsen we het modulatie model van de SPRYSECs in een breder context door te kijken naar paralellen elders in de literatuur.

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List of Publications

Chen, Q., **Rehman, S**., Smant, G., and Jones, J. T. 2005. Functional analysis of pathogenicity proteins of the potato cyst nematode *Globodera rostochiensis* using RNAi. Mol. Plant-Microbe Interact. 18:621.

In preparation

Rehman, S., Butterbach, P., Popeijus, H., Overmars, H., Davis, E. L., Jones, J. T., Helder, J., Goverse, A., Bakker, J., Smant, G. 2007. The identification and characterization of novel and most abundant cellulases in potato cyst nematode stylet secretions.

Rehman, S., Qin, L., Overmars, H., Petrescu, A.J., Thomma, B., Helder, J., Goverse, A., Baker, J. Smant, G. Overexpression of a nematode SPRYSEC-gene in potato promotes the virulence of a nematode, a fungus, and a virus.

Rehman, S., Qin, L., Overmars, H., Vossen, J., Helder, J., Goverse, A., Bakker, J., Smant, G.
The nematode effector SPRYSEC-19 interacts with the Leucine-Rich-Repeat-domain of a CC-NB-LRR protein from Solanaceae.

Published abstracts

Rehman, S. (2002) The recombinant expression of and the generation of antibodies against RanBPM homologues from potato cyst nematode *Globodera rostochiensis*. *In: Disease Resistance in Plants : Autumn school 2002, Wageningen 14-16 October, pp. 66.*

Rehman, S., Qin, L., Overmars, H., Goverse, A., Helder, J., Bakker, J., Smant, G. 2004. Functional analysis of the RanBPM-like gene family in *Globodera rostochiensis*. *In: XXVII European Society of Nematologists (ESN) International Symposium, Rome, pp. 80 - 81*.

Rehman, S., Qin, L., Overmars, H., Goverse, A., Helder, J., Bakker, J., Smant, G. 2005. Functional analysis of the RanBPM-like gene family in Potato cyst nematode *Globodera rostochiensis*. *In: 12th International Congress on Molecular Plant-Microbe Interactions, Cancún, Mexico, pp. 160.*

Rehman, S., Qin, L., Overmars, H., Goverse, A., Helder, J., Bakker, J., Smant, G. 2006. Functional analysis of the DGL-2-gene family in potato cyst nematode *Globodera rostochiensis*. *In: European Society of Nematologists XXVIII International Symposium, Blagoevgrad-Bulgaria, 5-9 June 2006.*

Rehman, S., Overmars, H., Qin, L., Goverse, A., Bakker, J., Smant, G. 2007. The identification and characterization of the sprysec - gene family as effecters in *Globodera rostochiensis* Plant –parasitism. In*: XIII International Congress on Molecular Plant-Microbe Interactions. July 21-27,2007, Sorrento, Italy. Book of Abstracts, p p. 77.*

Curriculum Vitae

Sajid Rehman was born on 24th of April 1975 in Suianwala, Pakistan. He studied at University of Agriculture, Faisalabad from 1994-to-1999 and obtained his BSc. (Hons.) Agriculture degree with specialization in agricultural entomology. In 2000, he received fellowship from Nuffic (Netherlands organization for cooperation in higher education) to do MSc in Crop Science in Wageningen University with specialization in Integrated pest management (IPM). He obtained his MSc degree with distinction (cum laude) from Wageningen University in March 2002 with a thesis entitled "Recombinant expression of and generation of antibodies against RanBPM homologues (A41, A18, E19) from the potato cyst nematode *Globodera rostochiensis*". From June 2002 to June 2007 he was appointed as Assistant in opleiding (as PhD researcher) at Laboratory of Nematology, Wageningen University. His research was focused on studying the interactions between plant parasitic nematodes and their hosts at molecular level.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Sajid Rehman**
Date: **January 31, 2008**
Group: **Laboratory of Nematology, Department of Plant Sciences, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
The recombinant expression of and the generation of antibodies against RanBPM homologues (A41, A18, E19)	Nov 12, 2002
► **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, Utrecht University	Mar 27, 2003
EPS Phd student day, Vrij University, Amsterdam	Jun 03, 2004
EPS PhD student day, Radboud University, Nijmegen	Jun 02, 2005
EPS PhD student day, Wageningen University	Sep 19, 2006
► **EPS theme symposia**	
EPS theme 2 symposium 'Interactions between plants and biotic agents', Wageningen University	Dec 12, 2003
EPS theme 1 symposium 'Developmental Biology of Plants', Wageningen University	Feb 17, 2004
EPS theme 2 Symposium 'Interactions between plants and biotic agents', Utrecht University	Sep 17, 2004
EPS theme 1 symposium 'Developmental Biology of Plants', Wageningen University	Apr 26, 2005
EPS theme 2 symposium 'Interactions between plants and biotic agents', Leiden University	Jun 23, 2005
EPS Symposium on Intracellular Signaling, University of Amsterdam	Feb 02, 2006
► **NWO Lunteren days and other National Platforms**	
NWO-ALW Experimental plant sciences meeting, Lunteren 2003	Apr 07-08, 2003
NWO-ALW Experimental plant sciences meeting, Lunteren 2004	Apr 06-07, 2004
NWO-ALW Experimental plant sciences meeting, Lunteren 2005	Apr 04-05, 2005
NWO-ALW Experimental plant sciences meeting, Lunteren 2006	Apr 03-04, 2006
► **Seminars (series), workshops and symposia**	
Flying Seminar Jeff Dangl	May 09, 2003
Workshop RNA interference, Vrij University , Amsterdam	May 27, 2004
Symposium on Systems Biology in honor of Prof.Dr. Pierre de Wit, Wageningen	Nov 04, 2004
Lecture Dr. René Ketting, Wageningen University	Mar 01, 2005
Lecture Dr. Jan W. Vos, Wageningen	Apr 26, 2005
Real time PCR & gene expression analysis, seminar series from Bio-Rad, Wageningen	Jun 07,2005
Flying Seminar Prof.dr. Joseph R. Ecker	Sep 26, 2005
► **Seminar plus**	
► **International symposia and congresses**	
XXVII International Symposium of the European Society of Nematologists, Rome, Italy.	Jun 14-18, 2004
► **Presentations**	
Molecular Basis of Microbe-Plant interactions, Summer school, Leiden (Poster presentation)	Jun 05-07, 2003
XXVII International Symposium of the European Society of Nematologists, Rome, Italy (Poster presentation)	Jun 14-18, 2004
MPNI- meeting, Wageningen (Oral presentation)	Feb 10, 2005
EPS-Theme 2: linteractions between Plants and Biotic Agents, Leiden (Oral presentation)	Jun 23, 2005
MPNI- meeting, Ghent Belgium (Oral presentation)	Mar 02, 2006
Bio-Exploit workshop on Rx1, WICC Wageningen (Oral Presentation)	Dec 7, 2006
► **IAB interview**	Jun 03, 2005
► **Excursions** Visiting Ghent University for scientific and technical insights (3 days)	2003-2005
Subtotal Scientific Exposure	*14.0 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Disease Resistance in Plants : Autumn school 2002, Wageningen	Oct 14-16, 2002
Molecular Basis of Microbe-Plant interactions, Summer school, Leiden	Jun 05-07, 2003
Workshop GFP and LUC, Applications of 'Light'-Reporters in Biology, Wageningen University	Apr 11-12, 2005
► **Journal club**	
Various literature discussions in research group of molecular plant-nematode-interactions	2002-2007
► **Individual research training**	
Practical training for RNA interference in Scottish Crop Research Institute, Scotland	Apr 27-May 04, 2003
Subtotal In-Depth Studies	*7.8 credits**

4) Personal development	*date*
► **Skill training courses**	
Bioinformation Technology-1, Wageningen	May 12-21, 2005
Molecular Phylogenies: Reconstruction and Interpretation, Wageningen	Oct 18-22, 2004
EMBO Course on Plant Cell Biology, Wageningen	Jun 18-24, 2006
Techniques for Writing and Presenting a Scientific Paper, Wageningen	Feb 20-23, 2003
Working with end-note 8	Feb 2003
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*8.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**31.4**

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 credits

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

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The research presented in this thesis was performed at the Laboratory of Nematology, Wageningen University, The Netherlands

Cover: The adult female of *Globodera rostochiensis* developing on potato leaf embedded in agar. The picture was taken by Hein Overmars at 7 weeks post inoculation.

Design: Sajid Rehman