“If you find a path with no obstacles, it probably doesn't lead anywhere”

Frank A. Clark

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Functional analysis of *Globodera pallida* SPRYSEC proteins

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Functionele analyse van *Globodera pallida* SPRYSEC-eiwitten

Cover illustration

Front: An confocal image taken at 2 days after transient expression of YFPc::GpSPRY-414-2 and YFPn::truncated StCLASP in leaf cells of wild type *Nicotiana benthamiana* via Agroinfiltration. Reconstituted YFP displaying in green indicates that these two proteins interact with each other *in planta*. Autofluorescence from chloroplasts is displayed in blue. As a whole, the picture resembles earth which implies *Globodera pallida* is a global challenge to tackle.

Back: Confocal images of transient expression of mRFP::StCLASP in leaves of transgenic CB13 *Nicotiana benthamiana* containing the α-tubulin marker *tua*-GFP at two days after Agroinfiltration. Purple color indicates the localisation of StCLASP, green color indicates the tubulin marker while the silver color indicates the co-localisation of these two proteins. Blue color is autofluorescence from chloroplasts.

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Contents

List of Abbreviations

AA	amino acid
ABA	abscisic acid
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
At	*Arabidopsis thaliana*
Avr	avirulence
BiFC	bimolecular fluorescence complementation
BLAST	basic local alignment search tool
bp	base pair
CCD4	carotenoid cleavage dioxygenase 4
CC-NB-LRR	coiled-coil nucleotide-binding (NB)-leucine-rich repeat (LRR)
cDNA	complementary DNA
CLASP	clip-assosiated protein
CLE	CLAVATA3 (CLV3)/Endosperm Surrounding region (ESR)
CLIP	cytoplasmic linker protein
DAMP	damage assosiated molecular pattern
DG	dorsal gland
DIG	digoxigenin
DMSO	Dimethyl sulfoxide
DMT	5-methoxy-N,N-dimethyl tryptamine oxalate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
dpi	days post inoculation
dsRNA	double stranded RNA
DTT	dithiotreitol
DUB	deubiquitinating enzymes
EDTA	ethylenediaminetetraacetic acid
EPPO	European and Mediterranean plant protection organisation
EST	expressed sequence tag
ETI	effector triggered immunity
ETS	effector triggered susceptibility
FITC	fluorescein isothiocyanate
FLS2	flagellin-sensing 2
For-primer	forward primer
FW	fresh weight
GFP	green fluorescent protein
GHF	glycosyl hydrolase family
h	hours
H_2O_2	hydrogen peroxide
HGT	horizontal gene transfer
HMM	hidden Markov model
HR	hypersensitive response

iNOS	nitric oxide synthase
ISC	syncytial cell
J2	second-stage juvenile
kDa	kilo Dalton
LB	Luria Bertani
LRR-RLK	leucine-rich repeat receptor-like kinase
MAMP	microbial-associated molecular pattern
MAPK	mitogen-activated protein kinase
min	minutes
ML	maximum likelihood
mRNA	messenger RNA
MW	molecular weight
NADPH	nicotinamide adenine dinuleotide phosphate
NFS	nematode feeding site
NO	nitric oxide
nt	nucleotides
ORF	open reading frame
PAMP	pathogen assosiated molecular pattern
PAO	polyamine oxidase
PCN	potato cyst nematode
PCR	polymerase chain reaction
PIN	PIN-FORMED proteins
PPN	plant parasitic nematode
PRR	pattern recognition receptor
PTI	PAMP triggered immunity
R	resistance
RanGAP2	Ran GTPase-activating protein 2
RBP-1	Ran-binding protein 1
Rev-primer	reverse primer
RIN4	RPM1-interacting protein 4
RKN	root knot nematode
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT	reverse transcription
RT-PCR	reverse transcriptase polymerase chain reaction
s	seconds
SDS	sodium dodecyl sulphate
SL	strigolactone
Sl	*Solanum lycopersicum*
SMART	simple modular architecture research tool
SP	signal peptide
SPRYSEC	secreted SPRY-domain containing protein
St	*Solanum tuberosum*
SVG	subventral gland

TIR	Toll-Interleukin receptor
TRIM	Tripartite motif
U	unit
UTR	untranslated region
VAP	venom allergen protein
WT	wild type
Y2H	Yeast two-hybrid

Chapter 1

General introduction

Nematodes are thought to form the largest Phylum in the animal kingdom, with more than 25,000 species described to date (Abad and Williamson, 2010; Williamson and Kumar, 2006). They can be found in almost every possible ecological niche throughout the world ranging from polar regions to tropics (Bongers and Ferris, 1999). Despite their huge abundance and varied habitats, most nematodes display relatively little morphological variation. Their bilaterally symmetric and unsegmented body consists of an external cylinder (the body wall or cuticle) and an internal cylinder (the digestive system) that are separated by a fluid filled pseudocoelomic cavity which contains other body tissues including the reproductive tissues and the nervous system (Decraemer and Hunt, 2006).

Nematodes can be non-parasitic or parasitic. Non-parasitic nematodes feed mainly on bacteria, fungi, dead organic matter or invertebrates, including other nematodes. They are important in soil nutrient turnover and serve as indicator species in ecological studies (Perry and Moens, 2011) .One free living nematode, *Caenorhabditis elegans*, was chosen as a model organism for genetics and developmental biology in the 1970s and has since become one of the most intensively studied organisms on the planet (The *C. elegans* sequencing Consortium 1998). Parasitic nematodes can infect a range of organisms, including humans and other animals as well as plants.

1.1 Plant parasitic nematodes: The underestimated enemy

Plant parasitic nematodes (PPNs) account for approximately 20% of the total number of described nematode species (Oliveira *et al.,* 2007). Although PPNs can feed on all parts of the plant including stems, leaves, flowers and seeds the majority are soil borne and attack roots. The severity of nematode damage to plants ranges from negligible injury to total crop loss. It is estimated that globally nematode damage causes losses of $US 80 billion per year (Nicol *et al.,* 2011). However, it is possible that this figure is a significant underestimate because many farmers, especially in developing countries, are not aware of PPNs or the damage that they can cause (Jones *et al.,* 2013). It is widely acknowledged that the most devastating plant parasitic nematodes are the root-knot (*Meloidogyne* spp) and cyst-forming (*Heterodera* spp. and *Globodera* spp.) nematodes. The removal of most effective nematicides due to concerns about their potential toxicity, coupled with the fact that biocontrol strategies for PPNs are rarely effective enough for widespread uptake mean that new control strategies are required. The most promising of these is the development of

nematode resistant plants. In this context, it is vital to understand the molecular interactions between nematodes and their host plants.

Plant parasitic nematodes are well-equipped for plant parasitism. Some plant parasitic nematodes are capable of suspending development in adverse conditions. For example, cyst nematodes enter a dormant stage as J2s and do not resume development until diffusates from the roots of a suitable host are detected. All PPNs have a stylet, a hollow, protrusible needle-like structure used to pierce plant cells and remove cell contents during feeding and through which secretions of the oesophageal gland cells are introduced into plant tissues (Baum *et al.,* 2007). PPNs have two sets of oesophageal gland cells, the dorsal and subventral, which are present in different sizes and numbers depending on the species. Products of these gland cells play important roles in the plant-nematode interaction (Hussey *et al.,* 2002).

1.2 Evolution and diversity of plant parasitism

An analysis of the patterns of evolution of plant parasitism and an understanding of how this lifestyle has originated require an accurate phylogeny of the Phylum Nematoda. However, the conserved body plan of nematodes coupled to the absence of a fossil record has made the establishment of an accurate phylogeny, particularly when based on morphological characters, very difficult. Several different hypotheses for how nematodes have evolved were put forward by taxonomists but with little agreement (Baldwin *et al.,* 2004). More recently these problems have been addressed by the analysis of small subunit ribosomal RNA, which has allowed a detailed molecular phylogeny of the Nematoda to be established (Blaxter *et al.,* 1998; Van Megen *et al.,* 2009). These studies have shown that plant parasitism by nematodes has arisen independently on at least four different occasions: one group of PPNs is present in Clade 1 (Trichodoridae), one in Clade 2 (Longidoridae), at least one is present in Clade 10 (*Bursaphelenchus* and Aphelenchoides) and the largest group, including migratory endoparasites, cyst and root knot nematodes is in Clade 12 (Van Megen *et al.,* 2009).

The modes of parasitism displayed by these PPNs are diverse. Ectoparasites mainly feed on epidermal cells of roots using their stylets while the whole body remains outside the host for the duration of the life cycle. The longer the stylet, the deeper the nematodes can feed.

While the majority of ectoparasites are migratory, some species are sedentary, in which case a feeding site may be initiated for part or the whole life cycle (Hofmann and Grundler, 2007). The migratory species usually have a broad host range and the damage that they cause is usually limited to necrosis of the cells upon which they feed. However, some migratory ectoparasites from the orders Triplonchida and Dorylaimida can act as virus vectors (Wyss, 2003). Endoparasitic nematodes invade the host for part or all of their life cycle and can also be migratory or sedentary. Migratory endoparasites migrate between or through plant cells causing extensive tissue damage. These nematodes feed on plant cells and often reproduce within the host. Some migratory endoparasites have more complex life cycles. For example, the pine wood nematode, *Bursaphelenchus xylophilus*, feeds on living trees as well as fungi that colonize dead trees and is transported to a new host by an insect vector from the Genus *Monochamus* (reviewed by Jones *et al.,* 2008). Sedentary endoparasites, such as root-knot and cyst nematodes, have developed very intimate and long-term feeding relationships.They induce a specialized feeding site, which can serve as a nutrient pool for the nematodes that are sessile inside the host. These nematodes are the most highly evolved and damaging species.

1.3 Potato cyst nematodes

1.3.1 History, host range and damage

The white potato cyst nematodes (*Globodera pallida)* are obligate sedentary endoparasites that parasite Solanaceous plants including potato, tomato and aubergine. They originate in the Andean region of South America but have now spread throughout the world. Potato syct nematode (PCN) is present across much of the EU and in many other important potato growing regions including the Ukraine and Idaho (Pylypenko *et al.,* 2005). Infected plants are stunted and yellow, and may die off entirely if heavily infested or subjected to additional abiotic stress. Yield of the potato crop is adversely affected by infection with PCN, with total yield loss related to the population density of nematodes in the soil at planting. *G. pallida* is considered a more serious pest than *G. rostochiensis* as the latter is readily controlled using potato varieties which carry the *H1* resistance gene. However, no similar monogenic resistance is currently available against *G. pallida* in economically viable cultivars. A survey has shown that over 60% of potato growing land in the UK is infested with PCN and that

over 90% of this land is infested with *G. pallida* (Minnis *et al.,*2002). *G. pallida* causes losses of more than £50 million in the UK each year (Jones and Perry 2004).

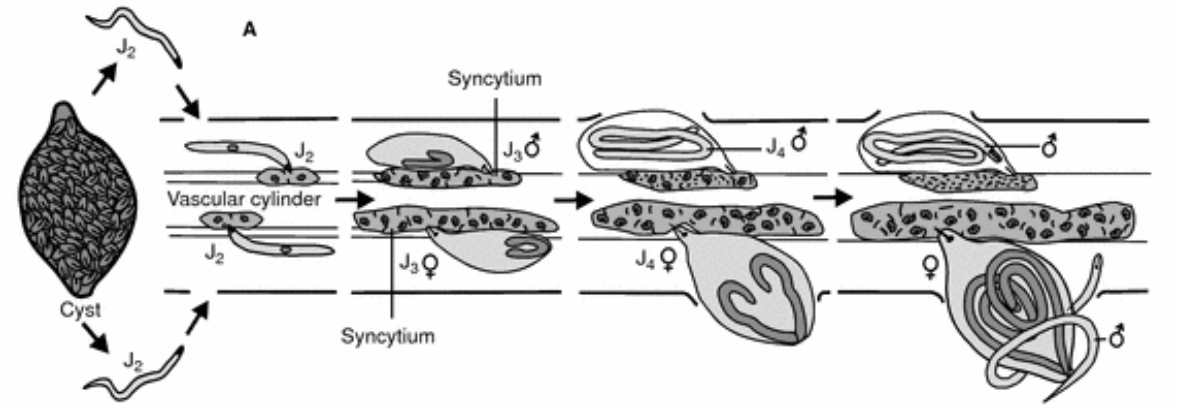


Figure 1.1 The life cycle of *Globodera pallida*. J2, J3, J4 juveniles in the second, third and fourth developmental stages (From Jung and Wyss, 1999)

1.3.2 Life cycle

G. pallida emerge from the egg as infective second stage juveniles (J2s) after being stimulated to hatch by host plant root diffusates. The hatched J2s penetrate the roots just behind the apex, using a combination of physical and chemical means, and migrate intracellularly through the elongation zone to a site near the vascular tissue, where the nematode chooses a cell that will become the initial syncytial cell (ISC). A cocktail of effector proteins released from esophageal glands is then injected into the cytoplasm of the ISC and initiates the development of the nematode's syncytial feeding site (below).The nematode produces a feeding tube which extends into the syncytium and acts as a molecular sieve through which it ingests symplastic contents (Eves-van den Akker *et al.,* 2014). The syncytium serves as a nutrient sink and remains the sole source of nourishment for the whole life of the nematode. After successive feeding cycles, J2s moult into J3s, J4s and eventually reach the adult stage in four to six weeks (Von Mende *et al.,* 1998). Sexual dimorphism is controlled by environmental factors such as nutrient supply, with abundant food sources leading to production of more females while a restricted availability of food gives rise to a higher proportion of male nematodes (Lilley *et al.,* 2005). Female adults develop a round body shape with the posterior part exposed outside root tissue. By contrast, adult males regain the vermiform body shape, exit from the root and mate with the exposed females. The female's body wall hardens to form a protective cyst which holds

several hundred eggs. Eggs enter a period of diapause and can remain viable in the soil for up to 20 years (Lilley *et al.,* 2005). The life cycle of *G. pallida* is summarised in Figure 1.1.

1.3.3 The syncytium

Like other cyst nematodes, *G. pallida* induces the formation of a complex feeding site in the roots of its hosts called a syncytium. Syncytia are large multinucleate cells that are generated as a result of controlled dissolution of cell walls and subsequent fusion of protoplasts. The syncytium begins with the fusion of the initial syncytial cells with its neighbors and further layers of cells are then incorporated (Jones, 1981) (Figure 1.2). The final syncytium can consist of several hundred fused cells. Syncytia are metabolically active and contain multiple enlarged nuclei, small vacuoles and highly proliferated mitochondria, free ribosomes and smooth endoplasmic reticulum (Gheysen and Jones, 2006). As a biotrophic pathogen, the cyst nematode needs to keep the feeding site alive for weeks in order to complete its life cycle.

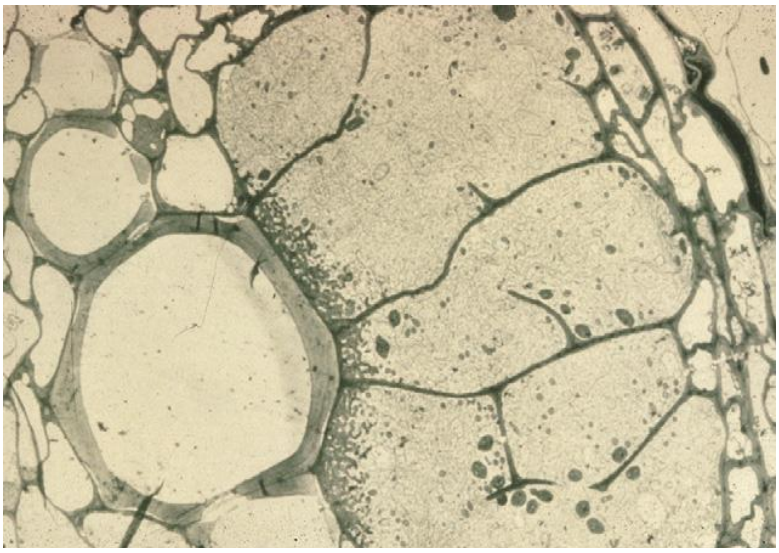


Figure 1.2 Syncytium induced by *G. pallida* in the roots of potato (from Jones *et al.,* 2013)

The development of the syncytium is accompanied by huge changes in host gene expression. Microarray studies have identified numerous differentially expressed genes within the syncytium when compared with uninfected roots (Ithal *et al.,* 2007; Klink *et al.,* 2007; Szakasits *et al.,* 2009). Gene ontology analysis shows that many of the up regulated

genes relate to the changes in structure or the high metabolic activity seen in the syncytium and include genes encoding ribosomal proteins, cell-wall modification proteins, transcription factors, signal transduction and cytoskeleton components.There are also genes that are down regulated in syncytia such as those related to defence responses, similar changes in gene expression are seen in giant cells induced by the root-knot nematode *M. graminicola* in rice (Ithal *et al.,* 2007; Ji *et al.,* 2013). These studies indicate that both up- and down regulation of genes are crucial parts of a general reprogramming of gene expression required for feeding site development. Plant hormones including auxins, cytokinins and ethylene are known to serve as regulatory signals in successful nematode infection (reviewed by Kyndt *et al.,* 2013). Collectively, these studies suggest that there are drastic shifts in the normal morphology and physiology of root cells during the formation of the syncytium.

1.3.4 Nematode genome and transcriptome analysis

Genome sequences have been reported for several plant-parasitic nematodes including *Meloidogyne incognita* (Abad *et al.,* 2008), *M.hapla* (Opperman *et al.,* 2008) and the pine wood nematode *Bursaphelenchus xylophilus* (Kikuchi *et al.,* 2011). More recently a detailed genomic analysis of *G. pallida* has been published including a draft genome assembly and transcriptomes for eight life stages that cover the whole life cycle (Cotton *et al.,* 2014).This resource allows expression profiles of all genes across the life cycle to be examined. The draft genome assembly is 124.7 Mb with a high rate of large-scale genome rearrangement and a greater proportion of non-repetitive, non-coding DNA when compared with other sequenced nematodes. Among the largest gene families are those encoding the SPRY domain proteins, a family of proteins similar to *Heterodera glycines* effectors 4D06 and G16B09, a family showing similarity to *Heterodera avenae* dorsal gland cell specific expression protein and a glutathione synthetase family. It is notable that each of these expanded gene families is likely to encode proteins that play a role in the interaction between the nematode and its host.

1.4 Plant defences

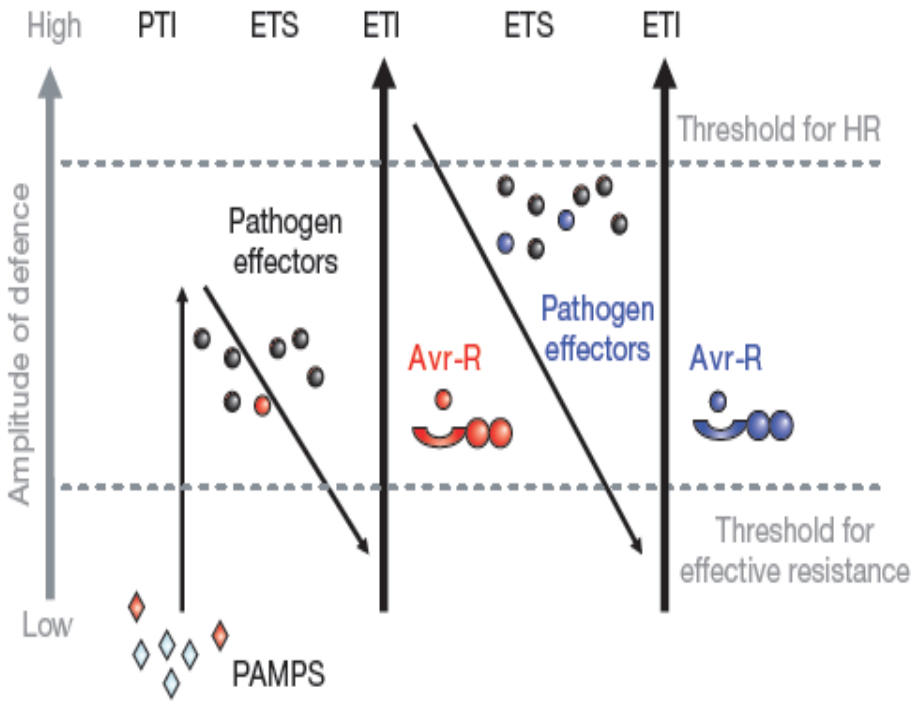


Figure 1.3 The zigzag model illustrates the quantitative output and evolution of the plant immune system (From Jones and Dangl, 2006)

Like all biotrophic pathogens, *G. pallida* needs to overcome plant defences. Plant defences can be represented by the 'Zig zag' model (Figure1.3) and can be simplified into two main branches (Jones and Dangl, 2006). The first line of active defence involves the recognition of pathogen- or microbial-associated molecular patterns (PAMPs or MAMPs), or self molecules (damage-associated molecular patterns, DAMPs) that are released on the perception of pathogen or pathogen induced cell damage. PAMPs or DAMPs are recognized by surface-localized plant Pattern Recognition Receptors (PRRs) (Macho and Zipfel, 2014). A range of different PAMPs and MAMPs have been identified from several pathogens, such as FLG22, a conserved 22 amino acid region of flagellin (Jones and Dangl, 2006). INF1 is an abundant secreted protein produced by the oomycete *Phytophthora infestans* (Bos *et al.,* 2010) while chitin is an essential component of the fungal cell wall and both are recognized as PAMPs. However, PAMPs of plant parasitic nematodes have not yet been discovered, even though local callose deposition was observed around the chitinous stylet when nematodes were attempting to initiate a feeding site (Hussey *et al.,* 1992). PRRs activate broad-spectrum

resistance defined as PAMP-triggered immunity (PTI). Early PTI responses include the rapid generation of reactive oxygen species (ROS), cell wall callose deposition, the activation of mitogen-activated protein kinases (MAPKs) and the up regulation of defence related genes (Macho and Zipfel, 2014).

PTI is generally effective to ward off most microbial invasions. However, successful pathogens suppress PTI by delivering effectors into the apoplast or cytoplasm of host cells (Jones and Dangl 2006). Examples of some of these effectors identified from bacteria, oomycete and nematodes are given in detail in 1.4.1. These effectors (or their activity) can be recognized by a second layer of plant defences mediated by highly specific immune receptors (resistance, or R proteins), which often results in a fast and strong, localized cell death known as hypersensitive response (HR) (Dodds and Rathjen 2010). Recognition between effector and immune receptor can be direct, but in most cases ETI receptors indirectly detect pathogen effectors through their effects on other plant proteins. This is known as the guard model. The majority of R proteins belong to the nucleotide-binding (NB)-leucine-rich repeat (LRR) super family, which can be further divided into two classes containing either a coiled-coil (CC) domain or a Toll-Interleukin receptor (TIR) domain in the N-terminal part. The first nematode resistance gene *Hs1*$^{pro-1}$conferring resistance to *Heterodera schachtii* was cloned from sugar beet in 1997 (Cai *et al.,* 1997). Since then, many other nematode resistance genes have been identified, including *Mi-1* and *Hero A* from tomato, *Gpa2* (*G. pallida* 2) and *Gro1-4* from potato and *CaMi* from pepper (Koropacka, 2010). Some of these genes are specific to a restricted subset of a nematode species (*e.g. Gpa2*) while others, such as *Hero* and *Mi-1*, have a much broader spectrum. For example, *Mi-1* confers resistance to root-knot nematodes, whiteflies, aphids and tomato psyllids (Roberts and Thomason, 1986; Casteel *et al.,* 2006; Nombela *et al.,* 2003; Rossi *et al.,* 1998). Resistant plants often restrict the development of feeding sites resulting in the nematode being unable to complete its life cycle or giving a shift in sex ratio towards males, which have reduced nutritional requirements compared to females. Despite promising progress in cloning of *R* genes, the mechanisms of nematode disease resistance signaling remain elusive. To date, the only effector from a plant parasitic nematode that is recognized in a nematode resistance response in plant is the *G. pallida* SPRYSEC protein RBP-1 (Ran-binding protein 1). Co-expression of *RBP-1* and *Gpa2* in *Nicotiana benthamiana* leaves results in

Gpa2-mediated cell death (Sacco *et al.,* 2009).The strength of the interaction is dependent on polymorphisms that are predicted to be located in the SPRY domain (Carpentier *et al.,* 2012). The presence of the effector MAP-1 from the root-knot nematode *M. incognita* is related to virulence against *Mi* but whether the triggered immune response is associated with Mi-1 resistance protein needs to be investigated. Likewise, Cg1 from *M. javanica* is also associated with the ability to trigger an immune response in host plants harboring the *Mi-1* resistance gene but the mechanisms behind this interaction also require further studies (Gleason *et al.,* 2008). There have been pathogen effectors identified to suppress this layer of plant defence with details explained below.

1.4.1 Suppression of plant defences by biotrophic pathogens

All biotrophic pathogens must suppress plant defences in order to survive. Bacterial and fungal pathogens have evolved a wide range of effectors or molecules that they release into plant cells to suppress PTI. Examples include the AvrPtoB effector from *Pseudomonas syringae* which interferes with perception of Flg22 by FLS2 by using the host ubiquitin-proteasome pathway to degrade the receptor (Xiang *et al.,* 2008). The *P. infestans* Avr3a effector suppresses PTI induced by the oomycete PAMP INF1 (Bos *et al.,* 2010). By contrast, suppression of PTI by nematodes is less well characterized although a few effectors were identified recently such as calreticulin Mi-CRT from *M. incognita* (Jaouannet *et al.,*2013) and GrCEP12 from *G. rostochiensis* (Chen *et al.,* 2013).

Pathogens may also suppress ETI as illustrated by *P. syringae* effectors and the *Arabidopsis thaliana* RPM1-interacting protein 4 (RIN4). Two effectors, AvrRPM1 and AvrB, were shown to interact with RIN4, leading to hyperphosphorylation of the RIN4 protein. Plants with the RPM1 immune receptor detect this modification and activate ETI against *P. syringae*. However, another effector (AvrRpt2) cleaves RIN4 and therefore suppresses the RPM1-induced hypersensitive response. Yet*, A. thaliana* has evolved another *R* gene called *RPS2* which monitors this cleavage (Smant and Jones, 2011). There is also evidence that suggests plant parasitic nematodes suppress ETI signaling. For example, some populations of *G. pallida* carrying the *RBP-1* allele that induces a Gpa2 dependent HR are virulent on potato plants carrying *Gpa2*. Nematode effectors involved in suppressing plant immunity are reviewed in section 1.5.5 below.

1.5 Molecular interactions between nematodes and host plants

1.5.1 Nematode gland cells

Plant parasitic nematodes produce numerous effectors which modulate plant immune responses, facilitate infection and initiate or maintain feeding sites (Gheysen and Mitchum 2011). Although some secreted proteins can originate from the hypodermis, amphids (Semblat *et al.,* 2001; Jones *et al.,* 2003) and phasmids (Bellafiore *et al.,* 2008), the majority of candidate effector molecules involved in parasitism are produced in the oesophageal gland cells and are secreted into the host plant through the stylet (Figure 1.4). Tylenchid plant parasitic nematodes have three oesophageal gland cells, one dorsal and two subventral. Each cell has a long cytoplasmic extension that connects through valves to the lumen. Inside the cells, secretory proteins are synthesized and packaged into granules that move through extensions and are released into the lumen via valves by exocytosis (Davis *et al.,* 2008). The adaptation of enlarged oesophageal secretory cells is also found in some animal parasitic nematodes but not in microbial-feeding *C. elegans* indicating their potential roles in parasitism. In root-knot and cyst nematodes, the two subventral gland cell extensions open into the oesophageal lumen inside the median bulb and are mainly active during nematode penetration and migration in the roots. In contrast, the dorsal gland cell empties through a valve at the base of the stylet and is mainly active during feeding site induction and maintenance (Haegeman *et al.,* 2012).

1.5.2 Nematode effectors

The study of effectors from nematodes has lagged behind similar work on oomycete and bacterial effectors, which have been studied extensively (reviewed by Pritchard and Birch, 2011). The first nematode effector, a cellulase, was identified in 1998 using antibodies raised against subventral gland cell components (Smant *et al.,* 1998). Since then, a range of different techniques have been employed to identify candidate effectors. The most popular technique was the analysis of expressed sequenced tags (ESTs) which were generated from various nematode species using materials from specific life stages, mixed life stages or even specific organs such as oesophageal gland cells (reviewed by Haegeman *et al.,* 2012). These sequence data were used to seek homologues of previously characterized effectors from other species or to look for genes encoding secreted proteins, characterized by the presence

of a signal peptide and the absence of transmembrane domain. The availability of nematode genome sequences has allowed expansion of this approach to a whole genome scale (Abad *et al.,* 2008; Cotton *et al.,* 2014; Opperman *et al.,* 2008). In more targeted studies a proteomic approach for identification of effectors (Bellafiore *et al.,* 2008; Shinya *et al.,* 2013) and micro-aspiration of oesophageal gland cells followed by EST analysis (Huang *et al.,* 2003) have expanded knowledge of effector repertoires. Recently, direct isolation of gland cells has allowed the identification of effectors from nematodes of different parasitic styles (Maier *et al.,* 2013). The data generated so far provide strong evidence that this technical advance can be used to discover plant parasitic nematode effectors relatively easily and expediently. An overview of nematode effectors mentioned in this thesis (1.5.3, 1.5.4 and 1.5.5) can be seen in table 1.1.

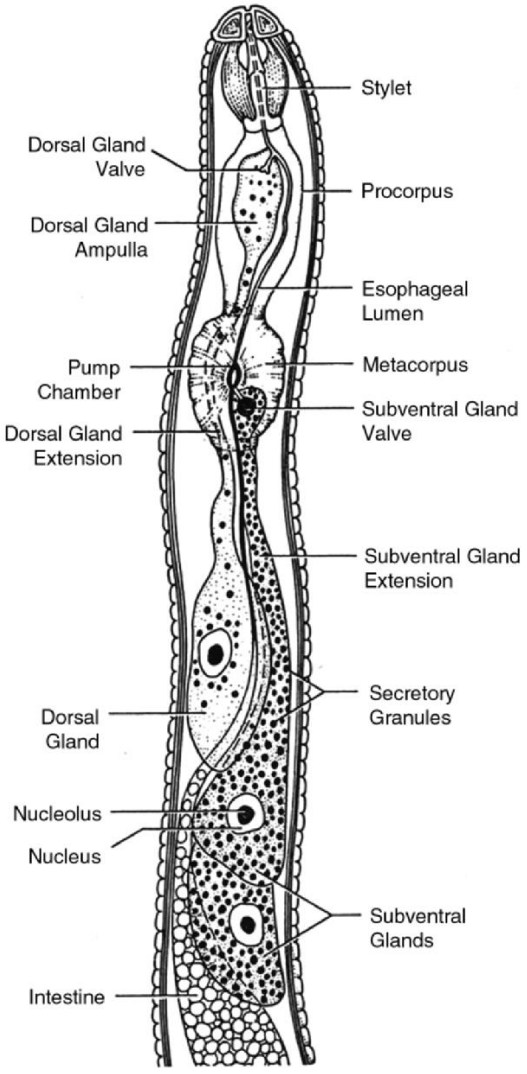


Figure 1.4 Anterior end of a second-stage juvenile cyst nematode. The anterior end of cyst nematodes harbors major adaptations for plant parasitism, particularly the stylet and the three oesophageal glands, from Baum *et al.,* 2007.

Table 1.1 Nematode effectors mentioned in this Chapter involved in plant cell wall modifying, induction of nematode feeding site and suppression of plant defences.

Effector	Predicted nature	Function	Identified plant target	Nematodes	Reference
GR-eng-1 and GR-eng-2	Beta-1,4-endoglucanase	Cell wall modifying	Cell wall	*G. rostochiensis*	Smant *et al*., 1998
HG-eng-1 and HG-eng-2	Beta-1,4-endoglucanase	Cell wall modifying	Cell wall	*H. glycines*	Smant *et al*., 1998
PEL-1	Pectate lyase	Cell wall modifying	Cell wall	*G. rostochiensis*	Popeijus *et al*., 2000
Mi-pel-1 and Mi-pel-2	Pectate lyase	Cell wall modifying	Cell wall	*M. incognita*	Huang *et al*., 2005
Hspel1 and Hspel2	Pectate lyase	Cell wall modifying	Cell wall	*H. glycines*	Vanholme *et al*., 2007
Mi-pg-1	Polygalacturonase	Cell wall modifying	Cell wall	*M. incognita*	Jaubert *et al*., 2002
Mi-xyl1	Xylanase	Cell wall modifying	Cell wall	*M .incognita*	Mitreva-Dautova *et al*., 2006
Gr-Exp-1	Expansin	Cell wall modifying	Cell wall	*G. rostochiensis*	Qin *et al*., 2004
HsCBP	Cellulose-binding protein	Cell wall modifying	Pectin methylesterase AtPME3	*H. schachtii*	Hewezi *et al*., 2008
Bx-eng-1	GHF45 endoglucanase	Cell wall modifying	Cell wall	*Bursaphelenchus xylophilus*	Kikuchi *et al*., 2004
Hs19C07	Unknown	Feeding site induction	Auxin influx transporter AtLAX3	*H. schachtii*	Lee *et al*., 2011
HgSYV46	CLE-like peptide	Feeding site induction	-	*H. glycines*	Wang *et al*., 2001
Mi16D10	CLE-like peptide	Feeding site induction	Scarecrow-like transcription factor AtSCL6 and 11	*M. incognita*	Huang *et al*., 2006
Mi-prx2.1	Peroxiredoxins	Defence suppression	-	*M. incognita*	Dubreuil *et al*., 2011
Gp-FAR-1	Retinol- and fatty acid-binding protein	Defence suppression	Linolenic and linoleic acids	*G. pallida*	Prior *et al*., 2001

Table 1.1 Nematode effectors mentioned in this Chapter involved in plant cell wall modifying, induction of nematode feeding site and suppression of plant defences (Continued)

Effector	Predicted nature	Function	Identified plant target	Nematodes	Reference
Gr-VAP-1	Venom allergen protein	Defence suppression	Papain-like cysteine protease $Rcr3^{pim}$	*G. rostochiensis*	Lozano-Torres *et al*., 2012
Hs30C02	Unknown	Defence suppression	β -1,3-endoglucanase	*H. schachtii*	Hamamouch *et al*.,2012
Mi-CRT	Calreticulin	Defence suppression	-	*M. incognita*	Jaouannet *et al*.,2013
Hs4F01	Annexin-like	Defence suppression	Oxidoreductase of the 2OG-Fe(II) oxygenase family	*H. schachtii*	Patel *et al*., 2010
HsCM	Chorismate mutase	Defence suppression	-	*H. schachtii*	Vanholme *et al*., 2009
MjCM-1	Chorismate mutase	Defence suppression	-	*M. javanica*	Doyle and Lambert 2003 Chronis *et al*., 2013
GrUBCEP12	Ubiquitin-like	Defence suppression	-	*G. rostochiensis*	Chen *et al*., 2013
Hs10A06	Unknown	Defence suppression	Spermidine synthase AtSPDS2	*H. schachtii*	Hewezi *et al*., 2008 Rehman *et al*.,2009
SPRYSEC19	SPRYSEC	Defence suppression	NB-LRR protein SW5F	*G. rostochiensis*	Postma *et al*., 2012

1.5.3 Cell wall modifying enzymes

The plant cell wall is the first physical barrier that plant parasitic nematodes have to overcome in order to invade plant tissue. It is composed primarily of cellulose crosslinked with hemicelluloses embedded in a pectin matrix. Nematodes overcome this barrier using physical thrusting of their stylet and by producing cell wall modifying enzymes in the pharyngeal glands that are released viathe stylet. The first nematode effector identified was a beta-1,4-endoglucanase from cyst nematodes capable of cellulose and xylogucan degradation (Smant *et al.,* 1998). Cell wall degrading enzymes from PPNs targeting other cell wall polymers, such as pectate lyase(Popeijus *et al.,* 2000; Huang *et al.,* 2005; Vanholme *et al.,* 2007; Wieczorek *et al.,* 2014), polygalacturonase (Jaubert *et al.,* 2002) and xylanase (Mitreva-Dautova *et al.,* 2006) were subsequently identified. In addition, nematodes also secrete proteins that do not have hydrolytic activity such as expansin and cellulose-binding proteins. Expansins can disrupt non covalent bonds between cellulose microfibrils in the cell wall and allow easy access of cell wall components to enzyme activity. The discovery of expansin in potato cyst nematode represented the first record of such a protein's existence outside the plant kingdom (Qin *et al.,* 2004).

The role of cellulose binding proteins is not yet fully understood. However, they may be involved in the control of syncytial cell wall modifications or enhancing plant enzyme activity as it was shown to interact with a host pectin methylesterase (Hewezi *et al.,* 2008). All cell wall modifying enzymes identified to date are expressed in subventral gland cells and they are thought to have been acquired through horizontal gene transfer from bacteria and / or fungi. While the majority of cellulases present in PPNs are from glycosyl hydrolase family (GHF)5, the cellulase from *Bursaphelenchus xylophilus* is a GHF45 endoglucanase. This suggests that multiple horizontal gene transfer events have occurred during the evolution of nematode plant parasitism (Kikuchi *et al.,* 2004).

1.5.4 Induction of the feeding site

Cyst nematodes develop specialized feeding cells called syncytia. Although the precise mechanisms underlying syncytial development remain obscure, it is likely that the process is initiated by effector proteins that are secreted from oesophageal gland cells via the stylet. Cyst nematodes have been shown to induce the redistribution of PIN-FORMED proteins

(PINs) that are transporters acting in the efflux of auxin from cells. In addition, a reduction in nematode infection has been seen in PIN-related mutants or after application of auxin transport inhibitors, demonstrating the importance of these proteins for feeding site development (Grunewald *et al.,* 2009). The effector Hs19C07 from *Heterodera schachtii* was proposed to increase auxin influx mediated by the auxin influx transporter LAX3 leading to increased auxin accumulation in the developing feeding site which stimulates cell wall hydrolysis to facilitate syncytia expansion and development (Lee *et al.,* 2011).

Other effectors involved may include the nematode CLE-peptide family. CLAVATA3 (CLV3)/Endosperm Surrounding region (ESR) (CLE) is a group of peptides from plants that are involved in shoot meristem differentiation, root growth and vascular development. Plant CLE peptides have a hydrophobic N-terminal signal peptide, a highly variable domain of unknown function and a conserved 14 amino acid consensus sequence at or near C-terminus called the CLE motif (Mitchum *et al.,* 2008). A protein including a CLE motif (HgSYV46) was identified from the soybean cyst nematode *H. glycines* (Wang *et al.,* 2001) and was the first record of CLEs outside plants. HgSYV46 encodes a small protein of 139 amino acids with an N-terminal signal peptide and is unique to *H. glycines* where it is expressed in the dorsal gland cells of parasitic stage nematodes. When overexpressed in wild type *Arabidopsis*, this nematode peptide caused shoot apical meristem differentiation. It could also rescue the *clv3* mutant phenotype of enlarged shoot and floral meristems. This suggests that the nematode protein has the same function as the plant CLE. Further CLEs have been identified from *H. schachtii* and *G. rostochiensis.* A short peptide resembling CLEs called 16D10 was also isolated from *M. incognita*. However, expression of 16D10 could not rescue the *clv3* mutant phenotype and in a yeast two hybrid screening it was shown to interact with a scarecrow transcription factor whose function is not related to the CLAVATA signaling pathway (Huang *et al.,* 2006). Nevertheless, recently CLE-like motifs were identified from secreted MAP family members of several root-knot nematode species. It is therefore likely that CLE signaling pathways have a common host node that are targeted by a diverse range of nematodes (Rutter *et al.,* 2014).

1.5.5 Suppression of host defences

Some plant parasitic nematode effector proteins are thought to act as suppressors of plant defences in order to protect themselves and their feeding structures. Several effectors that play a role in these processes have now been identified.

Protection of the nematode: A secreted peroxiredoxin capable of neutralizing reactive oxygen species produced in the oxidative burst was identified in all parasitic stages of *M. incognita*. RNAi silencing of these peroxiredoxins reduced the viability of preparasitic juveniles after an *in vitro* exposure to hydrogen peroxide and also affects the infectivity of this nematode on tomato plants (Dubreuil *et al.,* 2011). Another antioxidant enzyme, superoxide dismutase, was identified in secretions of preparasitic infective juveniles of *G. rostochiensis* (Robertson *et al.,* 1999). In addition to these enzymes that scavenge reactive oxygen species, a fatty acid- and retinol-binding protein was found in the surface coat of cyst nematode *G.pallida* at the early stages of parasitism (Prior *et al.,* 2001) as well as in rice white tip nematode *Aphelenchoides besseyi* (Cheng *et al.,* 2013). This protein may interfere with lipid-based signaling involved in host defence regulation.

Suppression of host defences: Effectors of plant parasitic nematodes also can directly target host defence processes. The venom allergen-like protein Gr-VAP-1 from *G. rostochiensis* specifically interacts with the cysteine protease $Rcr3^{pim}$ of the wild tomato species *Solanum pimpinellifolium* (Lozano-Torres *et al.,* 2012). $Rcr3^{pim}$ appears to be a common target of a range of different plant pathogens and acts as a node in defence-related signaling networks. Knocking out $Rcr3^{pim}$ in tomato significantly increased the susceptibility to pathogen infection. Interestingly, the interaction between Gr-VAP-1 and $Rcr3^{pim}$ is recognized by the tomato resistance gene *Cf-2* and leads to resistance to nematodes. Another effector from *H. schachtii* (Hs30C02) was shown to interact with a beta-1,3-glucanase of *A. thaliana,* a pathogenesis-related protein involved in defence responses against fungi. Overexpression of this effector in *A. thaliana* increased susceptibility to nematodes and knocking down the expression of this gene by host-derived RNA interference significantly reduces nematode development (Hamamouch *et al*., 2012).

Calcium is an important secondary messenger in the host defence responses in plants, with a flow of calcium from the apoplast into the cell an essential component of PTI signalling. It has recently been shown that root-knot nematodes secrete calreticulin which suppresses

PTI, presumably by binding apoplastic calcium. Calreticulins are normally intracellular proteins that bind calcium and that can control intracellular homeostasis and protein and glycoprotein folding in the endoplasmic reticulum. The Mi-CRT effector from *M. incognita* was localized in the cell wall of giant cells. Knocking down this effector in *M. incognita* dramatically reduced nematode infection. Stably transformed *A. thaliana* plants expressing this effector were more susceptible to nematode infection as well as another root pathogen, the oomycete *Phytophthora parasitica*. In addition, callose deposition induced by the PAMP elf18 was suppressed in the presence of the Mi-CRT effector (Jaouannet *et al.,* 2013).

Annexins also bind to calcium as well as phospholipid proteins in most eukaryotes. Plant annexins are associated with abiotic stress responses. An annexin-like effector Hs4F01 was identified in the dorsal gland of *H.schachtii* (Patel *et al.,* 2010). Overexpressing this effector in *A. thaliana* resulted in enhanced susceptibility to *H. schachtii* when compared to controls. It was also found that it interacts with an oxireductase of the 20GFe (II) oxygenase family. Knockout of *20GFe (II)* led to enhanced defence-related gene expression. Therefore, it is hypothesized that Hs4F01 may target host oxireductases to interfere with host defences, even though demonstration of a direct down-regulation of plant immunity by nematode annexins is still lacking.

Both sedentary and migratory endoparasitic nematodes are able to produce chorismate mutase suggesting that it may have a role in manipulating plant defences (Bauters *et al.,* 2013; Haegeman *et al.,* 2011; Jones *et al.,* 2003; Opperman *et al.,* 2008; Vanholme *et al.,* 2009). Chorismate mutase is a key enzyme of the shikimate pathway in bacteria, fungi and plants. This pathway is not present in animals and it is thought that plant parasitic nematodes have acquired this enzyme from bacteria via horizontal gene transfer (Haegeman *et al.,* 2011). Chorismate mutase converts chorismate into prephenate which can subsequently be converted into a variety of compounds that play critical roles in growth, development and defences of plants. It has been suggested that chorismate mutase -1 from *M. javanica* (MjCM-1) reduces cytoplasmic chorismate resulting in a flux of this compound from the plastid into cytoplasm (Doyle and Lambert, 2003). As IAA is synthesized from chorismate in the plastid, this may cause a depletion of IAA within plant tissues. This is backed up by the fact that transgenic soybean plants expressing MjCM-1 have a phenotype

of suppressed lateral root and vascular tissue formation, which is similar to the phenotype seen in the absence of IAA and was rescued by exogenous application of the same hormone. Chorismate mutase may contribute to the synthesis of flavonoids (Gheysen and Fenoll, 2002) which are auxin transport inhibitors and it has been suggested that changes in local flavonoid levels may allow nematodes to manipulate auxin concentrations. However, mutant plants lacking flavonoid biosynthetic pathways are susceptible to nematodes indicating that chorismate mutase is unlikely to manipulate flavonoid levels for this purpose (Jones *et al.,* 2007). Salicylic acid and several phytoalexins are also chorismate derivatives. It has therefore been suggested that nematodes use chorismate mutases to reduce the chorismate available for synthesizing salicylic acid in host cells (Doyle and Lambert 2003). However, no direct evidence is available to support this.

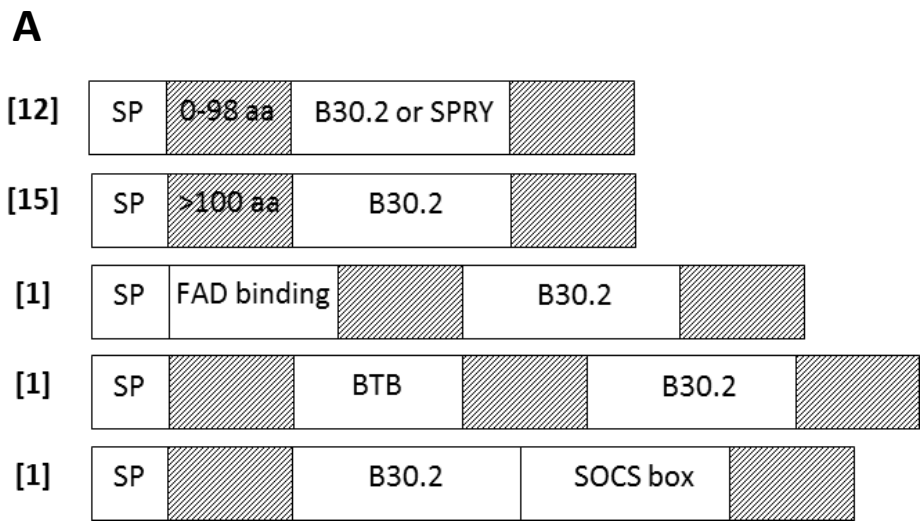
As is seen in other pathosystems, nematodes may exploit the host's ubiquitin-based proteasomal degradation system in order to facilitate parasitism. Ubiquitin is a highly conserved 76 amino acid protein that can be found in almost all tissues of eukaryotic organisms. Ubiquitination of a protein targets the protein for further processing which may include degradation by the 26S proteasome or changes in trafficking or protein functions depending on the topology of the ubiquitin chain formed on a protein (Kaiser and Huang 2005). The process of ubiquitination involves the sequential action of three different enzymes (Ye and Rape, 2009): ubiquitin activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3. The E1 protein is needed to form a high energy bond between itself and the C-terminal glycine residue of the ubiquitin. The E2 enzyme is the main mediator that determines assembly of the chain.The E3 enzyme specifically recognizes UBI-E2 complex and transfers ubiquitin from this complex to the target protein. It is important to note that the E3 enzymes determine the protein that is targeted for ubiquitination. Ubiquitination is reversible because of deubiquitinating enzymes (DUB) which release ubiquitin from their targets and thus can change the fate of a target protein (Vierstra, 2009). The ubiquitination system is involved in plant defence mechanisms through regulating levels of important signaling proteins. However, plant pathogens have also evolved to manipulate host ubiquitination systems for their own benefits. Ubiquitin proteins can be classified into polyubiquitin proteins that contain tandemly repeated ubiquitin monomers and ubiquitin carboxyl extension proteins (UBCEPs) which consist of a single ubiquitin monomer fused to a

carboxyl extension protein (CEP). The latter have been identified from the cyst nematodes *H. glycines* (Gao *et al.,* 2003), *H. schachtii* (Tytgat *et al.,* 2004), *G. pallida* (Jones *et al.,* 2009), *G. rostochiensis* (Chronis *et al.,* 2013) and ubiquitin-like proteins were identified in the stylet secretions of *M. incognita*. In *G. rostochiensis*, GrUBCEP12 consisting of a signal peptide, a mono-ubiquitin domain and a 12 amino acid carboxyl extension protein (CEP12) is expressed exclusively in the dorsal gland cell and is up-regulated in parasitic second-stage juveniles. Knockdown of *GrUBCEP12* via RNA interference reduced nematode infection while over-expression of this gene in potato resulted in increased nematode susceptibility indicating its roles in plant parasitism. In transient expression assays in *N. benthamiana*, GrUBCEP12 was processed into two functional units, one being free ubiquitin potentially affecting the host 26S proteasome to promote feeding cell formation and the one being a CEP12 peptide acting to suppress plant immunity (Chen *et al.,* 2013; Chronis *et al.,* 2013).

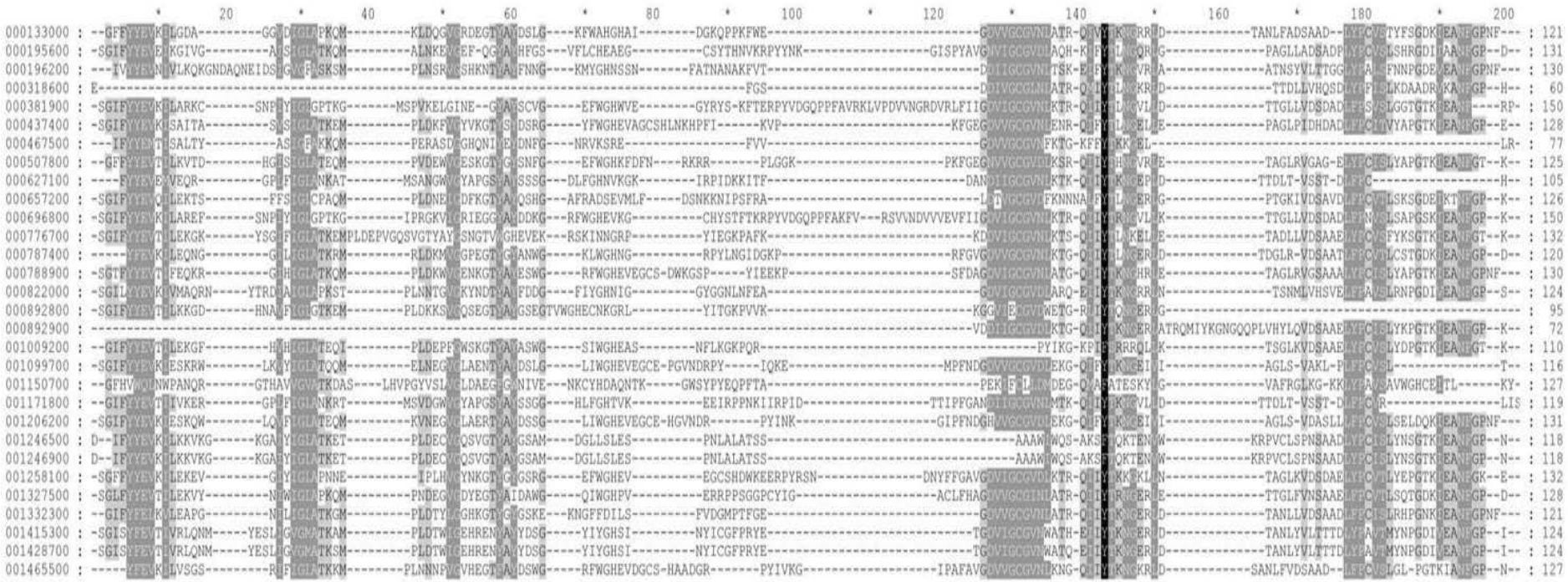
10A06 is a cyst nematode effector protein identified from the soybean cyst nematode *H. glycines* and its homolog *Hs10A06* was cloned from *H. schachtii* which is able to infect *A. thaliana* (Hewezi *et al.,* 2010). Transgenic plants overexpressing *10A06* showed hypersusceptibility to nematode infection and a significant down-regulation of several pathogenesis-related genes that are associated with the salicylic acid dependent pathway. In the yeast two-hybrid analysis and *in planta* bimolecular fluorescent molecular complementation assays, 10A06 interacted specifically with Spermidine Synthase 2 (SPDS2) which is a key enzyme involved in synthesis of polyamines. *In planta* expression of *10A06* or *SPDS2* gave rise to increased expression of several antioxidant genes upon nematode infection. It was speculated that the cyst nematode effector 10A06 could function through its interaction with SPDS2 to increase spermidine levels and subsequently polyamine oxidase (PAO) activity and that the increased PAO activity leads to stimulated induction of cellular antioxidant machinery in syncytia.

Recently, a nematode effector, SPRYSEC 19, has been identified that suppresses ETI induced by some but not all CC-NB-LRR resistance proteins. SPRYSECs are considered in detail below.

Figure 1.5 Schematic representations of *G. pallida* SPRYSEC proteins and alignment of the SPRY domains. (A) Schematic representation of the SPRYSEC proteins found in *G. pallida*, indicating the presence of a signal peptide (SP) and position of either a B30.2 or SPRY domain. The B30.2 domain contains residues in the N-terminus that form a distinct PRY domain structure such that the B30.2 domain consists of PRY (Pfam PF13765) and SPRY (Pfam PF00622) subdomains. Only 3 SPRYSEC proteins have a SPRY domain not associated with a PRY domain (GPLIN_000467500, GPLIN_001009200 and GPLIN_001246900). Hatched boxes represent variable protein sequences with no domain identified. Only 3 SPRYSEC proteins contain one specific extra domain besides the SPRY/B30.2 domain: GPLIN_001327500 with a FAD binding domain (Pfam PF01565), GPLIN_000788900 with a BTB domain (Pfam PF00651) and GPLIN_001150700 with a SOCS-box (Pfam PF07525). Bold numbers in brackets in front of each schematic protein structure indicate how many *G. pallida* SPRYSEC are represented by the model. (B) Alignment of the SPRY domain sequences from the 30 *G. pallida* SPRYSEC proteins identified in the nematode genome. Numbers in front of the sequences correspond to the gene loci (GPLIN_number). Conserved and most conserved residues are boxed (from black to light grey for most to less conserved).



B



1.5.6 The *SPRYSEC* gene family

The *SPRYSEC* (secreted B30.2/SPRY domain-containing protein; Figure 1.5) is a gene family that has been identified to be expressed in the dorsal gland cell of potato cyst nematode J2s and is upregulated in early parasitic stages (Jones *et al.,* 2009). More intriguingly, it is among the largest gene families in cyst nematodes but is absent from root-knot nematodes (Abad *et al.,* 2008; Opperman *et al.,* 2008; Cotton *et al.,* 2014).

The SPRY domain was first identified in the dual specificity kinase spore lysis SP1a in *Dictyostelium discoideum* and in three mammalian Ca^{2+}-release channel Ryanodine receptors, however, at the same time the term B30.2 was named for a domain encoded by an exon in the human class 1 major histocompatibility complex region. The B30.2 domain (also known as PRYSPRY domain) consists of a conserved C-terminal SPRY domain and a variable PRY domain in N-terminus, and was later defined by the presence of three highly conserved sequence motifs LDP, WEVE and LDYE (Henry *et al.,* 1998). In SMART database The B30.2 domain contains residues in the N-terminus that form a distinct PRY domain structure such that the B30.2 domain consists of PRY (Pfam PF13765) and SPRY (Pfam PF00622) subdomains (Figure 1.5). The SPRY domain appears to be evolutionarily ancient and has been found in animals, plants and fungi, whereas the B30.2 domain has been only found in vertebrates (Rhodes *et al.,* 2005). There is some confusion over the nomenclature of these two domains and their relationship. As B30.2 domains (~160 amino acids) are longer than SPRY domains (~130 amino acids) and only found in vertebrates, it is often described as being evolved from the more ancient SPRY domain by addition of PRY element. An alternative hypothesis was put forward that these two domains are derived from a common ancestor with the N-terminal having diverged more rapidly than the rest of the domain. This hypothesis was supported by the fact that SPRY domain-containing proteins that do not have a PRY domain have a similar structural motif in the corresponding position (Perfetto *et al.,* 2013).

The SPRY/B30.2 domains have no known enzymatic activity and are most likely involved in protein-protein interactions (Perfetto *et al.,* 2013), although in most cases their interacting proteins or the molecular determinants of the binding specificity are still unknown. Nevertheless, it has become increasingly apparent that these domains are involved in a range of different biological processes. For example, the SPRY-only DEAD box protein DDX1 in human is involved in 3'-end pre-mRNA processing (Bléoo *et al.,* 2001). The RyR1 protein

containing three SPRY domains can function to regulate excitation coupling in skeletal muscle. The B30.2 domain containing protein Tripartite motif (TRIM) 7 is involved in glycogen biosynthesis while TRIM18 (MID1) is thought to be associated with cytoplasmic microtubules, mutations of which can cause X-linked Optiz syndrome with midline abnormalities such as cleft lip and heart defects. In addition, many proteins with these domains appear to be involved in innate immunity. The SPSBS, so called SPRY domain-containing SOCS box proteins, can function as adaptor proteins to help substrate ubiquitination by E3 ubiquitin ligases. In mammals, the SPRY domain is responsible for binding N-terminus of inducible nitric oxide synthase (iNOS), targeting it for SOCS box mediated polyubiquitination and subsequent proteasomal degradation. The iNOS is a key effector of the innate immune response and in response to infection it can produce nitric oxide (NO) that is toxic to invading microbes. However, the regulation of NO production is of great importance because in a relatively large amounts it can be linked to numerous human pathologies including Alzheimer disease, asthma and cancer (Nishiya *et al.,* 2011). As another example, the B30.2 domain of TRIM5 alpha protein in Old World Monkeys binds to the capsid of the human immunodeficiency virus-1 (HIV1) to prevent reverse transcription of the viral genome in order to eventually confer immunity. In contrast, human TRIM5 alpha is unable to restrict HIV replication due to weak interaction with HIV-1 capsid (Stremlau *et al.,* 2006).

In SMART (Simple Modular Architecture Research Tool) database, there are 129 SPRY domain containing proteins in Viridiplantae that count for 1.66% of all SPRY proteins discovered in a range of organisms to date. However, none of the SPRY proteins have been characterized. Only recently, a homologue of the human RanBPM (Ran-binding protein) was identified in *A. thaliana* that is mainly cytoplasmic and has highly conserved SPRY, LiSH, CTLH and CRA domains. The authors showed that this protein physically interacts with LisH-CTLH domain-containing proteins but the function is as yet uncharacterized (Tomaštíková *et al*., 2012).

Like in plants, the function of SPRY domain-containing proteins in nematodes remains fragmentary. The first secreted B30.2/SPRY domain-containing protein from nematodes was identified from the dorsal gland of *G. rostochiensis* in an effector-finding approach using cDNA-amplified fragment length polymorphism (AFLP) expression profiling on various

developmental stages of nematodes(Rehman *et al.,* 2009). Members of this novel gene family code for secretory proteins comprising a single B30.2 or SPRY domain and the secondary structure includes highly conserved beta-strands interspersed with loops varying in length and sequences. Multiple SPRYSECs have been identified from potato cyst nematodes and the *G. pallida* genome is predicted to encode >300 different SPRY domain containing proteins (Cotton *et al.,*2014). However, many of these proteins have no signal peptide. SPRYSECs have been also shown to localize to a range of different subcellular localizations and may target many different host proteins through the SPRY domain (Jones *et al.,* 2009; Rehman *et al.,* 2009). SPRYSEC19 from *G. rostochiensis*is is able to interact with a disease resistance protein of the CC-NB-LRR type but does not activate effector-triggered immunity in host plants. In contrast, it was subsequently shown that SPRYSEC19 can suppress programmed cell death mediated by several coiled-coil (CC)-NB-LRR immune receptors. Furthermore, SPRYSEC19 reduced resistance to potato virus X as well as the fungal pathogen *Verticillium dahlia*. It was therefore speculated that SPRYSEC19 most likely disturbs immune signaling instead of effector recognition (Postma *et al.,* 2012). Another member of this family, GpRBP-1 from *G. pallida,* was demonstrated to provoke programmed cell death in *N. benthamiana* leaves when co-expressed with the Gpa2 resistance protein from potato. Recognition of GpRBP-1 by Gpa2 is associated with a single amino acid polymorphism at position 187 in the SPRY domain and Gpa2 mediated defences also require Ran GTPase-activating protein 2 (RanGAP2) that interacts with the N-terminus of Gpa2.

1.6 Thesis outline

This thesis aims to investigate the functions of six SPRYSEC effectors from *G. pallida* that are predicted to have signal peptides and that are highly expressed in second-stage juveniles. **In Chapter 2,** we will analyse the SPRY domain containing gene family in *G. pallida* that has almost 300 members. We will study the subcellular localisation of the SPRYSEC effectors in plant cells and if they play a role in suppressing host defences. We will also evaluate if they can target different host proteins in yeast two hybrid screens. **In Chapter 3**, we will characterise in more detail GpSPRY-17I9-1 and in **Chapter 4**, we will do the same for SPRYSEC GpSPRY-414-2. **In Chapter 5**, we will discuss the roles of SPRYSEC effectors in plant parasitism and outline future prospects.

Chapter 2

General insight into the *SPRYSEC* gene family of *Globodera pallida*[*]

[*] Adapted from:
Yuanyuan MEI, Peter THORPE, Athanas GUZHA, Annelies HAEGEMAN, Vivian C. BLOK, Katrin MACKENZIE, Godelieve GHEYSEN, John T. JONES and Sophie MANTELIN (2015). Only a small subset of the SPRY domain gene family in *Globodera pallida* is likely to encode effectors, two of which suppress host defences induced by the potato resistance gene *Gpa2*. *Nematology*.
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MYY performed SPRYSEC gene cloning, yeast two-hybrid screening, cell death suppression assay, and ROS assay. She also analysed the data from the Y2H and cell death suppression.

2.1 Introduction

The potato cyst nematodes (PCN), *Globodera pallida* and *Globodera rostochiensis*, are obligate sedentary endoparasites that infect a variety of solanaceous plants including potato, tomato and aubergine (Sullivan *et al*., 2007). They originate in the Andean region of South America but are now distributed almost everywhere that potato is grown (Turner and Evans, 1998). PCN is present across much of the EU and in many other important potato growing regions including Ukraine and the US state of Idaho (Hockland *et al.,* 2012). Infected plants are stunted and yellow, and may die off entirely if heavily infested or subjected to additional abiotic stress. Yield of the potato crop is adversely affected by infection with PCN, with total yield loss related to the population of nematodes in the soil at planting (reviewed by Schomaker and Been, 2013). *G. pallida* is considered a more significant problem than *G. rostochiensis* as the latter is readily controlled using potato varieties which carry the H_1 resistance gene (Ellenby, 1952). However, few economically viable cultivars carrying similar resistance are currently available against *G. pallida* for which resistance is most often controlled by several Quantitative Trait Loci (QTL), making it more difficult to breed than monogenic sources (Bakker *et al*., 2006).

G. pallida has complex, biotrophic interactions with plants. After invading a host and migrating through the root cells to the inner cortical layers, the nematode selects an initial syncytial cell, which is transformed into a large multinucleate syncytium (reviewed in Sobczak and Golinowski, 2011). Cell wall openings are formed, initially by widening of pre-existing plasmodesmata between the initial syncytial cell and its neighbours, followed by controlled breakdown of the plant cell wall in these regions. The cytoplasm of the initial syncytial cell proliferates, the central vacuole breaks down and the nucleus becomes enlarged. These changes are also observed in the cells surrounding the initial syncytial cell. The protoplasts of the initial syncytial cell and its neighbours fuse at the cell wall openings. This process is repeated with further layers of cells until up to 200-300 cells are incorporated into the syncytium.

The interactions between *G. pallida* and its host are mediated by effectors, which can be defined as secreted pathogen proteins or peptides that manipulate the host plant to the benefit of the nematode. Effectors play a variety of roles in the host-parasite interaction

including induction and maintenance of the feeding site and suppression of host defences. Effectors of plant-parasitic nematodes (PPNs) originate mainly from the subventral and dorsal pharyngeal gland cells, from where they can be secreted via the stylet into host cells, but may also be secreted into the apoplast from the amphids or the nematode surface (Eves-van den Akker *et al*., 2014; Vieira *et al*., 2011). Effectors have been identified from a variety of cyst and root-knot nematodes and this topic has been the subject of several recent, extensive reviews (Haegeman *et al*., 2012; Jones *et al*., 2011; Mitchum *et al*., 2013). While many effectors still have no clear function ascribed to them, a few others that are important in the induction or development of the feeding site (Lee *et al*., 2011; Wang *et al*., 2005) and suppression of host defences (Jaouannet *et al*., 2013; Lozano-Torres *et al*., 2012; Postma *et al*., 2012) have been functionally characterised.

Until recently identification of effectors from PPNs relied mainly on analysis of partial sequence datasets, most often from Expressed Sequence Tag (EST) analysis of cDNA (complementary DNA) libraries from whole nematodes or aspirated gland cell contents (*e.g.* Huang *et al*., 2003; Jones *et al*., 2009; Maier *et al*., 2013). However, the availability of genome sequences for several PPNs including *G. pallida* (Abad *et al*., 2008; Cotton *et al*., 2014; Kikuchi *et al*., 2011; Opperman *et al*., 2008) allows analysis of the full effector complements of these species. In particular, the availability of a genome sequence allows the full extent of effector gene families present in a species to be analysed, something that EST analysis, which is inherently biased towards abundantly expressed genes and which only targets genes expressed in the stages used for the library construction, does not permit. In keeping with this, analysis of the *G. pallida* genome sequence has allowed identification of several hundred putative effectors many of which are related proteins encoded by substantial gene families (Cotton *et al*., 2014; Thorpe *et al*., 2014). One of the most notable of these is a family of approximately 300 genes encoding SPRY domain proteins. These proteins appear to be evolutionarily ancient and have been found in animals, plants and fungi. The SPRY domain was first identified in the dual specificity kinase spore lysis SP1a protein from *Dictyostelium discoideum* and in three mammalian Ca^{2+}-release channel RYanodine receptors (SPRY domain; Ponting *et al*., 1997). The SPRY domain has no known enzymatic activity and is most likely involved in protein-protein interactions (Perfetto *et al*., 2013), although in most cases their interacting proteins or the molecular determinants of

the binding specificity are still unknown. Most nematode species encode between 8 and 25 SPRY domain proteins, none of which are predicted to be secreted. By contrast, over 300 SPRY domain-containing protein sequences are present in *G. pallida*. A subset of these putative proteins is predicted to be secreted (SPRYSEC) and these SPRYSEC proteins are thought to be deployed as effectors. Some of the SPRYSEC effectors have been shown to be expressed in the dorsal gland cell in both *G. rostochiensis* (Rehman *et al*., 2009) and *G. pallida* (Jones *et al*., 2009). Notably, one *G. rostochiensis* SPRYSEC (SPRYSEC19) has been shown to suppress defence responses induced by several resistance (*R*) genes in plants (Postma *et al*., 2012). In addition, some members of the *G. pallida* RBP-1 subgroup (protein showing homology with the Ran Binding Protein to microtubules) in the SPRYSEC family are recognised by the Gpa2 resistance protein (Sacco *et al*., 2009). The recognition of RBP-1 by Gpa2 is determined by a single amino acid polymorphism in RBP-1, suggesting that the diversity in the *SPRYSEC* gene family may be due to selection pressure to evade recognition by the host. The *SPRYSEC* gene family is therefore important in the biology of *G. pallida* in terms of both the susceptible and resistant interactions.

Like all biotrophic pathogens *G. pallida* needs to overcome plant defences in order to successfully infect its host. The function and evolution of plant defences can be summarised by the zigzag model (Jones and Dangl, 2006). In this model, conserved pathogen molecules (Pathogen-Associated Molecular Patterns; PAMPs) are detected by host cell surface pattern recognition receptors (PRRs) which activate PAMP-triggered immunity (PTI). While PTI is sufficient to ward off most potential pathogens, adapted pathogens deliver effectors that suppress PTI, and other defence responses. To counter this, plants possess a second layer of immune receptors (encoded by *R* genes), that detect the presence of effectors, leading to effector-triggered immunity (ETI). Although the zigzag model was originally developed in terms of the interactions between microbial pathogens and plants, several lines of evidence suggest that it is also relevant to plant-nematode interactions including the availability of resistance genes against PPNs and the identification of resistance mediated by gene-for-gene interactions that are effective against PPNs (Janssen *et al*., 1991; Kaloshian *et al*., 2011), as well as the discovery of nematode effectors that suppress PTI (Jaouannet *et al*., 2013).

Here we further characterise the SPRY domain/*SPRYSEC* gene family in *G. pallida*. We use sequence analysis and expression profiling to demonstrate that a small proportion of the SPRY domain proteins are likely to be deployed as effectors. Through *in planta* transient expression assays and yeast two-hybrid screening we found that these proteins localise to different subcellular structures and putatively interact with different plant proteins. In addition, we demonstrate that the ability of SPRYSEC effectors to suppress plant defence responses is a feature of several, but not all, of these proteins.

2.2 Materials and Methods

2.2.1 Nematode material and sequence resources

The standard Pa2/3 population "Lindley" of *G. pallida* was used for all work described here (Phillips and Trudgill, 1998). This is the same population used for generation of the genome sequence of *G. pallida* (Cotton *et al.,* 2014). However, two of the SPRYSEC sequences characterised in detail (*GpSPRY-12N3* and *GpSPRY-414-2*) originated from cDNA of other *G. pallida* populations (Eric Grenier pers. comm.). Nematodes were grown on the susceptible potato (*Solanum tuberosum*) cultivar Désirée in a glasshouse. Cysts were extracted using standard protocols (Caswell *et al*., 1985) and stored at 4^{o}C for at least 6 months before use. Second stage juveniles (J2) were hatched in tomato root diffusate prepared as previously described (Jones *et al*., 1996).

The SPRY domain proteins identified in the *G. pallida* predicted protein set version 1.0 (16th May 2012) were used in this analysis and expression profiles of SPRY domain proteins across the life cycle were determined analysing the RNAseq information available for *G. pallida* (Cotton *et al*., 2014) replicated RNAseq datasets from eggs (containing unhatched J2), invasive stage J2, parasitic nematodes at 7, 14, 21, 28 and 35 days post infection (dpi) and adult males. Normalised RPKM expression data (reads per kilobase per million; Cotton *et al.,* 2014) was subjected to expression clustering analysis using MBClusterseq (Si *et al*., 2013). Clusters were then manually assigned into categories: egg, J2, J2 and male, constitutive, parasitic, male only and no expression based on the cluster data.

All *G. pallida* proteins that contained a SPRY domain (Pfam accession number PF00622) were identified. Phylogenetic analysis was performed on a protein multiple alignment comprising the SPRY domains extracted, using the Pfam database search facility (Finn *et al*., 2014), from each of these *G. pallida* sequences. The alignments were generated using

MAFFT (Katoh and Standley, 2013) using the G-INS-1 method (a slow progressive method with an accurate guide tree). Columns in the alignment were then deleted if less than 10% of the characters were amino acid characters (<34 out of 349 sequences). Sequences were removed if more than 45% of the sequence was missing (69aa out of 130aa). The multiple sequence alignments were visualised using Jalview (Waterhouse *et al*., 2009). Model selection was done in TOPALi v2 (Milne *et al*., 2009) and the WAG+G substitution model of protein evolution was selected based on the BIC criterion.

Phylogenetic trees were estimated with TOPALi v2 using the maximum likelihood method PhyML (Guindon *et al*., 2010) and the substitution model WAG with GAMMA option and 100 bootstraps. The tree presented in Figure 1 was mid-point rooted. It is poorly resolved at basal nodes, with better resolution elsewhere in general. These resolution issues are probably mainly due to the short length (130aa) of the domain alignment which makes the estimation of a fully resolved tree a challenging task, due to the domain size setting being an upper limit for the phylogenetic signal. Another reason for low bootstrap support could also be the production of mosaic sequences by a recombination-like process.

Potentially secreted SPRY proteins from *G. pallida* were identified on the basis of the presence of a signal peptide (as predicted by SignalP 3.0; Bendtsen *et al*., 2004). The presence of nuclear localisation signals (NLS) was tested using the PSORT version 6.4 prediction tool (http://psort.hgc.jp/form.html) and nucleolus localisation was predicted by NoD, Nucleolar Localization Sequence Detector (Scott *et al*., 2010; http://www.compbio.dundee.ac.uk/www-nod/). Potential N- and O-linked glycosylation sites were predicted using the NetNGlyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGly4.0 (Steentoft *et al*., 2013; http://www.cbs.dtu.dk/services/NetOGlyc/) tools respectively.

2.2.2 *In situ* hybridisation

The spatial expression patterns of some candidate effectors were examined by *in situ* hybridisation as previously described (Jones *et al*., 2003). In brief, J2 nematodes were fixed in 2% paraformaldehyde, cut with a razor and permeabilised with proteinase K. Nematode fragments were hybridised with digoxigenin labelled sense or antisense probes which were subsequently detected with an anti-digoxigeninin alkaline phosphatase conjugated

antibody. Gene specific primers were designed that yielded products of 200-250 bp for probe synthesis (Appendix 3).

2.2.3 Cloning of *SPRYSECs*

Messenger RNAs were isolated from J2s using a Dynabeads mRNA Direct Micro kit (Invitrogen) and treated with RQ1 DNase (Promega). The cDNA was synthesised using the Superscript III system (Invitrogen) with poly (dT) primers following the manufacturer's instructions. For cloning, the coding sequences of selected effector candidates were amplified by PCR from cDNA using gene specific primers (Appendix 3), excluding the predicted signal peptide sequence but with the ACCATG leader sequence in the forward primer. Reverse primers incorporated a stop codon where products were destined for vectors allowing N-terminal fusions with the enhanced green fluorescent protein (eGFP), the monomeric red fluorescent protein (mRFP) or the C-terminus of split-yellow fluorescent protein (YFP-C; Chapters 3 & 4) tags. For constructs to be generated with a HA tag as a C-terminal fusion, the sequence encoding the HA tag (TACCCTTATGATGTACCTGATTATGCC translated YPYDVPDYA) followed by a stop codon was incorporated in the reverse primer. PCR was performed using the proof reading KOD DNA polymerase (Novagen) and products were resolved on 1.5% (w/v) agarose gels. Amplification products of the expected size were purified from gels using the QIAquick Gel Extraction Kit (QIAGEN) and inserted into the pCR8/GW/TOPO Gateway ENTRY vector by TA cloning following the manufacturer's instructions (Invitrogen). Using LR clonase (Invitrogen) following the manufacturer's instructions, clones were subsequently recombined into the Gateway-compatible binary expression vectors pK7WGF2/pH7WGR2 and pK7FWG2/pH7RWG2 for eGFP/mRFP N-terminal and C-terminal fusions respectively, or the pK7WG2 vector for expression with the HA tag or without a tag (Karimi *et al*., 2002), or the pDEST32 vector to make a fusion with the GAL4 DNA-binding domain for yeast two-hybrid screening (Invitrogen ProQuest™ Two-Hybrid System). The integrity of the effector sequence (Appendix 1) in entry and destination vectors, as well as the fusion with fluorescent proteins or the GAL4 DNA-binding domain, was confirmed by sequencing. Detailed information of protein fusions and vectors are available in Appendix 4. Similarly, the coding sequence of the eGFP present in the fusion vectors was cloned into pK7WG2 to be used as free eGFP control. For *Agrobacterium*-mediated transient expression assays, the expression vectors (Spectinomycin 100 µg ml^{-1}

selection) were transferred by electroporation to *Agrobacterium tumefaciens* strain GV3101 that contains a helper plasmid encoding *virG*^{N54D} (Gentamicin 25 µg ml^{-1} selection; Van Der Fits *et al*., 2000).

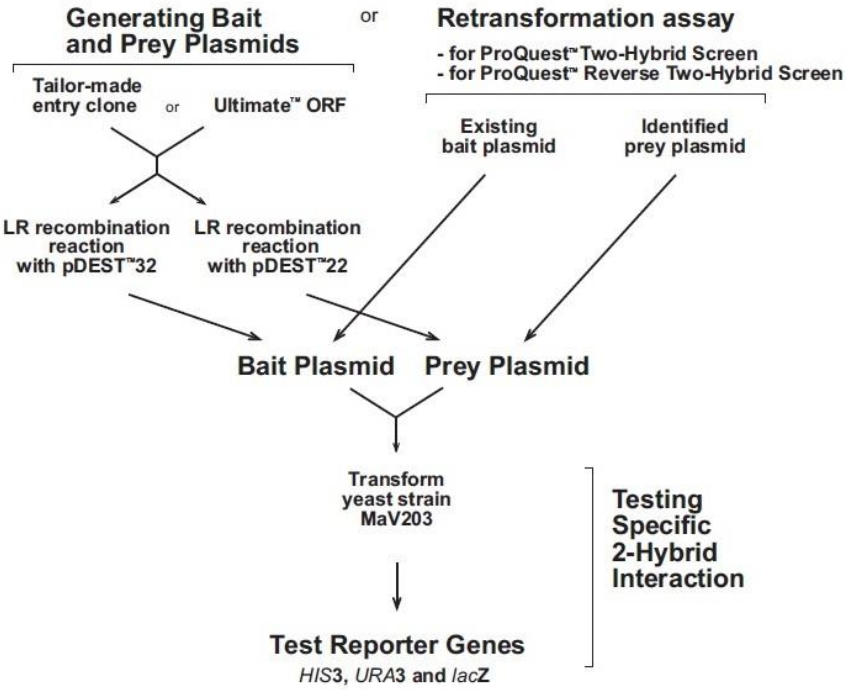


Figure 2.1 Flowchart with major steps necessary to verify an interaction using the ProQuest™ Two-Hybrid System (Catalog nos. PQ10001-01 and PQ10002-01, user manual version A, 2005)

2.2.4 Yeast two-hybrid screening

The ProQuest system (Invitrogen) was used to construct all the bait and prey constructs, and protocols provided by the manufacturer were followed for the yeast two-hybrid screening (Figure 2.1). The cDNA library, made commercially from *P. infestans* infected Désirée potato leaf material at 15h (early biotrophic phase) and 72h (necrotrophic phase) post inoculation, was cloned into pDEST22 (Bos *et al*., 2010). Yeast Mav203 cells were co-transformed with 1µl potato cDNA library (1µg µL^{-1}) and 2µl SPRYSEC bait (50ng µl^{-1}) cloned in pDEST32. Yeast transformants were plated out on synthetic Leu and Trp dropout media and colonies were picked from these plates to test interactions in the subsequent reporter gene assays. Candidate transformants were regarded as positive if they grew on triple dropout media (Leu, Trp and His) with 10mM 3-Amino-1, 2, 4-triazole which was added to suppress self-activation at the *HIS3* gene and turned blue in the X-gal assay. Each interacting prey

candidate clone was then purified from yeast, rescued into *E. coli* and sequenced. Preys for which the sequence was not cloned in frame with the GAL4 activation domain were discarded. In order to confirm interactions for the selected prey clones, each unique identified potato prey clone was then co-transformed one-to-one with its cognate SPRYSEC bait into Mav203. From each transformation, at least 3 independent clones were selected that were tested with the same reporter gene assays as described above.In addition, each prey and bait was individually tested for absence of auto-activation by co-transformation into Mav203 together with empty bait or prey vector, respectively.

2.2.5 Cell death suppression assays in *N. benthamiana*

Agrobacterium clones were grown overnight at 28°C in 5 ml Luria Bertani (LB) medium containing 25 µg ml^{-1} Rifampicin, 25 µg ml^{-1}, Gentamicin and 100 µg ml^{-1} Spectinomycin. Bacterial cells were pelleted by centrifugation, rinsed and resuspended in infiltration buffer containing 10 mM $MgCl_2$, 10 mM MES (2-[*N*-Morpholino] ethane sulfonic acid), and 200 µM acetosyringone, and adjusted to an optical density at 600 nm (OD_{600}) of 1.5, or 0.6 for R3aand $Avr3a^{KI}$. Bacteria were then incubated for at least 3h in the dark at room temperature prior to further dilution in infiltration buffer. Infiltration was then done in one-month-old *N. benthamiana* on the abaxial side of the leaves using a 1 ml needleless syringe. *Agrobacterium* clones carrying either a *SPRYSEC* construct or eGFP as a control were spot co-infiltrated at a final OD_{600} of 0.5 in combination with *R/Avr* constructs mixed in 1:1:1 ratio at a final OD_{600} of 0.5 each except *R3a* or $Avr3a^{KI}$for which final OD_{600} was 0.2. The *R/Avr* gene combinations tested in this study were *R2/Avr2* (Saunders *et al*., 2012), $R3a/Avr3a^{KI}$ (Armstrong *et al*., 2005), *Gpa2/RBP-1* (Sacco *et al*., 2009), *Rx/*PVX-CP (Slootweg *et al*., 2010), *Cf-4/Avr4* and *Cf-9/Avr9* (Thomas *et al*., 2000). In addition, the assay was conducted with an autoactive form of Mi-1.2 ($Mi\text{-}1.2^{T557S}$ at OD_{600} = 0.5; Gabriëls *et al*., 2007) and the *P. infestans* PAMP elicitor INF1 (OD_{600} = 0.5; Kamoun *et al*., 2003). For each combination of effector and cell-death inducer assayed, 3 blocks of 12 plants were used which were infiltrated on 2 leaves with 1 spot per leaf for effectors and 1 spot on the same leaf for the eGFP control. The presence of a macroscopic hypersensitive response (HR) was recorded daily until most eGFP control spots got necrotic. The HR was scored as positive if greater than 50% of the infiltrated area showed cell death, as described by (Gilroy *et al*., 2011). Each assay was done at least twice.

2.2.6 Reactive oxygen species (ROS) suppression assay

Free eGFP control (in pK7WG2) and eGFP-tagged effector constructs (in pK7WGF2) were transiently expressed in *N. benthamiana* leaves using *Agrobacterium*-mediated expression system as described above (paragraph 2.2.5) except that bacteria suspended in infiltration buffer were incubated overnight in the dark at room temperature prior to further dilution in infiltration buffer. Next morning, bacteria were then spot-inoculated at OD_{600nm}=0.3. About 30 h post inoculation, leaf discs (16 mm^2) were sampled and floated on water overnight in 96-well plates (8 to 24 replicates per construct sampled from at least 8 different plants depending of experiments). Active oxygen species production was then concomitantly elicited with the bacterial PAMP flg22 peptide (synthetic peptide QRLSSGLRINSAKDDAAGLAIS; PeptideSynthetics, UK) and measured by a Luminol-dependent assay 48 h post inoculation. Briefly, water was replaced by a solution containing flg22 (100 nM), horseradish peroxidase (20 µg/mL HRP; SIGMA) and L-012 (0.5 mM; Waco Chemicals, Germany). The HRP combines with hydrogen peroxide (H_2O_2) and the resultant complex can oxidize a wide variety of hydrogen donors such as Luminol or its derivative L-012, which is a highly sensitive chemiluminescence probe. Luminescence was measured (as relative luminescence units; RLUs) using a plate-reader luminometer (SpectraMax-M5; Molecular Devices) over time (60 min kinetic with measures taken every second) with 750 ms integration. With these kinetic and sensitivity parameters only half of a 96-well plate can be measured at a time.

2.2.7 *In planta* localisation and confocal microscopy

For subcellular localisation *in planta* of the SPRYSEC effectors fused to fluorescent tags, the constructs were transiently expressed in leaves of 4-week-old *N. benthamiana* using *Agrobacterium*-mediated transformation. Agrobacteria cultures were prepared as described above. Bacteria were then incubated for at least 3h in the dark at room temperature prior to further dilution in infiltration buffer to OD_{600nm} of 0.01 per construct and infiltration on the abaxial side of the leaves using a 1-mL needleless syringe. For co-localisation analysis, bacteria were infiltrated in leaves of transgenic *N. benthamiana* line (CB157) expressing a nuclear histone marker fused to mRFP (mRFP-H2B; Martin *et al*., 2009). Localisations were imaged 48h post inoculation using a Zeiss LSM 710 or a Leica SP2 confocal laser-scanning microscope. The eGFP was imaged with an excitation wavelength (λ) of 488 nm and

emission at λ495-530 nm (λ505-530 nm for SP2). Autofluorescence from chlorophyll generated by excitation at this wavelength was collected at λ657-737 nm (SP2 λ650-700 nm). The mRFP was imaged sequentially with an excitation at λ561 nm and emission at λ592-632 nm (SP2 λ580-610 nm).

2.3 Results

2.3.1 Phylogenetic analysis of SPRY domain proteins and identification of candidate SPRYSEC effectors

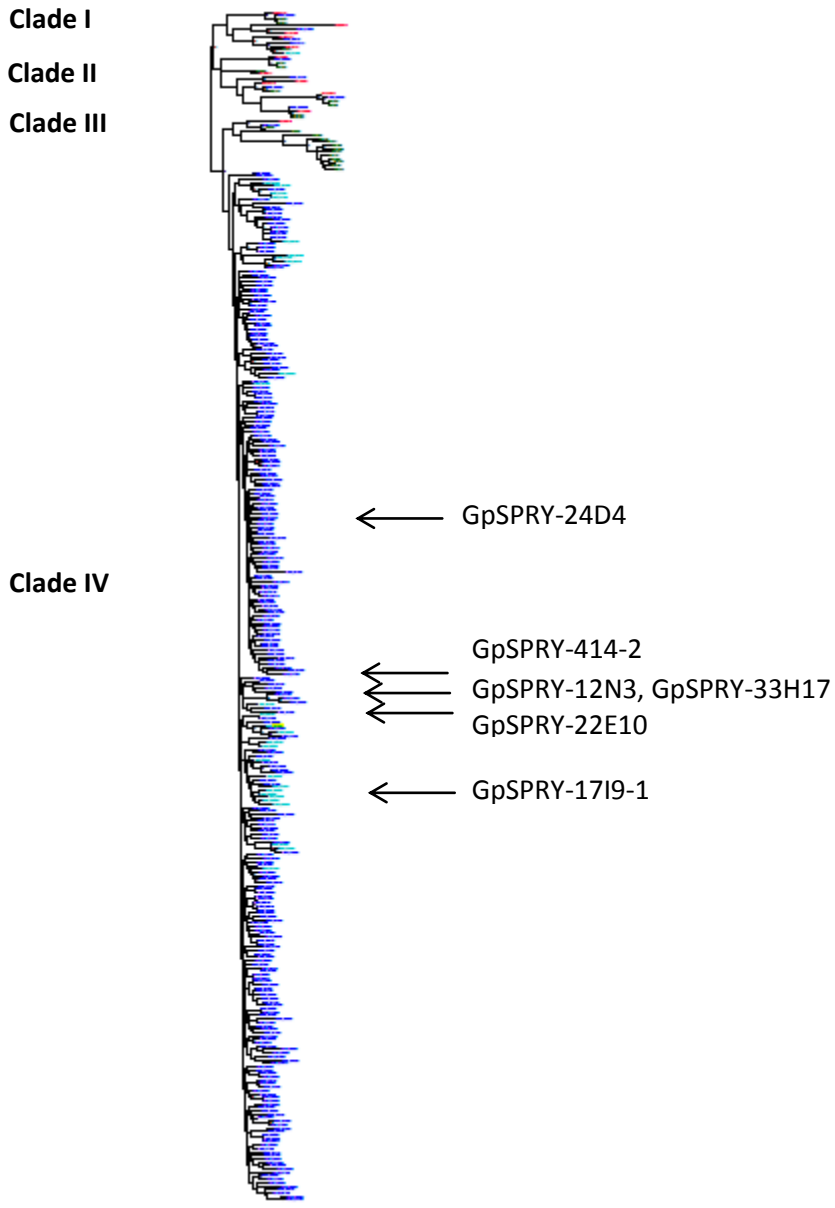
The SPRY domain protein family in *G. pallida* is greatly expanded compared to other nematodes, including other plant-parasitic nematodes. A Pfam search of the *Caenorhabditis elegans*, *Meloidogyne incognita* and *Bursaphelenchus xylophilus* genomes showed that these species contain 8, 25 and 14 proteins with one or more SPRY domains respectively, in contrast with the 299 SPRY domain proteins identified in *G. pallida*. A phylogenetic analysis of the SPRY domain proteins from *G. pallida* and other plant-parasitic nematodes is presented in Figure 2.1. The tree is split into four clades, one major and three minor. The *G. pallida* orthologues of the SPRY domain proteins from other nematodes are present within the upper three minor clades of the tree, along with the SPRY domain proteins from other organisms. A substantial expansion of the SPRY domain gene family containing only *G. pallida* sequences forms the lower major clade. Analysis of the SPRY domain protein sequences for the presence of a signal peptide showed that none of the SPRY domain proteins from any species except *G. pallida* has a signal peptide. Surprisingly, only 30 of the *G. pallida* SPRY domain proteins are predicted to have a signal peptide, suggesting that the vast majority are unlikely to be secreted and are thus unlikely to be SPRYSEC effectors. The 30 sequences of SPRYSEC candidate effectors (with a predicted signal peptide) are labelled in the phylogenetic tree in light blue colour in Figure 2.2. The effectors are distributed across this clade suggesting independent origins for effectors (rather than all effector sequences being derived from a singleprecursor). However, there are also clusters of similar effector sequences suggesting diversification after evolution of an ancestral effector.

Identifying the true N-terminus of a predicted protein in a genome assembly is one of the more challenging parts of the annotation process. It was therefore important to check whether more of the SPRY domain sequences might have signal peptides present on an

upstream region not called by the annotation software.We therefore analysed the upstream regions (1 and 2Kb) of all SPRY domain encoding genes in order to determine whether a region encoding a potential signal peptide could be present. As a control, the downstream regions (1 and 2Kb) of the SPRY domain encoding genes were analysed in the sameway; this showed that the number of potential signal peptides potentially encoded by the genome regions up and down stream of the SPRY domain proteins was identical. It was therefore concluded that the lack of signal peptides on the majority of sequences is not due to gene calling errors and that it is unlikely that a large number of additional SPRYSEC proteins are present among the SPRY domain family of *G. pallida*. Further support for this finding was obtained from a comparison of the expression profiles of the *G. pallida* SPRY domain proteins with and without predicted signal peptides. SPRYSECs were always specifically upregulated at J2 or at the very early stages of parasitism (Figure2.3A). By contrast, SPRY domain proteins with no signal peptide showed different expression profiles and were frequently constitutively expressed across the life cycle (often at a very low level) or, in some cases, upregulated in adult males (Figure 2.3B). The presence of a signal peptide is therefore correlated with expression at the early stages of the parasitic process. Taken together these lines of evidence strongly suggest that SPRY domain proteins lacking a signal peptide are unlikely to be effectors and that the number of SPRYSEC effectors is relatively small compared to the size of the SPRY domain gene family.

SPRYSECs as secretory proteins are most likely undergo post-translational modification such as glycosylation, a process that may help prevent proteins from degrading quickly. All SPRYSEC sequences from the genes cloned in this study were checked for the presence of glycosylation sites (see discussion section). The results showed that all sequences had potential O-glycosylation sites while four of the SPRYSECs, GpSPRY-12N3, GpSPRY-33H17, GpSPRY-22E10 and GpSPRY-24D4, also have predicted N-glycosylation sites (Table 2.1).

Figure 2.2 Phylogeny for SPRY domain proteins of *Globodera pallida* and other plant-parasitic nematodes. The distance tree was mid-point rooted. Distances used were based on maximum-likelihood estimated parameters (see text for details) for the SPRY domain only. Numbers at branching points indicate bootstrap percentages (when ⩾50 %) derived from 100 replicates. Sequences of SPRY domain proteins from *G. pallida* (blue), *Meloidogyne incognita* (green) and *Bursaphelenchus xylophilus* (red) are represented. SPRYSEC candidate effectors from *G. pallida* are shown in light blue and *G. pallida* proteins lacking a predicted signal peptide shown in dark blue. A major clade is present at the top of the tree containing the SPRY domain proteins from some *G. pallida* sequences and other nematodes while a large *G. pallida* specific expansion is present below this clade. The full sized tree is accessible on the following website: http://www.molecularbiotechnology.ugent.be/publications/yuanyuanmei2015A/ or through the online version of the published paper Mei *et al*., 2015. SPRYSECs studied in this thesis are indicated with black arrows.



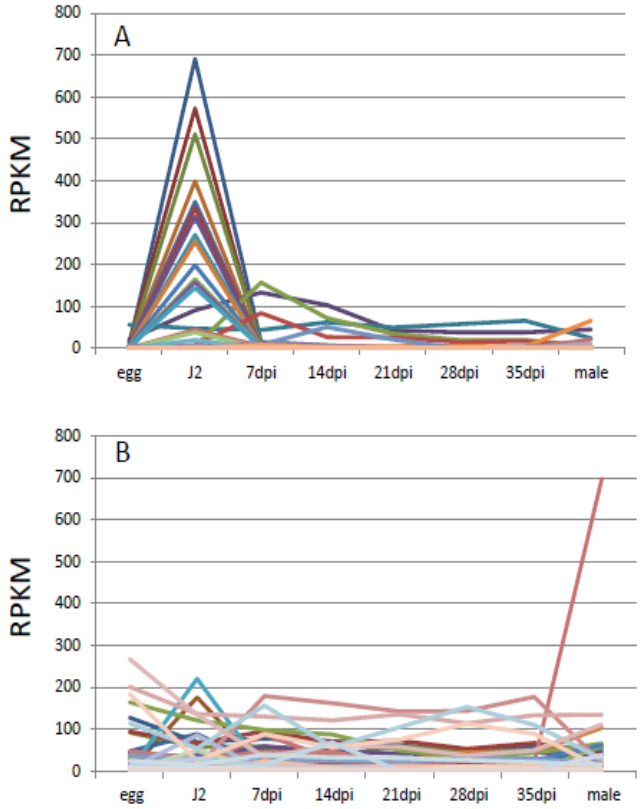


Figure 2.3 Comparison of expression profiles, inferred from RNAseq data, of *G. pallida* SPRY domain proteins with (A) and without (B) a predicted signal peptide. Y axis figures represent reads per kilobase per million (RPKM). All SPRY domain proteins predicted from the *G. pallida* genome are included in this analysis. Each line represents the expression pattern of an individual sequence. Sequences with a predicted signal peptide (30 sequences) are upregulated at J2 or early parasitic stages while the vast majority of sequences without a predicted signal peptide (269 sequences) are expressed constitutively or at the adult male stage.

2.3.2 Spatial expression profiles of SPRYSEC effectors in *G. pallida*

Previous studies have demonstrated that SPRYSEC effectors are expressed in the PCN dorsal gland cell including GpSPRY-12N3, GpSPRY-33H17 and GpSPRY-22E10 (Jones *et al*., 2009; Thorpe *et al*., 2014). The expression patterns of three additional SPRYSEC proteins were analysed by *in situ* hybridisation in *G. pallida*; each of these was also expressed in the dorsal gland cell (Figure 2.4). Expression within the dorsal gland cell is therefore a common property of all *G. pallida* SPRYSEC candidate effectors analysed to date.

Figure 2.4 Localisation of expression of some *SPRYSEC* candidate effector genes by *in situ hybridisation* to *Globodera pallida* preparasitic stage juveniles (J2s). Sections of nematodes were incubated with antisense probes designed based on DNA coding sequence for the following gene loci (A) *GPLIN_000892900*, (B) *GpSPRY-17I9-1* and (C) *GpSPRY-414-2*. All are expressed in the dorsal gland cell (arrows). No staining was observed with sense control probes (not shown). *G. pallida*J2s are approximately 30 µm in diameter.

Table 2.1 Predicted N- & O-linked glycosylation sites in SPRYSECs.

Protein[a]	N-Glycosylation[b]				O-Glycosylation[c]
	Sequon with predicted Glycosylated Asn (N)	Potential	Jury agreement	Result	Number of positive sites
GpSPRY-12N3	98 NCSS	0.5547	(7/9)	+	13
GpSPRY-17I9-1	*No N-Glycosylation site predicted in this sequence*			-	5
GpSPRY-22E10	98 NCSS	0.5301	(3/9)	+	12
GpSPRY-24D4	19 NESS	0.6252	(7/9)	+	2
	39 NRSN	0.6306	(7/9)	+	
	75 NSSK	0.6706	(9/9)	++	
GpSPRY-414-2	*No N-Glycosylation site predicted in this sequence*			-	8
GpSPRY-33H17	98 NCSS	0.5582	(7/9)	+	12

[a] The amino acid sequences used for the prediction correspond to the SPRYSEC proteins without the signal peptide.

[b] Prediction by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/)

N-Glycosylation results: The 'potential' score is the averaged output of nine neural networks. The jury agreement column indicates how many of the nine networks support the prediction.

- Potential < 0.5

+ Potential > 0.5

++ Potential > 0.5 AND jury agreement (9/9) OR potential > 0.75

[c] Prediction by NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/)

O-Glycosylation results: Only the sites with prediction confidence scores higher >0.5 are predicted as glycosylated. A safe interpretation of a positive prediction is that the protein in that local region is more likely to carry O-GalNAc modifications.

2.3.3 Subcellular localisations of SPRYSECs in plants

Effectors from *G. pallida* including SPRYSECs have been shown to target a range of subcellular structures in plant cells (Jones *et al*., 2009; Thorpe *et al*., 2014). In agreement with this, current study showed that six SPRYSECs localised to slightly different cell compartments when tested as eGFP-fusions in a transient expression assay in *N. benthamiana* CB157 plant with an mRFP marker targeted to the nucleoplasm. As shown in Figure 2.5, GpSPRY-24D4 localised mainly to the cytoplasm while GpSPRY-17I9-1 and GpSPRY-414-2 localised to both cytoplasm and nucleoplasm even though neither of them were predicted with a nuclear localisation signal (NLS). The other three SPRYSECs GpSPRY-12N3, GpSPRY-33H17 and GpSPRY-414-2 with NLS predicted showed localisations in cytoplasm as well as in nucleoplasm. The localisation patterns of GpSPRY-33H17 and GpSPRY-24D4 were in agreement with previous study (Jones *et al*., 2009). None of the SPRYSECs in this study was predicted with nucleolus localisation signal. However, we observed that GpSPRY-22E10 may slightly accumulate in nucleolus (Figure 2.5). Previous report in Jones *et al*., 2009 showed an even more clear nucleolar localisation of this effector when it was expressed via a tobacco rattle virus (TRV) RNA2 vector. It is worthy to note that GpSPRY-12N3 clearly localised in the nucleolus when tagged on the C-terminus (Figure 2.5), but the position of the tag didn't seem to influence the localisation patterns of other SPRYSECs (data not shown) at all. Furthermore, for all SPRYSECs used in this study no difference in localisation patterns was observed with fusion to a different flurorescent protein such as mRFP (data not shown).

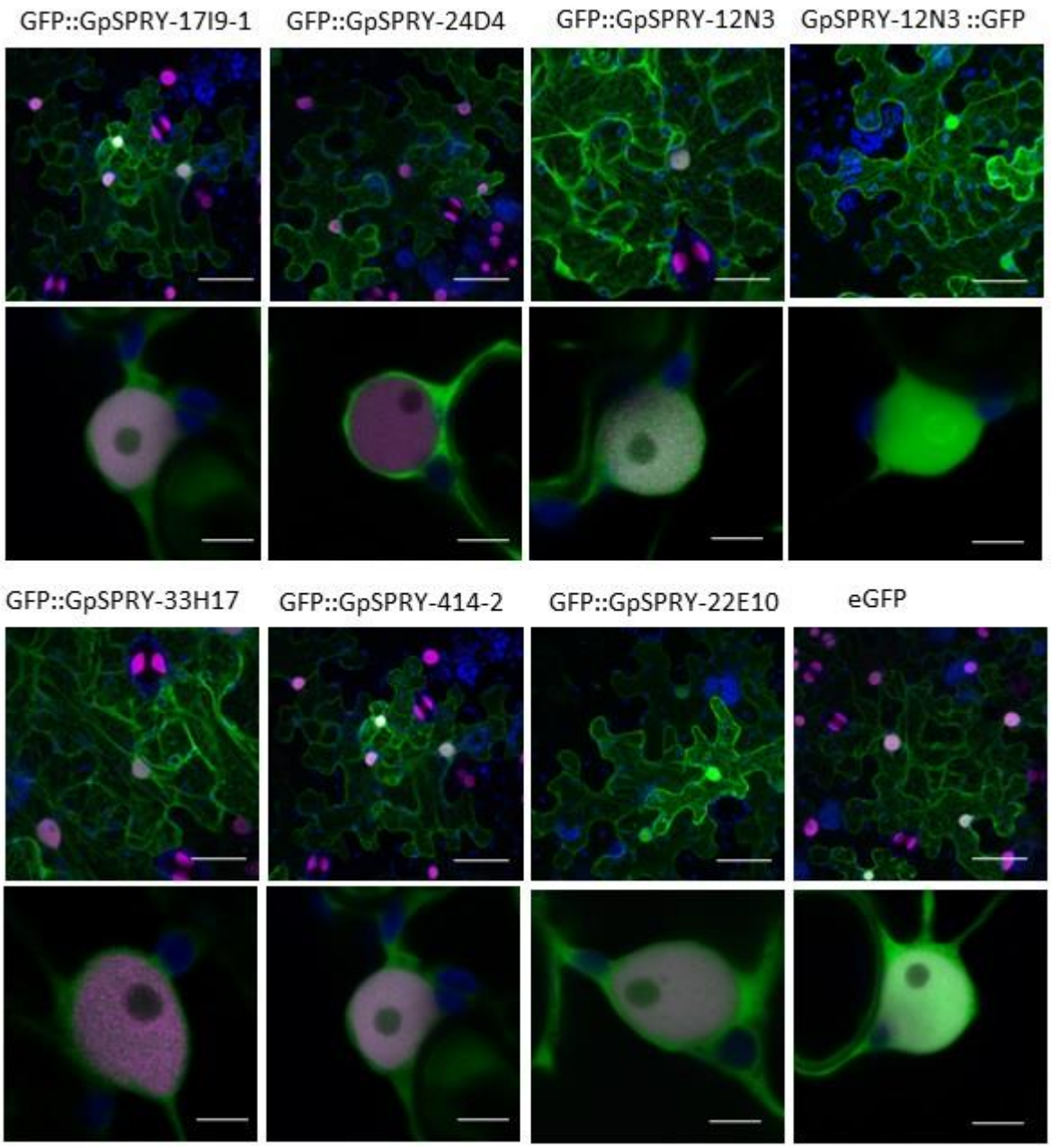


Figure 2.5 Different *G. pallida* SPRYSEC proteins show slightly different subcellular localisations within plant cells. GpSPRY-17I9-1 and GpSPRY-33H17 were localised in cytoplasm, nucleoplasm and excluded from nucleolus. GpSPRY-24D4 was localised mainly in cytoplasm with a limited amount of signal in the nucleoplasm. GpSPRY-414-2 and GpSPRY-12N3 showed localisation in cytoplasm and nucleoplasm. When tagged on C-terminus, GpSPRY-12N3 showed accumulation in the nucleolus. GpSPRY-22E10 was localised in cytoplasm, nucleoplasm and maybe slightly in nucleolus. All constructs were infiltrated on CB157 *N. benthamiana* plants with red nucleoplasm marker except GpSPRY::GFP which was infiltrated on wild type plants. eGFP is displayed in green, mRFP in magenta and autofluorescence of chloroplasts in blue. Scale bars in the nuclear detail pictures represent 5µm while others represent 50µm. Each imaging was done at least twice with three replicates.

2.3.4 Suppression of elicitor and R-mediated plant defences by SPRYSEC effectors

It has previously been shown that SPRYSEC19 from *G. rostochiensis* suppresses cell death induced by co-expression of the resistance gene *Gpa2* and its cognate avirulence factor *RBP-1* (Postma *et al*., 2012). SPRYSEC19 was also able to suppress cell death induced by the related *Rx* gene in the presence of the potato virus X coat protein (PVX-CP) that it recognises but SPRYSEC19 did not suppress cell death induced by several other *R/Avr* gene combinations or by the presence of the *P. infestans* elicitor INF1 (Postma *et al.,* 2012). We therefore investigated whether the ability of SPRYSEC effectors to suppress elicitor-mediated cell death *in planta* is specific to SPRYSEC19 or is a more general property of the SPRYSEC proteins.

To achieve this, six SPRYSECs (GpSPRY-12N3, GpSPRY-17I9-1, GpSPRY-22E10, GpSPRY-24D4, GpSPRY-33H17, GpSPRY-414-2), either tagged with eGFP (N- or C-terminal fusion) or without tag, were tested for their ability to suppress the cell death response induced in *N. benthamiana* by the transient expression of *R2/Avr2, R3a/Avr3a*^{KI}*, Rx/PVX-CP, Cf-4/Avr4, Cf-9/Avr9, Gpa2/RBP-1,* an autoactive mutant of *Mi-1.2* resistance gene *(Mi-1.2*^{T557S}*)* or *INF1*. In addition, they were also tested for their ability to suppress the production of reactive oxygen species (ROS) in *N. benthamiana*, which is one of the earliest PTI responses, when exposed to another PAMP flg22.

None of the SPRYSECs, either tagged or untagged, could suppress INF1-mediated PTI. By contrast, one SPRYSEC (GpSPRY-414-2) showed clear suppression of flg22-Mediated ROS production. As indicated in Figure 2.6A, ROS production in *N. benthamiana* leaves expressing GpSPRY-414-2 was significantly lower compared to *N. benthamiana* leaves expressing eGFP over a time course of 60min after exposure to flg22 peptide. This difference remained the same in terms of ROS peak production at 16min post-elicitation (Figure 2.6B) and total amount of ROS produced over the period of the experiment (Figure 2.6C). It is noteworthy that the suppression of flg22 mediated ROS production was only observed with GFP N-terminally tagged GpSPRY-414-2, while no significant effect was seen when the effector was not tagged or tagged on the opposite side (data not shown).

No suppression of ETI was observed except for GpSPRY-414-2 (presented in Chapter 4) and two closely related SPRYSECs most similar to the *G. pallida* predicted GPLIN_0001082900 sequence (GpSPRY-12N3 and GpSPRY-33H17) that suppressed cell death induced by

Gpa2/RBP-1, irrespective of the position of the eGFP tag in the fusion (Figure 2.7A). The ability to suppress *R*-mediated HR in plant is therefore not restricted to SPRYSEC19 but is not a conserved feature of the whole SPRYSEC protein family. Based on the kinetics of the appearance of the macroscopic symptoms (Figure 2.7B-2.7C), the suppressor effect of the *G. pallida* SPRYSECs was more of a strong delay of the HR than a total inhibition of the plant cell death response mediated by Gpa2/RBP-1.

During the process of testing the *G. pallida* SPRYSECs for their ability to suppress the Gpa2/RBP-1 mediated HR, we noticed that the presence of a tag was required in order to observe the suppressor effect of the SPRYSECs (Figure 2.7). However, the nature of the tag (eGFP or HA tag) did not significantly affect their capacity to suppress the plant response although the kinetics of development of the HR symptoms was slightly different (illustrated for GpSPRY-12N3; Figure 2.8).

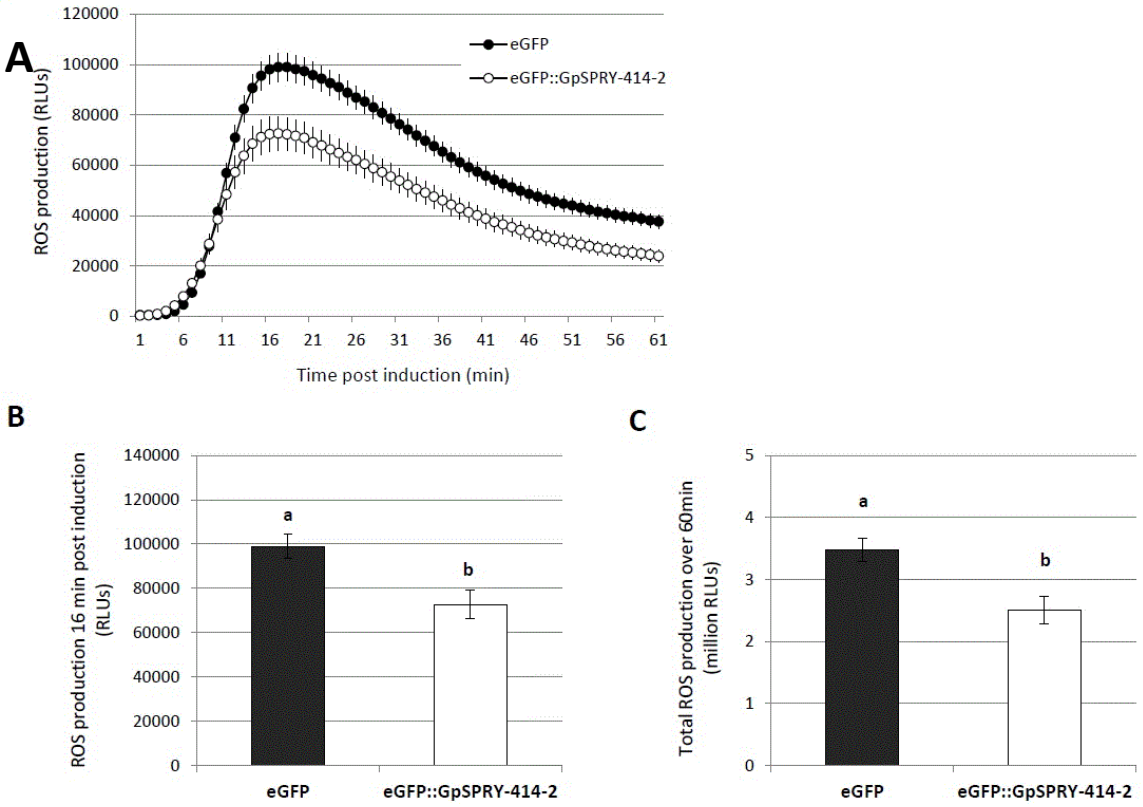


Figure 2.6 Transient ROS production in response to flg22 in *Nicotiana benthamiana* leaves expressing eGFP::GpSPRY-414-2 (white) or free eGFP (black) as control.ROS production shown as time-course after elicitation by flg22 in panel (A), shown at peak ROS production (16 minutes post-elicitation) in panel (B) or expressed as total Relative Light Units (RLUs) over 60 minutes following elicitation in panel (C).Values are mean ± SE. Means with different letters denote a significante difference (*t*-test at $P< 0.05$, n=24) in panels (B) or (C).

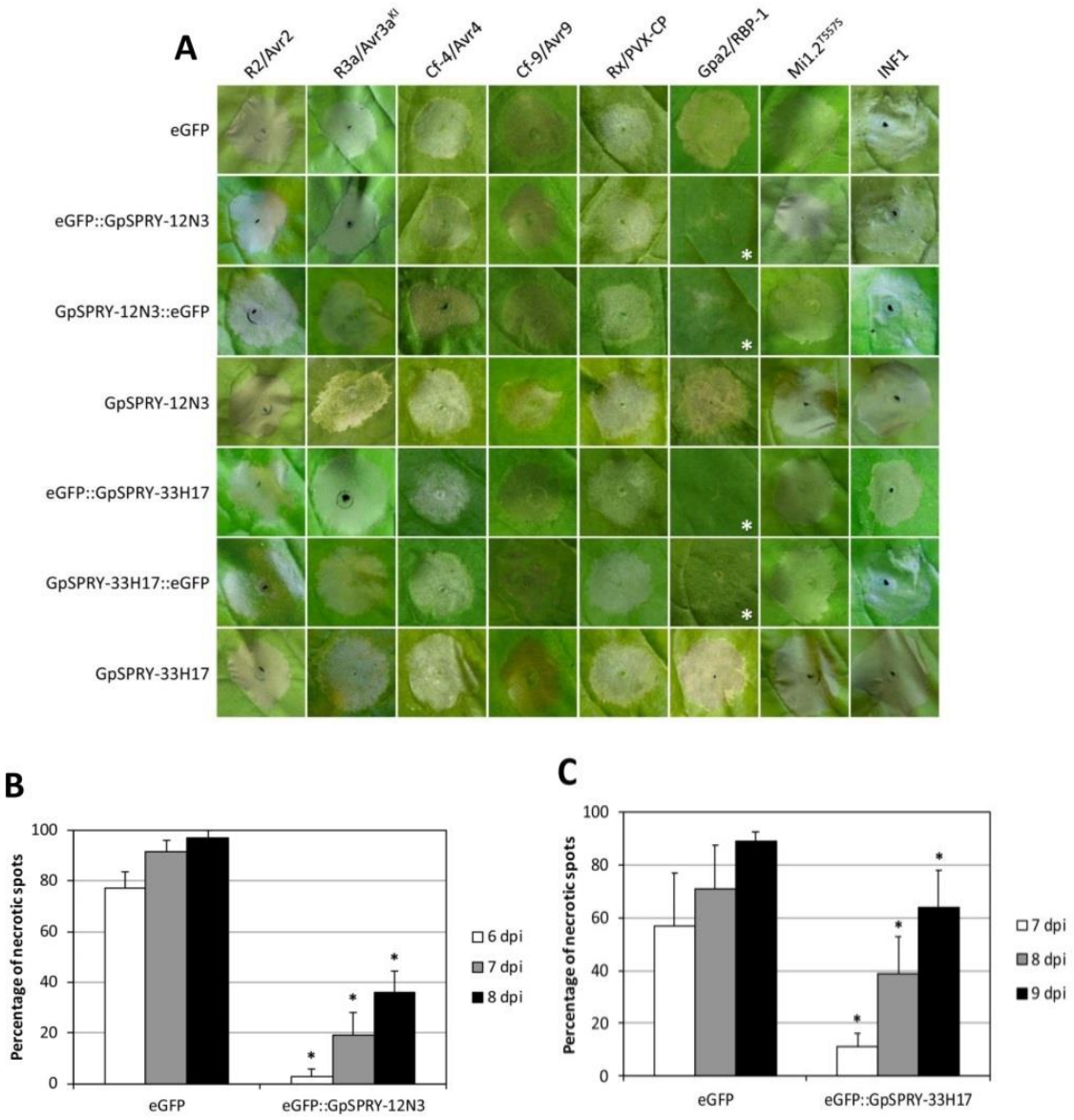


Figure 2.7 SPRYSEC effector candidates GpSPRY-12N3 and GpSPRY-33H17 suppress the hypersensitive response induced by Gpa2/RBP-1.A: Cell death symptoms induced in *N. benthamiana* by co-expression of R2/Avr2 (3 days post infiltration (dpi)), R3a/Avr3a^{KI} (2 dpi), Cf-4/Avr4 (4dpi), Cf-9/Avr9 (4dpi)Rx/PVX-CP (3 dpi), Gpa2/RBP-1 (7 dpi), an autoactive form of Mi-1.2 (Mi-1.2^{T557S}; 3 dpi), or the *Phytophthora infestans* PAMP elicitor INF1 (3 dpi) in leaves expressing either the free enhanced green fluorescent protein (eGFP) as a control or *G. pallida* SPRYSEC candidate effectors GpSPRY-12N3 or GpSPRY-33H17 fused to eGFP at the N or C terminus or lacking a GFP fusion. Asterisks indicate combinations where the symptoms are significantly suppressed by the candidate effector compared to eGFP. B & C: Graphs show the percentage of infiltration sites developing a clear hypersensitive response (HR) over time, at 6 to 9 dpi depending on the experiment, mediated by Gpa2/RBP-1 in *N. benthamiana* leaves expressing either free eGFP as a control or *G. pallida* SPRYSEC candidate effectors GpSPRY-12N3 (B) or GpSPRY-33H17 (C) fused to eGFP at the C-terminus. Experiments were done at least two times with blocks of 12 plants infiltrated on two leaves each; error bars indicate ± SE. Asterisks above the error bars indicate a significant difference (*t*-Testat $P<0.05$) from the free eGFP control evaluated at the same time point.

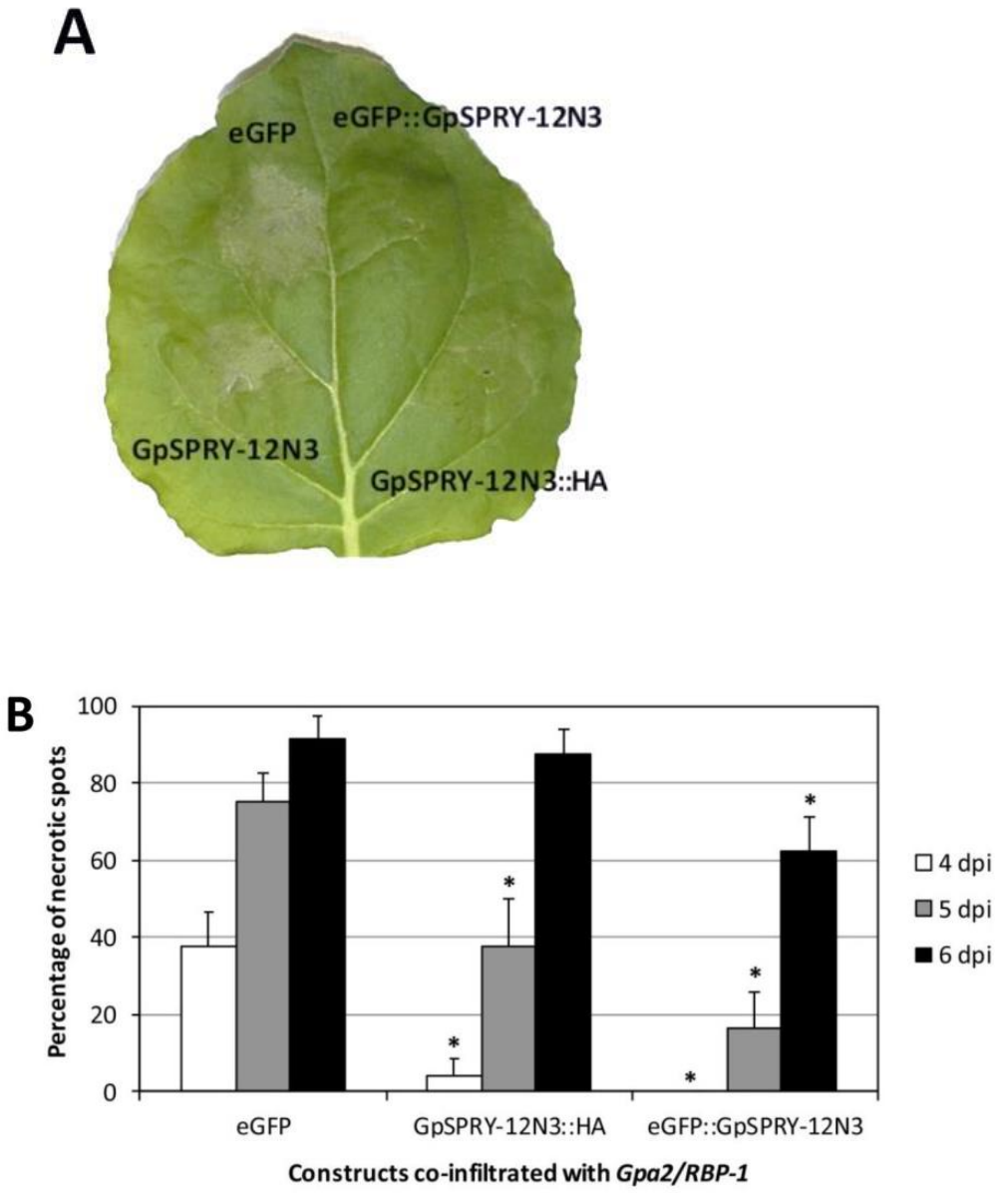


Figure 2.8 A tag is required for SPRYSECs to suppress the hypersensitive response induced by Gpa2/RBP-1. A: No suppression of the hypersensitive response (HR) induced by Gpa2/RBP-1 in *N. benthamiana* leaves is observed at 4 days post infiltration (dpi) in the presence of free enhanced green fluorescent protein (eGFP) or untagged SPRYSEC GpSPRY-12N3 compared with spots expressing GpSPRY-12N3 tagged with either eGFP or HA tag. Infiltrated regions are approximately 1cm in diameter. B: Graph shows the percentage of infiltration sites developing a clear HR over time (4 to 6 dpi) mediated by Gpa2/RBP-1 in *N. benthamiana* leaves expressing either free eGFP as a control or *G. pallida* SPRYSEC candidate effector GpSPRY-12N3 tagged with either eGFP or HA tag. Experiments were done at least two times with blocks of 12 plants infiltrated on two leaves each; error bars indicate ± SE. Asterisks above the error bars indicate a significant difference (*t*-Test at $P<0.05$) from the free eGFP control evaluated at the same time point.

2.3.5 SPRYSECs putatively interact with various host proteins

In order to identify the potential host proteins targeted by the SPRYSEC effectors, we carried out a yeast two-hybrid screening using a potato cDNA library made from late blight infected potato leaves (see 2.2.4). First we made sure that all SPRYSEC proteins as baits were not autoactiving any of the three reporter genes with empty prey vector. Then we started the real screening process for each of the baits. In most cases, clones of the interacting proteins were identified more than once with insert in the prey vectors corresponding to more or less extended 3'end truncated coding sequence of the corresponding gene. The overview of the screening results can be found in table 2.2. For GpSPRY33H17 and GpSPRY-22E10, we screened about 2.78 million and 1.69 million transformants respectively but didn't find any positive ones that could activate both *His3* and *LacZ* reporter genes. For GpSPRY-12N3, 1.19 million transformants were screened in total and 288 potential positive transformants were picked for further evaluation with the reporter gene assay. However, only three colonies were confirmed, that turned again positive in *His3* and *LacZ* reporter gene assay. Prey interactors that were present in these yeast cells were then sequenced but none of them were in the correct reading frame except one that corresponded to the ethylene-responsive factor 1. This interactor was finally re-transformed together with the bait GpSPRY-12N3. However, it turned out to be negative with no activation on either *His3* or *LacZ*. For GpSPRY-24D4, approximately 0.58 million transformants were screened and 96 promising colonies were picked for further reporter assays. It was seen that 35 of them turned positive in both *His3* and *LacZ* reporter gene assay. All interactors in these yeast cells were then isolated and sequenced. As a result, five different interactors were identified, mostly captured many times. For example, the N-ethylmaleimide sensitive fusion protein was captured once, a uncharacterized protein was captured 3 times, a probable Leucine-Rich repeats receptor-like protein kinase was captured 15 times, a HIV-1 rev binding protein was captured 7 times and CDK5 regulatory subunit associated protein 1-like was captured 9 times. Each of the individual clones was transformed together with the bait GpSPRY-24D4. It was then shown that only the interactions with a probable Leucine-Rich repeats receptor-like protein kinase and a uncharacterized protein turned out to be positive again. Unfortunately, the corresponding full length coding gene sequence of the Leucine-Rich repeats receptor-like protein kinase

could not subsequently be cloned. For GpSPRY-17I9, in total 1.96 million transformants were screened and 96 potential positive ones were picked out for further reporter gene assay. Unfortunately only 4 of them showed positive signals in *His3* and *LacZ* reporter gene assay. Further sequencing of these interactors revealed that all constructs corresponded to the potato carotenoids cleavage dioxygenase 4 (CCD4) protein. The interactions between GpSPRY-17I9-1 and CCD4 were further investigated in Chapter 3 of this thesis. For effector GpSPRY-414-2, 0.196 million yeast transformants were screened and 192 colonies were selected for a second-time reporter assay. It was shown that only 3 colonies turned blue in X-gal assay and grew on HIS3 dropout medium. Sequencing of these interactors revealed three different genes that were Ethylene-responsive factor 1, a hypothetical protein SORBIDRAF as well as a CLIP-assosiated protein like. Only the last interactor was shown positive in the one-to-one yeast transformation. Further study was carried out for the interactions between GpSPRY-414-2 and CLIP-associated protein and the details are presented in Chapter 4.

A summary of the analysis of all six SPRYSECs described in this thesis is shown in Appendix 5. Similarity matrices of these SPRYSECs are available in Appendix 2 and the alignments between defence related SPRYSECs is in Appendix 6.

Table 2.2 The list of putative interactors found for different SPRYSECs in the yeast two-hybrid screening

SPRYSECs	Blastp against Genbank	Captured times	Results of one-to-one transformation*
GpSPRY-12N3	Ethylene-responsive factor 1 (ERF)	2	-
GpSPRY-414-2	Ethylene-responsive factor 1 (ERF)	1	-
	Hypothetical protein SORBIDRAF	2	-
	CLIP-associated protein like	1	+
GpSPRY-24D4	CDK5 regulatory subunit associated protein 1-like	9	-
	HIV-1 rev binding protein	7	-
	Probable Leucine-Rich Repeats receptor-like protein kinase	15	+
	Uncharacterized proteins	3	+
	N-ethylmaleimide sensitive fusion protein, etc	1	-
GpSPRY-17I9-1	Carotenoid cleavage dioxygenase 4 (CCD4)	4	+

*Note that the result of one-to-one transformation is considered positive (+) when at least two reporter genes were activated (*His 3* and *LacZ*) in the reporter assay. Otherwise, the result is labelled as negative (-).

2.4 Discussion

SPRY domain proteins are commonly found in many organisms but expansion of this gene family appears to be specific to a subset of PPNs, although the absence of genome information for many PPNs hampers the determination of the precise evolutionary patterns of the family. No similar expansion of the SPRY domain protein family is observed in the genomes of the root-knot nematodes *M. incognita* and *M. hapla* or in the transcriptome of *Radopholus similis,* a migratory endoparasitic nematode closely related to cyst nematodes (Jacob *et al*., 2008), suggesting that this may be an adaptation to parasitism by cyst nematodes. Sequencing of the genomes of further cyst nematode species including *G. rostochiensis* (J. Jones, pers. comm.) and *H. glycines* is currently underway and will allow comparisons of the *SPRYSEC* gene families in a range of cyst nematodes.

A surprising finding in the current analysis of the *G. pallida* SPRY domain proteins is that only a minority (approximately 10%) have a predicted signal peptide present at the N-terminus. Our analysis suggests that it is unlikely that the remaining SPRY proteins are secreted into the host. Although it is known that some proteins lacking a signal peptide can be secreted from nematodes, including *G. rostochiensis* (Robertson *et al*., 2000), a comparison of the expression profiles of the SPRY domain proteins with and without signal peptides does not support the idea that the SPRY domain proteins lacking a signal peptide act as effectors; our analysis showed that the presence of a signal peptide is strongly correlated with the corresponding gene being upregulated in the J2 or in the early stages of parasitism. It is possible that the remaining, non-secreted, SPRY domain proteins have an as yet uncharacterised role in cyst nematode internal metabolism. However, it is known that at least one of the *G. pallida* SPRYSEC proteins, RBP-1, is recognised by a host resistance protein (Sacco *et al.,* 2009) and this suggests that the gene family may be under strong diversifying selection to evade recognition. The presence of many non-secreted forms in the gene family may allow for diversification while avoiding the potential for recognition by the host. New SPRYSECs may subsequently be generated by recombination, allowing the nematode to maintain a pool of potential effector sequences with only a subset in each nematode being exposed to host defences.

Given the fact that SPRYSECs are strong candidate effectors (expressed in the dorsal gland cell in J2 or in early stages of parasitism and predicted to be secreted), we further examined

their localisations in plant cells using eGFP-fusions by Agrobacterium-mediated transient expression in *N. benthamiana* leaves. Our current data confirms the localisations of several SPRYSECs that were reported before. Of particular note is the observation of GpSPRY-22E10 which is consistent to some extend with a previous study performed in roots (Jones *et al*., 2009). This suggests that assays on *N. benthamiana* leaves are of value to examine the localisation of nematode effectors that are secreted in plant roots in reality.

Several SPRYSECs in this thesis showed localisations in the nucleus. Although it should be noted that any sequence below a certain molecular mass will diffuse into the nucleus, as observed for free GFP, it is possible that nuclear effectors may manipulate gene expression and could therefore be associated with feeding site formation or alternatively may act directly interfering with host cell transcription. It is noteworthy that PSORT NLS predictions and NoD detections were not always consistent with what was seen in experiments. In order to confirm that diffusion into nucleoplasm or nucleolus is not simply due to passive diffusion the effector could be fused to a larger tag than eGFP such as GFP::GUS which would create a protein too big to be able to passively diffuse. In the current study, GpSPRY-22E10 and C-terminally tagged GpSPRY-12N3 showed a slight and strong accumulation inside the nucleolus respectively. Despite the absence of nucleolus targeting signal in these effectors, this kind of localisation is likely to represent a genuine presence of the effector rather than passive diffusion in the nucleolus. Lines of evidence in other research indicate that proteins may need a targeting signal or as alternatives need another protein forming a complex with, or interacting with, or being chaperoned by something else which moves into the nucleolus (Thorpe *et al*., 2014; Torrance *et al*., 2011; Wright *et al*., 2010).

Despite of the diverse localisations within the plant cell, the biological function of SPRYSECs during plant-nematode interactions are largely unknown. We identified in this study three SPRYSEC proteins that were able to suppress the HR induced by co-expression of Gpa2 and its cognate avirulence factor RBP-1 in *N. benthamiana.* However, a further three SPRYSECs were not able to suppress this defence response. Taken with the previously published data for SPRYSEC19 of *G. rostochiensis* this suggests that the function of some SPRYSEC effectors is suppression of host defences, other SPRYSECs are likely to have different functional roles or may suppress a different part of the defence signalling pathway not tested in these assays. Furthermore, none of the SPRYSECs tested here suppressed the cell death response mediated by a range of other R proteins, suggesting that both GpSPRY-12N3 and GpSPRY-

33H17 target a part of the defence signalling pathway specific to the *Gpa2/RBP-1* gene combination. Interestingly, and in contrast to SPRYSEC19 (Postma *et al.,* 2012), both of the *G. pallida* SPRYSECs identified here as suppressor of the *Gpa2*-mediated HR did not suppress the HR mediated by the Gpa2-closely related R protein Rx. This may be a reflection of differences in the ability of the two SPRYSECs to target the R proteins or their downstream signalling pathways.

We also tested the ability of six SPRYSECs to suppress PTI provoked by INF1 or flg22. The observation that GpSPRY-414-2 can suppress flg22-mediated ROS production is interesting. Production of ROS is one of the many PTI responses that are triggered after the perception of bacterial flagellin or its derivative flg22. A few nematode effectors have been reported previously for their roles in PTI suppression including a ubiquitin carboxyl extension effector protein derived GrCEP12 peptide that can suppress flg22-mediated ROS production and a RKN calreticulin (Chen*et al*., 2013; Jaouannet *et al*., 2013). However, this is the first evidence that a SPRYSEC effector has this biological function.

The finding that the presence of a tag is required for observing the biological activity of the SPRYSECs in terms of ETI suppression is surprising, given that the proteins are unlikely to be secreted by the nematode with any tag. However, it should be borne in mind that the experimental system used here will give rise to much higher levels of proteins being present (ETI components) than would be the case when a nematode infects a plant and introduces effectors. The most likely explanation for these data is that the tag improves stability of the protein, in what is an artificial system, and allows the biological effect to be observed. Testing this hypothesis would require generation of specific antisera against individual effectors. It is also possible that the nematode effectors are glycosylated when secreted into the host by the nematode and that this glycosylation (which is most likely absent or different under the *in planta* transient expression assay) provides stability to the effector. Analysis of the sequence of the SPRYSECs analysed here suggests that sites for glycosylation are present on many of these sequences (Table 2.1). Alternatively, several different effectors may be secreted concomitantly by the nematode that acts in concert to form a stable, biologically active complex in plants. If this is a common effect for proteins tested in this system, which is widely used in plant pathology, it will be important to ensure that effectors are tested for biological activity both with and without a tag in order to avoid false negatives.

Several cyst nematode effectors have now been identified that suppress cell death induced by plants in response to activation of their defences. These include SPRYSECs, as described here and by Postma *et al.* (2012), and an ubiquitin extension protein (Chronis *et al*., 2013). The ability to suppress a cell death response may be reflected in the manner in which some nematode resistance genes operate. The cell death response triggered by activation of some nematode R proteins seems to be targeted at the cells surrounding the syncytium, preventing further spread and development of this structure (reviewed by Sobczak and Golinowski, 2011). This in turn leads to a shift in sex ratio towards a greater proportion of males, as sex is determined by food availability in some cyst nematodes (Grundler *et al*., 1991). One explanation for this could be that the nematode is able to protect the syncytium itself from host defence responses through the secretion of effectors that suppress cell death, but that the cells further from the nematode that do not contain the effectors are not protected. Effectors that suppress host defences may therefore allow development and protection of the syncytium on susceptible plants and may also permit survival of the genotype, in the form of males, on resistant plants. These would then be able to locate and mate with virulent female nematodes on the same plant or with nematodes on another (susceptible) plant in the vicinity.

The interacting protein candidates identified in the yeast two-hybrid screens provide new insights into the putative functions of SPRYSECs. None of the interactors identified here were similar to those identified for *P. infestans* effectors (Paul Birch, The James Hutton Institute, pers. comm.). Subsequent *in planta* assays will allow confirmation of the interaction as well as a deeper understanding of how these proteins work during the interplay between *G. pallida* and its host plant (see Chapters 3 and 4). There are three effectors for which no interactors were identified. Among those, GpSPRY-12N3 and GpSPRY-33H17 showed the capability of suppressing plant defences while the other effector, GpSPRY-22E10, was localised in the nucleus and nucleolus. These biological activities suggest that they are very likely to interact with host proteins. It is possible that the sequences encoding the proteins that they interact with are not present (or not abundant) within the library that was screened. For example, they may interact directly with *the* R-protein Gpa2 or with a root-specific protein, neither of which would be expressed using the cDNA library made from susceptible Désirée potato leaves. In this case, a screen against an alternative library may be

beneficial to find true interactors and further unravel the mechanisms of their observed functions.

Chapter 3

The SPRYSEC effector candidate GpSPRY-17I9-1 from *Globodera pallida* may modulate host biochemistry to improve nematode dietary intake

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Y.Y. Mei, K. Wright, M. Taylor, A. Haegeman, G. Gheysen, J.T. Jones and S. Mantelin
Manuscript in preparation

MYY performed the gene cloning, Y2H screening, nematode infection assays and RNAi experiment. She was partly involved in the confocal assays. In addition, she took part in data analysis and wrote this chapter.

3.1 Introduction

Cyst nematodes, including the potato cyst nematode *Globodera pallida,* form intimate relationships with their host plants. In the UK alone, the economic losses of more than £50 million are caused by *G. pallida* on potato (Jones & Perry, 2004). Second stage juveniles (J2) nematodes invade plants in the elongation zone above root tips and migrate intracellularly to the vascular cylinder. In the vascular cylinder, the nematode identifies a cell that can serve as an initial syncytial cell (ISC) using stylet probing. The oesohageal bulb functions as a pump that allows for the exchange of fluids between the nematode and host plant. A cocktail of effector proteins are secreted from the nematode stylet into the ISC, transforming this cell into a multinucleate, metabolically active feeding site called a syncytium (Gheysen & Jones, 2006). The syncytium is formed by controlled breakdown of the plant cell wall followed by fusion of neighboring protoplasts. Nutrients are withdrawn from the syncytium through a feeding tube, a molecular sieve that extends from the stylet into the syncytium (Eves-van den Akker *et al*., 2014). The feeding nematode goes through three moults to reach the adult stage. Sex determination is controlled by environmental factors such as nutrient supply, with plentiful food sources leading to production of more female nematodes while inadequate nutrition gives rises to a higher proportion of males (Lilley *et al*., 2005). For example, a reduction of female numbers developing on transgenic *A. thaliana* plants expressing cowpea trypsin inhibitor was observed, presumably due to inhibition of digestive serine proteinases (Urwin *et al*., 1998). It has been also shown that syncytia associated with male nematodes are smaller and with have fewer cell wall ingrowths in the region bordering the vascular tissues than those associated with females (Muller *et al*., 1981; Sobczak *et al*., 1997).

Nutritional requirements are variable between nematodes and in most cases they are not well defined (Braeckman *et al*., 2009; Goheen *et al*., 2013). Although ingestion of appropriate nutrients is essential for completion of the life cycle, studies in this area are still fragmentary. Nematodes are known to require carbohydrates, vitamins, amino acids and lipids in their diets (Goheen *et al*., 2013). It is also likely that carotenoids are important nutritional components for nematodes. These are isoprenoid molecules that animals are generally unable to synthesize but that need to be obtained from their diets in order to meet

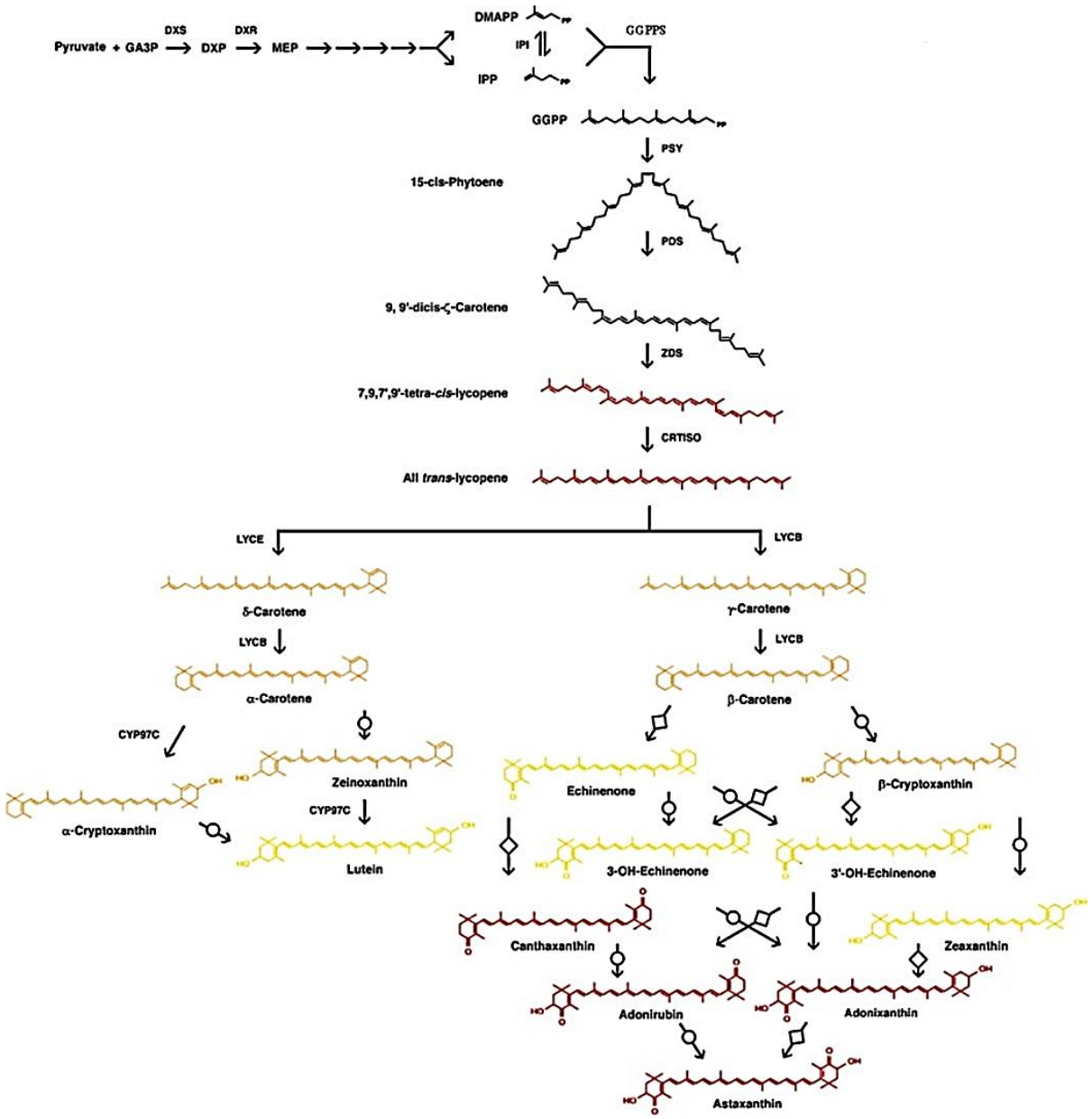


Figure 3.1 The carotenoid biosynthetic pathway in plants. The precursor for the first committed step in the pathway is GGPP (geranylgeranyl pyrophosphate), which is converted into phytoene by phytoene synthase (PSY). GGPP is formed by the condensation of IPP (isopentenyl pyrophosphate) and DMAPP (dimethylallyl pyrophosphate) which are derived predominantly from the plastidial MEP (methylerythritol 4-phosphate) pathway as depicted in the upper part of the figure. The pathway is linear until between phytoene and lycopene, and there are three steps that are catalyzed by separate enzymes in plants. Lycopene is the branch point for the α- and β-carotene pathways, which usually end at lutein and zeaxanthin, respectively, through the expression of β-carotene hydroxylases (arrows with circles). An elaborated ketocarotenoid pathway can be introduced by expressing β-carotene ketolases (arrows with diamonds) since these compete for substrates with β-carotene hydroxylases and generate diverse products. Other abbreviations: GA3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; DXR, DXP reductoisomerase; IPI, IPP isomerase; GGPS, GGPP synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; HydE, carotene ε-hydroxylase. Modified from Farré *et al*. (2010).

health demands (Ruiz-Sola & Rodríguez-Concepción, 2012). A biosynthetic pathway of carotenoids in plants is presented in Figure 3.1. Dietary carotenoids in most animals can be processed to form precursors for vitamin A biosynthesis and play many physiological roles, including immunostimulants and antioxidants, and thus help promote good health (Cazzonelli, 2011). Carotenoids can determine the coloration of animal ornaments that function as reliable quality signals indicating good body condition or parasite resistance. For example, it has been shown that reduced *Trichostrongylus tenuis* parasitism could increase carotenoid concentration and redness of red grouse ornament, indicating the involvement of nematodes in manipulating carotenoid-based signals (Martínez-Padilla *et al*., 2007). In plants, carotenoids derived from zeaxanthin and beta-carotene can function as substrates from which phytohormones such as abscisic acid (ABA) and strigolactone (SLs) are synthesized respectively (Figure 3.14).

Apocarotenoids such as retinal, ABA and SLs are generated through oxidative cleavage of carotenoids catalyzed by a family of carotenoid cleavage dioxygenases (CCDs). The presence of a range of different CCD catalytic products implies their various roles in many aspects of plant growth and development (Auldridge *et al*., 2006). Here we show that the SPRYSEC effector protein GpSPRY-17I9-1 secreted from *G. pallida* may interact with the potato carotenoid cleavage dioxygenase 4 protein (StCCD4). CCD4 was reported as a negative regulator of beta-carotene content in *Arabidopsis* seeds (Gonzalez-Jorge *et al*., 2013) and reduced potato *CCD4* expression led to accumulated carotenoids in storage organs (Campbell *et al*., 2010). In our current study, silencing *CCD4* in potato significantly increased nematode susceptibility. Further investigations were performed to examine whether this effect is due to changed levels of precursors of defence-related plant hormones or differences in carotenoids for dietary requirements of the nematodes.

3.2 Materials and Method

3.2.1 Plant growth conditions

Potato plants used in this study were grown from internode cuttings and cultured in a compost and sand mixture (1:1) in root trainers in a glasshouse. Wild-type potato (cv. Désirée), and transgenic silenced lines, supplied by Mark Taylor (The James Hutton Institute), for *CCD4* (RNAi-15 & RNAi-38; Campbell *et al.*, 2010) and *CCD8* (RNAi-1 & RNAi-8; *Pasare et*

al., 2013) as well as a control line (RNAi-EV4) were used for this work. *N. benthamiana* plants were cultured in the glasshouse in potting soil. The temperature in the glasshouse was maintained around 20°C/15°Cday/night with 16h day light.

3.2.2 *GpSPRY-17I9-1* and *StCCD4* cloning

Previous work in our group has identified a substantial number of *G. pallida SPRYSEC* genes from a large scale expressed sequence tags (ESTs) analysis (Jones *et al.*, 2009 and chapter 2). One of these effectors, GpSPRY-17I9-1, was selected for further study due to its abundance in the transcriptome. The full-length coding sequence of this gene (Appendix 1) was PCR amplified without signal peptide from cDNA of J2s, using gene specific primers (Appendix 3) and cloned in the pCR8/GW/TOPO Gateway ENTRY vector (Invitrogen) as described in Chapter 2. Several fusion clones were made (Appendix 4 and Chapter 2) for functional analysis studies.

A yeast two-hybrid (Y2H) screen (Chapter 2) identified the potato StCCD4 as putative target of GpSPRY-17I9-1. The full-length coding sequence of this gene, cloned from potato cultivar Désirée into pGEM-T vector (Promega), was obtained from Mark Taylor (The James Hutton Institute). The 1770-bp coding sequence was subcloned into pDONR221 (Invitrogen; Kanamycin 50 µg ml^{-1} selection; see Appendix 3 for primer information) and then transferred into a variety of expression vectors using Gateway technology according to the protocol provided by the manufacturer (Invitrogen). The resulting yeast prey and binary vectors (Appendix 4) were transformed into yeast Mav203 cells or *A. tumefaciens* strain GV3101 as appropriate, as described in Chapter 2. Protein domain architecture analysis was done using SMART (Schultz *et al*., 1998) and ChloroP tool (Emanuelsson *et al*., 1999) for plastid-targeting sequence prediction.

3.2.3 *In situ* hybridisation of the *G. pallida* effector candidate GpSPRY-17I9-1

The *in-situ* hybridization was done as previously described (Jones *et al*., 2000; Chapter 2) with primers designed to amplify a 232bp fragment from nucleotide 251 to nucleotide 482 (Appendix 3). The clone *GpSPRY-17I9-1* in pCR8/GW/TOPO was used as template for the PCR.

3.2.4 Yeast two-hybrid (Y2H) analysis

G. pallida SPRYSEC bait *GpSPRY-17I9-1* cloned in pDEST32 and prey interactors Y2H clones identified as *StCCD4* (I1-1, I3-12, I4-2, I5-2) or full length *StCCD4* coding sequence cloned in pDEST22 were simultaneously co-transformed into Mav203 strain following the Invitrogen ProQuest™ Two-Hybrid System protocol. The transformants were first plated out on synthetic Leu and Trp dropout media. From each transformation, at least 3 independent clones were then selected that were tested to confirm interaction based on two reporter gene assays: colonies that grew on triple dropout media (Leu, Trp and His) with 10mM 3-Amino-1,2,4-triazole and turned blue in X-gal assay were selected as positive candidates.

3.2.5 *In planta* localization and bimolecular fluorescence complementation (BiFC) analysis

The subcellular localization of GpSPRY-17I9-1 and its putative plant target StCCD4 (as full length or truncated versions corresponding to the Y2H insert fragments) were investigated using proteins fused to fluorescent tags (eGFP and mRFP; Appendix 4) for confocal analysis. *Agrobacterium*-mediated transient expression in *N. benthamiana* and imaging by confocal microscopy were performed as described in Chapter 2. For BiFC analysis, the *YFPc::GpSPRY-17I9-1* construct in pCL113 and *GpSPRY-17I9-1::YFPc* in pBatTL-B-sYFPC, the *YFPn::StCCD4* construct in pCL112 and *StCCD4::YFPn* in pBatTL-B-sYFPN, as well as 4 partial *StCCD4* clones (corresponding to the Y2H clone insert sequence) in pCL112 were used (Appendix 4; Split-YFP vectors pCL112 and pCL113 as well as pBatTL-B-sYFPC and pBatTL-B-sYFPN were provided by Sean Chapman (The James Hutton Institute). Complementary split-YFP constructs in the *A. tumefaciens* strain GV3101 were co-infiltrated in *N. benthamiana* leaves at OD_{600nm} 0.02 and 0.1 for the *SPRYSEC* and the *StCCD4* clones respectively. The YFP was imaged 48hpi with an excitation wavelength (λ) of 514 nm and emission collected at λ530-575 nm on Zeiss LSM 710 confocal.

3.2.6 Nematode infection assay

Invasive-stage juveniles of *G. pallida* were obtained by soaking dried cysts in sterile distilled water for 5 days followed by incubation in tomato root diffusate (Chen *et al*., 2005) at room temperature. Nematodes collected for infection assays were used within 24 hours of hatching. Two-week old potato plants derived from internodal cuttings and cultured in compost:sand mixture (1:1) in root trainers in glasshouse were inoculated with about 400 *G.*

pallida J2s from the standard Pa2/3 population "Lindley". The degree of infection was evaluated 3 weeks after infection in roots stained with acid fuchsin by counting the number of female and early stage nematodes per plant. To visualize the nematodes, roots were first soaked in 1% bleach for 5 min, washed intensively with tap water and then boiled for 4 min in 60 times diluted acid fuchsin solution (0.35g acid fuchsin, 25ml glacial acetic acid and 75ml water). The stained roots were washed again with tap water and kept in destaining solution (glycerol containing 0.1% glacial acetic acid).

3.2.7 Chemical treatment

ABA and Fluridon (which inhibits ABA and carotenoid biosynthesis) were purchased from Sigma-Aldrich and dissolved in separate vaporizers in a few drops of ethanol and DMSO respectively before diluting in water. The concentrations used were 100µM ABA and 30µM Fluridon. For the chemical application, 2-week old potato plants were sprayed on the leaves with vaporizers until runoff with a fine mist of either compound at the indicated concentrations (100mL solution prepared). Distilled water containing a drop of either dissolvent was used as a control treatment. In infection experiments, the chemicals were sprayed 24h before nematode inoculation.

3.2.8 Silencing *GpSPRY-17I9-1* in *G. pallida* by RNA interference

A fragment of 232bp from nucleotide 251 to nucleotide 482 of the *GpSPRY-17I9-1* cDNA sequence was selected for silencing. Two PCR products were amplified with the T7 promoter sequence incorporated at the 5' end of either the sense or antisense strand using primers described in Appendix 3 using the Megascript RNAi kit, following the manufacturer's instructions. A fragment of GFP was created as a control using primers described in Whisson *et al.* (2005). Silencing was achieved by soaking J2 nematodes in the dsRNA solution as previously described (Chen *et al.*, 2005). For each silencing construct tested, 10 three-week old potato plants (cultivar Désirée) derived from internodal cuttings and cultured in compost:sand mixture (1:1) in root trainers in glasshouse were inoculated with about 200 *G. pallida* J2s from the standard Pa2/3 population "Lindley" soaked in dsRNA. Plants were rinsed and roots stained in acid fuchsin as described above three weeks later in order to assess nematode infection.

Gene silencing in dsRNA-treated worms was checked by reverse-transcription polymerase chain reaction (RT-PCR) following procedures described in Chen *et al.* (2005) and using gene specific primers designed outside the region chosen for the silencing (Appendix 3). Each PCR reaction contained 1 µl of cDNA (prepared using a Superscript III Kit (Invitrogen) from mRNA extracted using an Invitrogen Micro Fast Track kit from the soaked or control nematodes, 5µl 10x GoTaq PCR buffer (Promega), 2µl 10mM dNTPs, 1.5µl of each primer at 10µM, 0.2µl GoTaq DNA polymerase (Promega) and water to 50µl. Cycling conditions consisted of one cycle of denaturing at 95°C for 2min followed by 35 cycles of 95°C denaturing for 45 seconds, 53°C or 59°C annealing for 30 seconds (for *GFP* and *SPRYSEC* genes or *EF1α* control respectively) and 72°C extension for 20 seconds. A final extension was done for 3 minutes at 72°C. Aliquots of reactions were removed after 22, 26 and 30 cycles. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

3.2.9 Statistical analysis

All statistical analyses were performed using either SPSS (IBM) or STATISTICA (StatSoft) analytic packages. For pair-wise comparison of sample means, Student's *t*-test at $P<0.05$ was applied. For more complex sets of data to be analysed, one-way or two-way analysis of variance (ANOVA) was applied unless otherwise stated. ANOVA was applied only if assumptions of normality and homogeneity of the variance were fulfilled. Normality of the data was checked by applying the Shapiro Wilk test ($\alpha = 0.05$). Homoscedasticity of the data was checked by applying the Levene test ($\alpha = 0.05$). Significant differences between means were evaluated using the Fisher's LSD or Tukey's HSD tests. If ANOVA could not be applied, robust test of equality of means, i.e., Welch and Brown-Forsythe tests were used to determine the difference between groups.

3.3 Results

3.3.1 GpSPRY-17I9-1 is a putative SPRYSEC effector

GpSPRY-17I9-1 was initially discovered in an EST project for *G. pallida* (Jones *et al*., 2009) and was selected for study due to its abundant expression in J2s (Chapter 2). Subsequently, this gene was cloned from cDNA and used for further functional analysis. The *GpSPRY-17I9-1* sequence without signal peptide encodes a 217 amino acid protein with a predicted molecular mass of 24 KDa. It has one SPRY domain spanning the region from amino acid 73

to 206 according to SMART protein sequence analysis results. An *in situ* hybridization assay demonstrated that *GPSPRY-17I9-1* is expressed in the dorsal gland cell of J2 indicating a potential role in plant parasitism (Figure 3.2).

Treatment of nematodes with dsRNA of *GpSPRY-17I9-1* seem to reduce the capability of the nematodes to parasite host plants. As shown in Figure 3.3A, the average number of *GpSPRY-17I9-1* soaked nematodes three weeks after inoculation was around 20 per gram of root while the average of *GFP* dsRNA treated nematodes was approximately 40. This indicates a 50% reduction in infection after exposure to *GpSPRY-17I9-1* dsRNA, although this figure was not statistically significant due to the small sample size. No significant difference was observed between both treatments with respect to the percentage of females (Figure 3.3B).

To confirm that RNAi was successful, the transcript levels of *GpSPRY-17I9-1* were measured by RT-PCR on total RNA extracted from all samples, using *GpEF1α* as a control gene (Figure 3.4). After 22 cycles, bands of the expected size were amplified for *GpEF1α* but no amplification was seen for *GpSPRY-17I9-1*. After 26 cycles, the *GpSPRY-17I9-1* bands are present in all samples but at higher level in the control sample. After 30 cycles, a higher level of *GpSPRY-17I9-1* amplification was seen in the control sample compared to *SPRYSEC* silenced samples. Amplification of a band from *GpEF1α* is similar in both control and *GpSPRY-17I9-1* dsRNA treated sample. These data indicate a specific reduction in *GpSPRY-17I9-1* transcript in nematodes exposed to *GpSPRY-17I9-1* dsRNA.

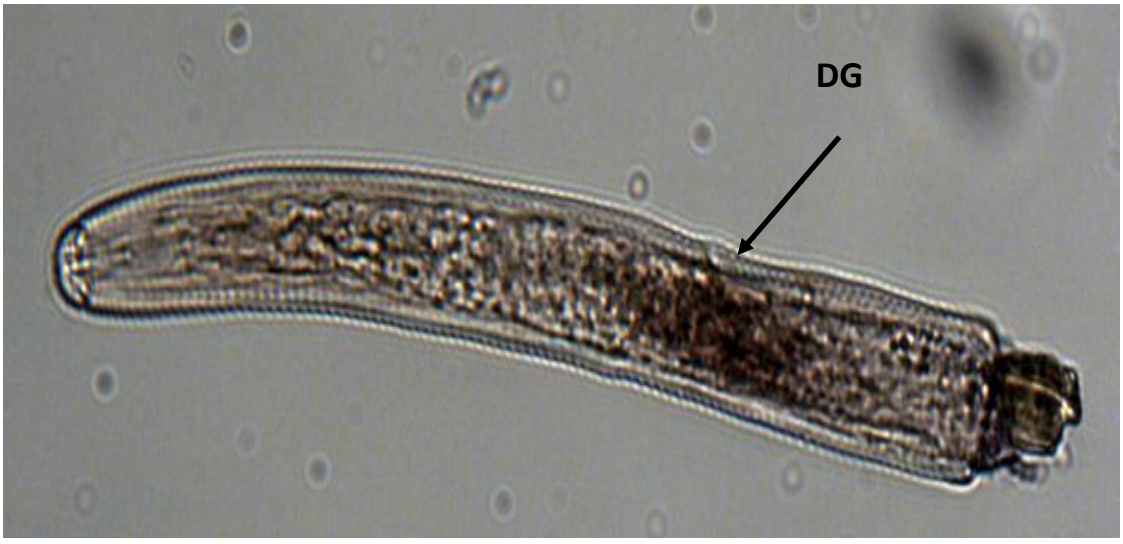


Figure 3.2 Localisation of the *G. pallida* candidate effector *GpSPRY-17I9-1* expression in the nematode dorsal gland cell (DG) by *in situ* hybridisation to preparasitic second stage juveniles (J2s). Sense control probe showed no binding to nematode structures (not shown). *G. pallida* J2s are approximately 30 µm in diameter.

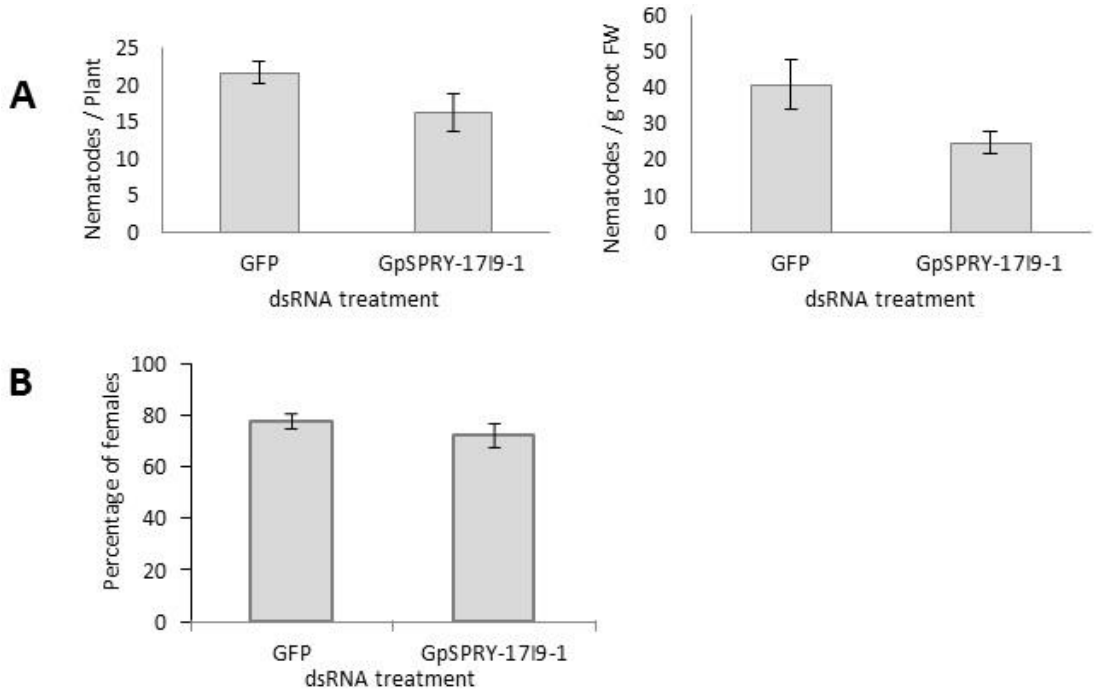


Figure 3.3 Silencing effect of the nematode effector GpSPRY-17I9-1 on parasitic success. Nematodes were soaked in dsRNA generated from eGFP or from GpSPRY-17I9-1. (A) Total number of *G. pallida* per plant or per gram (fresh weight) of potato roots was slightly but not significantly lower after exposure to GpSPRY-17I9-1 dsRNA compared to GFP control treatment. (B) There is no difference in the percentage of females between GpSPRY-17I9-1 dsRNA soaked samples compared with the control. Error bars indicate the standard error of the mean (n=10).This experiment was carried out once.

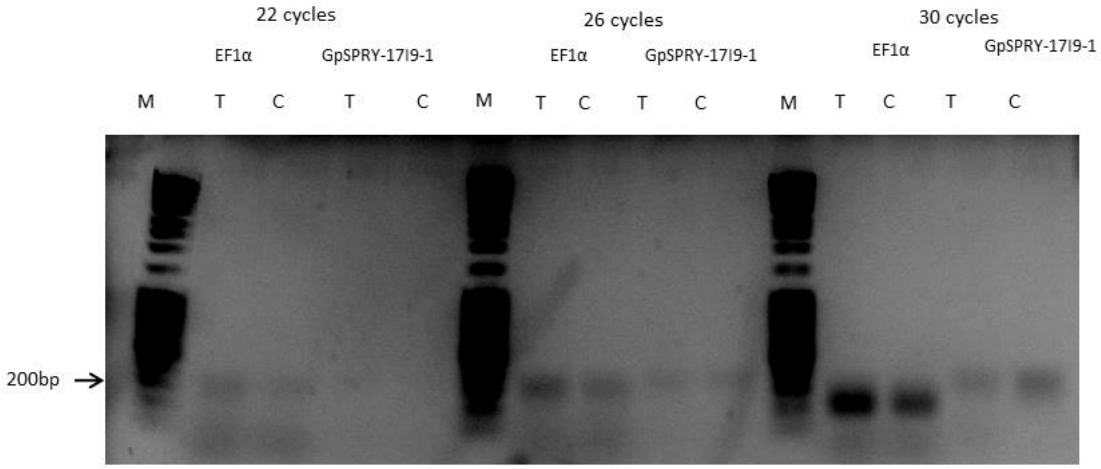


Figure 3.4 Reverse-transcription polymerase chain reaction (RT-PCR) analysis showing that levels of *GpSPRY-17I9-1* are reduced in nematodes exposed to dsRNA from *GpSPRY-17I9-1*. Gel shows amplification products from *GpSPRY-17I9-1* and Elongation factor 1 alpha (*GpEF1α*) in control nematodes soaked in *GFP* dsRNA (C) and nematodes exposed to *GpSPRY-17I9-1* dsRNA (T). Reactions were stopped after 22, 26 and 30 cycles. M=1Kb ladder. This experiment was carried out once.

3.3.2 GpSPRY-17I9-1 localises in the plant cytoplasm where it may interact with the potato carotenoid cleavage dioxygenase 4 (StCCD4) protein

GpSPRY-17I9-1 is expected to be delivered into the host cell by nematodes through the stylet. To examine its subcellular localization within plant cells, a construct was generated in which the effector protein was N- or C-terminally fused to eGFP under the control of a cauliflower mosaic virus (CaMV) 35S constitutive promoter. The construct was transiently expressed in *N. benthamiana* by Agroinfiltration. In both cases, eGFP signal was observed in the cytoplasm as well as in nucleoplasm but was excluded from the nucleolus (Figure 3.5 A-D). The same localization pattern was seen when the effector was tagged with mRFP (data not shown). Interestingly, with N-terminal tagged eGFP it was occasionally shown that GpSPRY-17I9-1 localised to some small cytoplasmic vesicles (0.5-1µm) of unknown identity (Figure 3.5 E-F).

To identify the host target of GpSPRY-17I9-1, we screened this effector as bait against a prey cDNA library made from potato leaves infected with the foliar pathogen *P. infestans*. Four independent prey clones encoding potato carotenoid cleavage dioxygenase 4 (*StCCD4*) were identified. Through one-to-one Y2H transformation, the interactions between bait GpSPRY-17I9-1 and these truncated StCCD4 clones were confirmed. However, no positive interaction was observed with the full-length StCCD4 in yeast (Figure 3.6).

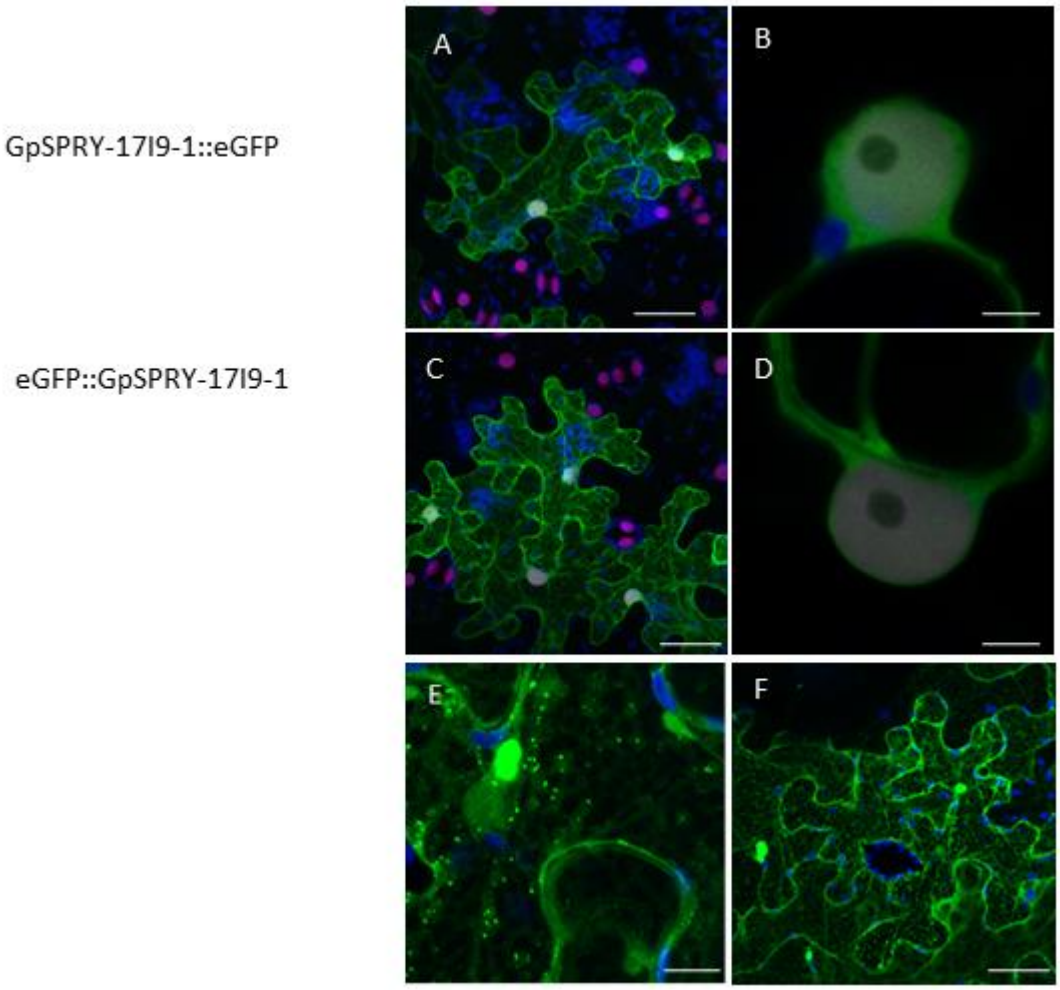
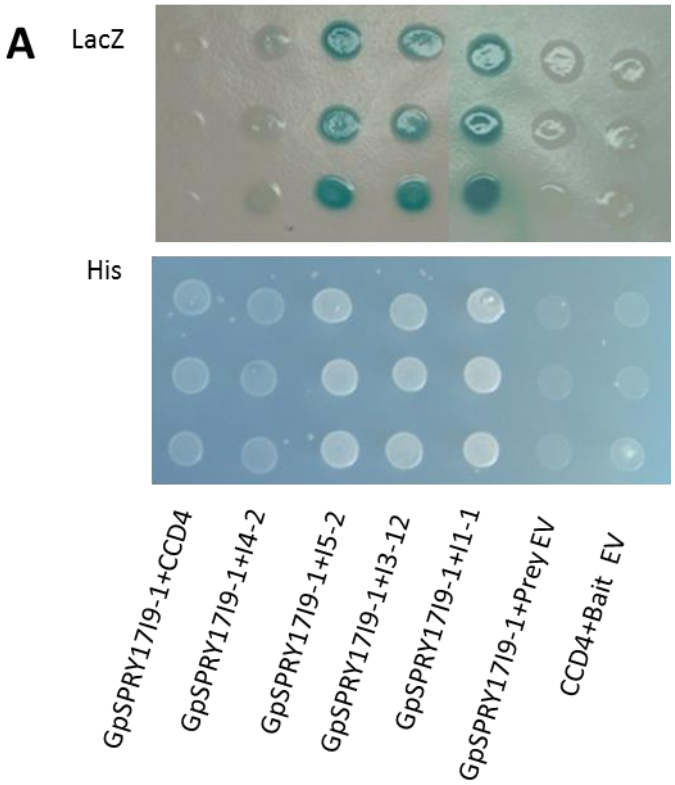


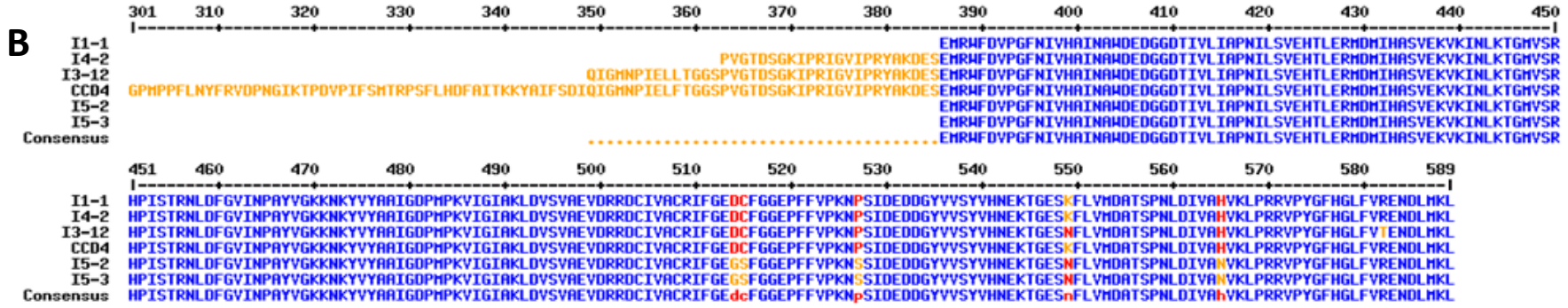
Figure 3.5 GpSPRY-17I9-1 mainly localizes in the cytoplasm and nucleoplasm, but not in the nucleolus, irrespective of the position of the GFP tag. Constructs were infitrated into *N .benthamiana* strain CB157, which contains a histone H2B-mRFP marker in the nucleus. A&B: localization of GpSPRY-17I9-1 with eGFP fused to the C-terminus. C&D: localization of GpSPRY-17I9-1 with eGFP fused to the N-terminus. E&F: Very occasional labelling of eGFP::GpSPRY-17I9-1 in unidentified cytoplasmic vesicles. eGFP signal displayed green, mRFP signal displayed magenta, silver color shows areas where green and red signals are overlaid. Autofluoresence from chloroplasts is displayed as blue. Scale bars in A, C and F represent 50 µm, in B and D represent 5 µm while the one in E represents 10 µm. Each experiment was repeated at least twice with three technical replicates.



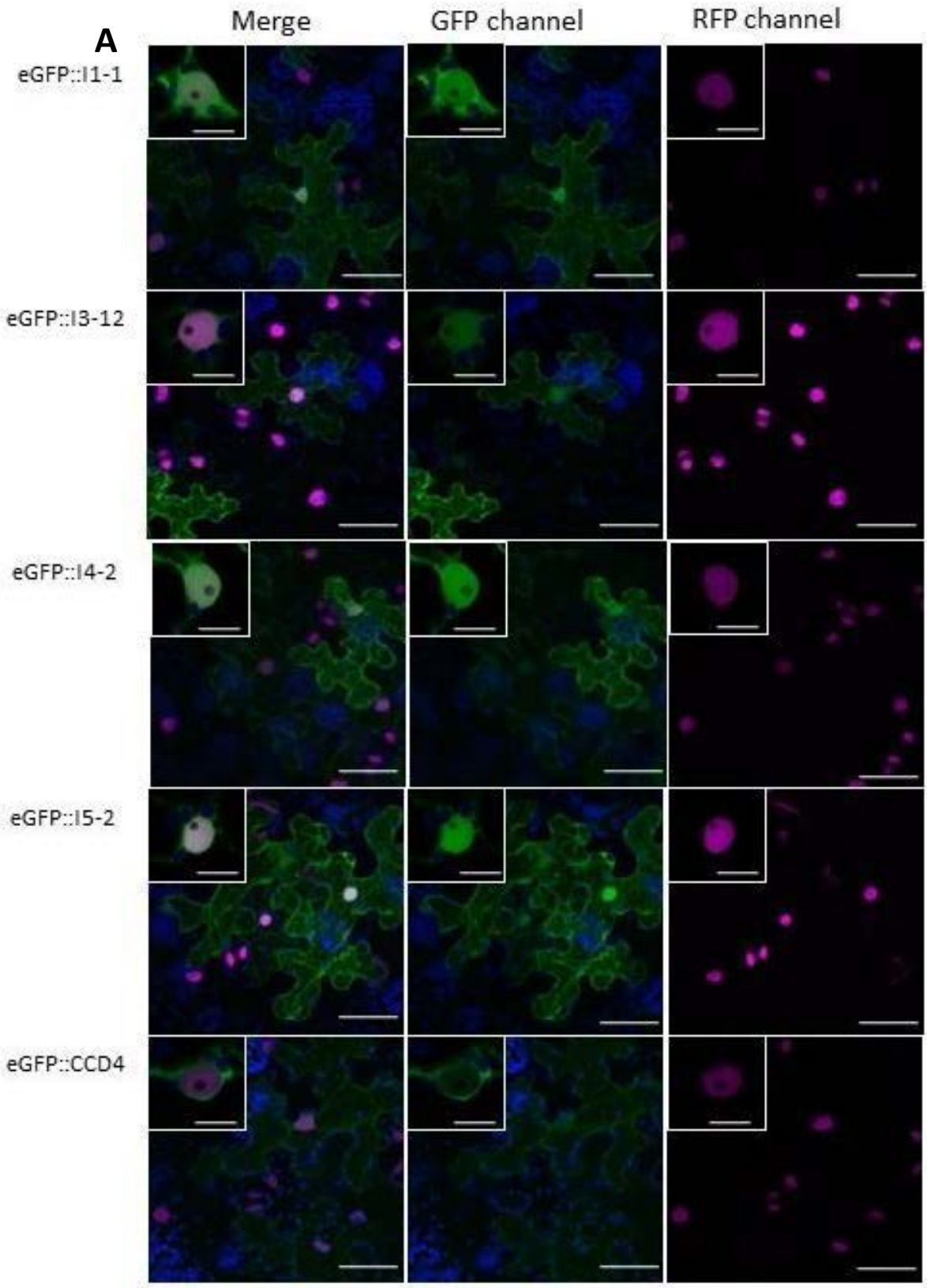
Gal4-DBD	GpSPRY-17I9-1

Gal4-AD	StCCD4

Figure 3.6 The effector GpSPRY-17I9-1 was seen to interact with StCCD4 in yeast. (A) Yeast two-hybrid analysis showing interactions between GpSPRY-17I9-1 and the full length (CCD4) or truncated (I1-1, I3-12, I4-2 and I5-2) StCCD4 proteins. Both *LacZ* (blue colouration) and *His3* (providing growth on medium lacking His [-his]) reporter genes were activated. Autoactivation tests for both bait *GpSPRY-17I9-1* and prey *CCD4* clones were negative. The scheme on the right represents details of both bait and prey constructs. (B) Protein sequence alignment of truncated StCCD4 clones and the full length CCD4 sequence. Alignment was generated using MultAlin (Corpet, 1988). Amino acids in blue are conserved among all sequences and amino acids in red are the most prevalent.



We subsequently examined the potential interaction between GpSPRY-17I9-1 and StCCD4 *in planta.* Both truncated and full length StCCD4s showed a cytoplasmic localization with some signal present in the nucleus but not in the nucleolus. Diffusion into nucleus was more obvious in case of the truncated CCDs probably due to their smaller size (Figure 3.7A). Interestingly, when tagged on the C-terminus, StCCD4 showed accumulation in chloroplasts as well as in the cytoplasm (Figure 3.7B). When co-expressed with effector GpSPRY-17I9-1, the localization of StCCD4s did not change and the two proteins co-localised in the cytoplasm (Figure 3.8A). Some co-localisation was also seen in the nucleoplasm with the truncated CCD4s (data not shown). A bimolecular fluorescence complementation (BiFC) assay was then carried out to examine whether GpSPRY-17I9-1 and StCCD4 interact with each other *in planta*. The N- or C-terminus-encoding portions of YFP were fused to full length or truncated StCCD4 or GpSPRY-17I9-1 and constructs containing complementary parts of YFP were co-expressed in *N. benthamiana*. When the tags were placed at the N-terminus of the fusions the YFP signal was observed in cytoplasm (Figure 3.8B), suggesting a positive interaction between GpSPRY-17I9-1 and StCCD4 (both full length or truncated) in plant cells. Stronger interactions between GpSPRY-17I9-1 and the four different truncated CCD4s were also seen in the nucleoplasm compared to the interaction with full length StCCD4. In both cases, no YFP signal was observed in the nucleolus. When the split YFP was tagged on the C-terminus of StCCD4, no YFP signal could be detected.


A
Merge
GFP channel
RFP channel
eGFP::I1-1
eGFP::I3-12
eGFP::I4-2
eGFP::I5-2
eGFP::CCD4

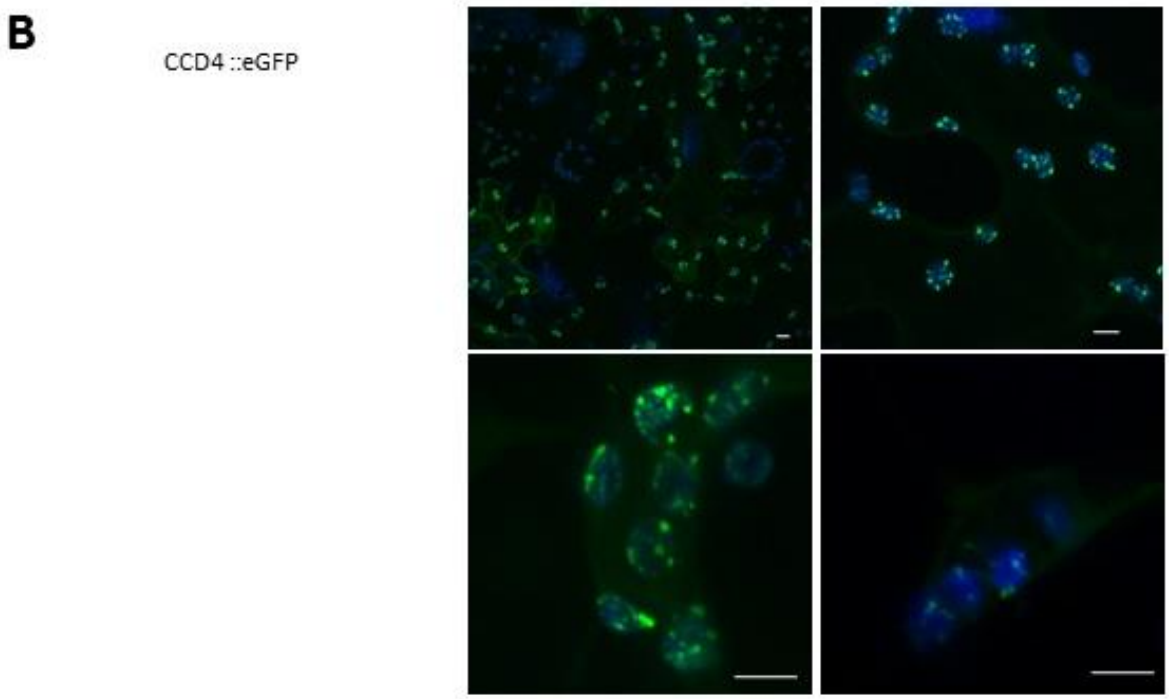


Figure 3.7 Subcellular localisations of truncated and full length StCCD4. A) All four truncated CCD4 interactors identified in the Y2H screens (I1-1, I3-12, I4-2 and I5-2) are localised in the cytoplasm and nucleus, but seem excluded from the nucleolus. These constructs showed a greater signal in the nucleus when compared to the eGFP::full length CCD4 construct. All constructs were Agroinfiltrated into leaves of transgenic *N. benthamiana* CB157 which contains a Histone H2B mRFP nucleoplasmic marker. Scale bars on nuclei pictures represent 10µm while the others are 50 µm. B) When tagged on the C-terminus, CCD4 localizes to the cytoplasm but with aggregations inside chloroplasts. This is shown in four Images with different magnification. The construct was infiltrated in *N. benthamiana* wild type leaves. GFP signal displayed green, mRFP signal displayed magenta, silver color shows areas where green and red signals are overlaid. Autofluoresence from chloroplasts is displayed as blue. Scale bars in panel B all represent 5 µm. Each experiment was repeated at least twice with three replicates.

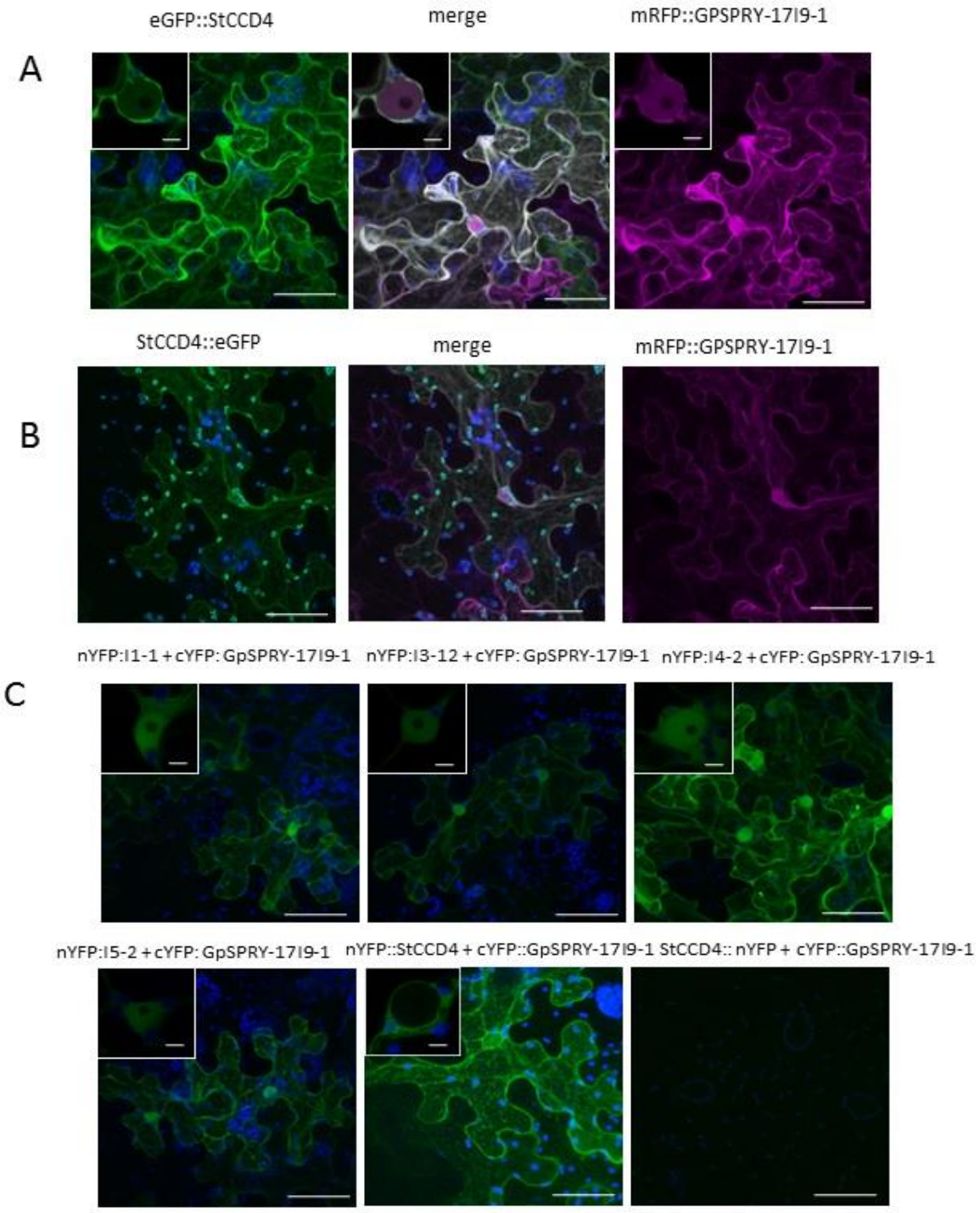


Figure 3.8 Co-localisation of, and interaction between, GpSPRY-17I9-1 and StCCD4 in *N. benthamiana* epidermal cells. A) When tagged on the N-terminus, StCCD4 (green signal) shows clear co-localisation with GpSPRY-17I9-1 (magenta signal) in the cytoplasm. B) When tagged on the C-terminus StCCD4 aggregates in chloroplasts but still shows some colocalisation with GpSPRY-17I9-1 in the cytoplasm. C) *In planta* interaction of StCCD4s with GpSPRY-17I9-1 analysed by BiFC. All truncated StCCD4s interact with GpSPRY-17I9-1 in the cytoplasm when nYFP tag is N-terminal. However, no signal was seen with nYFP tagged StCCD4 on C-terminus. A & B: GFP signal displayed green, mRFP signal displayed magenta, silver color shows areas where green and red signals are overlaid. Autofluoresence from chloroplasts is displayed as blue. C: Reconstituted YFP displayed green, autofluoresence from chloroplasts displayed blue. Scale bars all repsent 50µm except those in nuclei that represent 10µm. Each experiment was repeated at least twice with three replicates.

3.3.3 RNAi silencing of *StCCD4* dramatically increases nematode susceptibility independently of an indirect increase in abscisic acid

To examine the role of StCCD4 in the interaction between *G. pallida* and host potato plant, *S. tuberosum* cv. Désirée mutant plants (*CCD4*-RNAi-15) that carry a RNAi construct with a 324bp portion of the potato *CCD4* cDNA under the control of a CaMV 35S constitutive promoter (Campbell *et al*., 2010) were inoculated with *G. pallida* to assess the effects of reducing *CCD4* levels on nematode parasitism. *CCD4* RNAi plants showed significantly reduced shoot and root growth as well as heat-sprouting, chain tubers that are similar to heat-stress like phenotype (Campbell *et al*., 2010). At 3.5 weeks after inoculation, a significantly higher number of total nematodes as well as a bigger proportion of females were observed in the *StCCD4* RNAi line compared to the wild type control (Figure 3.9).

Further investigations were carried out to find the reasons for this increased susceptibility. Carotenoid cleavage dioxygenases (CCDs) are encoded by multigene families in plants and can catalyze the oxidative cleavage of carotenoids to generate a range of apocarotenoid products that fulfill various functions (Cazzonelli, 2011). Silencing the *CCD4* gene by RNA interference resulted in a large increase in violaxanthin and neoxanthin (Campbell *et al*., 2010). Violaxanthin is the main substrate for NCED type CCD enzymes (9-cis-epoxycarotenoid dioxygenases) that give rise to the defence-associated plant hormone ABA. We therefore examined whether the silencing effect of *StCCD4* on potato is due to an increase in ABA levels. To this end, we sprayed ABA on wild type Désirée plants and twenty-four hours later inoculated with *G. pallida*. The effect of this hormone treatment was then evaluated by counting the number of nematodes per gram of plant root three weeks after inoculation of treated and untreated (water sprayed) plants. The result is shown in Figure 3.10. Exogenous application of ABA resulted in a significant reduction in total nematode infection per gram of plant root compared with the control plant. Notably, the total number of females remained unchanged in ABA treated plants while the males were significantly reduced. This is also reflected in the dramatic increase in percentage of females in ABA treated plants (Figure 3.10B).

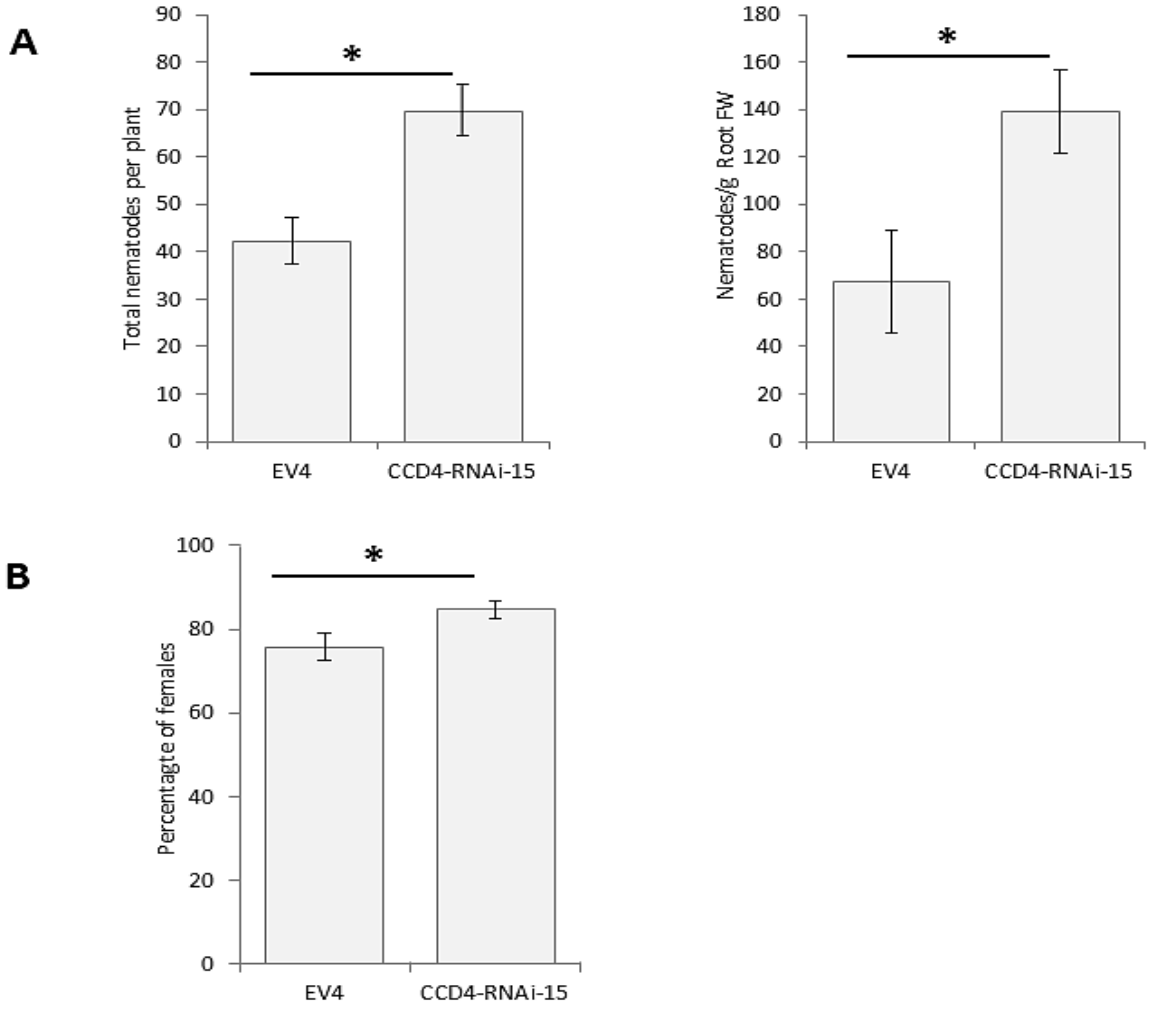


Figure 3.9 Transgenic plants containing an RNAi construct targeting *StCCD4* show increased susceptibility to *G. pallida.* RNAi line *CCD4*-RNAi-15 shows significantly increased total number of nematodes (A) and of females (B) compared to the empty vector control. Bars represent means ± SE of the number of nematodes per gram of root fresh weight at 3.5 weeks after inoculation recorded on 15 control plants and 19 *CCD4* RNAi plants. Asterisks indicate statistically significant differences between treatments (student's *t*-test with P<0.05). Data represent one of the three independent experiments with similar results.

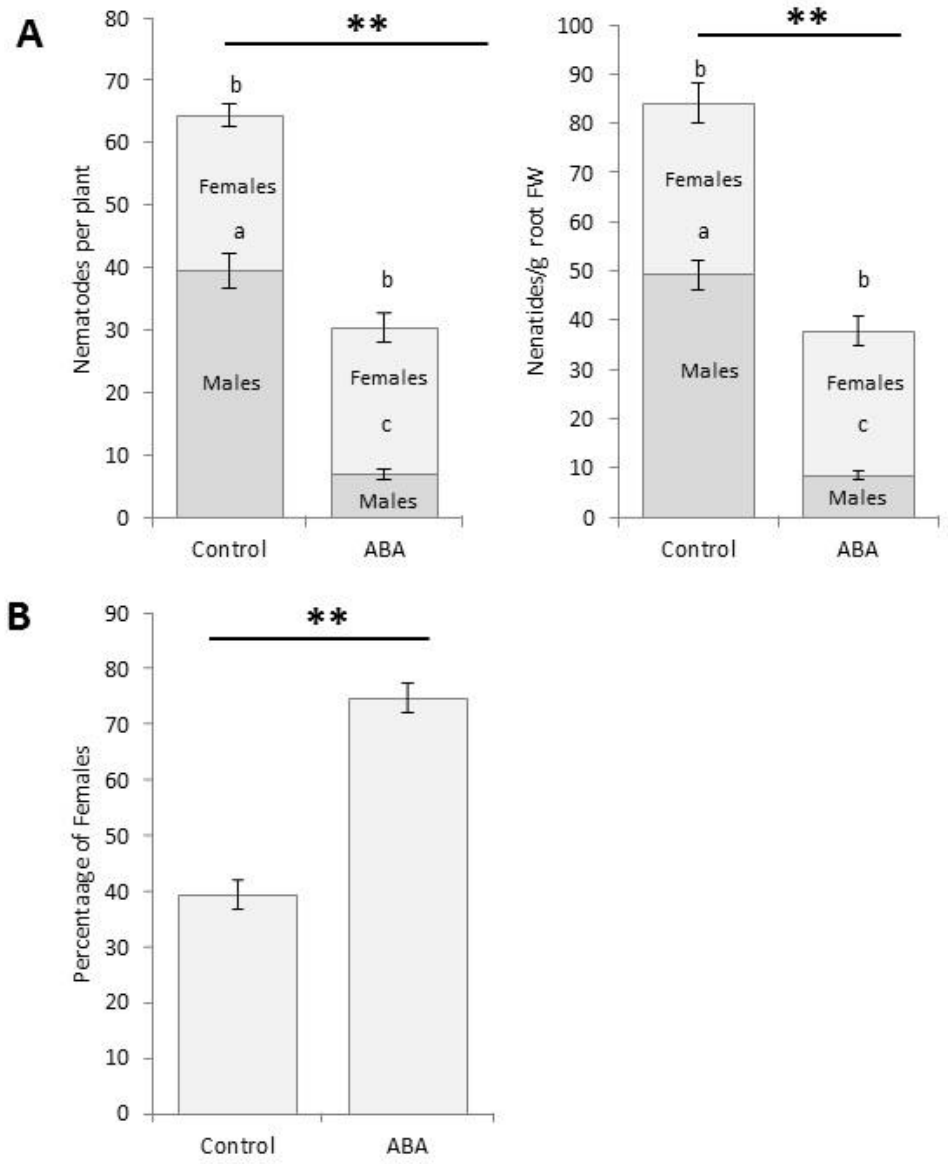


Figure 3.10 Effects of ABA treatment on nematode infection of *S. tuberosum* cv Désirée**.** (A) Exogenous ABA application significantly reduces nematode infection by decreasing the number of males that develop while having no effect on females. Bars represent means ± SE of the number of nematodes per gram of root fresh weight at 2.5 weeks after inoculation recorded on 24 control plants and 26 ABA treated plants. (B) Increase in percentage of females in ABA treated plants. Different letters and two asterisks show significant differences between treatments (student's *t*-test with P<0.01). Data represent one of the two independent experiments with similar results.

3.3.4 Increased susceptibility of *StCCD4* RNAi line may be associated with enhanced levels of carotenoids

3.3.4.1 *S. tuberosum* group Phureja that has naturally lower expression of *StCCD4* and higher content of carotenoids is highly susceptible to PCN

There is an apparent inverse relationship between *StCCD4* gene expression and carotenoid content (Campbell *et al*., 2010). High-performance liquid chromatography (HPLC) analysis revealed elevated carotenoid levels in the *StCCD4* RNAi line that was used in the current study (Campbell *et al*., 2010). Besides, tubers of plants of the *S. tuberosum* group Phureja have higher carotenoid content than other species (Burgos *et al*., 2009; Morris *et al*., 2004). Campbell *et al*. (2010) further investigated the relative expression levels of *StCCD4* transcripts using microarray data and identified two Phureja genotypes (cv. 333-16 and cv. Mayan Gold) that had approximately 5-fold lower *StCCD4* gene expression compared with Tuberosum types (cv. Désirée and cv. Maris Piper). The resistance of *Solanum phureja L. cv.* Mayan Gold to PCN is classified as very low to low (http://www.europotato.org/display_description.php?variety_name=Mayan%20Gold). We therefore carried out a *G. pallida* infection assay to compare the nematode colonization on Désirée and Phureja. Unfortunately the infective J2s used were old and gave a low overall infection rate. Although no significant difference in terms of total nematodes per gram of root was observed far higher levels of females were present in *S. phureja,* confirming that this species is more susceptible to *G. pallida* (Figure 3.11).

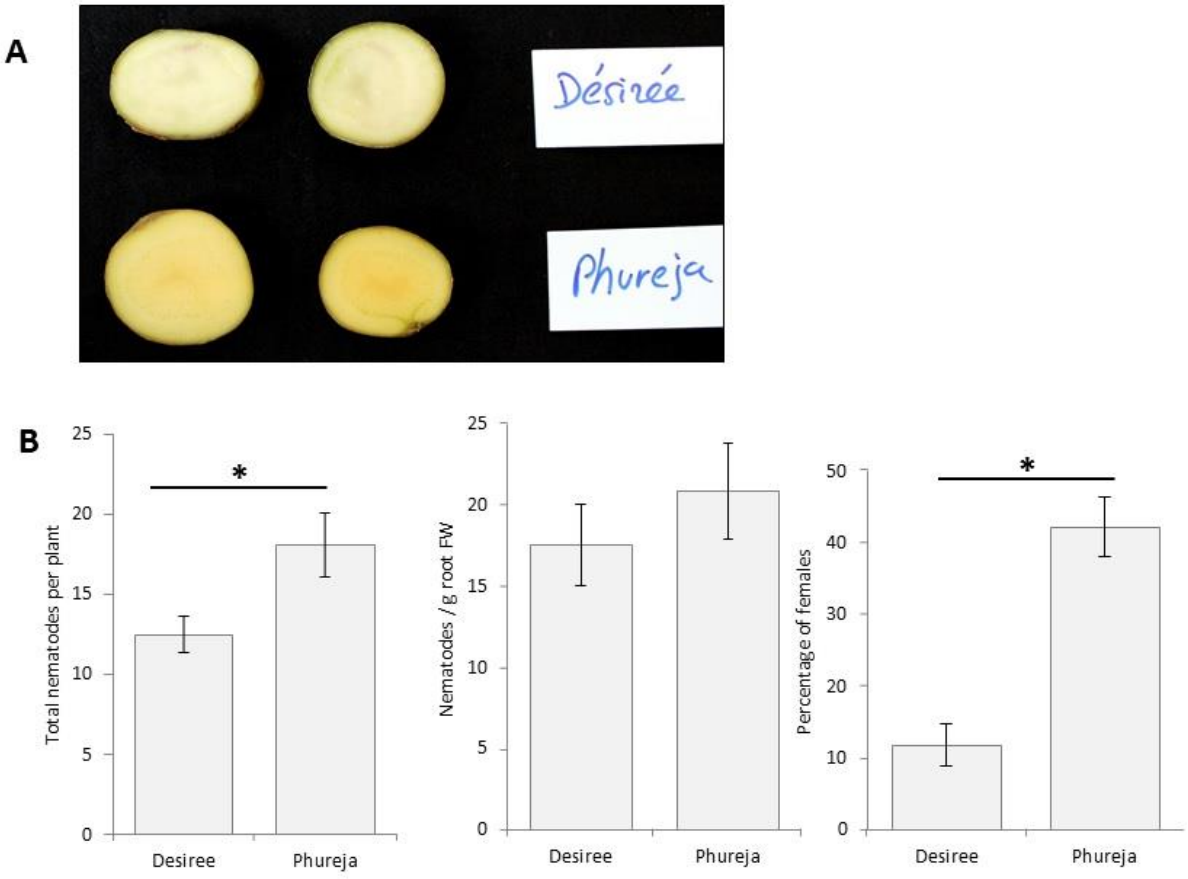


Figure 3.11 *Solanum phureja* has higher levels of carotenoids and is more susceptible to *G. pallida* compared to *S. tuberosum* cv Désirée. A) Cut tubers of *S. tuberosum* Désirée and yellow-fleshed *S. phureja* showing yellow coloration due to naturally higher carotenoid content. B) *S. phureja* supports more nematodes and a significantly higher percentage of females compared to cv Désirée. Bars represent means $\pm$ standard error of 18 Désirée or 22 *S. phureja*. Asterisk indicates statistically significant differences (student's *t*-test with $P < 0.05$).

3.3.4.2 Application of the carotenoid biosynthesis inhibitor Fluridon shifts nematode sex ratio towards males

To experimentally evaluate whether carotenoids can contribute to the susceptibility of *StCCD4* RNAi lines, we applied on wild type cv. Désirée plants the carotenoid biosynthesis inhibitor Fluridon and investigated the effect of this treatment on susceptibility to *G. pallida*. A bleaching phenotype was observed in all Fluridon treated plants while no such symptoms were seen on control plants (Figure 3.12 A), confirming the efficiency of the chemical treatment as Fluridon is blocking the Phytoene Desaturase (PDS) which is required for chlorophyll synthesis (Qin *et al.*, 2007). In addition, shoot dry weight was significantly decreased in Fluridon treated plants, although root growth was not affected (Figure 3.12B). Fluridon treated plants supported increased nematode numbers but with a significantly lower proportion of females (Figure 3.12 C).

The same experiment was repeated once with slightly different outcome. In terms of plant growth, experiment 2 (Figure 3.13 A) showed that there was significant reduction of shoot fresh weight, shoot dry weight and shoot length in Fluridon treated plants. In contrast, no significant difference was seen with regard to root fresh weight as well as the ratio of shoot/root fresh weight. Compared with experiment 1, this experiment (Figure 3.13 B) showed that after three weeks of infection there was no significant difference in terms of total number of nematodes per gram of root between two treatments, however, there was again a remarkable increase in male numbers and a dramatic shift of the sex ratio towards males.

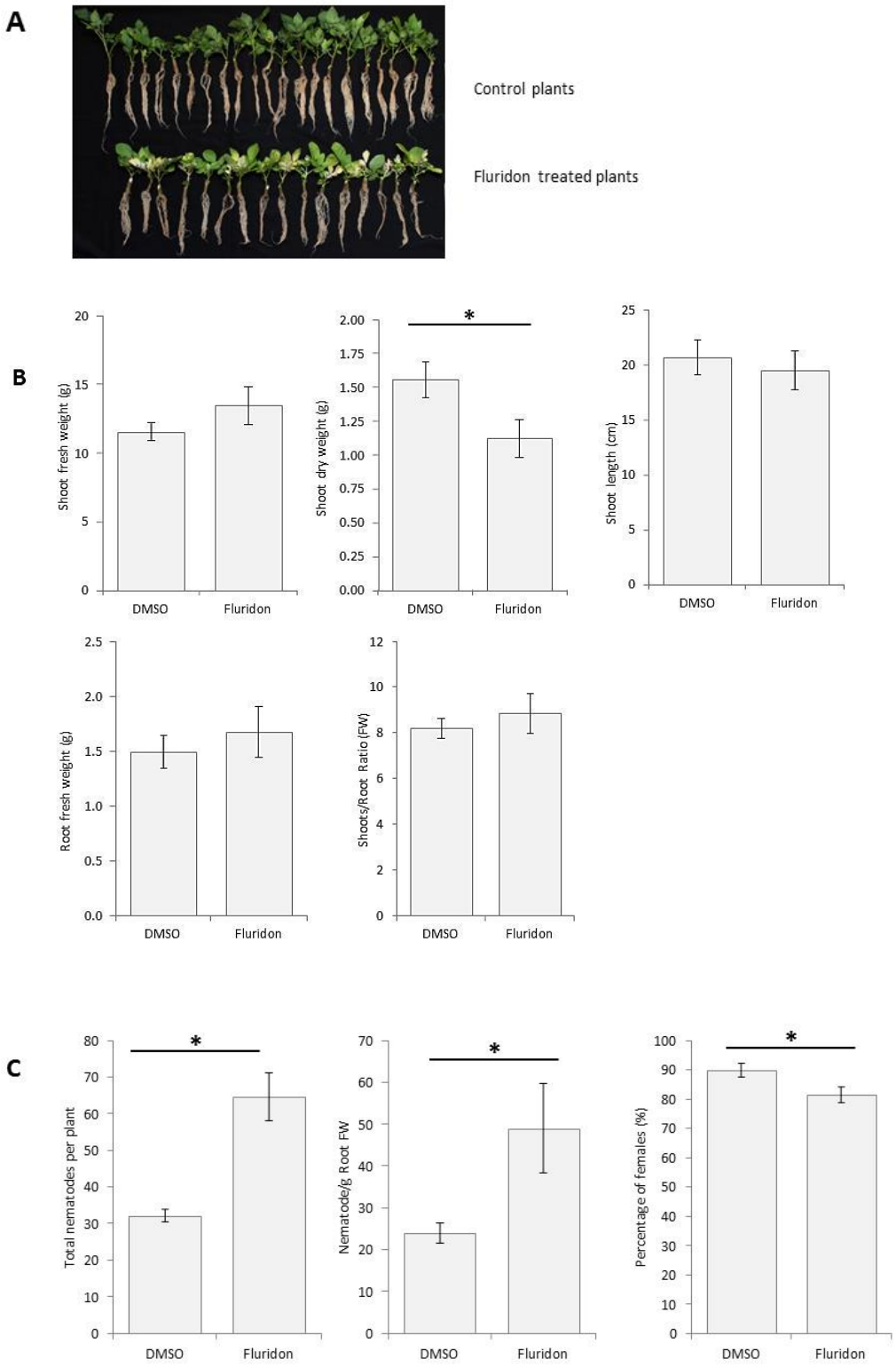


Figure 3.12 Effects of Fluridon on plant growth and nematode infection - experiment 1. A) Plants treated with Fluridon (right) showed bleaching on leaves compared with control plants (left). B) Growth of plant shoots, but not roots, is significantly affected by Fluridon application. C) Fluridon application significantly increased total nematode numbers and decreased the proportion of females. Bars represent means ± standard error of 19 control plants or 15 treated with Fluridon. Asterisks indicate statistically significant differences (student's *t*-test with $P < 0.05$).

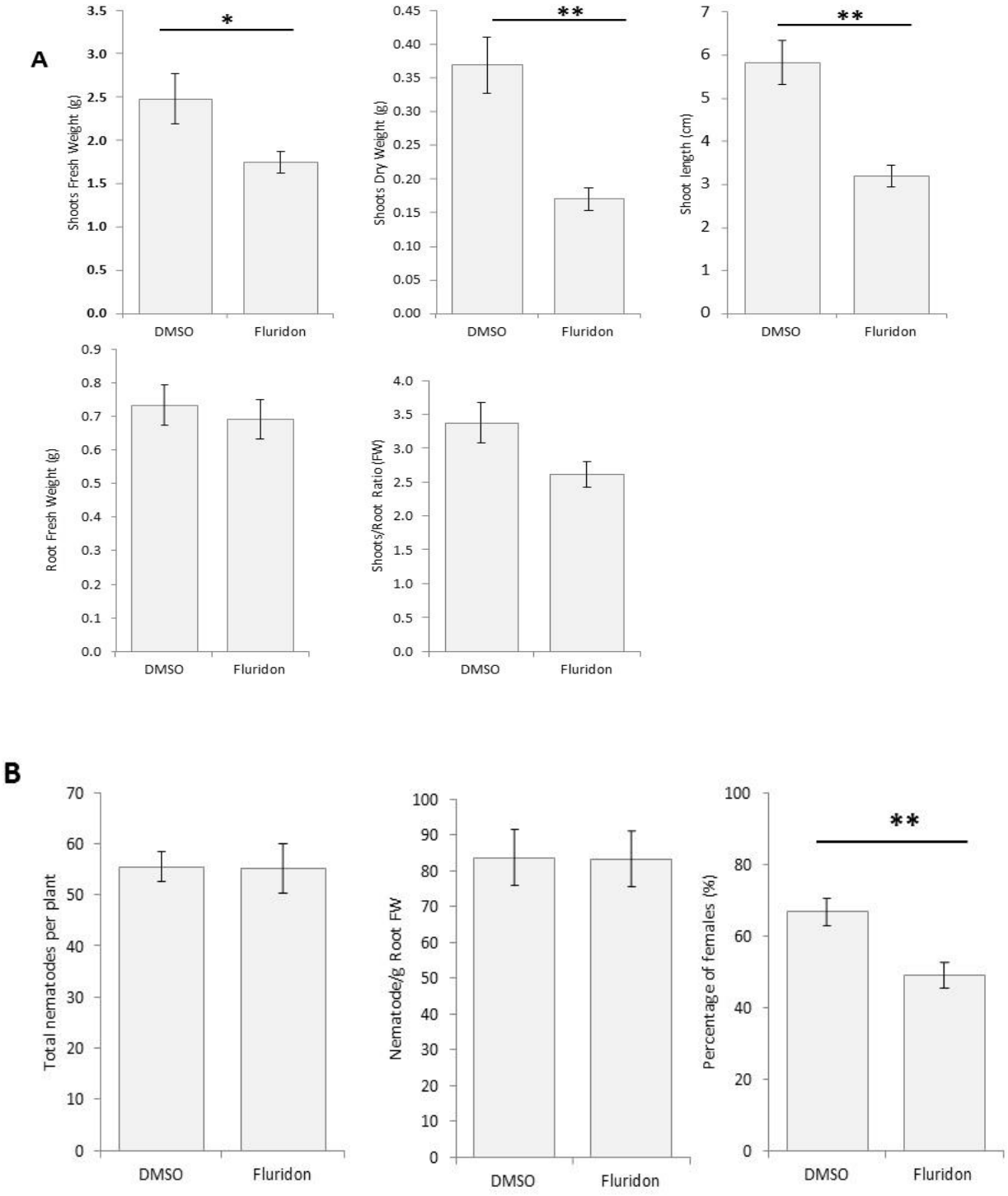


Figure 3.13 Effects of Fluridon on plant growth and nematode infection - experiment 2. A) Fresh weight of shoots, dry weight of shoots and shoot height were all significantly decreased after Fluridon treatment, while roots were not affected (n=21 for DMSO control treatment, n=16 for Fluridon treatment). B) Exogenous Fluridon application did not affect the total number of nematodes but significantly reduced the percentage of females. Bars represent means $\pm$ standard error. One asterisk indicates statistically significant differences (student's t-test with $P < 0.05$) while two asterisks indicate significance $P<0.01$.

3.4 Discussion

G. pallida deploys a suite of effector proteins that allow it to invade host plants and establish a feeding site. Nematode effectors can be defined as molecules that suppress host defences or manipulate the host to facilitate food acquisition (Bird *et al.*, 2014). The *SPRYSEC* gene family is of particular relevance due the large number of members in *G. pallida* and diverse roles in plant parasitism. In the present study, we have shown that one SPRYSEC effector, GpSPRY-17I9-1, may interact with potato carotenoid cleavage dioxygenase 4 protein (CCD4) in a Y2H screen. Silencing of the gene encoding this host protein significantly increased susceptibility to nematodes while silencing the effector appeared to slightly reduce the nematode's capability to parasitize plants, this was however not significant maybe due to the small sample size.

3.4.1 Roles of the plant hormone ABA and carotenoids in the potato – *G. pallida* interaction

Carotenoids are a group of isoprenoid molecules that are synthesized by all photosynthetic organisms, aphids, some bacteria and fungi. In animals they play fundamental roles in promotion of health and nutrition, sexual behavior, survival and reproduction (Cazzonelli, 2011). However, the majority of animals (including nematodes) are unable to synthesize carotenoids from endogenous precursors and rely on dietary uptake of these compounds. This seems true in case of *G. pallida* as no carotenoids biosynthesis pathway has been found with the whole genome being sequenced (J. Jones. pers. comm.). Carotenoid accumulation (Figure 3.14) is strongly influenced by carotenoids cleavage dioxygenases (CCDs) which catabolize enzymatic degradation of carotenoids leading to production of apocarotenoids that affect a wide range of biological processes (Auldridge *et al.*, 2006). CCD4s have been reported as negative regulators of carotenoids in several studies (Campbell *et al*., 2010; Gonzalez-Jorge *et al*., 2013). In potato, its main substrates are violaxanthin and neoxanthin, which are shared by 9-cis-epoxycarotenoids dioxygenases (NCEDs) that give rise to the biosynthesis of plant hormone ABA (Figure 3.14). ABA plays important roles in plant responses to various environmental stresses. ABA has also emerged as a complex signaling molecule in plant-pathogen interactions with promotion of resistance against some pathogens while increasing susceptibility to others (Asselbergh *et al*., 2008; Ton *et al*., 2009). ABA was reported to reduce reproduction of *M. incognita* on potato roots by lowering egg

masses up to 70% (Karimi *et al.*, 1995) but was shown to play a negative role in rice defence against *H*. *oryzae* (Nahar *et al.*, 2012). It was also reported that ABA-induced genes were up-regulated in syncytia of *H. glycines* resistant near-isogenic lines of soybean containing the *rhg-1* locus (Kandoth *et al*., 2011).

Silencing *CCD4* may divert violaxanthin and neoxanthin to the ABA biosynthesis pathway. We therefore investigated whether increased levels of ABA underpinned the enhanced susceptibility to nematodes of the *CCD4* RNAi plants. However, exogenous application of ABA led to increased resistance against *G. pallida*. In addition, a previous study (Campbell *et al*., 2010) has shown that there was no significant difference in ABA content in the *CCD4* RNAi mutant when compared to wild type cv. Désirée, further arguing against the idea that changes in ABA are involved in making this mutant more susceptible. Noticeably, in spite of the fact that there were remarkably reduced total number of nematodes in ABA treated plants, the reduction seemed to specifically target males while the number of females remained unchanged. Our findings suggest that ABA primarily affects *G. pallida* that were already having problems in establishing the feeding site.

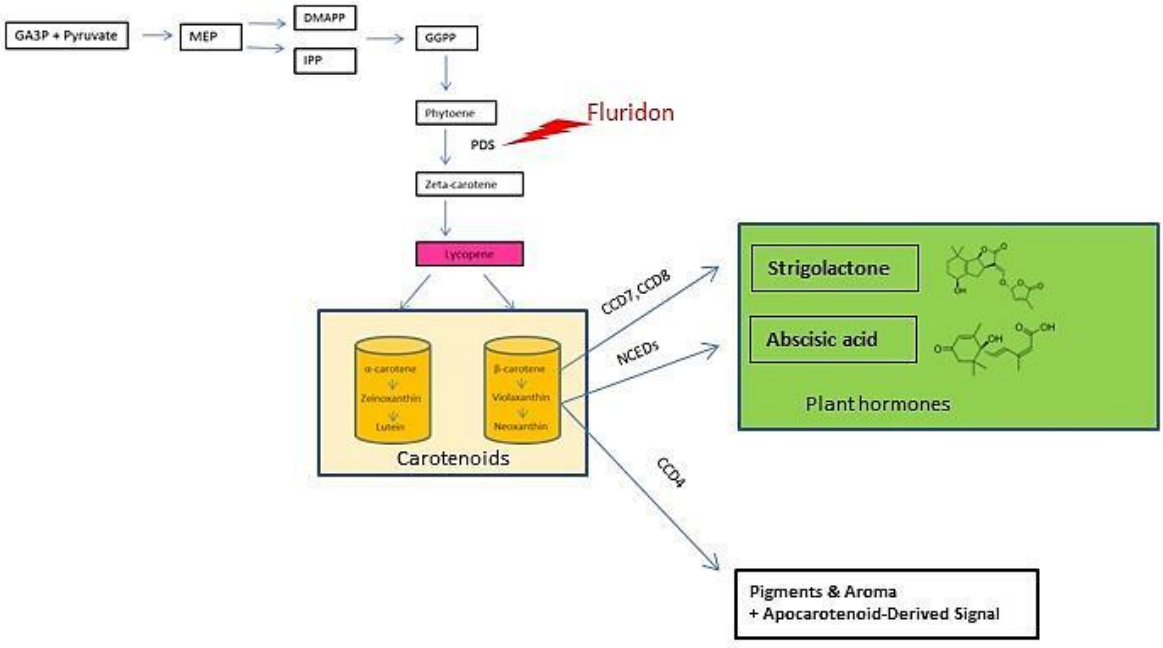


Figure 3.14 A simplified pathway of carotenoids biosynthesis and the various functions of carotenoid cleavage dioxygenases. Modified from (Cazzonelli & Pogson, 2010; Farré *et al*., 2010).

As the basic role of CCDs (including CCD4) is to break down carotenoids, it is possible that the enhanced susceptibility of the *CCD4* RNAi lines is due to elevated level of carotenoids,

although the involvement of downstream signals that are not yet well characterized cannot be totally discounted. Higher levels of carotenoids were measured in the *StCCD4* mutant line (Campbell *et al.*, 2010) and the yellow-fleshed potato *S. phureja* has naturally higher levels of carotenoids due to lower expression levels of *StCCD4*. This potato is more susceptible to PCN than other varieties that do not have a *S. phureja* background. The idea that nematodes benefit from enhanced carotenoid levels was further backed up by an experiment using exogenous application of the carotenoids biosynthesis inhibitor Fluridon. Two independent experiments were carried out that had slightly different outcomes in terms of total number of nematodes colonizing the plants treated with Fluridon compared with control untreated plants (Figure 3.12 and Figure 3.13). Experiment 1 showed exactly the opposite trend of ABA spray assay and this is in agreement with the fact that Fluridon is commonly used as an ABA biosynthesis inhibitor. Experiment 2 showed a much more severe phenotype on shoot development and did not show any significant difference with regard to total nematode colonization. Despite the differences between the replicates, both experiments showed a significant drop in percentage of female nematodes and a dramatic shift of sex ratios towards males indicating the potential importance of carotenoids in nematode growth and development.

3.4.2 Does the interaction between GpSPRY-17I9-1 and CCD4 underpin the effects of carotenoids on plant-nematode interactions?

GpSPRY-17I9-1 is most likely a functional nematode effector that targets CCD4 in his host. Indeed, different truncated CCD4 clones were identified to interact with GpSPRY-17I9-1 in Y2H. However, no interaction with full length StCCD4 was observed, possibly because the full-length protein was not folded properly in the yeast cell and the interaction domain is therefore altered or affected. *In planta*, GpSPRY-17I9-1 localised to the cytoplasm and nucleoplasm no matter where the fluorescent tag was positioned. In contrast, StCCD4 appeared to show different localization patterns depending on where the protein was tagged. This may be due to the fact that StCCD4 is predicted to have an N-terminal chloroplast transit peptide signal (cTP). When tagged on N-terminus, the cTP signal could therefore have been masked preventing the protein from translocating into the chloroplast and hence remaining in the cytoplasm. Conversely, cTP signal was not affected when the protein was tagged C-terminally and therefore the majority of the protein was addressed to chloroplasts. It was also reported that CCD4 in *Arabidopsis thaliana* localised in plastoglobule

which resides inside the chloroplasts (Lundquist *et al*., 2012; Ytterberg *et al*., 2006). Taken together, StCCD4 being localised in chloroplasts seems more genuine in potato cells.

Based on the protein localization study, the results of BiFC assays should be more reliable with the combination having StCCD4 tagged at the C-terminus. Unfortunately, no BiFC signal was detected in this case. This may indicate that there is no direct interaction between GpSPRY-17I9-1 and StCCD4 in the plant cell as they both mainly go to different compartments. Given the fact that silencing either *StCCD4* or the effector had an impact on nematode colonization, there must be other proteins that are involved in CCD4-dependent pathways. In case of StCCD4, it is possible that another as yet unidentified effector protein from *G. pallida* can interact with it. For GpSPRY-17I9-1, it also possibly interacts with another host protein(s) other than CCD4. To identify it, the effector protein could be screened against a different prey library such as one made from infected potato roots.

Since GpSPRY-17I9 and C-terminally tagged StCCD4 showed co-localisation in cytoplasm, even though the signal was very weak as majority of StCCD4 went to chloroplasts, there is still a possibility that these two interact with each other and that the interaction is too weak and transient to be detected using BiFC. If the proteins do indeed interact, our results suggest that GpSPRY-17I9-1 interferes with StCCD4 function, reducing its activity and thus keeping carotenoids at an elevated level in order to fulfill the nematode's dietary requirements. To further test this model, we could generate GpSPRY-17I9-1 overexpression potato lines and check if they have increased level of carotenoids as well as increased nematode susceptibility. In addition, *in vivo* and *in vitro* enzymatic assays have been reported that can be used to functionally characterize the cleavage activity of CCD4 enzyme (Huang *et al*., 2009; Lashbrooke *et al*., 2013). The effects of the GpSPRY-17I9-1 effector on the activity of the CCD4 enzymes could therefore be investigated. However, given the amount of work involved, it is beyond the scope of current study.

The fact that *G. pallida* secrets an effector (GpSPRY-17I9-1) that can target StCCD4, a host protein whose gene is mostly expressed in photosynthetic tissues and in flowers but has a much lower expression in roots (grown in the dark) is interesting (Campbell *et al*., 2010). However, a previous study in our group revealed a consistent strong induction of photosynthesis-related transcripts and transcripts involved in the biogenesis of chloroplasts in giant cells formed in root knot nematode-infected rice roots (Ji *et al*., 2013). In addition, Szakasits *et al*., 2009 also reported differentiation of plastids in the syncytia in *Arabidopsis*

plants infected with *H. schachtii*. It is very likely that these nematodes as biotrophic pathogens manipulate the regular metabolic pathways in their feeding sites to allow better growth and development. Light, if really needed in this process, can possibly come from two sources: ambient light filtering through the soil matrix and supplemental light piped downward and leaking outward from the xylem (Galen *et al*.,, 2007).

At the time of our research, there were also *CCD8* RNAi mutant lines available at the James Hutton Institute (Pasare *et al*., 2013). CCD8, is like CCD4, a carotenoids cleavage dioxygenase that uses carotenoids as substrate. It is involved in the biosynthesis of the important plant hormone strigolactones that were recently discovered to be also associated with plant defence (Cazzonelli, 2011; Torres-Vera *et al*., 2014). Silencing *CCD4* would increase the pool of carotenoids. This increase of carotenoids could possibly lead to the increase of SL even though the biosynthesis of this hormone using CCD8 doesn't require exactly the same substrate as the one metabolized by CCD4. Out of curiosity, we infected two potato *CCD8* RNAi lines with *G. pallida* and infection levels were assessed after three weeks using routine protocols. However, the results were quite variable in different biological replicates (Appendix 7). Therefore, whether or not SLs are involved in the silencing effect of *StCCD4* remains unclear.

Even though the work described here does not confirm that GpSPRY-17I9-1 can directly interact with the potato StCCD4, it indicates the potential importance of GpSPRY-17I9-1 and CCD4 in the compatible interaction between *G. pallida* and potato host plants.

Chapter 4

The SPRYSEC effector candidate GpSPRY-414-2 from *Globodera pallida* suppresses plant defences and interacts with a host CLASP protein

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Manuscript in preparation

MYY performed the gene cloning, Y2H screening, ROS assay, nematode infection assays and RNAi experiment. She was partly involved in the confocal assays and *in vitro* infection assay of *A. thaliana* mutant plants. In addition, she took part in data analysis and wrote the draft of this chapter.

4.1 Introduction

The white potato cyst nematode, *Globodera pallida*, is a sedentary endoparasite that causes yield losses on *Solanaceous* plants worldwide (Pylypenko *et al.*, 2005). It invades host plants in the elongation zone behind the root tip and then migrates through the inner cortex layers to initiate a feeding site near the vascular tissues (Lilley *et al.*, 2005). The specialized feeding site, or syncytium, is a large multinucleate cell formed by the breakdown of plant cell walls and subsequent fusion of adjacent protoplasts (Kyndt *et al.*, 2013). *G. pallida is* an obligate biotrophic pathogen and relies on the syncytium for all nutrients required for its growth and reproduction. The success of colonization by such biotrophs depends on their modulation of plant defences. These can be classified into two different branches, pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and effector triggered immunity (ETI; Dodds & Rathjen, 2010). Perception of PAMPs, such as the bacterial flagellin derivative flg22, provokes a range of downstream responses that include production of reactive oxygen species (ROS) to ward off pathogen attack. Adapted biotrophic pathogens release effectors that suppress PTI. In the second layer of plant defences, the products of resistance (*R*) genes recognise these effectors to trigger ETI. Effectors that are recognised are termed avirulence (*Avr*) genes and this frequently results in a hypersensitive reaction (HR). Like other biotrophs, *G. pallida* has to suppress plant defences in order to survive. It is widely accepted that both suppression of plant defences and successful establishment as well as maintenance of feeding site are mediated by effector proteins produced in the nematode oesophageal cells and secreted into the plant through the stylet (Gheysen & Jones 2006; Haegeman *et al.*, 2012).

A large number of effector proteins have been identified from *G. pallida* from expressed sequence tag (EST) and genome sequencing projects for this nematode (Cotton *et al.,* 2014; Jones *et al.,* 2009). Of particular note is a large family of secreted SP1a and Ryanodine receptor (SPRY) domain (SPRYSEC) proteins produced within the dorsal gland cell of J2s (Jones *et al.,* 2009). One member of this gene family, *RBP-1*, induces HR programmed cell death when co-expressed with the potato *Gpa2* resistance gene in *N. benthamiana* leaves (Sacco *et al.,* 2009). A similar gene family has been described from *G. rostochiensis* (Rehman *et al.,* 2009) and it was found that one *G. rostochiensis* SPRYSEC (SPRYSEC-19) could

physically interact with a CC-NB-LRR type disease resistance protein SW5F (Sacco *et al.,* 2009) and was subsequently shown to suppress ETI (Postma *et al.,* 2012). The size of this predicted gene family from *G. pallida* together with the various subcellular localization patterns of the secreted members suggest that the proteins encoded by *SPRYSECs* may play various roles in the interaction between pathogen and host plant (Jones *et al*., 2009). Characterising the functions of other members of the *SPRYSEC* gene family will be of great help to unravel their roles in plant parasitism.

Here we describe the identification and functional characterization of a new SPRYSEC protein GpSPRY-414-2 from *G. pallida*. We investigate the role of GpSPRY-414-2 in suppressing plant defences. Through yeast two-hybrid screening and *in planta* assays, we show that this effector protein can interact with a cytoplasmic linker protein (CLIP)-associated protein (CLASP) in potato. CLASPs are members of a conserved class of microtubule associated proteins (MAPs) that localise on the plus-end of microtubules which have various functions in cell motility and mitosis. They are needed for microtubule cytoskeleton polarization and contribute to microtubule stability and growth (Al-Bassam *et al*., 2010). Recent studies also indicate that CLASP1 may control some aspects of auxin transport by interacting with retromer component sorting nexin 1 (SNX1; Ambrose *et al*., 2013). A drastic distortion of microtubule organization and manipulation of the host's auxin response and transport have been previously shown to be required for nematode infection (Grunewald *et al*., 2009). Our research suggests that GpSPRY-414-2 may play dual roles in the plant-nematode interaction by suppressing plant defences and facilitating feeding site formation.

4.2 Materials and Methods

4.2.1 *GpSPRY-414-2* and *StCLASP* cloning

Previous work in our group identified a substantial number of *G. pallida SPRYSEC* genes from a large scale expressed sequence tags (ESTs) analysis (Jones *et al.,* 2009 and Chapter 2). One of these effector genes, *GpSPRY-414-2*, was selected for further study due to its abundance in the transcriptome. The full-length coding sequence of this gene (Appendix 1) was PCR amplified without signal peptide from cDNA of J2s, using gene specific primers (Appendix 3) and cloned into the pCR8/GW/TOPO Gateway ENTRY vector (Invitrogen) as described in

Chapter 2. Several fusion clones were made (Appendix 4 and Chapter 2) for functional analysis.

A yeast two-hybrid screen (Chapter 2) identified the potato StCLASP as putative target of GpSPRY-414-2. The full-length coding sequence of this gene was cloned from potato cultivar Désirée cDNA using gene specific primers (Appendix 3) that were designed based on the available genomic gene sequence. The coding sequence of the gene was identified based on an AUGUSTUS gene finding tool prediction (Stanke *et al.,* 2008) and by homology with the orthologous sequence from tomato which was annotated (Solyc09g063030). PCR was performed using the proof reading KOD DNA polymerase (Novagen) and products were resolved on 1% (w/v) agarose gels before purification with the QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer's protocol. The 4290-bp coding sequence was subcloned into pDONR221 (Invitrogen; Kanamycin 50 µg ml^{-1} selection) and then transferred into several expression vectors using Gateway technology according to the protocol provided by the manufacturer (Invitrogen). The resulting binary and yeast prey vectors (Appendix 4) were transformed into *A. tumefaciens* strain GV3101 and yeast Mav203 cells as appropriate, as described in Chapter 2. Protein domain architecture was analysed using SMART (Schultz *et al.,* 1998).

4.2.2 *In situ* hybridisation of the *G. pallida* effector candidate GpSPRY-414-2

The *in-situ* hybridization was done as described in Chapter 2 with primers designed to amplify a 263bp fragment targeting nucleotides 157 to 419 (cDNA sequence in Appendix 1 and primer sequence in Appendix 3). The clone *GpSPRY-414-2* in pCR8/GW/TOPO was used as template for the PCR.

4.2.3 Silencing of *GpSPRY-414-2* in *G. pallida* by RNA interference

A fragment of 263bp from *GpSPRY-414-2* cDNA sequence targeting nucleotides 157 to 419 was selected for silencing. The dsRNA synthesis (primer sequences in Appendix 3), nematodes soaking and inoculation procedures were carried out as described in Chapter 3. Gene silencing in dsRNA treated worms was checked by reverse-transcription polymerase chain reaction (RT-PCR) as described in Chapter 3, using *GpSPRY-414-2* and constitutive *GpEF1α* gene specific primers (Appendix 3).

4.2.4 Yeast two-hybrid analysis

G. pallida SPRYSEC baits cloned in pDEST32 and prey interactors Y2H clone G1-5, identified as *StCLASP*, or full length *StCLASP* coding sequences cloned in pDEST22 were simultaneously co-transformed into the Mav203 strain following the Invitrogen ProQuest™ Two-Hybrid System protocol. The transformants were first plated out on synthetic Leu and Trp dropout media. From each transformation, at least 3 independent clones were then selected that were tested to confirm the interaction based on two reporter gene assays: colonies that grew on triple dropout media (Leu, Trp and His) with 10mM 3-Amino-1, 2, 4-triazole and turned blue in X-gal assay were selected as positive candidates in comparison to the controls provided with the Invitrogen ProQuest™ Two-Hybrid System.

4.2.5 *In planta* localization and bimolecular fluorescence complementation (BiFC) analysis

The subcellular localization of GpSPRY-414-2 and its putative plant target StCLASP (as full length or truncated versions corresponding to the Y2H insert fragments) were investigated using proteins fused to fluorescent tags (eGFP and mRFP; Appendix 4) for confocal analysis. *Agrobacterium*-mediated transient expression in *N. benthamiana* and imaging by confocal microscopy were performed as described in Chapter 2. For co-localisation analysis with sub-cellular markers, bacteria were either infiltrated in leaves of transgenic *N. benthamiana* line CB157 expressing a nuclear histone marker fused to mRFP (mRFP-H2B; Martin *et al.,* 2009) or line CB13 expressing a microtubule marker fused to GFP (the α-tubulin *tua*-GFP; Gillespie *et al*., 2002).

For BiFC analysis, the *YFPc::GpSPRY-414-2* construct in pCL113 and *YFPn::G1-5* or *YFPn::StCLASP* in pCL112 were used (Appendix 4; Split-YFP vectors pCL112 and pCL113 were provided by Sean Chapmanat the James Hutton Institute). Complementary split-YFP constructs in *A. tumefaciens* strain GV3101 were co-infiltrated in *N. benthamiana* leaves at OD_{600nm} 0.02 and 0.1 for the *SPRYSEC* and either of the *StCLASP* clones respectively. At 48hpi, the YFP was imaged with an excitation wavelength (λ) of 514 nm and emission collected at λ530-575 nm on Zeiss LSM 710 confocal.

4.2.6 Flg22-mediated reactive oxygen species (ROS) production suppression assay

The ROS assay was performed as described in Chapter 2, with *N. benthamiana* leaf patches transiently expressing either the free eGFP as a control or *G. pallida* SPRYSEC GpSPRY-414-2 with an eGFP tag at the N-terminus of the fusion (construct in pK7WGF2). Data were analysed by ANOVA or Student's *t*-test using analytics software package STATISTICA (StatSoft).

4.2.7 Cell death suppression assay

The cell death suppression assay was performed as described in Chapter 2, with the same whole set of cell death inducers infiltrated in *N. benthamiana* leaves expressing either free eGFP as a control or *G. pallida* SPRYSEC candidate effectors GpSPRY-414-2 with eGFP tag at the N-terminus of the fusion. The experiment was repeated three times with similar results. Data were analysed by Student's *t*-test using the analytics software package STATISTICA (StatSoft).

4.2.8 Microtubule network disruption using drug treatment

A solution of 100µM colchicine was co-infiltrated with the *Agrobacteria* in leaf tissues to disrupt the microtubule network and the effects of this treatment on BiFC analysis as well as on ROS production and cell death suppression assays were examined. These assays and confocal imaging were performed as described above. To maintain the effects of the drug, a second application of colchicine was carried out at 4dpi during the cell death suppression assay.

4.2.9 *In vitro* infection assay of *A. thaliana clasp-1* mutant with the beet cyst nematode *H. schachtii*

Arabidopsis thaliana clasp-1 mutant seeds were obtained from TAIR (stock number CS67062; Ambrose *et al.*, 2007) and wild type *A. thaliana* Colombia (Col-0) seeds were provided by Aska Goverse (Wageningen University, The Netherlands). Sterilised seeds were germinated on Gamborg's B5 media supplemented with 2% sucrose (Gamborg *et al.,* 1968; Goverse *et al.,* 2000) on 6–well plates. In total, 4 plates of *clasp-1* mutant and 4 plates of wild type plants were grown at 22°C with 16h of light. Four weeks later, about 200 surface-sterilised (Postma *et al.,* 2012) beet cyst nematodes (*H. schachtii*) were drop-inoculated on each plant. The plates were sealed with Parafilm and grown in a growth chamber with 16h light / 8h

dark. Two and four weeks after inoculation, two plates of wild type plant and two plates of *clasp1* mutant were randomly removed from the growth chamber. Plants were washed to remove agar and nematodes that were not established in the roots. Growth parameters such as shoot weight, root length and root fresh weight were measured. Collected individual roots were rinsed briefly with tap water, covered with 1% household bleach for 5min with occasional stirring. Then roots were rinsed and incubated in fresh tap water for at least 10min. The water was then poured off and roots were covered with acid fuchsin work solution (30 times diluted from stock solution containing 0.35g acid fuchsin, 25ml glacial acid and 75ml water). The solution containing roots was brought to the boil for up to 1min in a microwave oven. The acid fuschin was allowed to cool and the samples were then rinsed extensively with tap water. Total numbers of nematodes and females were recorded by counting under a stereo microscope. Mean values were generated from 12 replicates and data were analysed by Student's *t*-test using IBM SPSS statistics software package.

4.3 Results

4.3.1 GpSPRY-414-2 is a putative SPRYSEC effector

Our previous work (Jones *et al.,* 2009; Thorpe *et al.,* 2014; Chapter 2) has allowed the identification of a substantial *SPRYSEC* gene family. The members of this gene family with a signal peptide, without transmembrane domain and highly expressed in J2s were prioritized for further detailed research. One of the highly expressed SPRYSEC genes *GpSPRY-414-2* was cloned from cDNA of J2s. The *GpSPRY-414-2* gene (without signal peptide) encodes a 211-amino acid protein with a predicted molecular mass of 23.3 KDa. It has one SPRY domain from amino acid 65 to 196 according to SMART protein sequence analysis. An *in situ* hybridization assay demonstrated that *GpSPRY-414-2* is expressed specifically in the dorsal pharyngeal gland cell of J2 indicating that it may encode a secreted protein that has potential roles in plant parasitism (Figure 4.1).

RNA interference mediated silencing of the *G. pallida GpSPRY-414-2* gene significantly reduced the total number of nematodes when compared with a dsRNA *GFP* control at three weeks after inoculation. As shown in Figure 4.2A, the average total number of nematodes per gram of root in the dsRNAi *GFP* control sample was around 44 while in *GpSPRY-414-2* dsRNA soaked samples the average number of nematodes was only 26. In addition, a significant (15%) reduction in the percentage of females was observed (Figure 4.2B) in

GpSPRY-414-2 silenced samples. In order to confirm the gene silencing, we carried out an RT-PCR reaction. The results (Figure 4.2C) showed that there was a specific reduction in the transcript levels of *GpSPRY-414-2* in the nematode sample soaked in the dsRNA derived from this gene. After 22 cycles, only *GpEF1α* was amplified. After 26 cycles, the bands for the effector gene started to emerge. After 30 cycles, both *GpEF1α* and effector bands were accumulating in higher amounts. The amplification of the *GpEF1α* gene appeared similar for both *GFP* and *GpSPRY-414-2* soaked samples, however, the latter sample showed lower level of amplification for the effector fragment with a slightly lighter band, indicating successful silencing.

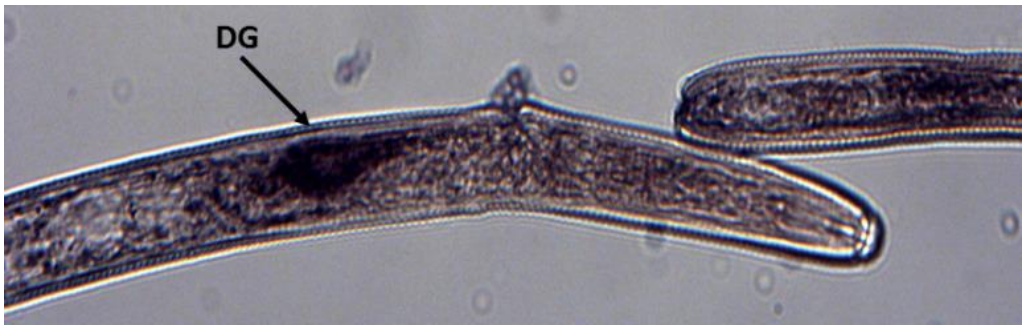


Figure 4.1 Localisation of the *G. pallida* candidate effector *GpSPRY-414-2* expression in the nematode dorsal gland cell (DG) by *in situ* hybridisation to preparasitic second stage juveniles (J2s). Sense control probe showed no binding to nematode structures (data not shown). *G. pallida* J2s are approximately 30 µm in diameter.

4.3.2 GpSPRY-414-2 is able to suppress plant defences

A burst of reactive oxygen species is one of the earliest PTI responses, and can be induced by a common PAMP (flg22). Compared with the negative control (*eGFP*), the expression of *eGFP::GpSPRY-414-2* dramatically suppressed flg22-induced ROS production in leaf discs (Chapter2, Figure 2.4).

The ability of GpSPRY-414-2 to suppress ETI was investigated using a range of resistance and avirulence gene pairs as described in Chapter 2. GpSPRY-414-2 only suppressed ETI mediated by Gpa2 and RBP-1 (Figure 4.3A). The statistical analysis from a large-scale infiltration confirmed that this suppression was statistically significant over a period of time from 7dpi to 9dpi with remarkably reduced percentage of necrotic spots in *eGFP::GpSPRY-414-2* treatment compared to the *eGFP* control (Figure 4.3B).

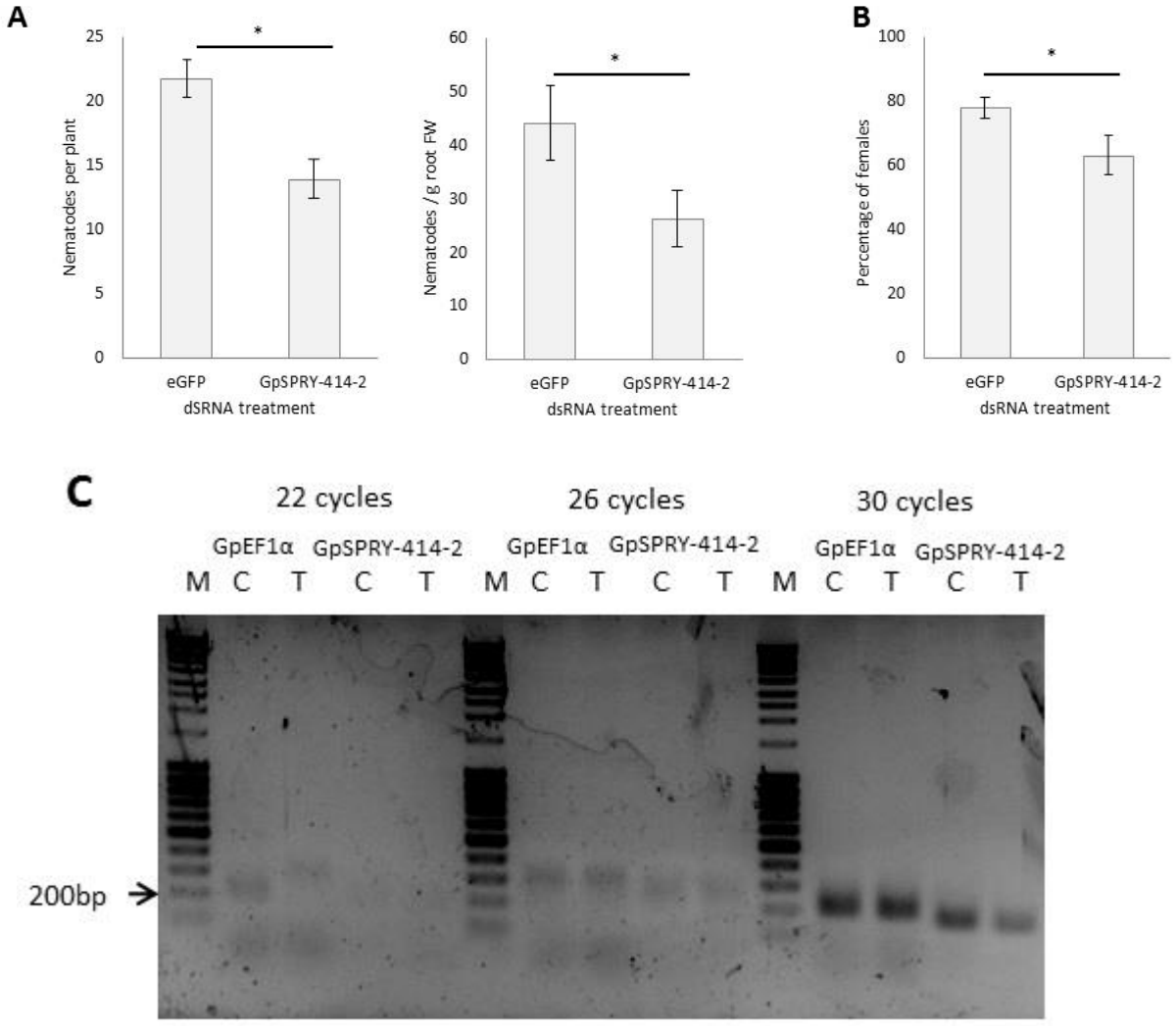


Figure 4.2 Silencing of the nematode effector gene *GpSPRY-414-2* reduces parasitic success and specifically reduces levels of *GpSPRY-414-2* transcripts (A) total number of nematodes per plant or per gram of root and (B) percentage of females are reduced after exposure of preparasitic J2s to dsRNA from *GpSPRY-414-2*. Bars represent mean ± SE. Asterisks indicate a statistically significant difference compared with control sample ($P < 0.05$, student's *t*-test, n=10). (C) Reverse-transcription polymerase chain reaction (RT-PCR) analysis showing that levels of *GpSPRY-414-2* are reduced in nematodes exposed to dsRNA from *GpSPRY-414-2*. Gel shows amplification products from *GpSPRY-414-2* and Elongation factor 1 alpha (*GpEF1α*) in control nematodes soaked in GFP dsRNA (C) and nematodes exposed to *GpSPRY-414-2* dsRNA (T). Reactions were stopped after 22, 26 and 30 cycles. M=1Kb ladder.

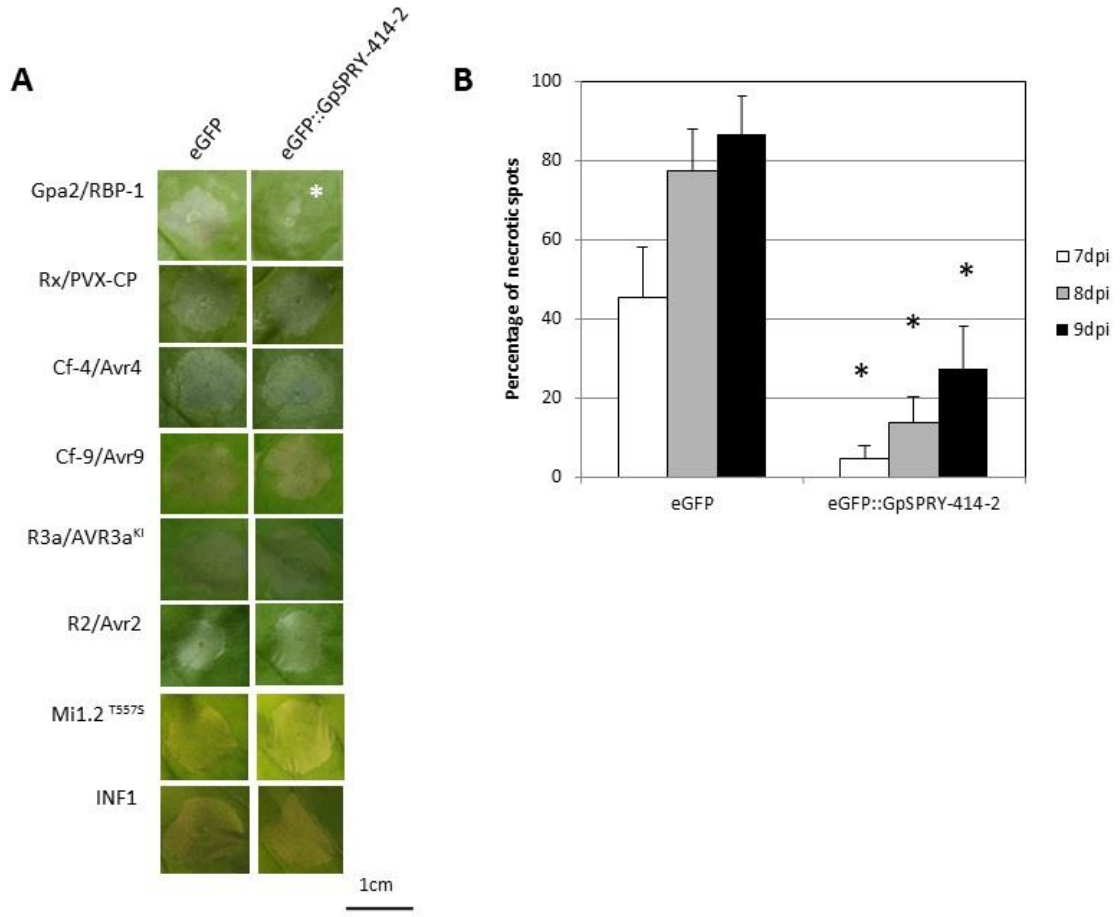


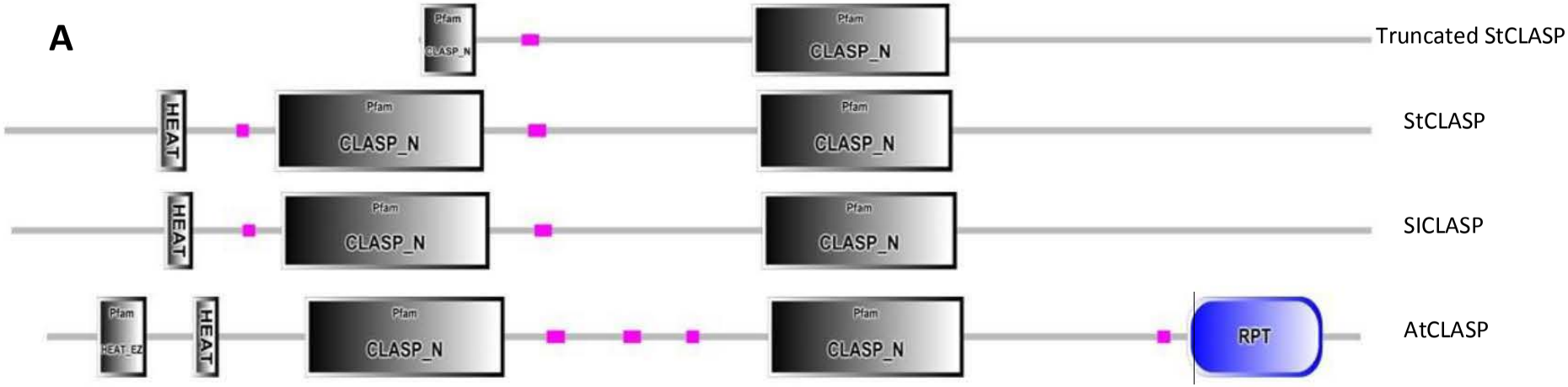
Figure 4.3 GpSPRY-414-2 specifically suppresses plant programmed cell death mediated by Gpa2 and RBP-1. (A) Cell death symptoms induced in *N. benthamiana* by co-expression of *R2/Avr2* (3 days post infiltration (dpi)), *R3a/Avr3a*^{KI} (2 dpi), *Cf-4/Avr4* (4dpi), *Cf-9/Avr9* (4dpi) *Rx/PVX-CP* (3 dpi*), Gpa2/RBP-1* (7 dpi), an autoactive form of Mi-1.2 (Mi-1.2^{T557S}; 3 dpi), or the *P. infestans* PAMP elicitor *INF1* (3 dpi) in leaves expressing either the free enhanced green fluorescent protein (eGFP) as a control or GpSPRY-414-2 with an eGFP tag at the N-terminus of the fusion. The asterisk indicates the combination where the symptoms are significantly suppressed by the effector compared to eGFP. (B) Percentage of infiltration sites developing a clear hypersensitive response (HR) from 7dpi to 9dpi mediated by Gpa2/RBP-1 in *N. benthamiana* leaves expressing eGFP or GpSPRY-414-2 with an eGFP tag at the N-terminus. Experiments were repeated three times with blocks of 12 plants infiltrated on two leaves each; error bars indicate ± SE. Asterisks above the error bars indicate a significant difference (student's *t*-Test at $P<0.05$, n=10) from the free eGFP control evaluated at the same time point.

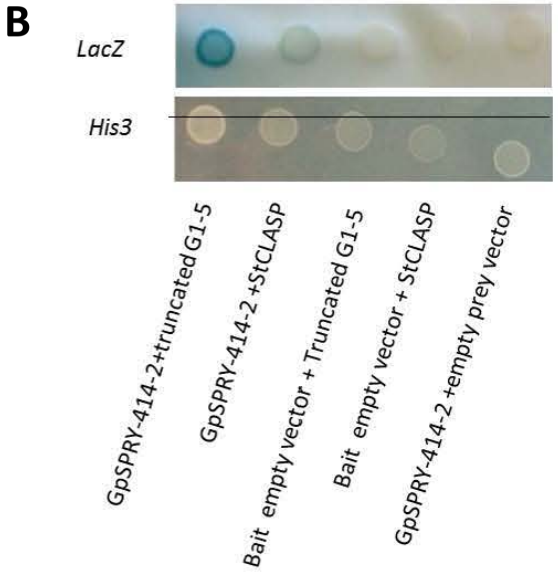
4.3.3 GpSPRY-414-2 interacts with a CLASP protein in yeast and *in planta*

A yeast two-hybrid screen was performed against a potato cDNA library to identify potential host interactors of GpSPRY-414-2. The primary screening (Chapter 2, table 2.2) showed that GpSPRY-414-2 could interact with a hypothetical protein SORBIDRAFT_06g027210, an ethylene-responsive factor 1 and a putative CLASP protein (clone G1-5). However, after one-to-one transformation, the *G. pallida* effector only interacted with the putative potato CLASP protein. The full length CLASP protein was therefore cloned from potato.

A BLASTn search with clone G1-5 sequence from the Y2H screen against the potato genome identified two scaffolds, PGSC0003DMB000000504 and PGSC0003DMB000000115, with the latter showing 99% sequence identity with the yeast clone (compared to 83% identity with the other matched sequence). However, gene loci were not yet annotated at these locations at the time of our research. Therefore, we used the closest annotated organism to potato, tomato, to help us predict the gene structure of our candidates. A tBLASTn search with the same sequence in the tomato genome identified two gene loci with 87% (Solyc09g063030) and 71% (Solyc06g008040) amino acid identity respectively. The sequences of the tomato gene locus Solyc09g063030 and the potato candidate gene were reciprocal best blast hits.

The potato *StCLASP* full length gene coding sequence has 97% and 72% identity with tomato and *A. thaliana CLASP* sequences respectively and has similar protein domain compositions (Appendix 8 and Figure 4.4). The coding region of the potato gene is 4290 bp long that translates into a 1429 amino acid protein containing two CLASP-N domains. The CLASP_N region is found at the N terminal end of CLIP-associated proteins (CLASPs), which are widely conserved microtubule plus-end tracking proteins that regulate the stability of dynamic microtubules. A sequence alignment indicates that truncated G1-5 covers more than 2/3 of the full length StCLASP from the C-terminus, including the second CLASP-N domain and a small part of the first CLASP-N domain (Figure 4.4A). The interaction between these two proteins and the SPRYSEC bait GpSPRY-414-2 activated two reporter genes of the Y2H system used in our study, even though the interaction with the full length interactor was weaker than the truncated clone (Figure 4.4B). Neither full length StCLASP nor truncated G1-5 could interact with three other SPRYSECs tested: GpSPRY-24D4, GpSPRY-12N3 and GpSPRY-17I9-1 (Figure 4.4C), with none of the combinations activating the *His3* or *LacZ* reporter genes.


A
Truncated StCLASP
StCLASP
SICLASP
AtCLASP
Pfam
CLASP_N
HEAT
HEAT_EZ
RPT


B
LacZ
His3
GpSPRY-414-2+truncated G1-5
GpSPRY-414-2 +StCLASP
Bait empty vector + Truncated G1-5
Bait empty vector + StCLASP
GpSPRY-414-2 +empty prey vector

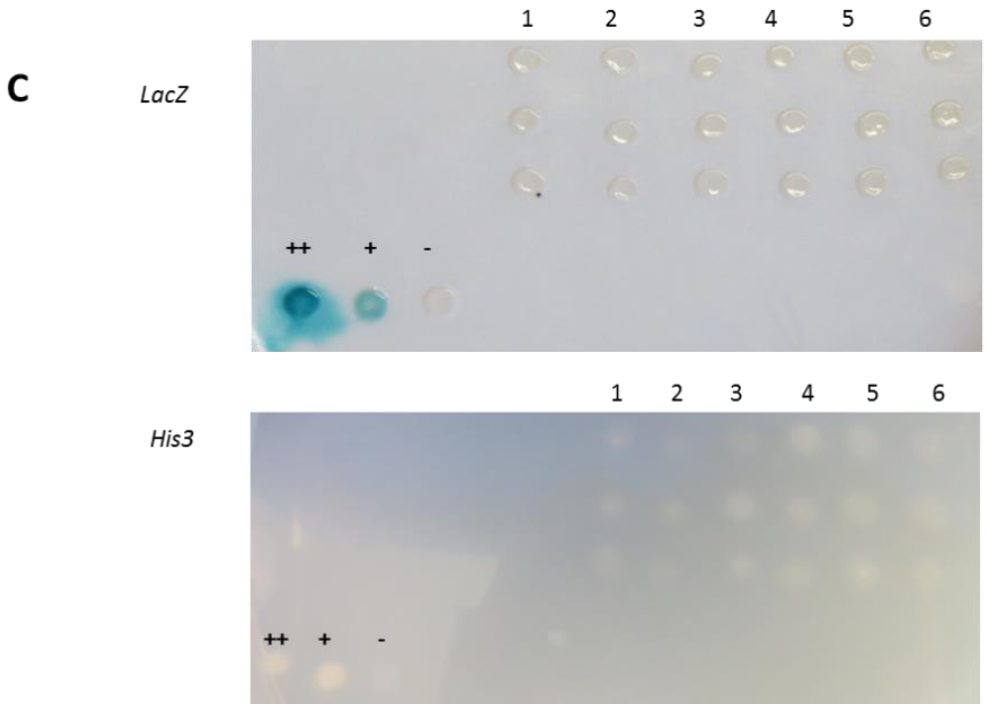


Figure 4.4 Interactions between GpSPRY-414-2 and CLASP (partial and full length) from potato (A) Schematic diagram showing the domain architecture of the potato truncated (partial protein clone G1-5) and full length CLASP from potato (St), *Arabidopsis* (At) and tomato(Sl) containing heat repeats and CLASP-N domain(s). Pink small blocks represent low complexity regions and lines indicate unknown regions according to SMART analysis. (B) GpSPRY-414-2 interacts with full length and truncated CLASP protein G1-5 with both *His3* and *LacZ* reporter genes activated in a Y2H screen. The empty vectors pDEST32 and pDEST22 were used as negative control. C) Full length StCLASP and truncated G1-5 both do not interact with other SPRYSECs. The bait and prey constructs in yeast transformants 1-6 are *GpSPRY-24D4+StCLASP* (1), *GpSPRY-24D4*+truncated *G1-5* (2), *GpSPRY-17I9-1*+ *StCLASP* (3), *GpSPRY-17I9-1*+ truncated *G1-5* (4), *GpSPRY-12N3*+*StCLASP* (5), *GpSPRY-12N3*+truncated *G1-5* (6), each with three replicates shown in three rows. ++ is the control yeast from the Invitrogen ProQuest™ Two-HybridSystem for strong interaction, + for weak interaction and – for no interaction. No activation of either reporter is observed with any combination. Each reporter assay was repeated at least twice with similar results.

To further investigate whether this interaction occurred within plant cells, we made fluorescent fusions for *GpSPRY-414-2* and its putative interactor *StCLASP* (truncated and full length clones) and examined their localization patterns using a transient expression assay in *N. benthamiana*. The effector GpSPRY-414-2 is localized in cytoplasm and nucleoplasm no matter which orientation it was tagged in (Figure 4.5A and B) or which fluorescent protein it was tagged with (data not shown). Co-expression of *mRFP::G1-5* with a microtubule marker indicates that the truncated StCLASP interactor G1-5 is also cytoplasmic but that it specifically labels microtubule strings (Figure 4.5C). The full length StCLASP showed the same localization pattern regardless of the position of the tag (Figure 4.5D and E). Further co-localisation and bimolecular fluorescence complementation assays showed that GpSPRY-414-2 and StCLASP were co-localized on microtubules (Figure 4.6A) and most probably interact with each other (Figure 4.6B). In addition, the same pattern was observed as in yeast in that the interaction with the truncated host protein G1-5 gave a stronger signal than the full length StCLASP.

The interaction between GpSPRY-414-2 and StCLASP (either full length or truncated) was not affected by the microtubule disturbing reagent colchicine. As shown in Figure 4.7, in absence of colchicine, GpSPRY-414-2 interacts with StCLASPs on microtubule strings. After colchicine treatment, the YFP signal was still positive but looked like fragmented. This was true for the interaction with either the full length StCLASP (Figure 4.7C and D) or its truncated version (Figure 4.7A and B).

4.3.3 The hypersensitive response mediated by Gpa2 and RBP-1 as well as its suppression by GpSPRY-414-2 do not require a functional microtubule network

In order to investigate whether CLASP could be responsible for the plant defence suppression that was observed, the microtubule disturbing agent colchicine was co-infiltrated with the *Agrobacteria* during an ETI assay. The results showed that colchicine did not affect the signalling triggered by Gpa2 and RBP-1 as the percentages of necrotic spots for both treated and untreated eGFP samples were not significantly different (Figure 4.8). Moreover, in colchicine treated samples, GpSPRY-414-2 was still significantly suppressing cell death induced by Gpa2 and RBP-1. In conclusion, a functional microtubule network is not required by both the hypersensitive response provoked by Gpa2 and RBP-1 and the suppression of this defence response by GpSPRY-414-2.

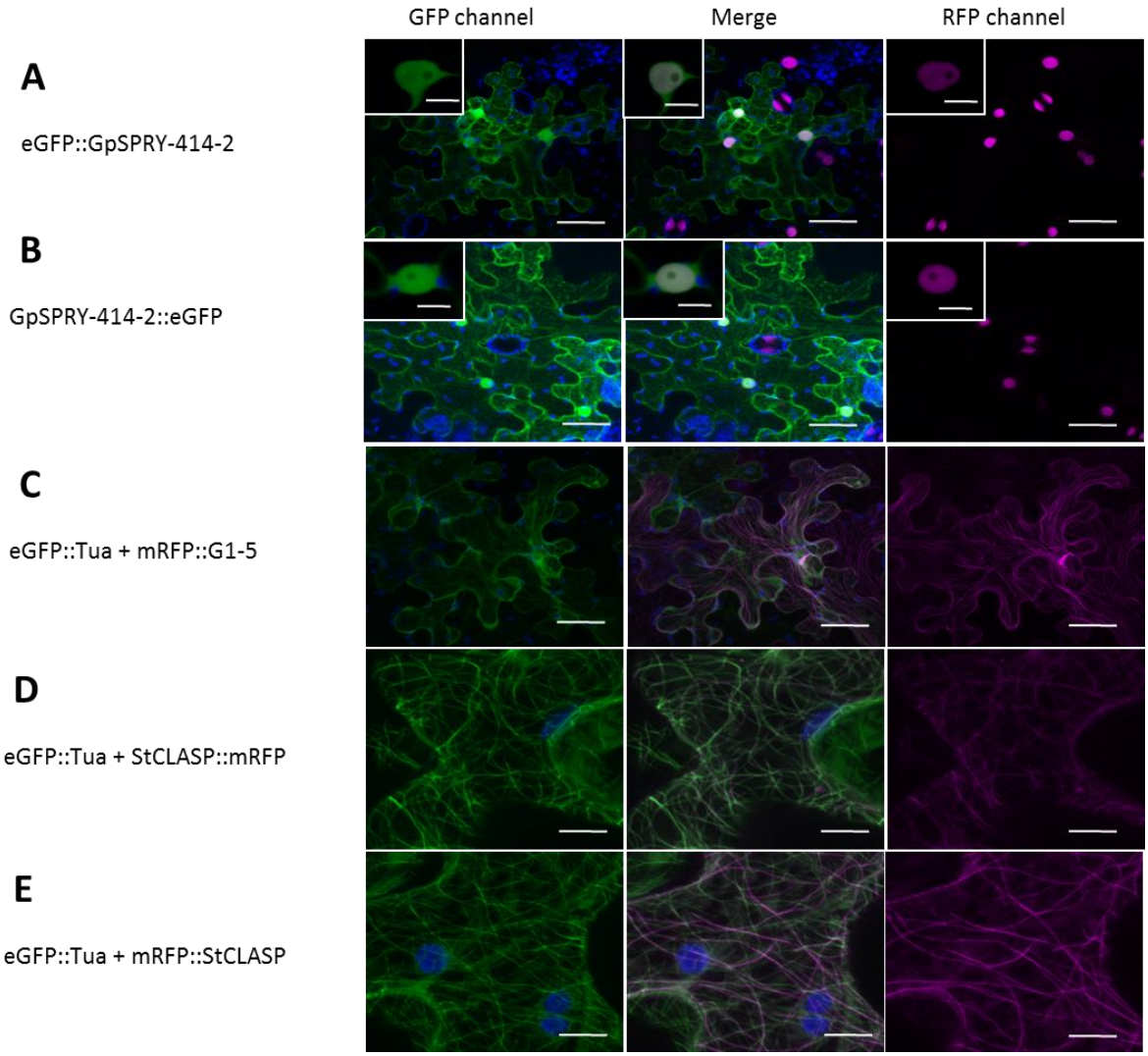


Figure 4.5 *In planta* localization of GpSPRY-414-2 and its putative plant target StCLASP. (A & B) GpSPRY-414-2 fused to eGFP at the N- (A) or C-terminus (B) and expressed in leaves of transgenic *N. benthamiana* strain CB157 containing a Histone H2B mRFP marker in the nuclei. (C-D-E) Truncated StCLASP corresponding to the yeast two-hybrid clone fragment G1-5 fused to mRFP at the N-terminus (C) or full length StCLASP (D-E) with eGFP tagged at the N (D) or C-terminus (E) and expressed in leaves of transgenic CB13 *N. benthamiana* (containing the α-tubulin marker *tua*-GFP). Pictures were taken 2 days post infiltration with the relevant *Agrobacterium* constructs. GFP signal displayed green, mRFP signal displayed magenta, silver color shows areas where green and red signals are overlaid. Autofluoresence from chloroplasts is displayed as blue. Each experiment was done at least twice with three replicates. Scale bars in A-C represent 50µm except those in the nuclei which represent 10µm instead. Scale bars in D and E represent 10µm.

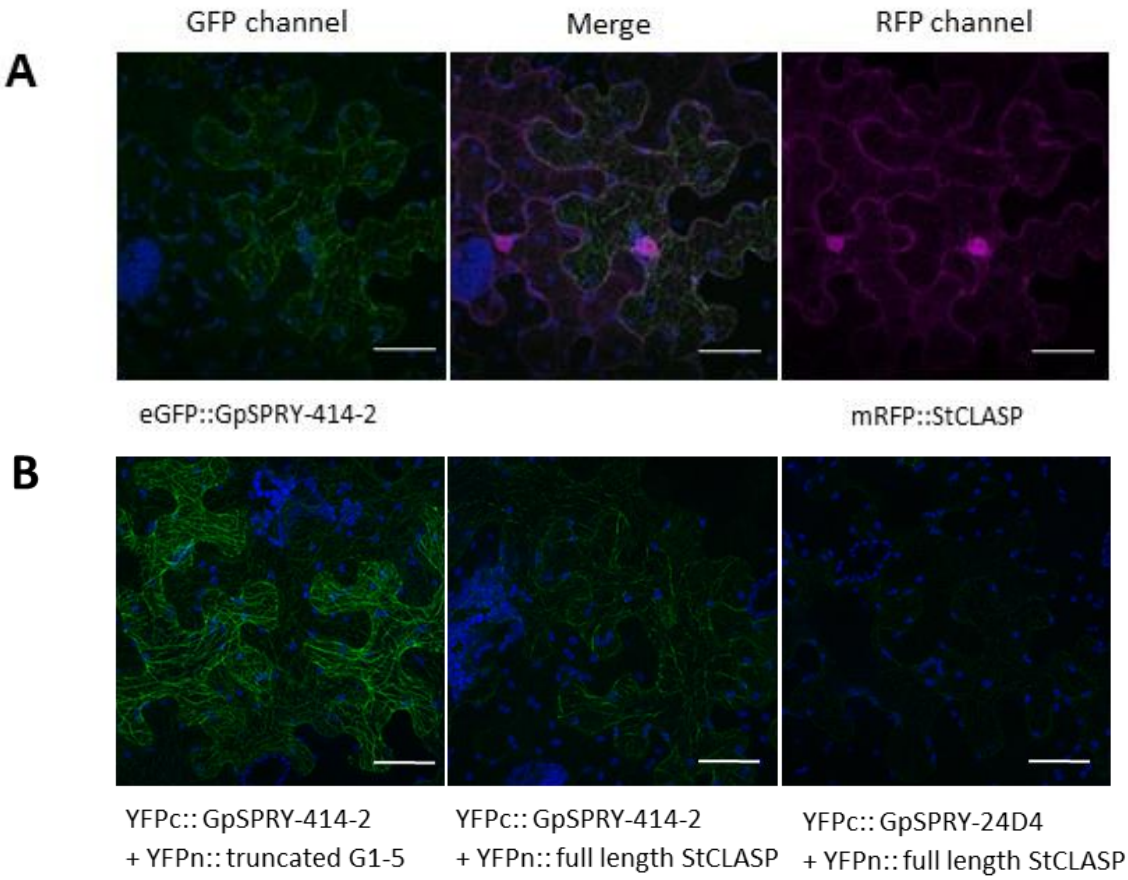


Figure 4.6 Co-localisation and interaction between GpSPRY-414-2 and StCLASP. A) GpSPRY-414-2 and StCLASP are co-localised on microtubules in agroinfiltrated *N. benthamiana* epidermal leaf cells. GFP signal displayed green, mRFP signal displayed magenta, silver color shows areas where green and red signals are overlaid. Autofluoresence from chloroplasts is displayed as blue. B) Both truncated and full length StCLASP interact with GpSPRY-414-2 but generate different signal intensity. No interaction between another SPRYSEC (GpSPRY-24D4) and G1-5 is seen under the same conditions. Reconstituted YFP is displayed green, autofluorescence from chloroplasts is displayed in blue. Each experiment was done at least twice with three replicates. Scale bars represent 50µm.

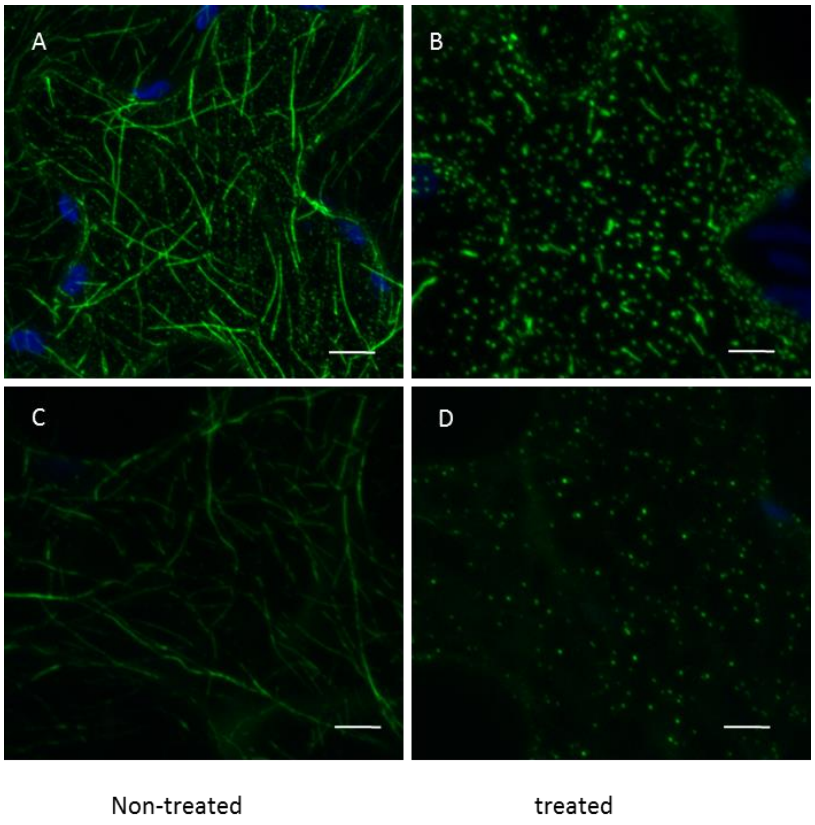


Figure 4.7 Effects of colchicine treatment on the interaction between GpSRPY-414-2 and StCLASP. Interaction between GpSPRY-414-2 and truncated StCLASP (A & B) and between GpSPRY-414-2 and full length StCLASP (C & D) is not affected by colchicine treatment. Reconstituted YFP is displayed in green. Each experiment was done at least twice with three replicates. Scale bars represent 10 µm.

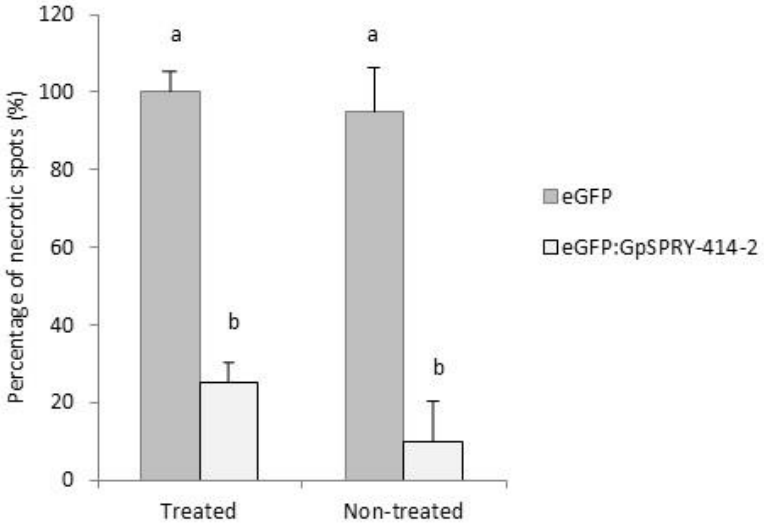


Figure 4.8 Disruption of microtubules using colchicine does not affect GpSPRY-414-2 suppression of cell death mediated by Gpa2 and RBP-1. HR is not affected by eGFP, but suppressed in the presence of GpSPRY-414-2, both with and without colchicine treatment. Scores were taken at 7dpi. Experiments were repeated three times, each with no less than 10 plants, and error bars are indicated ±SE. Asterisks indicate statistically significant differences compared with eGFP in either treated or non-treated plants respectively (*P < 0.05, Mann-Whitey U test, n=10).

4.3.4 The ROS production induced by flg22 may be partly dependent on the integrity of the microtubule network

Since StCLASP is involved in microtubule organization in plant cells, we tested whether disrupting the microtubule network could affect the suppression of flg22-induced ROS production mediated by GpSPRY-414-2. The microtubule-depolymerizing drug colchicine was infiltrated in *N. benthamiana* leaf tissues together with the *Agrobacteria* mediating transient expression of the eGFP::effector fusion or free eGFP. Plants infiltrated with the bacteria only were used as control. As seen in Figure 4.9A, there was a reduction of ROS production in leaves expressing GpSPRY-414-2 under control treatment, which is in agreement with the results in Chapter 2. However, ROS production was similar in both GpSPRY-414-2 and eGFP expressing leaves treated with colchicine. Compared with ROS production in *N. benthamiana* leaves expressing eGFP without colchicine treatment, all three other samples generated less ROS. Running all treatments and constructs on one plate allowed us to directly compare them, however, unfortunately probably due to a lack of replicates, none of the observed differences mentioned above were statistically significant.

We then performed similar experiments with only one gene and leaves treated or not with colchicine in order to have sufficient replicates for each combination. As shown in Figure 4.9 B, colchicine treatment significantly reduced the production of ROS in *N. benthamiana* leaves expressing eGFP compared with the control treatment. By contrast, ROS production in leaves expressing GpSPRY-414-2 showed no significant difference between colchicine treated and non-treated samples (Figure 4.9 C).

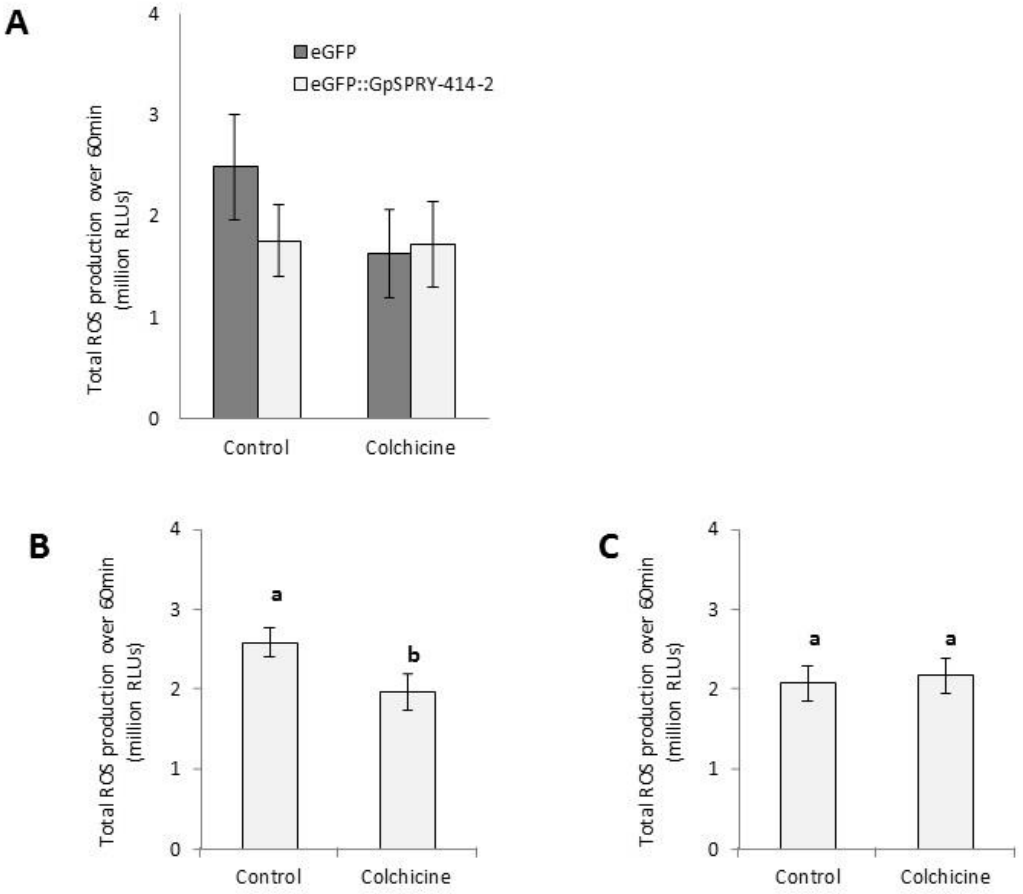
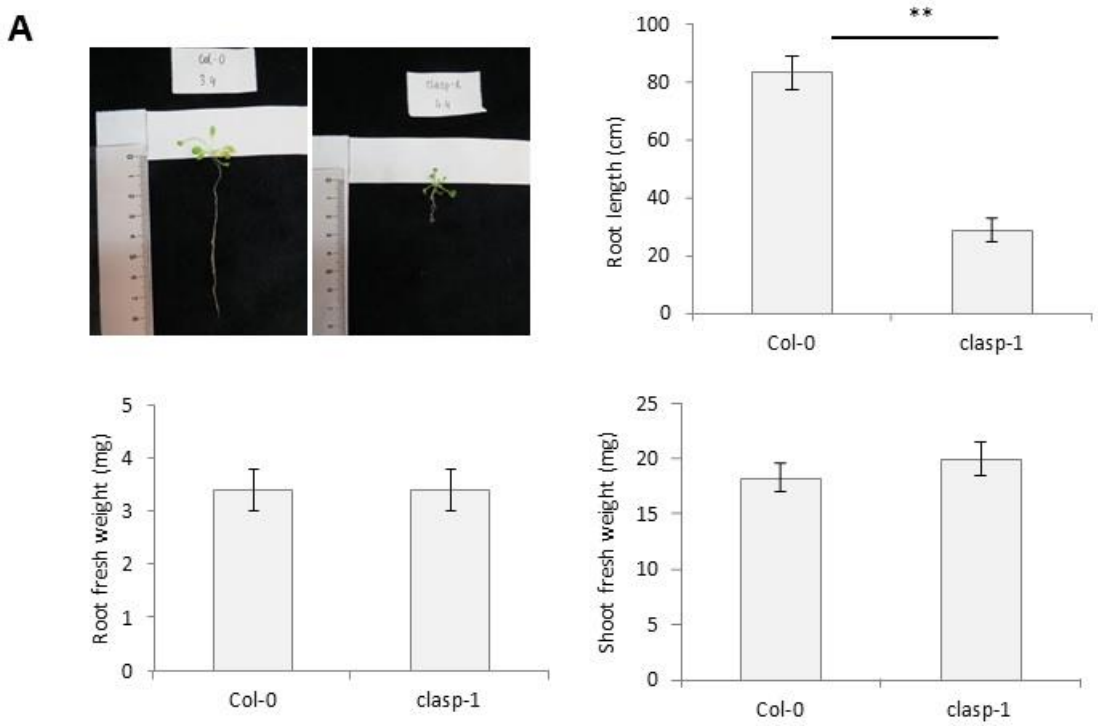


Figure 4.9 Effect of colchicine treatment on ROS production elicited by flg22 in *N. benthamiana* leaves. Reactive Oxygen Species (ROS) production induced by flg22 in *N. benthamiana* leaves expressing eGFP (black bar) or eGFP::GpSPRY-414-2 (white bar) treated or not with 100µM colchicine. ROS levels are expressed as total Relative Light Units (RLUs) over 60 minutes following elicitation with flg22. To allow direct comparisons, ROS experiment in panel (A) was performed in the same plate at the same time for all samples (n=8), while similar experiments presented in panels (B) for eGFP and (C) for eGFP::GpSPRY-414-2 were performed independently (n=24). Values are mean ± SE; two-way ANOVA analysis indicated no significant difference between treatments in panel (A); means with different letters denote a significant difference (student's *t*-test at $P< 0.05$) in panels (B) or (C).

4.3.5 A. thaliana clasp-1 mutant is susceptible to beet cyst nematode *H. schachtii*

In order to further explore the function of the CLASP protein in nematode parasitism, we switched to the pathosystem of beet cyst nematode *H. schachtii* and *A. thaliana*, as a mutant in the *AtCLASP* homolog to *StCLASP* was available (*clasp-1*; Ambrose *et al.*, 2007). Nematode colonization and plant growth parameters were examined at 14 and 28 days after infection. Compared to the wild type control Colombia, the *Arabidopsis clasp* mutant had similar root and shoot fresh weight but a dramatically shorter root system (Figure 4.10 A) as expected. In terms of nematodes, there were significantly more nematodes per mg of root in *clasp-1* than in wild type 14 days after infection. Even though the majority of nematodes in both samples were still J2s at this time point, the average percentage of J2s in *clasp-1* plants was lower than in wild type (Figure 4.10 B). After 28 days, there was a higher total number of nematodes observed in the mutant line. However, the mean percentage of well-developed females in the *Arabidopsis clasp-1* mutants was far smaller than in the wild type control plants (Figure 4.11).



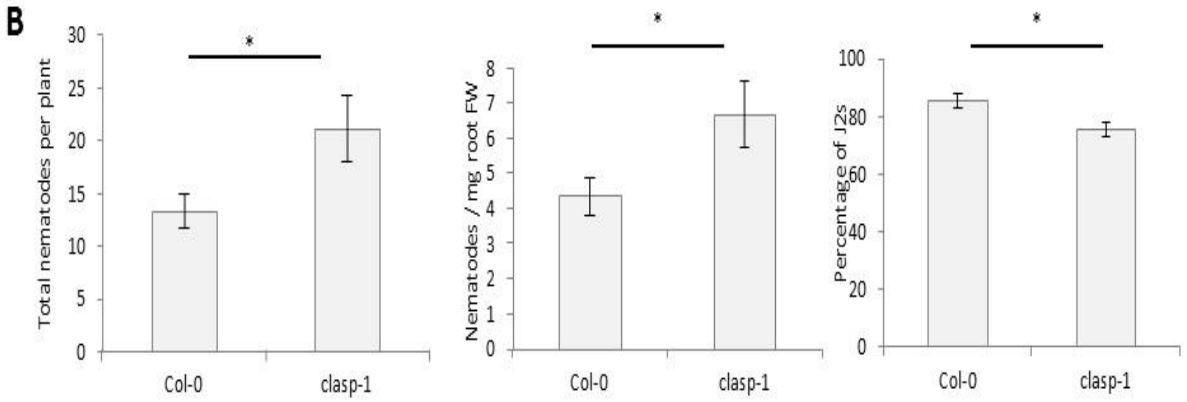


Figure 4.10 The *clasp-1* mutant plants of *A. thaliana* show reduced root length and are more susceptible to *H. schachtii.* A) Phenotypes of *Arabidopsis* wild type Colombia and *clasp-1* mutant when harvested at 14 days post inoculation. Fresh weights of roots and shoots are not affected in the *clasp-1* mutant but root length is significantly shorter in the *clasp-1* mutant. B) The *clasp-1* mutant of *Arabidopsis* is more susceptible to beet cyst nematodes at 14dpi. Bars represent mean±SE of 12 replicates. One asterisk indicates significance P < 0.05 while two asterisks implies significance P <0.01 in student's *t*-test.

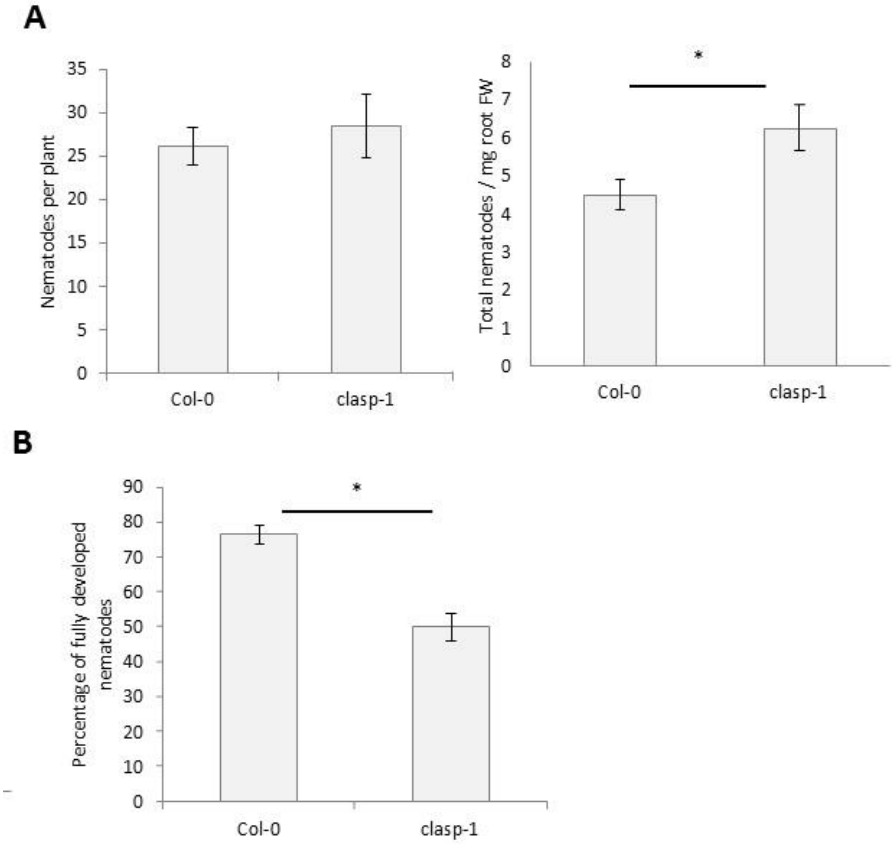


Figure 4.11 Development of nematodes in *A. thaliana clasp-1* mutant at 28 dpi. The *clasp-1* mutant plants contain a significantly higher number of total nematodes per mg of root fresh weight (A) but a lower percentage of fully developed females (B) at 28dpi. Bars represent mean + SE. Asterisks indicate that the difference between groups is significant (p < 0.05, student's *t*-test, n=12).

4.4 Discussion

Globodera pallida delivers effectors into host tissues to sustain its biotrophic life style. The SPRYSECs are a substantial gene family from this nematode (Cotton *et al*., 2014; Jones *et al*., 2009; Rehman *et al*., 2009). There is accumulating evidence that shows their importance in plant – cyst nematode interactions (Mei *et al*., 2015; Postma *et al*., 2012; Sacco *et al*., 2009). In this study, we focused on one new member, GpSPRY-414-2, and explored its detailed functions.

4.4.1 GpSPRY-414-2 is an effector protein contributing to successful parasitism of *G. pallida*

The gene encoding the GpSPRY-414-2 protein is expressed specifically in the dorsal gland cell of J2 nematodes and this SPRYSEC can suppress plant defences. Our data indicate that this protein can not only suppress PTI by reducing flg22-induced ROS production but also specifically suppresses ETI which is triggered by the potato *R* gene *Gpa2* and its cognate nematode avirulence factor *RBP-1*. Thirdly, to further directly test the function of GpSPRY-414-2 in nematode colonization, we carried out an RNAi assay to silence the expression of this effector. The nematode infection assay using the *G. pallida* nematodes soaked in dsRNA showed a significant decrease in nematodes' capability to colonise host plants with a reduced total number of nematodes and percentage of females compared to control treatment. Taken together, these data suggest that GpSPRY-414-2 is involved in parasitism.

4.4.2 CLASP protein is important in the plant – cyst nematode interaction

Microtubules are one of the three types of cytoskeleton elements in cells along with actin filaments and intermediate filaments. Microtubules are composed of alpha and beta tubulin dimers and play fundamental roles in a range of biological processes such as mitosis, cell migration, maintenance of cell shape and movement of cellular structures (Akhmanova & Steinmetz, 2008; Galjart, 2005). The microtubule is a polar tube with a slow-growing minus end and a fast-growing plus end and this leads to its most prevalent behaviour called dynamic instability, a process where it grows and shrinks at a rapid but constant rate through polymerization and depolymerization of tubulins (Horio & Murata, 2014; Howard & Hyman, 2003). Regulation of the dynamic behaviour of microtubules requires the

cooperation of microtubule-associated proteins (MAPs). Classic MAPs bind along the length of microtubules while others associate specifically with tubulin-subunit components. CLIPs (for cytoplasmic linker proteins) and CLASPs (for CLIP-associated proteins) target the plus end of microtubules and thus are called +TIPs, for 'plus-end tracking proteins' (Galjart, 2005). In plants, there are three +TIP families that have been studied: EB1 (end binding protein 1), TOG domain (tuber overexpression gene) proteins and plant specific kinesins (Young & Bisgrove, 2011). Through yeast two-hybrid screening, we found that the *G. pallida* GpSPRY-414-2 effector could interact with a potato TOG domain family protein, StCLASP. Transient expression of the full length potato *StCLASP* gene in *N. benthamiana* did not reveal a plus-end localization of the protein but showed labelling of the total microtubule. This may be due to the high expression levels that are generated in the experimental system used here and is in agreement with the report of Ambrose *et al.* (2007), who showed that plus end tracking could only be observed at low transgene expression levels; this is also consistent with reports from animal CLASPs and other +TIPs.

CLASPs are conserved in animals, yeast, fungi and plants (Gardiner, 2013) and are important in maintaining the stability of microtubules (Mimori-Kiyosue *et al.,* 2005). In *A. thaliana*, the *clasp-1* mutant was reported to have reduced cell expansion, decreased microtubule polymerisation and increased sensitivity to oryzalin (Ambrose *et al.,* 2007). In our current study, we showed that this mutant is far more susceptible to beet cyst nematode infection compared with wild type. Interestingly, at an early time point, absence of AtCLASP seemed to allow accelerated nematode growth while at the later stage it appeared to hamper the development of nematodes. The increased susceptibility at the early stage of nematode infection may be attributed to the reduced presence of microtubule bundles that may have facilitated initiation of the feeding sites. As nematodes preferably infect roots behind the root tip, the susceptibility could be also due to the increased formation of lateral roots in the *clasp* mutant (Kirik *et al.,* 2007) that may have provided more nematode infection sites. However, the *clasp-1* mutant was also reported to have a shorter elongation zone and fewer cells in that region (Kirik *et al.,* 2007). Since this is an area where the nematodes establish their feeding sites and obtain nutrients, these limitations could have restricted syncytium development and consequently reduce the percentage of females observed in the mutant line.

In addition, *A. thaliana clasp-1* mutant displayed a range of auxin-related defects such as abundant lateral roots, reduced apical dorminance as well as a reduction in root apical meristem size (Ambrose *et al*., 2007; Kirik *et al*., 2007). It was recently reported that CLASP promotes endocytic recycling of PIN2 and restricts its degradation via directly interacting with the retromer component sorting nexin 1 (SNX1) which was involved in maintaining PIN levels (Ambrose *et al*., 2013). Interestingly, it seems that plant parasitic nematodes have evolved to manipulate polarity shifts of PIN proteins to facilitate their establishment in the host plant. An enhanced auxin response was seen at the infection sites of both cyst and root-knot nematodes while auxin signaling mutants were shown to have significantly lower nematode infection (Goverse *et al*., 2000; Grunewald *et al*., 2009). It is therefore possible that the abberant auxin distribution in *clasp-1* mutants of the current study has influenced nematode infection.

Taken together, the poor development of nematodes in the *clasp-1* mutant at the later stage is perhaps not surprising. These findings imply that the *clasp* gene plays dual roles during the process of nematode infection. This is similar to a previous report of another microtubule associated protein, MAP65-3, which was expressed in the initial phase of giant cell formation but whose expression rapidly decreased before the development of fully mature giant cells (Caillaud *et al.,* 2008).

4.4.3 Does GpSPRY-414-2 effector function through CLASP?

Plant microtubules go through a range of reorganizations when plants are exposed to pathogens. As reviewed by Hardham (2013), pathogenic bacteria, fungi and oomycetes can induce a range of alterations in microtubule arrays and dynamics; viruses take advantages of microtubules to facilitate their movement and transmission; cyst nematodes and root knot nematodes manipulate microtubules as part of the process of enhancing mitosis and partial cytokinesis during the development of their feeding sites. In many cases, the depolymerization of plant cortical microtubule arrays is induced by pathogens. In this study we used a combination of yeast two-hybrid and *in planta* BiFC assays to show a specific physical interaction of the nematode effector with a CLIP-associated protein from potato, the StCLASP. Further investigations were carried out in order to determine whether CLASP is involved in the suppression of plant defences by GpSPRY-414-2.

Application of the microtubule disturbing reagent colchicine did not affect the cell death triggered by Gpa2/RBP-1 nor its suppression by the nematode effector. However, given the fact that colchicine did not affect the interaction between GpSPRY-414-2 and StCLASP, it is hard to conclude whether or not CLASP is involved in this process. Silencing the *clasp* gene in *N. benthamiana* by virus induced gene silencing (VIGS) prior to colchicine treatment might help to answer this question in the future.

The results of colchicine treatment on ROS suppression assays are intriguing. Our data showed that after treatment with colchicine, no significant difference of ROS production was seen in leaves expressing GpSPRY-414-2 compared to leaves expressing eGFP but in both cases ROS production was reduced compared to the production in *N. benthamiana* leaves expressing eGFP without colchicine treatment. In addition, GpSPRY-414-2 alone significantly suppressed flg-22-induced ROS production. Although the interaction between GpSPRY-414-2 and StCLASP was not affected by the colchicine treatment (see previous section of this chapter), the possibility remains that the nematode effector alters the function of CLASP and has an effect on the dynamics of the microtubule network which may be important for ROS signalling (Khairallah *et al.,* 2012). When adding colchicine to leaves expressing the effector, the microtubule network was already disturbed, so the effect of colchicine readily cannot be additive. Therefore, this could explain why no significant difference was observed. In conclusion, we speculate that GpSPRY-414-2 may suppress flg22-mediated ROS production by manipulating the microtubule network through CLASP.

Taken together, our data imply that the *G. pallida* effector GpSPRY-414-2 plays dual roles in the interaction with the host plant. It seems to be involved in both plant defence suppression and nematode feeding site establishment. The putative interactor protein CLASP appears to be engaged in the latter function while it may or may not be responsible for the former one. The interaction network looks far more complicated than expected and further investigations are needed to clarify the links between all the discoveries so far.

Chapter 5

General conclusions and perspectives

5.1 SPRYSECs form a small subset of effectors from a huge gene family with diverse functions in *G. pallida*

The related SPRY and B30.2 domains have been known for some time (Ponting *et al.*, 1997; Vernet *et al.*, 1993), but they only recently came to the attention of nematologists (Rehman *et al.*, 2009). An expression profiling approach led to the identification of an effector from *G. rostochiensis* containing a SPRY/B30.2 domain with similarity to human RAN-binding proteins involved in nuclear transport. The subsequent discovery of related effector sequences led to the novel gene family being named "*SPRYSEC*s". Since the discovery of this gene family numerous studies have been undertaken that aim to uncover their potential roles in plant – nematode interactions.

Despite their recent discovery and in spite of the fact that no biological function is associated with the SPRY domain itself, remarkable progress has been made on the characterisation of SPRYSECs. All SPRYSEC effectors studied to date are expressed specifically in the dorsal gland cell of cyst nematodes. Genome sequencing of *G. pallida* (Cotton *et al*., 2014; Jones *et al*., 2009) showed that the family of SPRY domain proteins in *G. pallida* is expanded to 299 sequences, a significant change compared to the normal complement of 12-25 sequences present in other nematode species such as *C. elegans*, *M. incognita* and *B. xylophilus* (Chapter 2). This gene family therefore represents almost 2% of the total protein encoding genes of *G. pallida,* strongly suggesting that it has an important role in the biology of this nematode. However, only 10% of the 299 *G. pallida* proteins that include a SPRY domain have a predicted signal peptide for secretion. A phylogenetic analysis (Chapter 2) showed that these secreted forms do not form a closely related subset of the full complement of SPRY domain proteins in the nematode but are instead found dispersed throughout the phylogenetic tree. A comparison of the expression profiles of sequences with and without signal peptides showed that the presence of a signal peptide is strongly correlated with expression being confined to the early stages of parasitism, thus confirming that the other sequences, that lack a signal peptide, are unlikely to encode functional effectors.

At present it is difficult to investigate the evolution of the SPRY domain gene family in other PPNs due to the absence of genome data for related species. Transcriptome or EST data are not suitable for this analysis, given the proportion of SPRY domain proteins in *G. pallida* that are not expressed, or only expressed at very low levels. Although root-knot nematodes (like all organisms) have SPRY domain proteins, there are no *SPRYSEC* genes (with signal peptides)

in the genomes of the two species sequenced to date (Opperman *et al.,* 2008; Abad *et al.,* 2008). It may therefore be the case that SPRYSEC effectors are an adaptation specific to cyst nematodes. A genome project for *G. rostochiensis* is currently being completed and an expanded SPRY domain family is present in this species (J. Jones, pers. comm.). It will be interesting to identify which of the *G. rostochiensis* SPRY domain proteins are SPRYSECs and to compare the SPRYSEC sequences from *G. rostochiensis* and *G. pallida*. Understanding whether the SPRYSECs in the two species are homologues, or whether an entirely different subset of the SPRY domain proteins are *SPRYSEC*s in *G. rostochiensis* compared to *G. pallida* may provide interesting information about the evolution of this gene family in cyst nematodes.

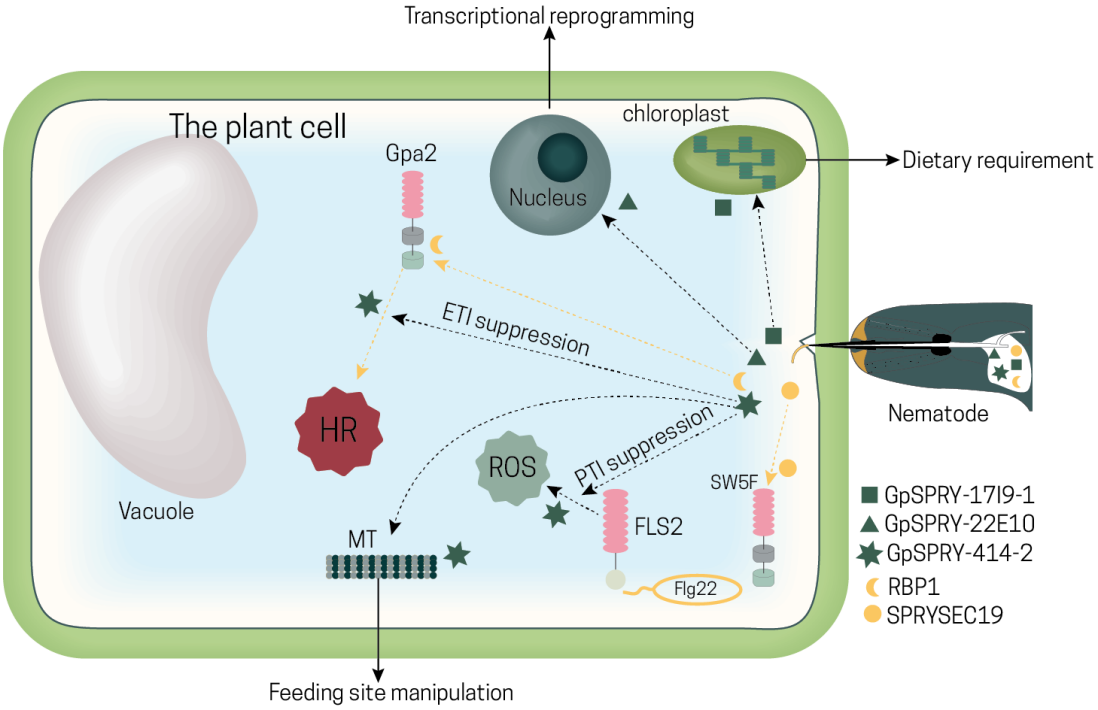


Figure 5.1 A scheme of the various functions of SPRYSECs discoverd in previous research as well as in this thesis. RBP-1 is recognised by the resistance protein Gpa2 leading to a hypersensitive response (HR) and SPRYSEC19 was shown to interact with the SW5F resistance protein. Pathways involved in these published effectors are indicated in yellow dashed lines. Pathways of three SPRYSECs in this thesis are indicated in black dashed lines. GpSPRY-22E10 appears to target the nuclei possibly to interfere with transcriptional reprogramming. GpSPRY-17I9-1 may interact with chloroplast-localised CCD4 to help fulfil nematodes' dietary requirement. GpSPRY-414-2 is involved in suppression of both PTI and ETI as well as interacting with microtubules to facilate feeding site formation.

Several lines of evidence suggest that SPRYSECs have diverse functions with respect to plant parasitism. First, SPRYSECs are localised to a range of different subcellular compartments including the cytoplasm and the nucleus in the plant cell (Jones *et al*., 2009). Secondly, host proteins shown to interact with SPRYSECs, either in yeast or *in planta,* function in a variety of plant metabolic pathways (Chapter 2, Chapter 3 and Chapter 4; Rehman *et al.,* 2009). As shown in Figure 5.1, one SPRYSEC *G. pallida* RBP-1, was reported to be recognised by the potato resistance protein Gpa2 thus leading to a hypersensitive response (Sacco *et al*., 2009). By contrast, other members such as *G. rostochiensis* SPRYSEC19 (Postma *et al.*, 2012) together with several newly discovered SPRYSECs - GpSPRY-12N3, GpSPRY-33H17 (Chapter 2) and GpSPRY-414 -2 (Chapter 4) - were seen to suppress host defences. All these proteins are clustered together in the phylogenetic tree while another SPRYSEC GpSPRY-22E10 is close to this cluster but did not show defence suppression (Chapter 2). A sequence alignment between two SPRYSECs that suppressed plant defences (GpSPRY-12N3 and GpSPRY-33H17) and GpSPRY-22E10 suggested that the SPRY domain may be important for mediating the suppression of plant defences (Appendix 5A). It is notable that the SPRYSECs identified as suppressors of ETI in this study are not able to suppress plant defences provoked by other R/Avr combinations including the closely related Rx/PVX-coat protein. This distinguishes them from SPRYSEC19 and suggests that they may suppress a different part of the defence signalling pathway. Sequence alignment between all three SPRYSECs in this study that suppressed plant defences and SPRYSEC19 revealed differences across the sequences (Appendix5B). These differences may explain their different functions compared to SPRYSEC19. Given the diverse and even opposite functions of SPRYSECs as well as the huge amount of uncharacterised non-secreted members, we speculate that SPRYSECs may undergo strong diversifying selection to help the nematode avoid being recognised by plants. The interaction between GpSPRY-24D4 and a putative LRR receptor-like kinase in yeast is interesting (Chapter 2). Transmembrane receptor – like protein kinases (RLKs) appear to be associated with both layers of plant defence and can link to PRRs in PTI and R proteins in ETI (Afzal *et al*., 2008; Greeff *et al*., 2012; Sun *et al*., 2004). It has been shown that one RLK (SlSERK1 - *Solanum lycopersicum* somatic embryogenesis receptor kinase 1) in tomato is required for Mi-1-mediated resistance to potato aphids (Mantelin *et al*., 2011). Another two members of the same gene family in tomato (*SlSERK3A* and *SlSERK3B*) were reported to be positive regulators of PTI, as silencing either gene resulted in enhanced susceptibility to root

knot-nematodes in a compatible host and to a strain of *Pseudomonas syringae* pv. tomato (*Pst*DC3000 *hrcC*) that is deficient in type III secretion system and that consequently cannot usually infect tomato (Peng & Kaloshian, 2014). It was also demonstrated that the receptor-like protein kinase 2 / Toadstool 2 (RPK2) together with the receptors CLV1 and CLV2 play a role in the perception of nematode CLEs, which act as ligand mimics of plant CLE peptides and are required for successful plant parasitism (Replogle *et al*., 2013). Further investigation on the interaction between SPRYSEC and the putatively interacting potato RLK will add more details to our knowledge of how *G. pallida* infects plants and will broaden our understanding of SPRYSECs.

In chapter 2, we also showed that the presence of a tag is important for SPRYSECs to suppress plant defence in the transient expression assay on *N. benthamiana* leaves. It was hypothesised that tags improved the stability of effector proteins in this artificial system. Given the fact that in reality nematode effectors secreted into plants are not tagged, we proposed that nematodes stabilize these proteins through glycosylation or forming biologically active complexes together with other effectors secreted concomitantly. It is also possible that the requirement for a tag is an artefact of the over-expression system used for these assays and that a tag would not be required for the comparatively small quantities of proteins introduced into a plant cell during a real nematode infection. Based on our data, we suggest that in future functional studies effectors should always be tested both with and without a tag in order to avoid false negatives.

In all the *in planta* subcellular localisation studies of this thesis, SPRYSECs were expressed without their signal peptide as this sequence will be cleaved during secretion of the protein from the gland cells. All SPRYSEC protein localisations here have therefore been performed within host cells. However, it has been demonstrated that effectors can also be possibly secreted into host extracellular spaces (Eves-van den Akker *et al*., 2014; Jaouannet *et al*., 2013; Mitchum *et al*., 2013). In order to determine whether SPRYSECs are introduced into the apoplast it would be necessary to use immunolocalisation which fixes both the host and pathogen tissues and allows for the localisation of nematode secreted proteins in both organisms (Vieira *et al*., 2011). However, given the requirement of developing protein specific antibodies, this technique is more expensive and time consuming than the *Agrobacterium*-mediated transient expression method used here.

5.2 GpSPRY-17I9-1 may modify host metabolism to fulfil the nematode's dietary requirements

We showed in Chapter 2 that GpSPRY-17I9-1 is not involved in suppressing plant defence provoked by two elicitors and a range of *R/Avr* combinations. However, in Chapter 3 we have primary evidence that this effector might be involved in promoting nematodes' pathogenicity on potato plants (Figure 5.1). Silencing this gene in *G. pallida* by dsRNA soaking of J2s appeared to slightly reduce the nematodes' capability to colonise potato, however, whether or not this reduction is significant needs further work probably with more replicates. Through yeast two-hybrid screening, four independent clones encoding a carotenoid cleavage dioxygenase 4 (CCD4) proteins were identified that interact with GpSPRY-17I9-1. Silencing *CCD4* in potato significantly increased the infection rate of nematodes. Notably, the effect was most pronounced on male nematodes. We further investigated the potential reasons behind this increase in host susceptibility to nematodes. First we analysed whether the effects could be due to changes in ABA levels as the ABA biosynthetic pathway uses the same substrates as CCD4. However, our data showed that the silencing effect of *CCD4* on nematode reproduction is not due to an increase in levels of ABA as application of this hormone on potato leaves significantly reduced the number of nematodes. An alternative hypothesis is that the effect of silencing *CCD4* on nematodes could be due to an increased level of carotenoids in these plants. Although the precise biochemical activities of many of the CCD proteins are not characterised in detail, it is known that CCD4 metabolises carotenoids and that wild *Solanum* species, such as *S. phureja,* that have lower levels of CCD4 activity have increased levels of carotenoids (Campbell *et al*., 2010). This is readily observed in tuber flesh, which has a golden yellow appearance in these species due to high carotenoid levels. It is interesting to note that *S. phureja* is more susceptible to PCN compared to *S. tuberosum*. We also demonstrated that the carotenoid biosynthesis inhibitor Fluridon shifted the sex ratio of nematodes towards males. These lines of evidence suggest that the role of GpSPRY-17I9-1 may be to prevent the normal operation of CCD4 thus increasing levels of carotenoids and improving the nutritional status of the plants to the nematodes. The differential effect of *CCD4* RNAi on males developing on the RNAi plants compared to females may also be consistent with this idea. Sex is determined environmentally in PCN, with nematodes that obtain a plentiful supply of food becoming female while those that are unable to induce a substantial feeding site, or that induce a

feeding site in an area of the root where it cannot make contact with the vascular tissue, become male (Sobczak & Golinowski, 2011). The stronger effect on males may be due to the fact that these nematodes can be considered to be under nutrient stress compared to the females. Lowering carotenoid levels may affect these nematodes to the point where they can no longer survive, while the females are better able to cope with the stress imposed by reduced carotenoids.

It is noteworthy that the *in planta* assay revealed a difference with regard to the subcellular localisation of CCD4 depending on the eGFP-fusion. When tagged at the N-terminus, CCD4 localised to the cytoplasm while it was more associated with chloroplasts when tagged at the opposite side. We speculate that the tag may interfere with the chloroplast transit peptide signal of CCD4 when it is at the N-terminus as localisation in chloroplasts seems more consistent with the literature on CCD4 (Lundquist *et al*., 2012; Ytterberg *et al*., 2006). However, when tagged in this way, the split YFP assay in our study showed a largely negative result with very weak signals in the cytoplasm. Therefore, whether GpSPRY-17I9-1 and CCD4 can interact with each other or not remains an open question. It could be that these two proteins do not interact with each other at all as they both locate into different cell structures. On the other hand, as they show weak interacting signal in the cytoplasm, the possibility exists that they do interact but in a very transient way which is hard to capture under our current experimental set up. To verify this, *GpSPRY-17I9-1* overexpression lines could be generated to measure their level of carotenoids, or *in vivo* and *in vitro* enzymatic assays could be performed to examine the cleavage activity of CCD4 in the presence of the effector protein (Huang *et al*., 2009; Lashbrooke *et al.*, 2013). Unfortunately given the amount of work, this is all beyond the scope of the current study.

5.3 GpSPRY-414-2 suppresses plant defence and facilitates feeding site formation

In Chapter 4, we presented our findings on GpSPRY-414-2 and showed that it is involved in successful plant parasitism. This SPRYSEC is able to suppress flg22-mediated ROS production (Chapter 2) and suppresses ETI induced by the combination of Gpa2 and RBP-1 (Figure 5.1). Furthermore, when *GpSPRY-414-2* expression in second stage juveniles was reduced by soaking in dsRNA, a significantly lower number of nematodes per gram of root and lower percentage of well-developed females were observed in potato compared to control treatment with dsRNA of *eGFP*.

In our yeast two-hybrid screening, GpSPRY-414-2 was found to interact with a potato CLASP protein which is associated with microtubules (Figure 5.1). The interaction was detected with both truncated and full length CLASP, although it was much stronger with the truncated version of the protein. The same interaction pattern was seen when the proteins were expressed *in planta*. CLASPs are a group of microtubule associated proteins that target the plus end of the microtubule strings and contribute to the stability of microtubule dynamics. It is also important for internal protein transportation, cell expansion as well as plant hormone distribution (Ambrose *et al*., 2013; Ambrose *et al*., 2007). All this implies that it may have a role in plant – cyst nematode interactions. Firstly, the cyst nematode feeding site is a specialized organ with disordered cytoskeleton and altered hormone distribution (de Almeida Engler *et al*., 2004; Kyndt *et al*., 2013). Secondly, protein transport inside the cell may be important for the translocation of some defence related proteins upon nematode attack. Analysing the role of the CLASP protein *in planta* in potato is difficult due to a lack of RNAi or overexpression lines. However, an *Arabidopsis* mutant that lacks the orthologue of the potato CLASP protein was available. We were able use this mutant to show that a lack of the CLASP protein promoted nematode infection at the early stage but impeded the development of nematodes at the later stage.

Using a microtubule disturbing reagent it was examined whether this interactor is responsible for the observed phenotypes of plant defence suppression. Even in plants with a strongly disturbed microtubule network, GpSPRY-414-2 was still able to significantly suppress the hypersensitive response mediated by Gpa2. However, a functional microtubule network does not seem essential for Gpa2 to induce cell death. Besides, the interaction itself between the effector and CLASP was not affected. It thus remains unclear whether CLASP is involved in the suppression by GpSPRY-414-2 of the Gpa2-dependent plant defence response. Future analyses such as silencing *CLASP* by virus-induced gene silencing, which can separate CLASP eventually from being recognized by GpSPRY-414-2, may provide an answer to this question.

Unlike what was observed for the ETI suppression assay, disrupting the plant microtubule network did slightly perturb the PTI response itself, triggered by the bacterial PAMP flg22. The colchicine treatment significantly reduced ROS production in *N. benthamiana* leaves control samples (about 25%) and that level of suppression was similar to the level of suppression achieved in leaves expressing GpSPRY-414-2 with or without colchicine

treatment. Both GpSPRY-414-2 and colchicine are thus involved in the suppression of flg22-mediated ROS production but with no additive effect. It is therefore likely that GpSPRY-414-2 suppresses ROS production through the disturbance on microtubule network via CLASP. Taken together, our results imply that GpSPRY-414-2 may play dual roles during the interaction with the host plant by suppressing plant defence and facilitating the establishment of nematode feeding sites.

It has been suggested (Rehman *et al.,* 2009) that SPRYSEC proteins could be components of multi-subunit E3 ligases in plants. E3 ligases are part of the ubiquitination system and specify proteins that are targeted for degradation by the proteasome system. One SPRYSEC in our research (Figure 1.5), even though not yet well characterised, was identified to contain a SOCS box which was suggested to function as adaptor proteins to help substrate ubiquitination by E3 ligase (Perfetto *et al*., 2013). It is also interesting to note that our functional data on GpSPRY-17I9-1 and GpSPRY-414-2 are consistent with this hypothesis as in both cases the data suggest that removing the host target increases parasitic success of the nematode. It is possible therefore that the *SPRYSEC* gene family has evolved to target host proteins that the nematode needs to remove in order to survive. There are other examples of pathogens that have evolved E3 ligases that target important host proteins for degradation, most notably the AvrPto effector of *P. syringae* (Abramovitch *et al*., 2006). Nematodes, like other pathogens, may therefore exploit the host ubiquitination system for their own benefit.

From a practical point of view, several SPRYSECs characterised in this thesis were shown to play roles in plant parasitism and thus may be good candidates for future control of *G. pallida*. For example, GpSPRY-12N3, GpSPRY-33H17 and GpSPRY414 were shown to suppress plant defences while GpSPRY-17I9 appeared to target the carotenoid pathway to improve the nematodes' food source. GpSPRY414 is of particular note as it may be involved in nematode feeding site formation. Silencing the plant targets of these effectors may however not be a good option to reduce nematode infection in the future as plant genes such as *clasp* are all vital for plant development. In current research, RNAi silencing by soaking nematodes in dsRNA was shown to successfully reduce nematodes' colonisation for two SPRYSECs GpSPRY-17I9 and GpSPRY-414-2. This suggests that an alternative novel control strategy in which *in planta* RNAi is used to silence nematodes' effector candidates during feeding could be considered.

Summary

The white potato cyst nematode *Globodera pallida* originated from South America but has now spread throughout the world including many important potato growing regions. Economic losses are valued at over 100 Million Euros within the EU alone. Control of this nematode is extremely difficult due to a lack of major resistance genes and increased public concerns about nematicides. New control strategies based on a better understanding of the molecular basis of the interaction between host plant and nematode will offer the prospect of sustainable control of this pathogen.

G. pallida is a biotrophic pathogen that interacts with its host plant through effector proteins, which are secreted from the nematodes into the host. Following the completion of the *G. pallida* genome sequence, many effectors have been identified from this nematode. Of particular note is the *SPRYSEC* gene family. SPRYSEC effectors are specific to cyst nematodes and are all secreted from dorsal gland cell. Although little is known about their function they all share the presence of a SPRY domain. While all organisms contain SPRY domain proteins, this gene family is remarkably expanded in *G. pallida* and consists of 299 members (compared to 12-25 in other nematode species). Our analysis shows that only 10% of the SPRY domain proteins in *G. pallida* are likely to be deployed as effectors. We demonstrated that SPRYSEC proteins localize to a range of subcellular structures and interact with many different host proteins. In this thesis we particularly focused on two members, GpSPRY-17I9-1 and GpSPRY-414-2, both of which were proven to be important in plant parasitism. GpSPRY-17I9-1 was shown to interact with potato carotenoids cleavage dioxygenase 4 (CCD4) protein. Silencing *CCD4* in potato resulted in significantly increased nematode susceptibility. It was further shown that this effect is not due to an indirect increase in the plant hormone ABA but instead that silencing *CCD4* may allow increased levels of carotenoids to accumulate that help nematodes to fulfil their dietary requirements. By contrast, we showed that the GpSPRY-414-2 effector may play dual roles in suppressing plant defences and helping establishment of nematode feeding site and that this is mediated through interactions with a host CLASP protein that regulates microtubule dynamics. With this thesis, the understanding of the function of SPRYSEC proteins has significantly progressed. Such advances will not only improve our fundamental knowledge of plant – nematode interactions, but also may lead to novel strategies to control *G. pallida*.

Samenvatting

Het aardappelcystenaaltje *Globodera pallida* heeft zich vanuit zijn oorsprongsgebied in Zuid-Amerika verspreid over de ganse wereld met inbegrip van veel regio's die belangrijk zijn voor de aardappelteelt. Economische verliezen bedragen alleen al in de EU meer dan 100 miljoen Euro. Controle van deze nematode is extreem moeilijk omdat er geen goede resistentiegenen voorhanden zijn en omdat de gebruikelijke nematiciden steeds meer verboden worden. Nieuwe controlestrategieën gebaseerd op een beter begrip van de moleculaire basis van de interactie tussen nematoden en de gastheerplant bieden het perspectief van een meer duurzame controle van deze pest.

G. pallida is een biotrofe pathogeen die interageert met de gastheer o.a. via effectoreiwitten, die gesecreteerd worden door de nematode in de gastheerplant. Door het bekomen van de *G. pallida* genoomsequentie konden heel wat effectorgenen geïdentificeerd worden, waarbij de *SPRYSEC*-genfamilie onmiddellijk opviel. SPRYSEC- effectors zijn specifiek voor cystenaaltjes en worden gesecreteerd door de dorsale kliercel. SPRYSECs hebben allemaal het SPRY-domein gemeenschappelijk maar verder is er heel weinig geweten over hun mogelijke functie. Alhoewel alle organismen SPRY-domeineiwitten bezitten, is deze genfamilie opvallend uitgebreid in *G. pallida* met 299 leden (in vergelijking met 12-25 in andere nematodenspecies). Onze analyse toont dat slechts 10% van de SPRY-domeineiwitten in *G. pallida* wellicht als effector functioneren. We hebben aangetoond dat SPRYSEC-eiwitten gelokaliseerd zijn in een verscheidenheid van subcellulaire structuren en interageren met zeer verschillende planteneiwitten.

Voor de verdere studie hebben we ons vooral toegespitst op twee leden, GpSPRY-17I9-1 en GpSPRY-414-2, beide belangrijk in plantenparasitisme. GpSPRY-17I9-1 interageert met *potato carotenoids cleavage dioxygenase 4* (CCD4)-eiwit. Gereduceerde expressie van *CCD4* in aardappel resulteert in significant verhoogde gevoeligheid voor nematoden. Bovendien werd aangetoond dat dit effect niet te wijten is aan een indirecte verhoging van het plantenhormoon ABA maar dat verminderen van CCD4 mogelijks resulteert in een verhoogd carotenoïdeniveau dat de nematoden helpt om aan hun voedingsvereisten te voldoen. GpSPRY-414-2 daarentegen speelt mogelijks een dubbele functie, enerzijds in de inhibitie van de plantenafweer en anderzijds als stimulerende factor voor de uitbouw van een

nematodenvoedingsplaats door interactie met het planteneiwit CLASP dat microtubuli reguleert.

Met dit doctoraatsonderzoek is de kennis over de rol van SPRYSEC-eiwitten bij nematodenparasitisme significant verbeterd. Deze vooruitgang kan ook leiden tot nieuwe controlestrategieën tegen *G. pallida* in aardappel.

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Curriculum Vitae

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Qualifications

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2008-2010	European Master of Science in Nematology, Ghent University
2006-2008	Master of Plant Pathology, Zhejiang University, China
2002-2006	Bachelor of Agriculture, Shandong Agriculture University, China

Publications

1. Mei YY, Thorpe P, Guzha A, Haegeman A, Block V, Mackenzie K, Gheysen G, Jones J & Mantelin S. (2015) Only a small subset of the SPRY domain gene family in *Globodera pallida* is likely to encode effectors, two of which suppress host defences induced by the potato resistance gene *Gpa2*. Nematology. DOI:10.1163/15685411-00002875.

2. Mei YY, Wright K, Haegeman A, Gheysen G, Jones JT & Mantelin S. (2014)Insight into the functions of *Globodera pallida* SPRYSEC proteins. In: Abstracts book of the 2nd annual conference of the COST SUSTAIN action, page 31-32.

3. Mei YY, Wright K, Haegeman A, Gheysen G, Jones JT & Mantelin S. (2014)Investigating the function of *Globodera pallida* SPRYSEC effectors. Proceedings of 66th International Symposium on Crop Protection, Page 46.

4. Mei YY, Wright K, Haegeman A, Gheysen G, Jones JT & Mantelin S. (2014) Functional analysis of *Globodera pallida* SPRYSEC proteins. Journal of Nematology, June 2014, Vol 46, No.2, Page 204.

5. Mei YY, Mantelin S, Haegeman A, Gheysen G& Jones JT. (2013) Functional analysis of *Globodera pallida* SPRYSEC proteins. Acta Phytopathologica Sinica, Vol 43, Supplement 2013, Page 381.

6. **Mei YY**, Mantelin S, Haegeman A, Gheysen G& Jones JT (2012). Functional analysis of *Globodera pallida* SPRYSEC proteins. Proceedings of 31st International Symposium of the European Society of Nematologists, Page 10.

Conferences and symposia contributions

Mei YY, Wright K, Haegeman A, Gheysen G, Jones JT & Mantelin S. (2014) Insight into the functions of *Globodera pallida* SPRYSEC proteins. The 2nd annual conference of the COST SUSTAIN action. Zakopane, Poland, 15th-17th October, 2014. Oral Presentation

Mei YY, Wright K, Haegeman A, Gheysen G, Jones JT & Mantelin S. (2014) Investigating the function of *Globodera pallida* SPRYSEC effectors. The 66th International Symposium on Crop Protection. Ghent, Belgium, 20th May, 2014.Oral Presentation

Mei YY, Wright K, Haegeman A, Gheysen G, Jones JT & Mantelin S. (2014) Functional analysis of *Globodera pallida* SPRYSEC proteins. The 6th International Congress of Nematology. Cape Town, South Africa, 4th – 9th May, 2014.Oral Presentation

Mei YY, Mantelin S, Haegeman A , Gheysen G& Jones JT. (2013) Functional analysis of *Globodera pallida* SPRYSEC proteins. The 10th International Congress of Plant Pathology. Beijing, China, 25th - 31st August, 2013. Oral Presentation

Mei YY, Mantelin S, Haegeman A, Gheysen G& Jones JT (2012). Functional analysis of *Globodera pallida* SPRYSEC proteins.The 31st International Symposium of the European Society of Nematologists. Adana, Turkey, 23rd -27th September, 2012. Oral presentation.

Mei YY, Mantelin S, Haegeman A, Gheysen G& Jones JT (2012). Functional analysis of *Globodera pallida* SPRYSEC proteins. International Molecular Plant-Nematode Interactions Group (IMPNIG) meeting aka SPIT meeting. Ghent, Belgium, 23rd -24th May, 2012.Oral presentation.

Major Awards

2014.10 Fellowship to attend the 2nd COST sustain conference

2014. 5 Student bursary from European Society of Nematologists to attend 6th ICN

2013. 8 Mobility grant from Ghent University Research Council

2013. 4 Student bursary from International Congress of Plant Pathology (ICPP)

2012. 9 Student bursary from the 31stinternational ESN symposium

2011. 8 Award for long leave study abroad, Faculty of Bioscience Engineering, UGent

2010-2014 Special Research Fund for PhD from developing countries, BOF-UGent

2008-2010 Erasmus Mundus scholarship from European Union

Master student supervision

Athanas Guzha (2012-2013) Functional analysis of nematode secreted proteins. Ghent University, Belgium. Thesis was submitted in partial fulfillment to obtain the degree of European Master of Science in Nematology.

Appendix

Appendix 1. FASTA sequences corresponding to the six cloned *SPRYSEC* effector candidates from *Globodera pallida*. The gene coding sequences were cloned without the signal peptide. Sequences provided here after are native sequences plus ATG start and stop codon added during the cloning process.

>GpSPRY-12N3

ATGTCGCCAAAACCGTCAAACACCAAACTCAAGAATAAAGGACCAACCAGTTCTGGCAATGCTAAACTAAACG
CAGATCCATCGCCAAAAACGTCAGTATCAAACACAAAACTCGAAAATGAACCAGCTGCACAAAAAAACCCAGG
ACTAACCGTTGAAAATCAATGGAATTCCAAAGCCGATGCATGCCATGCGGACCTTACGCTTTCTCAGCCCGTAC
AACCGTCCGACCCCAAACTGTCTAAGCCCAAACGATTCTTGGTTGTCAAACATAAGCCAGGCCAATCGAAGAAT
TGCAGCTCTGTGTTCGCGGTTCAGCCAATTCCAAAAGAAGGAATTTTCTACTATGAAGTGACAATTTTAGGGAA
AACAGGCGTTGTTTCTATTGGACTTGGTCCAAAACAAATGCCATTGGCCAAAGAAATTGGATTTGAAGGCTAC
GCGTACCAAAGCTGCGGTACCTTTTTGAATCACGAGGCGCCGGGATGTTACTACAGTGACATGGACGATAAAC
CACGTGCTTTGTTCGAAGAAGGAATTGAAGGTATTCGTCCCGGCAACGTCGTCGGATGCGGCGTGGATTTTAA
AAATAAAAAAATCTTTTACACGCTGAACGGAGAGCGTTTGGGTCCTGCCGGTGAATTTGTCGATTCTAGCGTA
AGATTGTTTCCGTGCGTTTCGTTGGCACGAGATGGCGACGAAATTGAAGCAAACTTTGGACCGAATTTTGAATT
CAAACATTGTTGA

>GpSPRY-17I9-1

ATGTCGCCAAAACCAGACAAAAAACGCGAAAAAGGACCTTCCAGTGCTGGCAATGCTGAATCAACCCCAGCTC
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TTGATTGTCCAATCTACTGGAGAAAAAAAATGATTGTCGCTCTGTCCGCGCCAAACTGCCAATTCCAGAATCCGG
CATTTTCTACTACGAAGTGACCATCTTAGAGAAAGGAGAGCACAACGGTATTTTCATTGGACTTGGGACGAAA
GAAACACCATCGGACAAAAAATCGGTTGGACAGAGCGAAGGCACTTACGCATACTCAAACAGGGGCAGTTTT
TGGGGACACGAAGTTAAGGACTGTTCCCATTGCAACAAAGGACGTCCTTTGATCACTGGAAATCTCAAATTTA
ACCGTAACGACGTCATCGGCTGCGGCGTGGATTGGGCAAAGAGCCAAATCATTTACACGCTAAACAAAGAGCT
TTTGAAAACTACCGATTTGAAAGTCGATTCTGCCGCCGATTTGTACCCGTGCGTTTCGTTGTTCCATTCTGGCGC
CAAAATTGAAGCGAATTTTGGCAAGAAAAAATTCATATTAGACATTGCCAAGGCATTTGAAAACTGA

>GpSPRY-22E10

ATGTCGCCAAAACCGTCAAACACCAAACTCAAGAATAAAGGACCAACCAGTTCTGGCAATGCTAAACTAAACG
CAGATCCATCGCCAAAAACGTCAGTATCAAACACAAAACTCGAAAATGAACCAGCTGCACAAAAAAACCCAGG
ACTAACCGTTGAAAATCAATGGAATTCCAAAGCCGATGCATGCCATGCGGACCTTACGCTTTCTCAGCCCGTAC
AACCGTCCGACCCCAAACTGTCTAAGCCCAAACGATTCTTGGTTGTCAAACATAAGCCAGGCCAATCGAAGAAT
TGCAGCTCTGTGTTCGCGGTTCAGCCAATTCCAAAAGAAGGAATTTTCTACTATGAAGTGACAATTTTAGGGAA
AACAGGCGTTGTTTCTATTGGACTTGGTCCAAAACAAATGCCATTGGCCAAAGAAATTGGATTTGAAGGCTAC
GCGTACCAAAGCTGCGGTACCTTTTTGAATCACGAGGCGCCGGGATGTTACTACAGGTGCGCTTTGACCGGGA
TTTCGACTTTTACCGGGACCGGGATTTCGATATCTTTGTGA

>GpSPRY-24D4

ATGAATGAACAAAATGCATATGGTTTCAACATAAATGATCAACAAAAAGAAATGAACGAATCGTCTGGTCAAG
CGATGGTCGTCGCCAAATTGGAGAAGCATCAGAACACCCAAAATCGAAGCAATGAACGTGAAGGGCAACTGA
ACGACATTTTGAAGCAGTTTGTTGCGGAACAGAAGGAAGCGAACAGAATGCTTCAGAAGCAAATGGACGAAT
TAGGAAACAGTTCGAAAGAGCTCGAAAAGGGAATCAATCAGTTGAAGGAAGAGATAGCAAAGATGGAGCAG

TATCAGAAAGAACAGCAGAATACAGTTGCCGTTGCCGTGTTAAATGGAATACAAATAATGCGTCAACAAAACC
GATGGGATTCCGCTGCAAGTCACGAGAACCTCATACTCTCTGAGCCCGATCGATTGGTTGTTCAATTTAATGGA
GAGGTGTGGGGCTCTGTCCGCGCTGAAAAGCGAATGCTGGAAAATCCTTACTTCGAAGTGAAAATCCTAGTGA
GCGGAAGCCGTATTTTCATTGGACTTGCGACCAAAAAAATGCCATTGAACAACAACCCGGTTGGAGTTCACGA
AGGCACTTTCGCATACGACAGCTGGGGCAGATTTTGGGGTCATGAGGTCGACGGATGTTCCCACGCCGCCGAT
GGACGTCCGTACATCGTAAAAGGAATACCCGCGTTTGCCGTCGGCGACGTCGTTGGCTGCGGCGTCAATTTAA
AAAATGGCCAAATTATTTACACAAAAAATGGAAAGCGTTTGGACAGCGCCAATTTATTTGTCGATTCTGCCGCC
GATTTATTTCCGTGCGTTTCGTTGGGGCTGCCTGGCACCAAAATTGCGAATTTTGGGCCGAACTTCAAATACAA
CTTTGCCGATGGCATTTGA

>GpSPRY-33H17

ATGTCGCCAAAACCGTCAAACACCAAACTCAAGAATAAAGGACCAACCAGTTCTGGCAATGCTAAACTAAACG
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ACTAACCGTTGAAAATCAATGGAATTCCAAAGCCGATGCATGCCATGCGGACCTTACGCTTTCTCAGCCCGTAC
AACCGTCCGACCCCAAACTGTCTAAGCCCAAACGATTCTTGGTTGTCAAACATAAGCCAGGCCAATCGAAGAAT
TGCAGCTCTGTGTTCGCGGTTCAGCCAATTCCAAAAGAAGGAATTTTCTACTATGAAGTGACAATTTTAGGGAA
AACAGGCGTTGTTTCTATTGGACTTGGTCCAAAACAAATGCCATTGGCCAAAGAAATTGGATTTGAAGGCTAC
GCGTACCAAAGCTGCGGTACCTTTTTGAATCACGAGGCGCCGGGATGTTACTACAGTGACATGGACGATAAAC
CACGTGCCTTGTTCGAAGAAGGAATTGAAGGTATTCGTCCCGGCAACGTCGTCGGATGCGGCGTGGATTTTAA
AAATAAAAAAATCTTTTACACGCTGAACGGAGAGCGTTTGGGTCCTGCCGGTGAATTTGTCGATTCTAGCGTA
AGATTGTTTCCGTGCGTTTCGTTGGCACGAGATGGCGACGAAATTGAAGCAAACTTTGGACCGGATTTTGAAT
TCAAAACATTGTTGAAATGGGATGAAATTGTAAACAAGAATTTATTGCCAAAAGATTGA

>GpSPRY-414-2

ATGTGGCCGCCAAAAACGACATCAAACAACAACCCAGGGCTAACTACTGGAAATAAATGGGATTCAAAAGCC
GATTCGTGTCACCGGGACCTGACGCTCTCGGAGCCCGATCAATTGACTGCCAAGGTTACAGGAAAGAATTTGG
GGTATCGCAGCGCTGTCTTCGCTGTTCAGTCAGTTCCACAAATTAATTCCGGCATTTTCTACTACGAAGTGGAA
ATAAAAGGGAGAGTAGGTTACATCTCCATTGGACTTGCGACCAAACAAATGGCATTGAACAACGAAGTTGGA
GAATTTCAAGGCTACGCATACCACTTCGGCGACGGTTTTCGTCGCCACGAGGCGGAGGGATGTTCCTACACGC
ACAACGTCAAACGTCCTTACTACAATAAAGGAATATCACGGTATGGTGTCGGCAACGTCATCGGATGCGGCGT
GGATTTAGCAAAGCGCAAAATCTTTTACACGCTGGACGGCCAGCGTTTGGGTCCTGCCGGTTTGTTAGCCGATT
CTGCCGACCCATTGTATCCATGCGTTTCGTTGTCACACCGTGGCGACATAATTGCAGCGAACTTTGGAGCGGAC
TTCCTATTCAAATTCGACATTGCCAAATGGAATTTAGAAACTGAAAAATGA

Appendix 2. Similarity matrices of the sequences of the six SPRYSECs studied. The comparison was done pairwisely with protein sequences (the whole mature proteins or only SPRY domains) using (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=blasttab) BlastP. Numbers in the matrices show the identies (%) beween the two sequences. Numbers in brackets indicate the percentage of coverage.

Identity (%) matrix of six mature SPRYSEC proteins

	GpSPRY-12N3	GpSPRY-33H17	GpSPRY-22E10	GpSPRY-24D4	GpSPRY-17I9	GpSPRY-GPE414
GpSPRY-12N3	100	-	-	-	-	-
GpSPRY-33H17	99	100	-	-	-	-
GpSPRY-22E10	100(68)	100(69)	100	-	-	
GpSPRY-24D4	41	40	33	100	-	-
GpSPRY-17I9	40	40	38	49	100	-
GpSPRY-GPE414	49	49	42	43	44	100

Identity (%) matrix of SPRY domains

	GpSPRY-12N3	GpSPRY-33H17	GpSPRY-22E10	GpSPRY-24D4	GpSPRY-17I9	GpSPRY-GPE414
GpSPRY-12N3	100	-	-	-	-	-
GpSPRY-33H17	99	100	-	-	-	-
GpSPRY-22E10	100(39)	100(39)	100	-	-	-
GpSPRY-24D4	48	47	46	100	-	-
GpSPRY-17I9	46	46	51	53	100	-
GpSPRY-GPE414	54	54	58	49	48	100

Appendix 3. Primer sequences.

Primer	Sequence (5'–3')	Gene identity & Primer use
17I9_ISH_F	AGAAAGGAGAGCACAACGGT	*GpSPRY-17I9-1in situ* hybridisation
17I9_ISH_R	CTCTTTGCCCAATCCACGC	*GpSPRY-17I9-1in situ* hybridisation
GPLIN_000892900_ISH_F	ACCATGTCGCCAAAACCAAACAAAAAAC	*GPLIN_000892900in situ* hybridisation
GPLIN_000892900R_ISH_R	ACAGAACGCCACTCCCTTTT	*GPLIN_000892900in situ* hybridisation
GPE414_ISH_F	GCTGTCTTCGCTGTTCAGTC	*GpSPRY-414-2in situ* hybridisation
GPE414_ISH_R	TTGCCGACACCATACCGT	*GpSPRY-414-2in situ* hybridisation
SPRY12N3-F	ACCATGTCGCCAAAACCGTCAAAC	*GpSPRY-12N3* cloning
SPRY12N3-R	TCAACAATGTTTGAATTCAAAATTCGG	*GpSPRY-12N3* cloning with stop codon
SPRY12N3-R3	ACAATGTTTGAATTCAAAATTCGG	*GpSPRY-12N3* cloning without stop codon
SPRY12N3-HA-R	TCA***GGCATAATCAGGTACATCATAAGGGTA***ACAATGTTTGAATTCAAAATTCGG	*GpSPRY-12N3* cloning with HA tag
SPRY17I9-F	ACCATGTCGCCAAAACCAGACAAA	*GpSPRY-17I9-1* cloning
SPRY17I9-R	TCAGTTTTCAAATGCCTTGGCA	*GpSPRY-17I9-1* cloning with stop codon
SPRY17I9-R3	GTTTTCAAATGCCTTGGCA	*GpSPRY-17I9-1* cloning without stop codon
SPRY17I9-HA-R	TCA***GGCATAATCAGGTACATCATAAGGGTA***GTTTTCAAATGCCTTGGCA	*GpSPRY-17I9-1* cloning with HA tag
SPRY22E10-F	ACCATGTCGCCAAAACCGTCAAA	*GpSPRY-22E10* cloning
SPRY22E10-R	TCACAAAGATATCGAAATCCCGGT	*GpSPRY-22E10* cloning with stop codon
SPRY22E10-R3	CAAAGATATCGAAATCCCGGT	*GpSPRY-22E10* cloning without stop codon
SPRY22E10-HA-R	TCA***GGCATAATCAGGTACATCATAAGGGTA***CAAAGATATCGAAATCCCGGT	*GpSPRY-22E10* cloning with HA tag
SPRY24D4-F	ACCATGAATGAACAAAATGCATATGGTTTC	*GpSPRY-24D4* cloning
SPRY24D4-R	TCAAATGCCATCGGCAAAGTT	*GpSPRY-24D4* cloning with stop codon
SPRY24D4-R3	AATGCCATCGGCAAAGTT	*GpSPRY-24D4* cloning without stop codon
SPRY24D4-HA-R	TCA***GGCATAATCAGGTACATCATAAGGGTA***AATGCCATCGGCAAAGTT	*GpSPRY-24D4* cloning with HA tag
SPRY22E10-F	ACCATGTCGCCAAAACCGTCAAA	*GpSPRY-33H17* cloning
SPRY33H17-R	TCAATCTTTTGGCAATAAATTCTTGTTTA	*GpSPRY-33H17* cloning with stop codon

SPRY33H17-R3	ATCTTTTGGCAATAAATTCTTGTTTA	*GpSPRY-33H17* cloning without stop codon
SPRY33H17-HA-R	TCA***GGCATAATCAGGTACATCATAAGGGTA***ATCTTTTGGCAATAAATTCTTGTTTA	*GpSPRY-33H17* cloning with HA tag
SPRYGpE414-F	ACCATGTGGCCGCCAAAAACG	*GpSPRY-414-2* cloning
SPRYGpE414-R	TCATTTTTCAGTTTCTAAATTCCATTTG	*GpSPRY-414-2* cloning with stop codon
SPRYGpE414-R3	TTTTTCAGTTTCTAAATTCCATTTG	*GpSPRY-414-2* cloning without stop codon
SPRY414GpE-HA-R	TCA***GGCATAATCAGGTACATCATAAGGGTA***TTTTTCAGTTTCTAAATTCCATTTG	*GpSPRY-414-2* cloning with HA tag
GFP-ATG-FOR	ACCATGGTGAGCAAGGGC	*eGFP* cloning
GFP-TGA-REV	TCACTTGTACAGCTCGTCCATG	*eGFP* cloning
G1-5_F1	AAAGCCTGCTCAAAGGTCTG	Y2H clone interactor G1-5 sequencing
G1-5-F2	GGGCCTAGAGGTTTTCCAGA	Y2H clone interactor G1-5 sequencing
G1-5-F3	CCCCTCGTATAGAAGTGGATTT	Y2H clone interactor G1-5 sequencing
G1-5_F2240	TGAACCAAGCATTCCTCAGA	Y2H clone interactor G1-5 sequencing
G1-5_F2477	AGATGCCATGGAGGATTCAG	Y2H clone interactor G1-5 sequencing
AttB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT	Cloning Y2H insert clone G1-5 into pDONR221
AttB2	GGGGACCACTTTGTACAAGAAAGCTGGGT	Cloning Y2H insert clone G1-5 into pDONR221
G1-5-Cloning-For	ATGGATGGAGGAGGCACTGGAAT	Potato full length *clasp* CDS cloning
G1-5-Cloning-Rev	CTAACTGCGGTTAGCATCTATGG	Potato full length *clasp* CDS cloning
G1-5-800F	AGCCCAAAAATCCCTTAG	Potato full length *clasp* CDS 5'end sequencing
G1-5-1600R	GCATCTCCTACACAACACTTT	Potato full length *clasp* CDS 5'end sequencing
CCD4-F	ATGGATGCTTTGTCTTCAAC	Potato full length *ccd4* CDS cloning
CCD4-R	TAGCTTCATAAGATCAT	Potato full length *ccd4* CDS cloning
CCD4-M794	ACATTTTTACCCTCGGCCGTCAC	Potato full length *ccd4* CDS sequencing
CCD4-F471	TTTCGACGGTGATGGAATGC	Potato full length *ccd4* CDS sequencing
I1-pDONR221-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGGAAATGAGGTGGTTTG	Cloning Y2H insert clone I1-1 into pDONR221
I145-pDONR221-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATAGTTTCATAAGATCATTTTCCCTC	Cloning Y2H insert clone I1-1 into pDONR221
I3-pDONR221-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACAAATTGGAATGAACCCAA	Cloning Y2H insert clone I3-12 into pDONR221

I3-pDONR221-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTATAGTTTCATAAGATCATTTTCCGT	Cloning Y2H insert clone I3-12 into pDONR221
I4-pDONR221-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCGGTGGGTACTGACT	Cloning Y2H insert clone I4-2into pDONR221
I145-pDONR221-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATAGTTTCATAAGATCATTTTCCCT	Cloning Y2H insert clone I4-2into pDONR221
I5-pDONR221-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGGAAATGAGATGGTTTGAT	Cloning Y2H insert clone I5-2into pDONR221
I145-pDONR221-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATAGTTTCATAAGATCATTTTCCCTC	Cloning Y2H insert clone I5-2into pDONR221
RLK-insert-F1	CTGACAATTTATCTGAGCGTAG	RLK Y2H clone interactor sequencing
RLK-insert-F2	TTGAGTAGTATGGAGGAAAGC	RLK Y2H clone interactor sequencing
RLK-Cloning-For	ATGGCGTGGTTTGGTG	Potato full length *RLK* cloning
RLK-Cloning-Rev	TCAGGTGGCACTCAGTGAT	Potato full length *RLK* cloning
pDEST-small-F2	CGACATCATCATCGGAAGAG	pDEST32 , Y2H bait insert forward sequencing
pDEST32-BaitBD-F	AACCGAAGTGCGCCAAGTGTCTG	pDEST32, Y2H bait insert forward sequencing
pDEST22-PreyAD-F	TATAACGCGTTTGGAATCACT	pDEST22, Y2H prey insert forward sequencing
pDEST-R	AGCCGACAACCTTGATTGGAGAC	pDEST22 & pDEST32, Y2H insert reverse sequencing
p35S-FOR	AAGGAAGTTCATTTCATTTGGAGAGGA	35S promoter, forward sequencing
t35S-REV	CAACACATGAGCGAAACCCTATAAGAA	35S terminator, reverse sequencing
M13-F(-20)	GTAAAACGACGGCCAG	M13, forward sequencing
M13-R	CAGGAAACAGCTATGAC	M13, reverse sequencing
M13-REV(-24)	AGGAAACAGCTATGACCATG	M13, reverse sequencing for pENTRY clones
pBatTL-CYFP-R	TGGTAGTGGTCGGCGA	YFP-C fusion, reverse sequencing in pBatTL-B-sYFP-C
pBatTL-NYFP-R	CCGTAGGTGGCATCGC	YFP-N fusion, reverse sequencing in pBatTL-B-sYFP-N
pCL112-NYFP-F	CAACTACAACAGCCACAACG	YFP-N fusion, forward sequencing in pCL112
pCL113-CYFP-F	CCGACAACCACTACCTGAG	YFP-C fusion, forward sequencing in pCL113
C-mRFP-FOR2	CCTACAAGACCGACATCAAG	mRFP fusion, forward sequencing in pH7WGR2
N-mRFP-REV	TTCAAGTAGTCGGGGATGT	mRFP fusion, reverse sequencing in pH7RWG2
Cterm-GFP-FOR	ACAACCACTACCTGAGCAC	eGFP fusion, forward sequencing in pK7WGF2
Nterm-GFP-REV	CGGACACGCTGAACTTG	eGFP fusion, reverse sequencing in pK7FWG2

GpEF1α-F	AACATCTCTGTGAAGGACATTCG	*G. pallidaEF1α* RT-PCR
GpEF1α-R	TCTCCTTAAGTTCGGCGAATTTGC	*G. pallidaEF1α* RT-PCR
17I9F	AGAAAGGAGAGCACAACGGT	*GpSPRY-17I9-1* dsRNA silencing
17I9T7R	GTAATACGACTCACTATAGGGCTCTTTGCCCAATCCACGC	*GpSPRY-17I9-1* dsRNA silencing
17I9R	CTCTTTGCCCAATCCACGC	*GpSPRY-17I9-1* dsRNA silencing
17I9T7F	GTAATACGACTCACTATAGGGAGAAAGGAGAGCACAACGGT	*GpSPRY-17I9-1* dsRNA silencing
17I9testF2	GCATACTCAAACAGGGGCAG	*GpSPRY-17I9-1* RT-PCR
17I9testR2	GTACAAATCGGCGGCAGAAT	*GpSPRY-17I9-1* RT-PCR
GPE414F	GCTGTCTTCGCTGTTCAGTC	*GpSPRY-414-2* dsRNA silencing
GPE414T7R	GTAATACGACTCACTATAGGGTTGCCGACACCATACCGT	*GpSPRY-414-2* dsRNA silencing
GPE414R	TTGCCGACACCATACCGT	*GpSPRY-414-2* dsRNA silencing
GPE414T7F	GTAATACGACTCACTATAGGGGCTGTCTTCGCTGTTCAGTC	*GpSPRY-414-2* dsRNA silencing
GPE414testF2	GGATGCGGCGTGGATTTAG	*GpSPRY-414-2* RT-PCR
GPE414testR2	GGAAGTCCGCTCCAAAGTTC	*GpSPRY-414-2* RT-PCR
GFPF	GCTGGAGTACAACTACAACT	*GFP* dsRNA silencing, Whisson *et al.*, 2005
GFPT7R	GTAATACGACTCACTATAGGGGGCAGATTGCGTGGACAGGT	*GFP* dsRNA silencing, Whisson *et al.*, 2005
GFPR	GGCAGATTGCGTGGACAGGT	*GFP* dsRNA silencing, Whisson *et al.*, 2005
GFPT7F	GTAATACGACTCACTATAGGGGCTGGAGTACAACTACAACT	*GFP* dsRNA silencing, Whisson *et al.*, 2005

HA tag and sequence leader used in *SPRYSEC* cloning

Appendix 4. Summary of GATEWAY recombination constructs generated.

Expression clone	Gateway ENTRY clone	Destination vector	Promoter	Antibiotics selection	Note
eGFP::GpSPRY-12N3	pCR8/GW/TOPO GpSPRY-12N3^{+stop}	pK7WGF2	35S	Spectinomycin	
GpSPRY-12N3::eGFP	pCR8/GW/TOPO GpSPRY-12N3^{-stop}	pK7FWG2	35S	Spectinomycin	
mRFP::GpSPRY-12N3	pCR8/GW/TOPO GpSPRY-12N3^{+stop}	pH7WGR2	35S	Spectinomycin	
GpSPRY-12N3::mRFP	pCR8/GW/TOPO GpSPRY-12N3^{-stop}	pH7RWG2	35S	Spectinomycin	
GAL4-DNA-BD::GpSPRY-12N3	pCR8/GW/TOPO GpSPRY-12N3^{+stop}	pDEST32 (Y2H bait)	ADH1	Gentamicin	
GpSPRY-12N3 (no tag)	pCR8/GW/TOPO GpSPRY-12N3^{+stop}	pK7WG2	35S	Spectinomycin	
GpSPRY-12N3::HA	pCR8/GW/TOPO GpSPRY-12N3^{+HA}	pK7WG2	35S	Spectinomycin	
eGFP::GpSPRY-24D4	pCR8/GW/TOPO GpSPRY-24D4^{+stop}	pK7WGF2	35S	Spectinomycin	
GpSPRY-24D4::eGFP	pCR8/GW/TOPO GpSPRY-24D4^{-stop}	pK7FWG2	35S	Spectinomycin	
mRFP::GpSPRY-24D4	pCR8/GW/TOPO GpSPRY-24D4^{+stop}	pH7WGR2	35S	Spectinomycin	
GpSPRY-24D4::mRFP	pCR8/GW/TOPO GpSPRY-24D4^{-stop}	pH7RWG2	35S	Spectinomycin	
GAL4-DNA-BD::GpSPRY-24D4	pCR8/GW/TOPO GpSPRY-24D4^{+stop}	pDEST32 (Y2H bait)	ADH1	Gentamicin	
GpSPRY-24D4 (no tag)	pCR8/GW/TOPO GpSPRY-24D4^{+stop}	pK7WG2	35S	Spectinomycin	
GpSPRY-24D4::HA	pCR8/GW/TOPO GpSPRY-24D4^{+HA}	pK7WG2	35S	Spectinomycin	
CYFP::GpSPRY-24D4	pCR8/GW/TOPO GpSPRY-24D4^{+stop}	pCL113 (BiFC)	35S	Spectinomycin	
GpSPRY-24D4::CYFP	pCR8/GW/TOPO GpSPRY-24D4^{-stop}	pBatTL-B-sYFPC (BiFC)	35S	Spectinomycin	
eGFP::GpSPRY-33H17	pCR8/GW/TOPO GpSPRY-33H17^{+stop}	pK7WGF2	35S	Spectinomycin	
GpSPRY-33H17::eGFP	pCR8/GW/TOPO GpSPRY-33H17^{-stop}	pK7FWG2	35S	Spectinomycin	
mRFP::GpSPRY-33H17	pCR8/GW/TOPO GpSPRY-33H17^{+stop}	pH7WGR2	35S	Spectinomycin	
GpSPRY-33H17::mRFP	pCR8/GW/TOPO GpSPRY-33H17^{-stop}	pH7RWG2	35S	Spectinomycin	
GAL4-DNA-BD::GpSPRY-33H17	pCR8/GW/TOPO GpSPRY-33H17^{+stop}	pDEST32 (Y2H bait)	ADH1	Gentamicin	
GpSPRY-33H17 (no tag)	pCR8/GW/TOPO GpSPRY-33H17^{+stop}	pK7WG2	35S	Spectinomycin	
GpSPRY-33H17::HA	pCR8/GW/TOPO GpSPRY-33H17^{+HA}	pK7WG2	35S	Spectinomycin	
eGFP:: GpSPRY-22E10	pCR8/GW/TOPO GpSPRY-22E10^{+stop}	pK7WGF2	35S	Spectinomycin	
GpSPRY-22E10::eGFP	pCR8/GW/TOPO GpSPRY-22E10^{-stop}	pK7FWG2	35S	Spectinomycin	
mRFP::GpSPRY-22E10	pCR8/GW/TOPO GpSPRY-22E10^{+stop}	pH7WGR2	35S	Spectinomycin	
GpSPRY-22E10::mRFP	pCR8/GW/TOPO GpSPRY-22E10^{-stop}	pH7RWG2	35S	Spectinomycin	
GAL4-DNA-BD::GpSPRY-22E10	pCR8/GW/TOPO GpSPRY-22E10^{+stop}	pDEST32 (Y2H bait)	ADH1	Gentamicin	
GpSPRY-22E10 (no tag)	pCR8/GW/TOPO GpSPRY-22E10^{+stop}	pK7WG2	35S	Spectinomycin	
GpSPRY-22E10::HA	pCR8/GW/TOPO GpSPRY-22E10^{HA}	pK7WG2	35S	Spectinomycin	
eGFP:: GpSPRY-17I9-1	pCR8/GW/TOPO GpSPRY-17I9-1^{+stop}	pK7WGF2	35S	Spectinomycin	
GpSPRY-17I9-1::eGFP	pCR8/GW/TOPO GpSPRY-17I9-1^{-stop}	pK7FWG2	35S	Spectinomycin	
mRFP::GpSPRY-17I9-1	pCR8/GW/TOPO GpSPRY-17I9-1^{+stop}	pH7WGR2	35S	Spectinomycin	

GpSPRY-17I9-1::mRFP	pCR8/GW/TOPO GpSPRY-17I9-1^{-stop}	pH7RWG2	35S	Spectinomycin	
GAL4-DNA-BD::GpSPRY-17I9-1	pCR8/GW/TOPO GpSPRY-17I9-1^{+stop}	pDEST32 (Y2H bait)	ADH1	Gentamicin	
GpSPRY-17I9-1 (no tag)	pCR8/GW/TOPO GpSPRY-17I9-1^{+stop}	pK7WG2	35S	Spectinomycin	
GpSPRY-17I9-1::HA	pCR8/GW/TOPO GpSPRY-17I9-1^{+HA}	pK7WG2	35S	Spectinomycin	
CYFP::GpSPRY-17I9-1	pCR8/GW/TOPO GpSPRY-17I9-1^{+stop}	pCL113 (BiFC)	35S	Spectinomycin	
GpSPRY-17I9-1::CYFP	pCR8/GW/TOPO GpSPRY-17I9-1^{-stop}	pBatTL-B-sYFPC (BiFC)	35S	Spectinomycin	
NYFP::I1-1	pDONR221 I1-1^{+stop}	pCL112 (BiFC)	35S	Spectinomycin	GpSPRY-17I9-1 Y2H interactor
NYFP::I3-12	pDONR221 I3-12^{+stop}	pCL112 (BiFC)	35S	Spectinomycin	GpSPRY-17I9-1 Y2H interactor
NYFP::I4-2	pDONR221 I4-2^{+stop}	pCL112 (BiFC)	35S	Spectinomycin	GpSPRY-17I9-1 Y2H interactor
NYFP::I5-2	pDONR221 I5-2^{+stop}	pCL112 (BiFC)	35S	Spectinomycin	GpSPRY-17I9-1 Y2H interactor
NYFP::StCCD4	pDONR221 StCCD4^{+stop}	pCL112 (BiFC)	35S	Spectinomycin	GpSPRY-17I9-1 interactor
StCCD4::NYFP	pDONR221 StCCD4^{-stop}	pBatTL-B-sYFPN (BiFC)	35S	Spectinomycin	GpSPRY-17I9-1 interactor
GAL4-AD::StCCD4	pDONR221 StCCD4^{+stop}	pDEST22 (Y2H prey)	ADH1	Ampicillin	GpSPRY-17I9-1 interactor
eGFP::StCCD4	pDONR221 StCCD4^{+stop}	pK7WGF2	35S	Spectinomycin	GpSPRY-17I9-1 interactor
StCCD4::eGFP	pDONR221 StCCD4^{-stop}	pKF7WG2	35S	Spectinomycin	GpSPRY-17I9-1 interactor
mRFP::StCCD4	pDONR221 StCCD4^{+stop}	pH7WGR2	35S	Spectinomycin	GpSPRY-17I9-1 interactor
StCCD4::mRFP	pDONR221 StCCD4^{-stop}	pH7RWG2	35S	Spectinomycin	GpSPRY-17I9-1 interactor
eGFP::GpSPRY-414-2	pCR8/GW/TOPO GpSPRY-414-2^{+stop}	pK7WGF2	35S	Spectinomycin	
GpSPRY-414-2::eGFP	pCR8/GW/TOPO GpSPRY-414-2^{-stop}	pK7FWG2	35S	Spectinomycin	
mRFP:: GpSPRY-414-2	pCR8/GW/TOPO GpSPRY-414-2^{+stop}	pH7WGR2	35S	Spectinomycin	
GpSPRY-414-2::mRFP	pCR8/GW/TOPO GpSPRY-414-2^{-stop}	pH7RWG2	35S	Spectinomycin	
GAL4-DNA-BD::GpSPRY-414-2	pCR8/GW/TOPO GpSPRY-414-2^{+stop}	pDEST32 (Y2H bait)	ADH1	Gentamicin	
GpSPRY-414-2 (no tag)	pCR8/GW/TOPO GpSPRY-414-2^{+stop}	pK7WG2	35S	Spectinomycin	
GpSPRY-414-2::HA	pCR8/GW/TOPO GpSPRY-414-2^{+HA}	pK7WG2	35S	Spectinomycin	
CYFP::GpSPRY-414-2	pCR8/GW/TOPO GpSPRY-414-2^{+stop}	pCL113 (BiFC)	35S	Spectinomycin	
GpSPRY-414-2::CYFP	pCR8/GW/TOPO GpSPRY-414-2^{-stop}	pBatTL-B-sYFPC (BiFC)	35S	Spectinomycin	
NYFP::G1-5	pDONR221 G1-5^{+stop}	pCL112 (BiFC)	35S	Spectinomycin	GpSPRY-414-2 Y2H interactor
G1-5::NYFP	pDONR221 G1-5^{-stop}	pBatTL-B-sYFPN (BiFC)	35S	Spectinomycin	GpSPRY-414-2 Y2H interactor
NYFP::StCLASP	pDONR221 StCLASP $^{+stop}$	pCL112 (BiFC)	35S	Spectinomycin	GpSPRY-414-2 interactor
StCLASP::NYFP	pDONR221 StCLASP $^{-stop}$	pBatTL-B-sYFPN (BiFC)	35S	Spectinomycin	GpSPRY-414-2 interactor
GAL4-AD::StCLASP	pDONR221 StCCD4^{+stop}	pDEST22 (Y2H prey)	ADH1	Ampicillin	GpSPRY-414-2 interactor
eGFP::StCLASP	pDONR221 StCLASP^{+stop}	pK7WGF2	35S	Spectinomycin	GpSPRY-414-2 interactor
StCLASP::eGFP	pDONR221 StCLASP^{-stop}	pKF7WG2	35S	Spectinomycin	GpSPRY-414-2 interactor
mRFP::StCLASP	pDONR221 StCLASP^{+stop}	pH7WGR2	35S	Spectinomycin	GpSPRY-414-2 interactor
StCLASP::mRFP	pDONR221 StCLASP^{-stop}	pH7RWG2	35S	Spectinomycin	GpSPRY-414-2 interactor

Appendix 5. **Overview of the results obtained from the functional assays for the six SPRYSECs assessed**.

SPRYSECs	ISH	Plant cell-death suppression									Glycosylation prediction	
		INF1	Flg22	R2/Avr2	R3a/Avr3a^{KI}	Cf-4/Avr4	Cf-9/Avr9	Rx/PVX-CP	Gpa2/RBP-1	Mi1.2^{T557S}	N-Gly	O-Gly
GpSPRY-12N3	DG[#]	-	-	-	-	-	-	-	+	-	+	+
GpSPRY-24D4	DG[×]	-	-	-	-	-	-	-	-	-	+	+
GpSPRY-33H17	DG[#]	-	-	-	-	-	-	-	+	-	+	+
GpSPRY-17I9-1	DG	-	-	-	-	-	-	-	-	-	-	+
GpSPRY-22E10	DG[#]	-	-	-	-	-	-	-	-	-	+	+
GpSPRY-414-2	DG	-	+	-	-	-	-	-	+	-	-	+

SPRYSECs	Confirmed interactor (Y2H)	*In planta* localisation	Note
GpSPRY-12N3	None	Cytoplasm, nucleoplasm, accumulates in nucleolus	
GpSPRY-24D4	Probable receptor-like protein kinase (RLK) and an uncharacterised protein	Cytoplasm mainly, nucleoplasm, excluded from nucleolus	
GpSPRY-33H17	None	Cytoplasm, nucleoplasm, probably excluded from nucleolus	
GpSPRY-17I9-1	Carotenoid cleavage dioxygenase 4 (CCD4)	Cytoplasm, nucleoplasm, excluded from nucleolus	Details in Chapter3
GpSPRY-22E10	None	Cytoplasm, nucleoplasm, maybe slightly in nucleolus	
GpSPRY-414-2	Potato clip-associated protein (CLASP)	Cytoplasm, nucleoplasm	Details in Chapter4

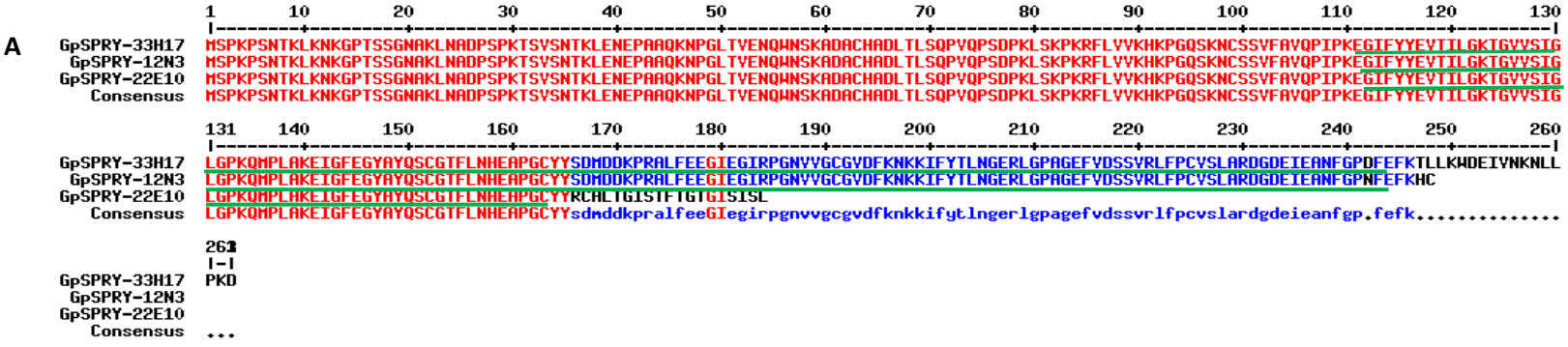
Note 1: For plant cell-death suppression and glycosylation prediction, '+' indicates that the SPRYSEC effector candidate can suppress the plant defence response or that glycosylation sites were predicted, while '-' indicates no suppression of plant defence or no predicted glycosylation sites.

Note 2: Some ISH data are from independent studies; '#' refers to Thorpe *et al.* (2014) while 'x' refers to Prof. John T Jones personal communication.

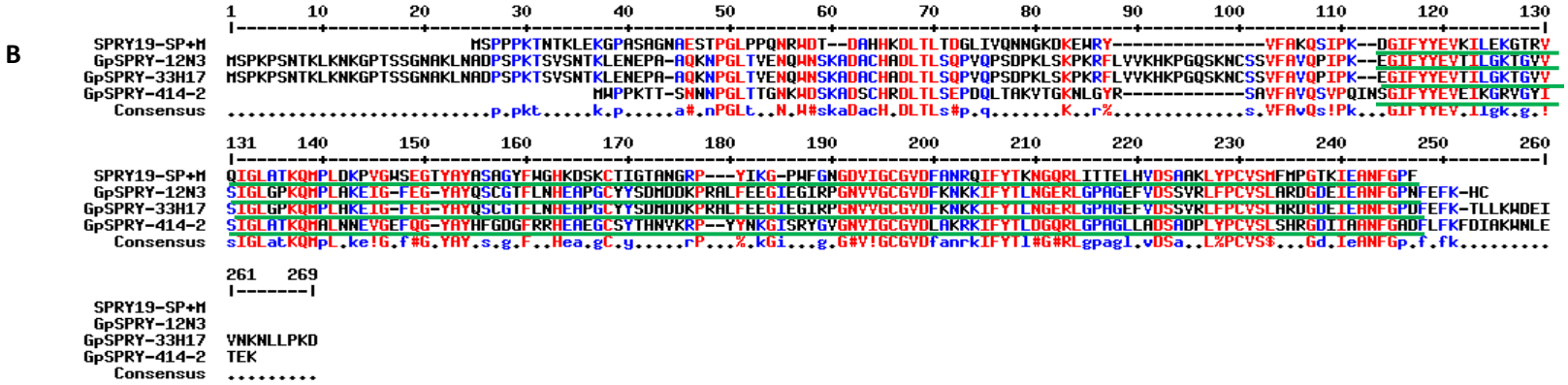
Abbreviations: DG = Dorsal Gland, ISH = *In situ* Hybridisation, Y2H = Yeast Two-Hybrid, N-Gly = N-linked glycosylation, O-Gly = O-linked glycosylation

Appendix 6. SPRYSEC sequence alignment. (A) Amino acid sequence alignment of the two *Globodera pallida* SPRYSECs that have been shown to suppress the plant hypersensitive reaction mediated by Gpa2 and RBP-1 recognition (GpSPRY-33H17 and GpSPRY-12N3) and the closest related *G. pallida* SPRYSEC investigated that didn't suppress plant defences (GpSPRY-22E10). (B) Amino acid sequence alignment of the three *G. pallida* SPRYSECs that have been shown to suppress plant defences and SPRYSEC-19 of *G. rostochiensis* that supresses the plant hypersensitive reaction mediated by resistance genes (Postma *et al*., 2012). In both (A) and (B) panels the sequences are presented without signal peptide and the SPRY domains are underlined in green. The consensus sequence is shown below the SPRYSECs sequences. Amino acids in red are conserved among all sequences and amino acids in blue are the most prevalent.

A

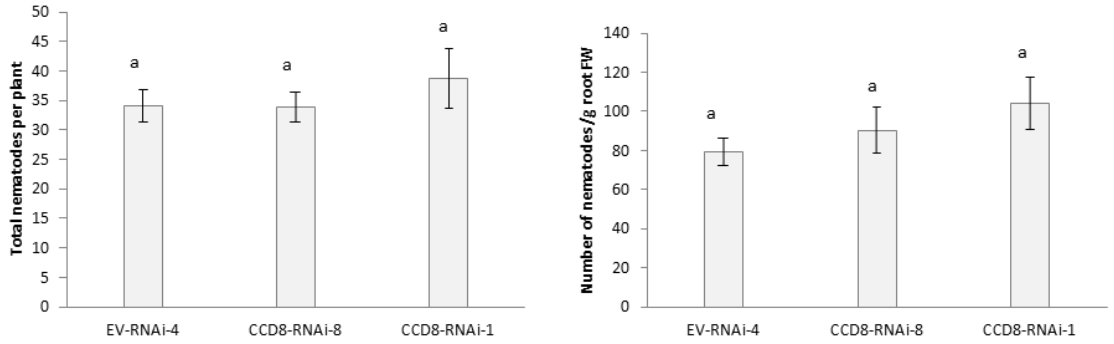


B

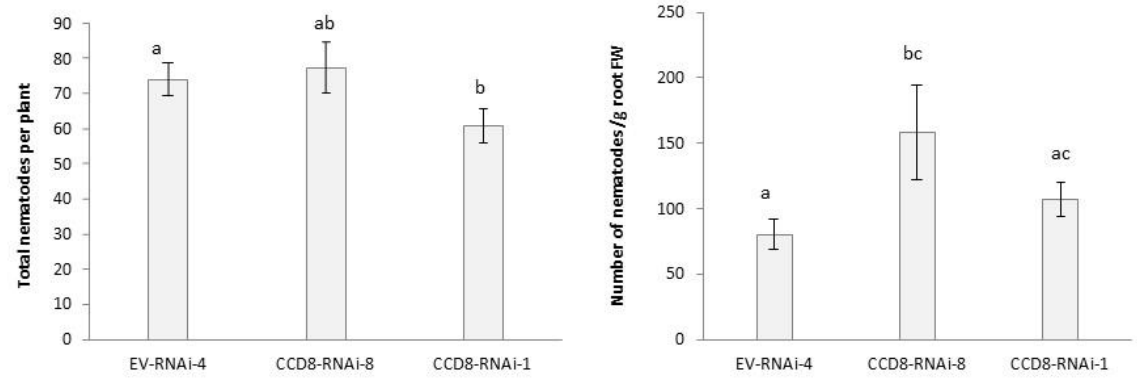


Appendix 7. The role of strigolactone in the interaction between potato and *G. pallida* remains unclear in this thesis. Three biologically repeated infection assays were carried out using two *CCD8*-RNAi mutant lines and one empty vector control. Experiment 1 was done with 10 plants of each control and *CCD8*-RNAi lines. No significant difference was observed among any of them. Experiment 2 was performed with 8 plants of each. No difference was significant except for *CCD8*-RNAi line 1 that showed significant reduction as to nematodes per gram of root compared with control. Experiment 3 appeared to give a different result with *CCD8*-RNAi line 8 giving significantly higher number of nematodes per gram of root while line 1 not showing any clear difference. As to the total nematodes per plant, line 8 didn't show a significant difference but there was a significant reduction in line 1. This experiment was carried out with 20 plants of each line. Bars show mean with standard error. Different letters above indicate the significance with $p<0.05$. Analysis was done by ANOVA analysis in SPSS.

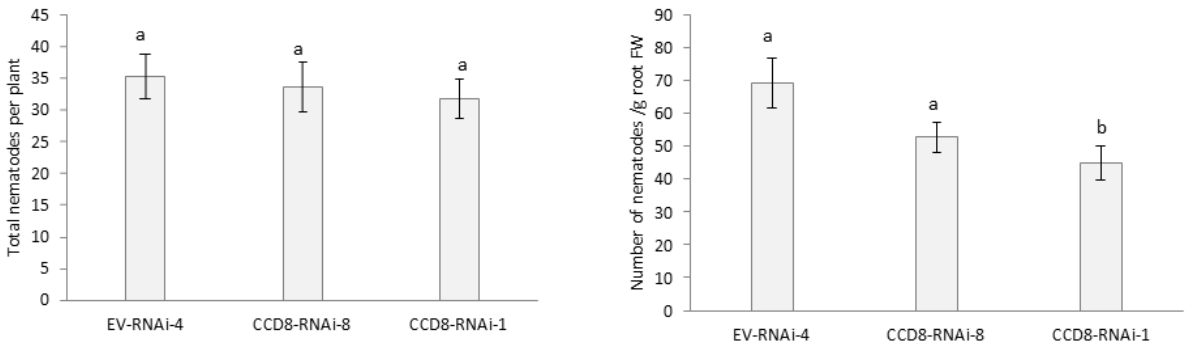
Experiment 1



Experiment 2



Experiment 3



Appendix 8. CLASP protein sequences alignments. Amino acid sequence alignment of the potato cytoplasmic linker protein (CLIP)-associated protein (StCLASP) with its tomato and Arabidopsis homologs, SlCLASP (Solyc09g063030) and AtCLASP (At2g20190), respectively. CLASP-N domains are underlined in green in the consensus sequence shown below the CLASP sequences. Amino acids in red are conserved among all sequences and amino acids in blue are the most prevalent.