Venom allergen-like proteins in secretions of plant-parasitic nematodes activate and suppress extracellular plant immune receptors

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To my mother Luz Marina Torres

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General Introduction

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Chapter 1

1.1 Introduction

Parasitic nematodes threaten human, animal and plant health in essentially all parts of the world. Soil-transmitted helminths infect over 1 billion people, mostly living in the tropics and the subtropics (*7-10*). Farmers spend billions of dollars on prevention and control of diseases caused by nematodes in their livestock and crops (*11-17*). Nematode infections can affect the host both directly and indirectly. Directly, an acute infection with filarial nematodes can lead to blindness and chronic disabling disfigurations in humans (*18, 19*), while an attack of soybean cyst nematodes can reduce plant yield up to 60% (*20*). Indirectly, nematode infections can alter the immune competence of a host for an extended period of time, making it more vulnerable to secondary infections by other opportunistic pathogens (*21*). Bacteremia caused by gram-negative bacilli, for example, is common in humans infected with parasitic roundworms (*22*), while *Fusarium oxysporum* wilt disease is more severe in cotton infected with root-knot nematodes (*23*).

It is important to understand how the immune system of plants and animals deals with parasitic diseases and how parasites overcome host immunity. This understanding is crucial if we want to protect our health and that of our livestock and if we want to secure global food supply in the future. The molecular mechanisms in host-parasite interactions have become the last frontier in the quest for understanding diseases and defense responses. Effectors are broadly defined as proteins and other small molecules produced by pathogenic organisms that alter host-cell structure and function. In parasitic nematodes, the effectors determine whether these roundworms can or cannot successfully infect a host (*24-30*). The objective of this thesis is the identification and functional characterization of a group of effector molecules known as venom allergen-like proteins (VAPs) from plant-parasitic cyst nematodes.

1.2 Cyst nematodes

Cyst nematodes (Box 1) are obligatory endoparasites that have a relatively small host range when compared to the polyphagous root-knot nematodes. After death, fertilized female cyst nematodes transform into a dark-colored, thick-walled protective structures known as cysts. The offspring of cyst nematodes, the infective juveniles, reside dormant in eggs within the confined space created by these remnants of a parent female. Root exudates released by host plants stimulate hatching of the second stage juveniles (J2s) from the eggs, which then break through the eggshell and emerge from the cyst. The environmental cues that lead to the escape of the juveniles from the thick-walled cyst are not well understood. When infective juveniles reach a host root, the parasites invade it by using the physical force of an oral stylet and

the biochemical activity of cell wall-degrading enzymes released through this needle-like structure. Next, endoparasitic infective juveniles migrate some time through the cortical cell layers of the root towards the vascular cylinder (*31-33*).

After this short migratory phase, the cyst nematode selects a host cell to begin the formation of a feeding site within or near the vascular cylinder. With its stylet, the nematode punctures the cell wall of this host cell –without breaking the plasma membrane– and releases effectors into the apoplast and the cytoplasm of the host cell. These effectors are thought to initiate the transformation of this host cell and other adjacent host cells into a large multicellular feeding site called a syncytium (*34*). The syncytium appears by the nematode-induced partial dissolution of cell walls between the initial syncytial cell and neighboring cells, resulting in a large joint protoplasm with high metabolic activity (*35*). This extraordinary increase in metabolic activity results from multiple duplications of the chromosomes and subcellular organelles inside syncytial cells caused by repetitive endoreduplication and an extensive transcriptional reprogramming (*36, 37*).

Cyst nematodes depend on the syncytium for all the nutrients they need for their development, growth, and reproduction. The establishment of the syncytium is associated with the loss of somatic muscles in the nematode, rendering the nematode from that moment onward immobile. Early in their development inside the root of a host plant, epigenetic factors such as food quality and availability determine the sex of the juveniles (*38*). After a few weeks of feeding on the syncytium, male cyst nematodes regain their mobility, which allows them to inseminate adult females. Females, on the contrary, stay sedentary and increase in size as their bodies fill with fertilized eggs. The eggs develop for approximately two more weeks, after which the

Box 1. A brief note on cyst nematode parasitism

Plant parasitism arose at least three times as an independent evolutionary innovation within the Phylum Nematoda (*1*). The most advanced lineage of plant parasites includes the sedentary root-knot and cyst nematodes, i.e. members of the family Heteroderidae (*2*). The potato cyst nematode *Globodera rostochiensis* originates in the South American Andes, where the nematode co-evolved with potato and other closely related Solanaceous plants species (*3*). There are more than ninety different Solanaceous species that can be parasitized by *G. rostochiensis*, including food crops such as potato, tomato, and eggplant (*4*). The beet cyst nematode *Heterodera schachtii* was the first nematode discovered as a plant parasite in Europe by the botanist H. Schacht causing a disease known as "beet fatigue" (*5*). More than 200 plant species in the families *Amaranthaceae* (sugar beets and spinach) and *Brassicaceae* (broccoli, cauliflower, cabbage, radish, mustard, and the model plant Arabidopsis) are hosts for *H. schachtii (6)*.

females die and become cysts. The eggs remain dormant in the cyst until the root system of a new host appears in the vicinity of the eggs (*39*).

1.3 Cyst nematode effectors

Effectors enable parasitic nematodes to invade host plants, modulate host immune responses, and initiate and maintain the feeding site (*26*). Although an effector functions to promote the virulence of a nematode, its recognition by the host immune system may also trigger a defense response (*40*). This thesis describes the nematode effector Gr-VAP1 from *G. rostochiensis* that is important for nematode virulence, but in some plant genotypes also activates plant defense responses.

Most of the current knowledge on the actions and functions of effectors comes from studies on bacterial, fungal and oomycete pathogens of the areal parts of plants. This is due to the fact that laboratories dedicated to research in plant pathology have mostly focused on fast-replicating pathogens causing diseases with overt symptoms in the leaves and stems. However, infections by sedentary plant-parasitic nematodes develop slowly and are confined to the root system of plants, and therefore nematode effectors have, figuratively speaking 'remained in the dark for a long time'. Only since the late 1990s have the number of functionally characterized nematode effectors and knowledge about their functions increased significantly (*25-27*).

Three pharyngeal glands (i.e. one dorsal and two subventral) produce the majority of effectors in cyst nematodes (*41, 42*). The subventral pharyngeal glands are most active at the onset of infections and produce, among others, effectors that facilitate plant cell wall degradation i.e. β-1,4-endoglucanases (*43*), pectate lyases (*44*), and expansins (*45*). The role of other effectors produced in the subventral pharyngeal glands, such as the venom allergen-like proteins, in parasitism is less well understood. The synthesis of effectors in the dorsal pharyngeal gland coincides primarily with feeding site initiation and maintenance inside the host plant (*46*). Perhaps because feeding sites are uniquely associated with infections by sedentary plant-parasitic nematode in host plants, many effectors produced in the dorsal pharyngeal gland have no similarity to effectors from other plant microbes. To date, the best-studied effectors from cyst nematodes are the CLE peptides that mimic plant CLAVATA-3/Endosperm surrounding region (CLE) peptides involved in shoot meristem differentiation, root growth, and vascular development (*47, 48*).

Besides their involvement in the initiation and maintenance of feeding sites, effectors from the dorsal pharyngeal gland have also been associated with alterations in host immune responses. Heterologous expression of the effector 10A06 from *H. schachtii* increases antioxidant protection, interrupts salicylic acid signaling in *Arabidopsis thaliana* and alters the disease

susceptibility of these transgenic plants (*49*). Similarly, the expression in plants of the *H. schachtii* effector Hs4F01, a homologue of plant annexins, increases nematode susceptibility by a mechanism that has thus far remains unclear (*50*). A SPRYSEC effector from *G. rostochiensis* physically interacts with SW5F, a coiled-coil nucleotide-binding leucine-rich repeat (CC-NB-ARC-LRR) protein. Unexpectedly, this interaction does not elicit a defense-related programmed cell death nor are plants harboring the SWF5 resistant to *G. rostochiensis*. In fact this SPRYSEC effector seems to have the opposite effect, as it suppresses programmed cell death and disease resistance mediated by CC-NB-ARC-LRR-type of resistance proteins (*51*). However, another SPRYSEC effector named RBP-1 from *G. pallida* activates a defense-related programmed cell death when it is co-expressed with the nematode resistance protein Gpa2 in the leaves of *Nicotiana benthamiana* (*52*). These findings suggest that cyst nematodes deliver effectors into host cells that can both activate and suppress defense responses mediated by cytoplasmic plant immune receptors.

1.4 Venom allergen-like proteins

The venom allergen-like proteins (VAPs) belong to the SCP/TAPS (Pfam accession number PF00188) protein family. The name SCP/TAPS is short for the acronym SCP/Tpx-1/Ag-5/PR-1/Sc7, and refers to several different functional classes of proteins such as sperm-coating proteins (SCP), mammalian testis-specific extracellular proteins (Tpx), venom-allergens from wasps and ants (Ag), and glioma and plant pathogenesis-related (PR) proteins. The SCP/TAPS protein family belongs to the cysteine rich secretory protein (CRISP) superfamily. Proteins from this superfamily play a role in reproduction, development, immunity, and several pathologies (*53, 54*). However, their exact functions and molecular actions have remained largely elusive thus far.

Members of the SCP/TAPS protein family are present in parasitic nematodes from the orders Strongylida, Rhabditida, Tylenchida, Spirurida and Ascaridida (Clades V, IV, and III within the phylum Nematoda). Three types of SCP/TAPS have been reported for nematodes, including double SCP domain, C-type single SCP domain, and N-type single SCP domain proteins. The latter two types are either similar to the carboxyl or amino terminus of the double SCP domain proteins. N- and C-type single and double sperm-coating domain-containing proteins occur in animal-parasitic nematodes. While only N-type single sperm-coating domain proteins have been found in plant-parasitic nematodes (*53, 54*).

Although signature sequences for the classification of members of the SCP/TAPS family have been identified, the nomenclatures in use for these proteins vary within different scientific communities (*53, 55*). The first

SCP/TAPS protein from animal-parasitic nematodes was found in secretions from the hookworm *Ancylostoma caninum* (*56*), and therefore animal parasitologists coined the term *Ancylostoma*-secreted proteins or activation-associated proteins (ASPs), to designate SCP/TAPS proteins from animal-parasites. On the other hand, in plant nematology, the most common name used to describe SCP/TAPS is venom allergen-like proteins (VAPs or VALs) because the first described SCP/TAPS protein of animal origin was found in snake venoms (*53, 54*). For the remainder of this thesis all SCP/TAPS proteins that have been identified in the secretions of plant-parasitic nematodes are referred to as venom allergen-like proteins (or VAPs).

The venom allergen-like proteins are probably the most abundant proteins in excretory-secretory products of many animal-parasitic nematodes (*29*). For their relative abundance at the onset of parasitism, venom allergen-like proteins are believed to be important for the establishment and persistence of infections by nematodes in animals (*57, 58*). It has been shown that *A. caninum* neutrophil inhibitor factor (NIF), an SCP/TAPS molecule, interferes with the adhesion of activated neutrophils to vascular endothelial cells and reduces the release of H_2O_2 from activated neutrophils (*59*). Furthermore, NIF blocks the binding of human platelet integrins to their ligands (*60*). The venom allergen-like protein Na-ASP2 from the hookworm *Necator americanus* may act as antagonistic ligand of the complement receptor 3, which may prevent chemotaxin binding and therefore may alter immune responses (*61, 62*). Ov-ASP-1 and -2 from *Onchocerca volvulus* stimulate an angiogenic response when injected in mice corneas (*63*). Although these venom allergen-like proteins are linked to innate immunity in animals, their exact functions in parasitism and the underlying molecular mechanisms are not known.

In plant parasites, venom allergen-like proteins have been identified in *Heterodera glycines* (Hg-VAP-1 and -2; (*64*)), *Meloidogyne incognita* (Mi-VAP-1 and Mi-VAP-2; (*65, 66*)), *Ditylenchus africanus* (Da-VAP-1; (*67*)), and in *Bursaphelenchus xylophilus* (Bx-VAP-1, -2, and -3; (*68*)). Genes coding for VAPs in plant-parasitic nematodes are specifically up-regulated in infective juveniles during the onset of parasitism in plants. Although the venom allergen-like proteins of plant-parasitic nematodes are thought to be involved in the establishment of persistent infections of these nematodes in plants, there is currently little experimental evidence to support this hypothesis.

1.5 Plant innate immunity

Plants defend themselves against a myriad of pathogens and pests by using a complex multilayered innate immune system. Most attackers are unable to invade a plant because of close to impenetrable constitutive physical and biochemical barriers. Invaders that manage to breach these barriers encounter

extracellular pattern-recognition receptors that recognize pathogen/microbe-associated molecular patterns (PAMPs or MAMPs) (*69-73*). A classic example of PAMP-mediated recognition is the perception of bacteria by the cell surface receptor FLS2 in *A. thaliana* that detects a short region of flagellin, which is the main component of a flagellum in many plant-pathogenic bacteria (*74, 75*). To date, little is known of highly conserved epitopes on the exterior of nematodes that act as PAMPs in plants. However, it has been suggested that highly glycosylated proteins in the cuticle of plant-parasitic nematodes may be recognized as PAMPs by the host (*76*). The defense response that plants mount following the recognition of PAMPs is referred to as PAMP-triggered immunity (PTI).

Some pathogens use effectors to suppress PTI, resulting in effector-triggered susceptibility. However, plants have evolved extracellular and intracellular immune receptors, known as resistance proteins, with novel recognition specificities towards these effectors. These resistance proteins confer to plants another layer of protection against specific strains of pathogens carrying matching effectors by a mechanism known as effector-triggered immunity (ETI) (*69, 70*).

The detection of effectors by plant resistance proteins is captured in the classical "gene-for-gene" model of recognition specificity that forms the basis of current disease resistance breeding in all major food crops (*77*). A small number of nematode resistance genes have currently been isolated, all of which encode intracellular nucleotide-binding leucine-rich repeat (NB-LRR) proteins (i.e. *Mi-1 (78, 79)*, *Hero A (80, 81)*, *Gpa2 (82)*, and *Gro1-4 (83)*). *Gpa2* and *Gro1-4* confer resistance to a narrow range of pathotypes from a single nematode species (*84-86*). Despite also being considered as classical major resistance proteins, the resistance proteins Mi-1 and Hero A from tomato plants display broad-spectrum recognition specificities to multiple unrelated invaders. The molecular mechanisms underlying the multiple recognition specificities of Mi-1 and Hero A are not clear.

1.6 Direct *vs.* indirect recognition of effectors

The most straightforward mechanism underlying the recognition of effectors by immune receptors involves a direct physical interaction between effector and receptor molecules. However, because of many fruitless attempts to demonstrate such ligand-like interactions, it seems that a direct recognition of pathogen effectors by resistance proteins might actually be an exception. This notion inspired the formulation of the 'guard' model to explain the specific recognition of effectors by resistance proteins via indirect physical interactions. In the 'guard' model, plant immune receptors monitor the "health" status of other host molecules that appear to function as virulence targets of plant pathogens. Thus, in this model immune receptors react to

disease-signaling perturbations induced by pathogen effectors in these virulence targets (*70, 87*). For example, the recognition of the effector Avr2 of the fungus *Cladosporium fulvum* by the extracellular-LRR resistance protein Cf-2 in tomato plants requires an apoplastic papain-like cysteine protease named Rcr3 (i.e. <u>R</u>equired for <u>C</u>ladosporium fulvum <u>r</u>esistance 3) (*88*). It is thought that Cf-2 activates defense responses to *C. fulvum* when it senses Avr2-induced perturbations in Rcr3. Thus, Cf-2 recognizes the effector Avr2 indirectly via its actions on other host molecules.

1.7 Papain-like cysteine proteases in plants

In chapter 3 of this thesis, we demonstrate that a venom allergen-like protein of a plant-parasitic nematode physically associates with apoplastic papain-like proteases of host plants. Proteases cleave peptide bonds of other proteins and play a role in development, signaling cascades, and regulation of defense responses (*89-91*). Cysteine proteases hydrolyze peptide bonds in proteins by using a nucleophilic cysteine residue in their active site (*91*). There are 70 different families of cysteine proteases, divided over 12 different clans (*92*). Papain-like cysteine proteases (PLCPs) are relatively small proteins of 23-30 kDa that are produced with an N-terminal signal peptide for secretion and an autoinhibitory domain. This autoinhibitory prodomain covers the active site rendering the protease inactive until it is cleaved off (*93*). PLCPs in plants have been associated with senescence and defense (*90, 94*). The PLCPs Rcr3 and Pip1 (*Phytophthora* inhibited protease 1) are expressed in tomato plants upon pathogen attack (*95, 96*). The PLCP C14 of *N. benthamiana*, was recently shown to play an important role in plant immunity, as an altered expression of this protease affected plant susceptibility to *P. infestans* (*97*). The question of whether PLCPs also play a role in nematode-plant interactions will also be addressed in this thesis.

1.8 Outline of this thesis

A common characteristic of plant- and animal-parasitic nematodes is their phenomenal persistence inside the host, presumably resulting from their ability to suppress host immunity. However, the mechanisms of immunomodulation by parasitic nematodes in plants and animals are not well understood. The overall objective of this thesis is to study the role of the venom allergen-like effector proteins (VAPs) from plant-parasitic nematodes.

In **chapter 2** of this thesis, we review the morphological, molecular and physiological adaptations of plant-parasitic nematodes within the context of plant immunity. In **chapter 3**, we focus on the identification of host targets of venom allergen-like proteins. We show that the venom allergen-like protein Gr-VAP1 from the potato cyst nematode *G. rostochiensis* targets among others

the papain-like cysteine protease Rcr3[pim] and that this interaction activates a Cf-2-mediated defense response in tomato plants. However, in the absence of Cf-2, Rcr3[pim] seems to enhance nematode susceptibility. In **chapter 4**, we used plant- and cell-based assays to investigate the virulence function of venom allergen-like proteins and we show that these effectors suppress defense responses specifically mediated by extracellular immune receptors. In **chapter 5**, we broadened the scope of our research and focused on the role of papain-like cysteine proteases, including Rcr3[pim], in nematode-plant interactions. We show, with Arabidopsis knockout mutants that papain-like cysteine proteases from diverse subfamilies are important in plant immune responses to cyst nematodes. Finally, in **chapter 6**, we further discuss a model of our findings and indicate future directions for research on venom allergen-like proteins.

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Chapter 2

Survival of Plant-parasitic Nematodes inside the Host

Chapter 2

2.1 Introduction

Plant parasitism arose at least three times as an independent evolutionary innovation within the phylum Nematoda during a period of ~350 million years (*1*). The most advanced lineage of plant-parasites includes the sedentary root-knot nematodes and cyst nematodes, i.e. members of the family Heteroderidae (*2*). After a migratory phase of host finding and invasion, root-knot and cyst nematodes transform host cells into complex metabolically active structures from which they acquire their nutrients. Feeding site formation is associated with loss of mobility in root-knot and cyst nematodes. Thus, these parasites have evolved towards an absolute dependency on the food provided by a single feeding structure. Although perhaps not so advanced, several parasites from the other two lineages also transform host cells to some degree prior to feeding.

Feeding on plants has led to convergent morphological adaptations in all three lineages of plant–parasitic nematodes. Herbivory in nematodes is, for example, always associated with specialized morphological adaptations in the outer surface of the nematode and, most notably, in the feeding apparatus. In this chapter we review the recent literature on various aspects of nematode survival in plants. We briefly summarize morphological, molecular, and physiological adaptations to plant-parasitism in nematodes (see also (*1, 3, 4*). We also describe the life support structures induced by nematodes in host plant tissues and, because plants appear to 'tolerate' feeding nematodes, we examine evidence that nematodes must have evolved ways to modulate the innate immunity of the host. We summarize current concepts of innate immunity in plants, which are mostly founded on work done with plant pathogenic fungi and bacteria. Finally, we address the molecular tools used by plant-parasitic nematodes to modulate host defense responses.

2.2 Morphological Adaptations to Plant Parasitism

2.2.1 Cuticle, surface coat and cuticular camouflage

The external cuticle of nematodes is an unusual and complex multilayered structure that acts as an exoskeleton (*5-7*). The cuticle is made of collagens and a variety of other proteins, lipids, and carbohydrates (*8*). The transitions through successive developmental stages in all members of the phylum Nematoda are marked by a moult. During each moult the cuticle is replaced with a new one, which is assembled from components produced in the underlying hypodermis. In plant-parasitic nematodes the rigid cuticular lining of the pharynx, including the feeding apparatus, is also renewed during moulting.

Besides maintaining the shape of the nematode, the cuticle also constitutes a strong protective barrier. The cuticle provides an impervious interface between nematode and host cells, which may provide protection against host defense responses. The outer surface of the nematode cuticle is covered with a negatively charged carbohydrate-rich surface coat (*8*). Glandular cells in the head and tail region of the nematode are believed to secrete the components that make up the surface coat. However, the exact origin and composition of the surface coat is not clear. Binding of lectins, human red blood cells, and gold-labelled glycoproteins to the surface coat of root-knot nematodes indicate that the surface coat includes carbohydrate-binding proteins (*8-11*).

The surface coat of animal-parasitic nematodes is implicated in two distinct immune evading strategies. The first strategy involves the shedding of the surface coat to ward off immune cells attacking the worm. For example, when immune cells attack *Toxocara canis*, the nematode sheds its mucin-based surface coat to escape from attached killer cells (*12, 13*). A question that needs further research is whether plant-parasitic nematodes also deploy surface coat shedding to evade host innate immunity. Plants do not have mobile immune cells that can be directed towards an invader. Instead, many sedentary plant parasites lay embedded within host tissue and have to deal with the defense responses from surrounding host cells. So it is difficult to envisage how surface coat shedding could provide plant-parasitic nematodes protection to host immunity. However, labelling experiments suggest that root-knot nematodes nonetheless shed their surface coat during host invasion. By shedding the surface coat during the transition from migratory to sedentary invader, the nematode may change the composition of the surface coat to adapt it to a new and hostile microenvironment (*14*). Mucin-like proteins that have been identified in the pharyngeal glands of plant-parasitic nematodes could be involved in changes of the surface coat ((*15*) GenBank accession AAC62109).

The second strategy deployed by animal parasitic helminths to evade the host's immune system is cuticular camouflage. To achieve cuticular camouflage animal parasites cover themselves with host molecules so that they are no longer recognized as non-self in the host. In the current concepts of cuticular camouflage a key role is assigned to C-type lectins (*16*). C-type lectins are capable of binding carbohydrate moieties of glycosylated host proteins, such as major histocompatibility complex class I antigens, C3 complement proteins, and IgG immunoglobulins (*16*). Because host C-type lectins direct diverse immunity related processes, parasite C-type lectins could also compete with the natural glycosylated ligands of the host C-type lectins. It has, for example, been proposed that parasite C-type lectins sequester alarm-signaling ligands of host C-type lectin receptors. Secretory C-type lectins have also been found in the pharyngeal glands of the soybean cyst nematode, *Heterodera glycines*, suggesting that these nematodes have at

least the potential to use a type of cuticular camouflage in plants (*17*). Some support for this hypothesis stems from a knock-down by RNA interference (RNAi) of a C-type lectin in *H. glycines*, which significantly reduced nematode survival inside host plants (*18*).

Another interesting case of cuticular camouflage occurs in the interaction between the entomopathogenic nematode, *Steinernema feltiae*, and its lepidopteran host *Galleria mellonella* (*19-21*). *Steinernema feltiae* penetrates the haemocoel of lepidopteran insects wherein it releases endosymbiosis bacteria that kill the host. The innate immune system of the lepidoptera consists of at least three components including antibacterial peptides, the prophenoloxidase activation system (humoral responses), and parasite encapsulation (cellular response). In an immunocompetent lepidopteran host, innate immunity is fully equipped to nip a bacterial outbreak in the hemocoel in the bud. However, *S. feltiae* manages to give its unleashed endosymbionts in the hemocoel a head start with the deployment of immune evasion and depression tactics centered on cuticular lipids. These cuticular lipids specifically bind various host hemolymph proteins involved in the synthesis of antimicrobial peptides and the proteolytic activation of prophenoloxidase. Further aspecific coating with host factors is believed to make the *S. feltiae* virtually undetectable as non-self, thus also preventing proper humoral and cellular responses that normally lead to parasite encapsulation.

2.2.2 The oral stylet — a multi-tool for nematodes

All plant cells are insulated by a rigid cell wall, which constitutes a protective container for the protoplast (*22*). In order to gain access to the host cell cytoplasm nematodes penetrate plant cell walls with a protrusible cuticular stylet, which is also used to inject secretions into host cells and to take up plant nutrients (*3*). Fierce outward movements of the stylet provide the necessary physical impact to perforate cell walls during host invasion. A more subtle behaviour is associated with feeding from plant cells, presumably to avoid the collapse of the host cell protoplasm (*23*). The feeding routine of the nematode includes repeated cycles of stylet insertion into host cell, release of secretions and uptake of plant solutes (*24*). Whether the stylet actually penetrates the cell membrane during feeding or the nematode uses an alternative mode of bidirectional transport over the cell membrane without disturbing the integrity of the membrane is still a matter of debate. Passive diffusion over the cell membrane into host cytoplasm can be excluded as a mode of transport, because the molecular mass of many of the nematode secretory proteins is too high.

Work on the plant pathogenic oomycete, *Phytophthora infestans*, has revealed a possible mechanism for the delivery of pathogen proteins into the host cells (*25, 26*). *Phytophthora infestans* forms haustoria in between the cell

wall and the cell membrane of host cells. The haustoria are enveloped by, but do not penetrate, the cell membrane of host cells. *Phytophthora infestans* releases secretions, so-called effectors, via its haustoria into the extracellular matrix of recipient host cells. One large class of *P. infestans* effectors, named RxLR-DEER effectors after a conserved sequence motif, are translocated over the cell membrane into the cytoplasm via a specific carrier/receptor protein (*27*). Although the translocation mechanism of RxLR-DEER effectors is not completely clear, there is a striking similarity with the translocation of effectors by the malaria parasite *Plasmodium* into human cells. Because biotrophic nematodes do not evidently penetrate the cell membrane a similar translocation system could deliver the nematode secretions into host cytoplasm. One approach to test this hypothesis is to systematically scan nematode secretory proteins for RxLR-DEER-like or other motifs that could function as tags for a translocation pathway.

2.2.3 Pharyngeal glands – the source of all evil

A quick glance through the lens of a microscope by students often raises questions about the huge nuclei in the pharyngeal region of plant-parasitic nematodes (*28-30*). These nuclei mark the position of large single-celled pharyngeal (or oesophageal) glands alongside the anterior section of the digestive tract. The sheer size of the nuclei and gland cells is a remarkable adaptation to plant-parasitism in nematodes. It was for this reason alone that secretions from the pharyngeal glands were associated with parasitism long before the nature of these secretions became known (*31, 32*). The pharyngeal glands in the advanced cyst and root-knot nematodes have been studied in particular detail. Therefore, most of our current knowledge of the pharyngeal gland cells and their secretions derives from these sedentary endoparasites (see the sections below for more details).

The pharyngeal glands are flask shaped cells with a large highly active nucleus, which lies embedded in an elaborate endoplasmic reticulum with numerous Golgi-bodies (*28, 29*). The nuclei are positioned in the posterior wider section of the gland cells, which narrows down into long extensions towards the head of the nematode. The pharyngeal gland cells are filled with secretory granules floating forward from the Golgi-bodies to the ampulla. In the collecting reservoir of the ampullae the secretory granules release their contents by means of exocytosis into the lumen of the pharynx. Inside the pharyngeal lumen the secretions move to the stylet base, and finally emanate from the orifice in the stylet tip.

Cyst and root-knot nematodes have three pharyngeal gland cells of which one is positioned in the dorsal sector, and two are located in the subventral sector of the pharyngeal region. Earlier studies have revealed that the subventral pharyngeal gland cells are mostly, but not exclusively, active

in migratory stages (*33, 34*). The dorsal pharyngeal gland is mainly active when the nematode is feeding. Remarkably, potato cyst nematodes can be fooled to believe that they are inside plants with a brief exposure to collected potato root exudates (*31*). These exudates activate gene transcription and the synthesis of secretions in both types of pharyngeal glands *in vitro* (*35*).

2.3 Molecular and Physiological Adaptations to Plant Parasitism

2.3.1 Host invasion

The first real direct encounter of the nematode and host plant, and more specifically the host's defenses, is during invasion of the host. Vertebrate animals have mobile defender cells to respond to invaders, and a nearly infinite diversity in binding potential in immunoglobulins to tag invaders as being foreign so that the defender cells can exterminate them (*36*). The immune system in vertebrates also builds on a memory of previous encounters with invaders, which allows them to respond quicker the next time the same type of invader makes an attempt to attack the host animal. Plants do not have such an adaptive immune system that can be directed towards invading parasites and, as far as is known, they do not have the capabilities to build a memory of previous encounters with microbes. Instead plants have evolved other unique features to protect themselves against the ingress of pathogens (*37*).

The first line of defense in plants is the cell wall, which is a nearly impenetrable physical barrier around each individual cell. The plant cell wall is an extremely complex structure including a variety of highly diverse carbohydrate polymers, mixed with extended hydroxy-proline rich glycoproteins, and aromatic compounds such as lignins (*22*). The main structural components of the cell wall are the cellulose microfibrils that, together with the xyloglycans and glucoarabinoxylans, provide the cell wall its rigidity. The structural network of cellulose and cross-linking glycans is embedded in a gel matrix made of pectins. The backbone of pectic polysaccharides consists of blocks of α-1,4 linked polygalacturonic acid residues interspersed with regions of alternating galacturonic acid and rhamnose residues, of which the rhamnose residues might be decorated with short galactans and arabinans (*38*).

The finding of various endogenous cell wall-degrading enzymes in nematodes (Table 1) over the last ten years implies that the physical impact of the stylet alone is not sufficient to perforate the cell wall. All nematode genera including plant-feeders studied to date appear to use cellulases to breakdown cellulose. Cellulases hydrolyze the cellulose polymers into oligosaccharides making the micofibrils significantly weaker. The cellulose microfibrils are tethered together by xyloglycans or glucoarabinoxylans depending on the

plant species. Cross-linking hemi-celluloses in dicotyledons have a glycan backbone, whereas non-commelinoid monocots use xylan-based polymers. Dicot xyloglycans are a substrate of cellulases, but xylan hydrolysis in monocots requires specific endoxylanases. The discovery of endoxyalanases in root-knot nematodes and burrowing nematodes (*Radopholus* spp.) feeding on monocots is therefore a possible adaptation to the cell wall composition of monocots.

Table 1. Cell-wall modifying proteins in plant-parasitic nematodes and their substrates[a]

Substrate	Enzyme-class	Genus
Cellulose	Cellulases	*Globodera, Heterodera Meloidogyne, Bursaphelenchus, Ditylenchus, Radopholus, Pratylenchus*
	Cellulose-binding proteins	*Globodera, Heterodera, Meloidogyne*
	Expansin (or expansin-like)	*Globodera, Heterodera, Bursaphelenchus, Meloidogyne, Ditylenchus*
Xyloglycans and Glucoarabinoxylans	Endoxylanases	*Meloidogyne, Radopholus*
Pectins	Pectatelyases	*Globodera, Heterodera Meloidogyne, Bursaphelenchus*
	Polygalacturonase	*Meloidogyne*

[a](*39-63*)

A novel class of cell wall modifying proteins targeting the hemicellulose/cellulose network are the expansins (*64*). Although the biochemistry of expansin activity is not completely clear, they are believed to weaken the non-covalent interactions between cellulose microfibrils and associated xyloglycan and glucoarabinoxylans. Expansins induce a measurable relaxation of the polymer interactions in the plant cell wall, allowing it to expand as a result of hydrostatic forces (*65*). For some time expansins were considered to be a plant-specific evolutionary innovation (*66*) but the recent discovery of functional expansins in pharyngeal gland secretions of plant-parasitic nematodes challenges this view (*51, 63, 67*). So far, all nematodes secreting cellulases also appear to release expansins, which suggests that the concerted action of cellulases and expansins may both be required to weaken the structural rigidity of the cell wall. The idea is that

expansins open up the hemi-cellulose/cellulose network to make it more accessible for cellulases.

The cellulose-binding proteins in nematode secretions are also associated with cellulose degradation. Cellulose-binding proteins have a Type II cellulose-binding domain attached to a short stretch of the amino acids with no match in the current sequence databases. The function of the ancillary domain in cellulose-binding proteins is not clear, other than that it likely acts on plant cell walls. Recent work on a cellulose-binding protein from *H. glycines* suggest that it may help to recruit plant cell wall degrading enzymes, not so much for host invasion but for cell wall degradation during feeding site development (*61*).

The structural hemicellulose/cellulose scaffold is embedded in pectic polysaccharides. Pectins are important for water retention, for determining the size exclusion limit of cell walls, and for the defense against a variety of plant pathogens. Pectins have extremely diverse decorations, and the degradation of pectins often involves enzymes to remove these decorations (i.e. esterase) and so-called backbone cutters (i.e. lyases and hydrolases). Two types of pectin degrading enzymes have been found in pharyngeal gland secretions of plant-parasitic nematodes at present. Both types, pectate lyases and polygalacturonases, are backbone cutters. Most of the plant-parasites studied to date secrete pectate lyases, while polygalacturonases have only been found in root-knot nematodes. Moreover, the overall picture arising from the current sequencing projects is that root-knot nematodes deploy a more diverse repertoire of cell-wall modifying proteins than cyst nematodes. This would make sense given that, in contrast to cyst nematodes, the majority of root-knot nematodes have extremely wide host ranges and are stealthy invaders of plants (*68*).

2.3.2 Feeding behaviour and structures

In plant pathology a classical division is made between necrotrophs feeding on dead cells of the host and biotrophs requiring living host tissues to feed on. The most advanced biotrophs are the obligate sedentary plant-parasites such as the cyst and root-knot nematodes. They transform host cells into specialized feeding structures to feed on for several weeks. One of the great mysteries in plant nematology is how these parasites can take up vast quantities of food from these feeding structures without actually killing the host cells. The answer probably is that the nematodes take full control over gene expression in these host cells, which changes them into life support systems, and essentially governs the host's innate immunity.

For more detailed descriptions of nematode-induced feeding structures the reader is referred to excellent reviews (*69-71*). Here we will only briefly summarize current insights in cyst and root-knot nematode feeding structure

development to emphasize the distinctive nature of these sites as a unique cellular and molecular phenomenon contributing to the survival and development of the parasite.

Shortly after host invasion cyst and root-knot nematodes start probing host cells for their competence to be adequate feeding structures. Following positional or development cues in the plant the nematodes carefully perforate the cell wall of a selected host cell and inject secretions into it. This behaviour sets off a series of cellular and molecular responses in the recipient host cells, either resulting in the formation of a syncytium in the case of cyst nematodes or giant cells in the case of root-knot nematodes. The ontogeny of the two types of feeding structures is fundamentally different, but both involve early manipulation of the mitotic cell cycle (*72-75*). The observed expression of cell cycle genes suggests that host cells prepare for a mitotic cell division while developing into a feeding structure. The chromosomes and the whole cellular machinery are duplicated to provide a viable legacy for the two daughter cells. However, the preparations for mitotis do not end in the completion of cell division. Instead, a shortcut in the cell cycle forces the cell into another round of preparations for cell division, which again is not completed. This process is repeated a couple of times resulting in large cells with high DNA contents. A key difference between a syncytium and a giant cell is the stage at which the mitotic cell cycle is aborted. In a syncytium abortion takes place just before nuclear division, whilst in giant cells the cell cycle progresses past nuclear division to be aborted just prior to cellular division.

The typical cellular phenotype of a syncytium then arises through progressive local cell wall degradation and subsequent fusion of the protoplasts (*76*). After a few weeks the syncytium consists of a large fusion complex of hundreds of hypertrophic cells. By contrast, the giant cells of root-knot nematodes remain as discrete cellular units while expanding into gigantic proportions over weeks (*77*). Typically a single root-knot nematode transforms 5-12 host cells into giant cells. Both the syncytium and the giant cells acquire the social status of a metabolic sink, which implies that the plant redirects much of its resources to these structures.

The cellular changes in the nematode-induced feeding structures are the outcome of extensively reprogrammed gene transcription in host cells (*69*). Our understanding of host gene regulation by nematodes has leapt forward over the last couple years because of amazing technological advances (*71, 78-92*). The *summum* of gene expression analysis in nematode-induced feeding structures at the moment is the application of laser-capture technologies to isolate individual host cells from microscopic cross sections of nematode-infected root material. From these and other studies it has become clear that nematodes regulate the expression of hundreds of host genes. It is a challenge to put all the pieces of this complex puzzle together, and to separate host genes that are under the direct control of the feeding nematode from

those that are merely responding indirectly to molecular and cellular changes. Nonetheless, the nematode-regulated host genes roughly fit into six functional categories, which help to draw an overall picture of the molecular phenomenon underlying feeding structures formation (*69*).

2.3.3 Plant innate immunity

It is remarkable that a parasite is capable of redirecting fundamental developmental programmes in host cells toward its nutritional and developmental needs. It is also remarkable that a host 'permits' a parasite to survive, to develop, and to reproduce while feeding from feeding structures. One would expect that the nematode and its feeding site are readily recognized as foreign bodies inside the plant, and that by default the plants respond to this with a series of deadly defense reactions. The fact that plant-parasitic nematodes remain inside the host for weeks suggests that they have evolved the means to modulate the innate immune system of the plant. Before detailing the tools used by nematodes to evade or suppress host defenses, we will first address current concepts of plant innate immunity to pathogenic microbes in general.

2.3.4 PAMP-triggered immunity

Despite the continuous presence of pathogenic microbes in their environment, disease in plants is still an exception. To protect themselves against the threats of pathogens and parasites, plants deploy a multilayered innate immune system (*37*). Basal defense responses – the first line of active defense – in plants are activated following the detection of non-self epitopes by extracellular pattern recognition receptors (*93-95*). These immune receptors perceive highly conserved pathogen-associated molecular patterns (PAMPs), which are parts of molecules accidently released by an invading pathogen or present as a structural component at the pathogen's surface (Fig. 1). Typically, PAMPs are required for the survival of the pathogen, which implies that they are not easily modified by mutations. PAMPs are therefore highly conserved across different taxonomic classes, which explains why basal defenses in a plant provide protection against a wide range of pathogens. Examples of PAMPs are chitin and lipopolysaccharides in the cell walls of fungi and bacteria (*93, 94, 96*).

A classical example illustrating that there is also a significant degree of convergent evolution in innate immune systems is the recognition of bacterial flagellin (*97, 98*). Flagellin is a principal motility component of flagellate bacteria, which is recognized by the extracellular leucine-rich repeat domain of both the receptor flagellin-sensing 2 in plants and a Toll-like receptor in vertebrates (*99*). Flagellin detection in both plants and vertebrates activates

signaling pathways leading to a type of immunity referred to as PAMP-triggered immunity (PTI). PAMP-triggered immunity involves, among others, altered ion fluxes, an increase in intracellular Ca^{2+} concentration, an oxidative burst, MAP kinase (MAPK) activation, protein phosphorylation, receptor endocytosis, defense gene induction, changes in protein-protein interactions, and callose deposition on cell walls (*100, 101*). These defense reactions will be discussed in more detail later on in this chapter. PTI activating signals can also originate from host tissues as products of the lytic activity of microbial enzymes. These host-born elicitors are known as damage-associated molecular patterns (DAMPs). Thus, basal defenses can be activated by pathogen molecules and by the perturbations pathogens induce in host molecules.

To date there is no nematode epitope identified as a PAMP in plant innate immunity, which is because scientists have not so far systematically addressed the role of PTI in nematode-plant interactions. Nonetheless, it seems indisputable that nematodes present many conserved epitopes on their cuticles that could act as PAMPs in plant innate immunity. Perhaps PAMP-triggered immunity is not so effective against plant-parasitic nematodes, because the response is simply not rapid enough to prevent host invasion and that migratory nematodes are able outrun this line of defense. In their migratory phase, even biotrophic nematodes exhibit necrotrophic behaviour, which makes them less vulnerable to PTI. But, in the sedentary biotrophic stage they need to have evolved other means to evade or suppress PTI. Perhaps, like some of the animal parasites, plant-parasitic nematodes use cuticular camouflage using host molecules to avoid being detected by extracellular pattern recognition receptors. Alternatively, the biotrophic plant-parasites may have evolved the means to modulate innate immunity downstream of PAMP recognition in host plants.

2.3.5 Effector-triggered immunity

Some strains of bacterial and fungal pathogens avoid PAMP-triggered immunity by deliberately releasing effectors into host cells (Fig. 1). These effectors can intercept PAMP detection by receptor proteins or suppress downstream PAMP-triggered signaling, which either way restores full virulence of the pathogen. Because PTI suppression poses an immediate threat to plants, they have evolved a second layer in their innate immune
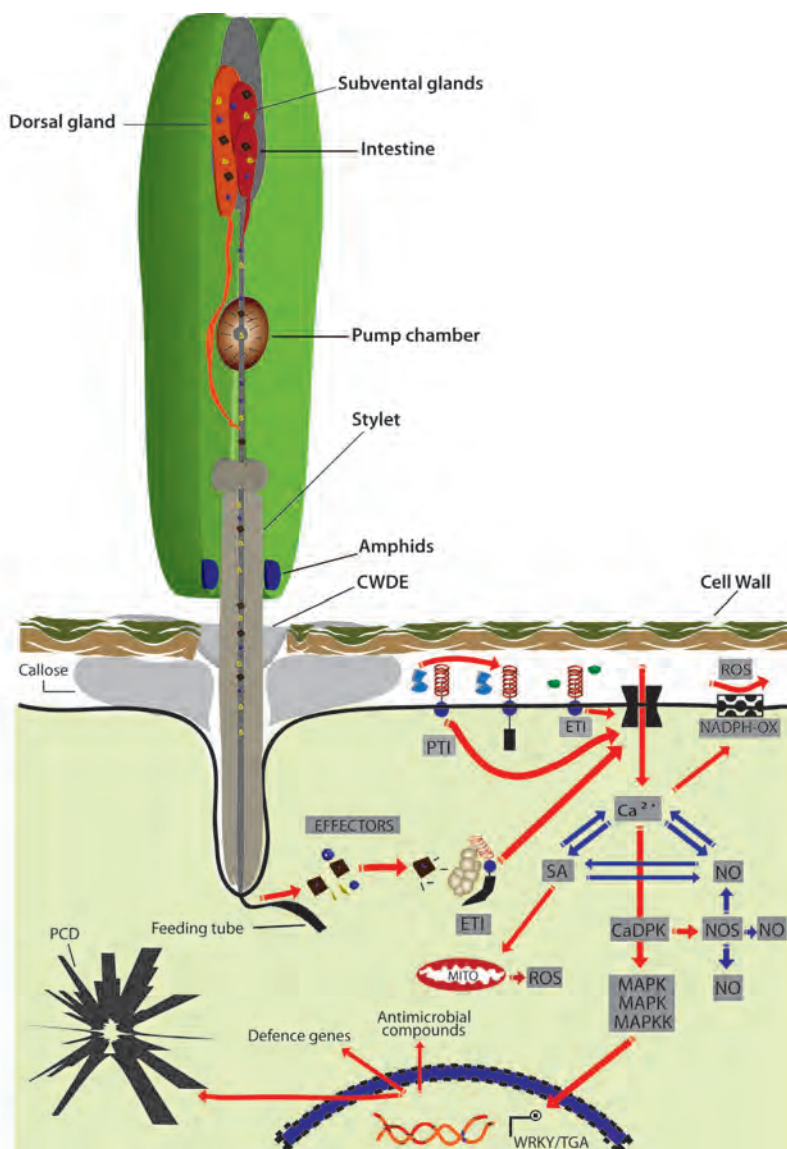
Fig. 1. The anterior section of a plant-parasitic nematode injecting effectors produced in the pharyngeal glands into a host cell. The nematode uses cell wall degrading enzymes (CWDE) to penetrate the host cell wall. Some of the nematode molecules may be recognized by the receptors of PAMP-triggered immunity (PTI) or effector-triggered immunity (ETI) in the host cell, which leads to defense signaling involving ion fluxes (Ca^{2+}), salicylic acid (SA), reactive oxygen species (ROS), and nitric oxide (NO). Defense signaling in innate immunity in plants often operates via mitogen-activated protein kinase (MAPK) cascades into the nucleus, wherein specific transcription factors (WRKY and TGA) regulate the expression of defense genes, PR proteins and antimicrobial compounds, eventually culminating in a hypersensitive response and programmed cell death (PCD).

system referred to as effector-triggered immunity (ETI) (*37, 102*). While PTI can be effective against a wide range of pathogens, effector-triggered immunity is highly specific to certain isolates of a single pathogen species. ETI receptors (formerly known as R proteins) are located as membrane-bound proteins on the cell surface and as intracellular receptors in the cytoplasm. The plant genes coding for the receptors in effector-triggered immunity are so-called major resistance genes. Thus, ETI occurs only in case the plant harbours an R protein that matches a specific effector in a pathogen.

Interestingly, some bacterial and fungal pathogens deliver '2nd generation' effectors into host cells that suppress the effector recognition or the signal transduction in ETI signaling pathways. These pathogens acquire full virulence again with their ETI suppressors. However, in their turn, some plant genotypes have evolved new recognition specificities in their repertoire of R proteins such that they are able to detect these ETI suppressing effectors, which restores disease resistance in these plants again. This co-evolutionary battle between pathogens and their host plants follows a *zig-zag* pattern of reciprocal adaptations either in the pathogen to acquire virulence or in the host plants to restore disease resistance (*37*).

R proteins are multi-domain receptor molecules that can be divided into several distinct structural classes (*103, 104*). The most abundant class consists of proteins with a nucleotide-binding domain and a leucine-rich repeat domain (NB-LRR). Some R proteins carry an additional amino-terminal domain with similarity to the Drosophila Toll or human Interleukin-1 Receptor (i.e. TIR-NB-LRR), while others include an amino terminal coiled-coil structure (i.e. CC-NB-LRR). All current members of the NB-LRR class are located in the cytoplasm. A second class of R proteins consists of an extracellular leucine-rich repeat domain linked to a transmembrane domain. Based on their domain structures the extracellular LRR proteins are divided further as receptor-like proteins or, in case they carry an additional carboxy-terminal kinase domain, they are named receptor-like kinases.

How exactly R proteins detect pathogen effectors and then activate ETI is still not fully understood. Initially the idea was that effector-receptor interactions followed classical direct ligand-receptor binding (*105*). However, this model is supported by experimental data for only a handful of effectors and matching R proteins. The prevailing opinion at the moment is that effector recognition in most of the cases does not involve direct binding between the effector and the receptor. Recognition by indirect interaction between effector and receptor is described in the guard model, which assumes that R proteins detect the perturbations brought about by pathogen effectors to other host proteins (*96*). The finding of various R proteins recognizing effector-induced cleavage or other types of modifications on host proteins supports the guard model. Remarkably, several R proteins may

guard the same host protein for different types of modifications brought about by diverse bacterial effectors (e.g. RIN4 in *Arabidopsis thaliana*) (*106*).

Mutant analysis and protein-protein interaction studies in *Arabidopsis* suggest that effector-triggered immunity constitutes an accelerated and amplified form of PAMP-triggered immunity. Effector-triggered immunity also leads to changes in ion fluxes, elevated intracellular Ca^{2+} concentrations, the production of reactive oxygen species, and defense genes expression (*37*). However, in most cases ETI ultimately directs the host cell into a hypersensitive response and programmed cell death (see below for more details). The actual molecular components in defense signaling downstream of R proteins and PAMP receptors have not been mapped out in great detail at the moment. But, a critical component in early defense signaling of both PAMP- and effector-triggered immunity seems to be the plant hormone salicylic acid (SA) (*107*). Exogenous application of SA to plants regulates defense gene expression and induces disease resistance to biotrophic pathogens. Salicylic acid is a derivative of chorismate in the Shikimate biosynthesis pathway. It is stored and can be released as different types of conjugates in various subcellular compartments. There is also substantial evidence for the involvement of mitogen-activated proteins kinase (MAPK) cascades in the early defense signaling (*108*). It has been shown that salicylic acid signaling in plant defenses depends on various components of MAPK cascades. Moreover, some of the MAPK pathways have been shown to feed into WRKY transcription factors controlling defense gene expression (*109*).

Breeding for resistance to plant-parasitic nematodes thus essentially aims to find genes encoding receptors for effector-triggered immunity in wild plants, and introducing these receptors into important food crops by genetic selection (*110*). At present six nematode resistance genes conditioning ETI have been cloned, and some of these were introduced into commercial cultivars. Most of the nematode resistance genes result in a hypersensitive response with increased defense gene expression and local cell death as the end result (see section below).

2.4 Molecular and Cellular Phenomena in Plant Innate Immunity to Nematodes

2.4.1 Defense genes: phytoalexins, PR proteins and protease inhibitors

Plants harbour a rich collection of chemical compounds capable of killing invading microbes (*111*). Here we briefly discuss three categories of defense genes, including phytoalexins, pathogenesis-related proteins, and protease inhibitors. Phytoalexins are low molecular weight antimicrobial compounds, which are produced in the secondary metabolic pathways. The presence of

these antimicrobial compounds in plants sometimes correlates with resistance to a pathogen. Virulence of pathogens in its turn can be determined by the ability to breakdown (pre)formed antimicrobial compounds. A textbook case of this type of chemical warfare in plants is the interaction of the fungal pathogen *Gaeumannomyces graminis* var. *avenae* and oat (*112*). Oat coleoptiles accumulate the broad-spectrum antimicrobial avenacin, which permeabilises cellular membranes. Virulence of the fungus *G. graminis* var. *avenae* on oat depends on the production of an avenacin-hydrolyzing enzyme.

Our current knowledge on the role of antimicrobial compounds in nematode-plant interactions is very limited. A few studies have addressed the importance of the isoflavonoid glyceollin in soybean during infection with *H. glycines*. Glyceollin was found to accumulate close to the nematode's head in a resistant cultivar but not in susceptible plants (*113*). Elliger *et al.* (185) have studied the accumulation of α-tomatine in susceptible and resistant tomato cultivars, and found no correlation with nematode resistance. However, α-tomatine is constitutively produced in tomato and more recent studies suggest that for virulence on tomato several fungal pathogens require the enzyme tomatinase (*114*).

Thus, experimental data pointing at a direct role of antimicrobial compounds in nematode-plant interactions is limited, mainly because these compounds have not received much attention from the scientific community. Indeed recent comprehensive transcriptome analyses show that many key enzymes in the biosynthetic pathways of antimicrobial compounds are regulated following nematode infections (*69*, *81*, *82*, *89*, *92*, *115*). Phenylalanine ammonia lyase, chalcone synthase, myrosinases, and hydroxy-methyl-glutaryl-CoA reductase are consistently up-regulated in nematode infected plants. Phenylalanine ammonia lyase is the key regulatory enzyme into the phenylpropanoid biosynthetic pathway leading to the production of precursors of four major classes of phenylpropanoid derivates (i.e. salicylates, coumarins, monolignols, and flavonoids). Chalcone synthase operates downstream of phenylalanine ammonia lyase in the production of flavonoids. The flavonoids and the coumarins include many phytoalexins, whilst the central defense signaling molecule salicylic acid belongs to the group of salicylates. Part of the phenylpropanoid pathway is dedicated to the synthesis of lignin, lignan and suberine, which are used in plants to fortify the cell walls. Myrosinases are involved in the hydrolysis of glucosinolates into extremely toxic isothiocyanates. Lastly, hydroxy-methyl-glutaryl-CoA reductase is the rate-limiting enzyme in a separate biosynthetic route into isoprenoids, which include the antimicrobial sesquiterpene phytoalexins. Thus, plants at least have the capability to deploy a broad arsenal of chemicals against parasitic nematodes. To what extent the phytoalexins contribute to immunity to nematodes is not clear and needs to be investigated in more detail.

2.4.2 Pathogenesis-related proteins

Pathogenesis-related (PR) proteins are loosely defined as microbe-induced proteins associated with plant defenses (*116, 117*). The concept of PR proteins was inspired by the finding of proteins specifically expressed during a hypersensitive response (see below) in tobacco plants resistant to Tobacco Mosaic Virus. The key identifier for a PR protein is indeed its expression pattern, and not an evolutionary relatedness or a similarity in biochemical activity. As a consequence of this relaxed criterion, the list of PR proteins now includes eighteen different families but it is likely to grow further. The PR proteins are believed to restrict pathogenicity of microbes on plants, but experimental evidence to substantiate their contribution in immunity is often weak, if present at all. Nonetheless, *in vitro* several PR proteins do have antimicrobial activities. For instance, some PR protein families breakdown fungal and bacterial cell walls, inhibit proteases, degrade ribonucleases or display antimicrobial toxicity. Given this wide spectrum of potential antimicrobial activity, and even though there is no robust experimental data to support this for many PR proteins, it seems likely that they provide at least partial protection against invaders.

In Table 2, we summarized the results of several transcriptome analyses of nematode-infected plant tissues including a category of defense-related genes. This category in fact includes several PR proteins that are locally and systemically expressed in plants infected with nematodes.

Table 2. Functional categories of nematode-regulated genes in feeding structures and examples of associated cellular and molecular processes (adapted from Gheysen and Fenoll, 2002 (69)).

Category	Examples of molecular processes and activities
Defense-related	PR-proteins, oxidative burst, wound-inducible proteins, lignin biosynthesis
Cell cycle and organization	Cyclin-dependent kinases, cyclins, tubulins
Plant hormones	Auxin-responsive genes, auxin transport, ethylene synthesis, jasmonic acid biosynthesis
Cell wall	Cell wall degrading enzymes, expansins, cellulose synthesis, cell wall proteins
Metabolism and water status	Metabolic enzymes, sugar transport
Gene expression	Transcription factors, protein turn-over

However, the expression patterns in the direct vicinity of the site of infection may be different from a systemic response in a plant (*118*). For example, *Arabidopsis* plants infected with the beet cyst nematode, *H. schachtii*, show elevated levels of PR-2 and PR-5 in infected roots, but not of PR-1 (*119*). However, in the shoots of these nematode-infected plants PR-1 expression was strongly induced. PR-1, PR-2, and PR-5 are all salicylic acid-induced defence genes, and are often simultaneously induced by microbes. The absence of PR-1 induction in roots, and the up-regulation of PR-1 in shoots, led to the hypothesis that perhaps cyst nematodes suppress local accumulation of PR-1 (*119*). Again, a systematic analysis of local PR-protein expression in the infection site, and in shoots at some distance from the infection site, could shed some light on the role of PR proteins in nematode resistance.

2.4.3 Protease inhibitors

The third group of defense genes involved in plant defense to nematodes encodes protease inhibitors. Microbe-induced protease inhibitors fit into the definition of a PR protein, but are often dealt with separately. The expression of protease inhibitors is regulated through signaling pathways that are activated by tissue injury (*120*). Plant protease inhibitors are also expressed in response to herbivorous animals, including plant-parasitic nematodes, possibly as a generic reaction to wounding. Animals feeding on plant tissue ingest host protease inhibitors, which are then believed to interact with proteases in the digestive tract. Inhibition of intestinal proteases likely disrupts normal uptake of protein fragments in the gut. Enzyme activity assays and proteomics on collected stylet secretions further suggest that plant-parasitic nematodes also secrete proteases that could be targeted by protease inhibitors (*121, 122*). To understand the role of these secreted proteases in nematode-plant interaction further investigations are needed. Because secreted proteases are among the few enzymes present in secretions from both animal- and plant-parasitic nematodes, novel insights may be acquired in a cross-disciplinary approach.

Efforts to engineer nematode resistant plants by constitutive overexpression of specific protease inhibitors demonstrates that proteases contribute to success in parasitism (*123*). Overexpression of a cysteine protease inhibitor, cystatin, from rice, targeting intestinal proteases in cyst nematodes, reduced the fecundity of female worms. Similarly, studies targeting the intestinal cysteine proteases of root-knot nematodes with RNAi led to a significant reduction in parasite development and reproduction (*124*). It may not be appropriate to translate the results obtained with transgenic protease inhibitors and RNAi to the natural situation in plants, but they do

show that protease inhibitors could constitute one more layer in plant defense to nematodes. However, a recent expression analysis of protease inhibitors in plants did not show a correlation with natural resistance, which challenges the view that protease inhibitors have a key role in nematode resistance (*125*).

2.4.4 Cell wall fortifications with callose deposits and lignin

Microbial plant pathogens, nematodes included, have evolved a repertoire of cell wall degrading enzymes to breakdown plant cell wall polymers. Plants sometimes respond to these invasions with the local deposition of callose (a β-1,3-glucan polymer) and lignin in between the cell wall and the cell membrane (*126, 127*). For example, cell wall fortifications with callose/lignin papillae in monocots can provide basal resistance against a variety of fungal pathogens (*126, 128*). Cell wall depositions make the cell wall more penetration resistant for two reasons. The deposits provide extra strength to resist the physical impact of the stylet, and lignin makes the cell wall less susceptible to cell wall degrading enzymes.

To what extent cell wall fortifications contribute to the penetration resistance to nematodes has not been studied in great detail. Callose deposits have been observed on plant cell walls penetrated by the stylet of biotrophic plant-parasites (*129, 130*). Callose accumulates on the cell wall between the site where the stylet is inserted and the invaginated cell membrane around the stylet tip (Fig. 1). However, callose deposits seem to occur in both susceptible and resistant plants, and there is no correlation between resistance to nematodes and callose deposition.

Lignin is one of the products of the phenylpropanoid biosynthesis pathways (see section above). Transcriptome analysis of susceptible soybean roots suggests that most of the rate-limiting enzymes in the phenylpropanoid pathways are up-regulated in nematode-infected root tissue (*81, 82*). Lignification of cell walls is observed in defense responses to biotrophic cyst nematodes in *A. thaliana*, but it is not clear to what extent lignification contributes to nematode resistance (*130*). Resistance to the necrotrophic nematode *Radopholus similis* in banana is correlated with high lignin content in cell walls (*131*). Besides having a higher constitutive level of lignin, resistant banana plants also respond to nematode infection with further lignification of the cell walls. Increasing penetration resistance with cell wall fortifications may affect migratory necrotrophic plant-parasites more significantly. Biotrophs can be hindered by lignified cell walls during their short migratory phase, but a slow down may not yield a major effect on their development and reproduction.

2.4.5 Hypersensitive response and programmed cell death

The ultimate defense layer in plant innate immunity is the generation of a hypersensitive response leading to local programmed cell death (HR-PCD) at the site of infection (*132-134*). The hypersensitive response in plant cells prevents further ingress of fungal and bacterial pathogens, and in the case of nematodes the development of a proper feeding structure (Fig. 2). It is still debated whether the programmed cell death or a barrage of cytotoxic compounds halts the pathogen. Cell death could also be induced by neighbouring cells to limit the damage caused by cytotoxic compounds to those cells that are in direct contact with the pathogen. So, local cell death could therefore be initiated in a cell by its neighbours to prevent a runaway autoactive response.
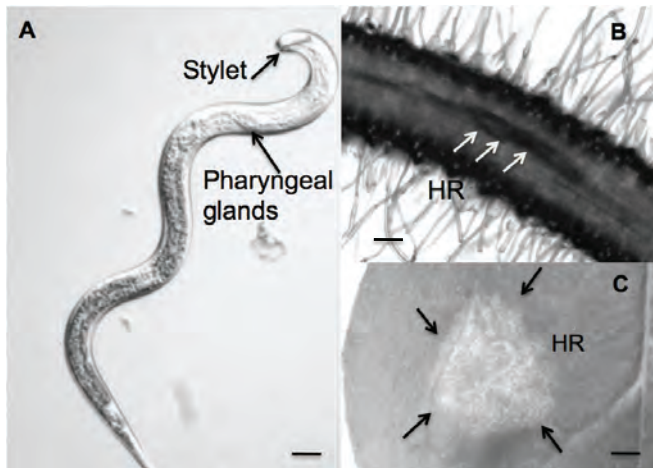
Fig. 2. (A) An infective second-stage juvenile of the potato cyst nematode *Globodera rostochiensis*. The translucent anterior section of the nematode includes the pharynx, pharyngeal glands, and the stylet. Scale bar = 20 μm. (B) A potato root harbouring the H1 resistance gene showing a hypersensitive response (HR) in host cells close to the nematode. Scale bar = 300 μm. (C) Transient co-expression of a nematode effector and a matching resistance gene in a *Nicotiana benthamiana* leaf results in a hypersensitive response and programmed cell death of plant cells (arrows). Scale bar = 0.25 cm.

The signaling cascades underlying the hypersensitive response are strictly controlled by highly specific immune receptors (*103*). These immune receptors, encoded by R genes, only activate downstream signaling when they detect the presence of matching pathogen-derived effectors (see Section 2.3.5). The pathways connecting activated immune receptors and the

hypersensitive response are currently the subject of intense research. Genetic studies with signaling mutants have revealed several critical nodes in signal-transduction routes downstream of immune receptors (*135-141*). For example, RAR1 and HSP90 are thought to act as chaperones to stabilize various immune receptors and to maintain them in an active configuration. A third component required for resistance, referred to as SGT1, is associated with linking pathogen recognition complexes to ubiquitination pathways. Similarly, EDS1 and PAD4 seem to operate downstream of TIR-NB-LRR receptors, whereas the NDR1 is required for and acts downstream of the CC-NB-LRR class of receptors. So, a growing number of molecular components in disease-signaling pathways has been discovered, but there still is a significant gap in our knowledge spanning these components and the early molecular and cytological phenomena associated with a hypersensitive response.

The earliest phenomena signaling the activation of a hypersensitive response are rapid ion fluxes across the cell membrane, the production of reactive oxygen species outside the cell and nitric oxide inside the cytoplasm, and the accumulation of salicylic acid (*142, 143*). Tests with specific inhibitors of ion channels and radiolabelled ions demonstrate that rapid changes in ion fluxes are required for the activation of a hypersensitive response. Of particular importance are influxes of Ca^{2+} ions that act as secondary messengers in a wide variety of signaling pathways in plants (*144*). ETI signaling apparently feeds into ion channels in the cell membrane, which increases the Ca^{2+} permeability of the cell membrane. The concentration of Ca^{2+} ions in the apoplast is in the order of 10,000-fold higher in the apoplast than in the cytoplasm. Rapid influxes of Ca^{2+} ions convey messages from outside the cell into the cytoplasm and the nucleus of the recipient cell. Spatiotemporal changes are believed to generate a Ca^{2+}-signature in the cytoplasm that is decoded by specific Ca^{2+} "sensors". Next, these Ca^{2+} sensor translate oscillations in cytoplasmic Ca^{2+} concentrations into the activation of downstream signaling-molecules involving calmodulins, calcium-dependent kinases, and MAP kinase cascades.

Calcium influx is a key regulator of plant defenses. For example, calcium-dependent phosphorylation activates membrane-associated NADPH oxidase, which generates extracellular superoxide radicals (*142, 143*). Apoplastic superoxide dismutases convert superoxide into hydrogen peroxide, and indirectly into hydroxyl radicals, resulting in what is known as the oxidative burst. Interestingly, apoplastic hydrogen peroxide also acts in a positive feedback loop, which further increases Ca^{2+} permeability and Ca^{2+} influx. The reactive oxygen species (ROS) are potentially toxic to invading microbes, but they also induce fortifications of the plant cell walls by lignification and cross-linking cell wall proteins.

Perhaps the strongest effect of ROS occurs through peroxidation of lipids in cellular membranes. Hydroxyl radicals subtract a proton from

unsaturated phospholipids to generate a lipid hydroxyperoxide radical. It is not difficult to see how extensive lipid peroxidation of lipids could lead to a loss of membrane integrity. Moreover, lipid peroxidation could also result from an increase in lipoxygenase action, which is strongly up-regulated in cells undergoing a hypersensitive response.

Influx of Ca^{2+} also induces the synthesis of nitric oxide through a calcium-dependent nitric oxide synthase (*145, 146*). Specific inhibitors of nitric oxidase and scavengers of nitric oxide point at an important role for nitric oxide in plant innate immunity. Exogenously applied nitric oxide to Arabidopsis cells regulates the expression of several pathogenesis-related proteins and other defense genes. To further add to the complexity evolving around signal transduction cascades in plant defenses, evidence suggests that nitric oxide is capable of mobilizing Ca^{2+} ions from intracellular calcium pools, which again creates an amplification cycle in the accumulation of secondary messengers.

Salicylic acid (SA) is also a key compound in early signaling of a hypersensitive response (*107*). Exogenous application of SA to plant cells can elicit a cell death response. SA is associated with the generation of cytoplasmic ROS, possibly through derailing the oxidative phosphorylation pathway in mitochondria such that ATP is no longer produced in the cells. SA is also held accountable for the changes in the cytoplasmic redox state, which activate other proteins downstream in the SA signaling cascade such as NPR1. SA-activated NPR1 moves into the nucleus, wherein it interacts with TGA type of transcription factors that regulate the transcription of various defense genes. Some of the redox-based reactions induced by SA likely involve post-translational modifications via a mechanism called S-nitrosylation that uses nitric oxide (*147*). In conclusion, it is a complex interplay among at least four critical messengers (i.e. Ca^{2+}, ROS, nitric oxide, and SA) that determine the onset of the hypersensitive response.

The cellular changes associated with the hypersensitive response and local programmed cell death seem to vary somewhat among individual pathosystems. Nevertheless, the HR-PCD roughly proceeds through the following sequence of events (*132*). The first observable change in cells undergoing a hypersensitive response is cessation of the cytoplasmic streaming, which coincides with the reorganization of the cytoskeleton. Next, the cytoplasm acquires a more granular appearance, and shrinks in volume. The mitochondria inside the cytoplasm swell, and then terminate normal oxidative phosphorylation and the production of metabolic energy. Chromatin DNA condensation is also observed in the nuclei of cells undergoing cell death. Increasing intracellular concentration of hydrogen peroxide and lipoxygenase are the likely cause of lipid breakdown in cellular membranes. This irreversible damage to the membranes leads to a loss of

semi-permeability, which is quickly followed by disintegration of the nucleus and collapse of the protoplast (Fig. 2).

The hypersensitive response is an extremely fast and powerful defense strategy to ward off biotrophic microbes. This seems to be especially true for sedentary endoparasitic nematodes (Fig. 2) because the transition from migratory to sedentary stage involves the breakdown of locomotory muscles, which leaves them completely dependent on the resources provided by their living feeding structure (*110, 148*). Plants seem to have exploited this vulnerability as they use the nematode-induced feeding structure as their primary battlefield to deploy a hypersensitive response to these nematodes (Fig. 2). The hypersensitive responses mediated by nematode resistance genes can be roughly divided into two types (*149*). First are the hypersensitive responses culminating in a classical fast and local cell death in and around the feeding structure. In addition, several nematode resistance genes induce a delayed-type hypersensitive response mostly involving cell death in cells surrounding the feeding structure, and in cells between the feeding structure and nearby vascular tissue of the plant. The delayed-type hypersensitive response develops over days to weeks and allows the infective nematodes to feed on their feeding structures for a significant amount of time.

Root-knot nematodes invading tomato harbouring the *Mi-1* gene induce a rapid and local hypersensitive response and cell death in the initial feeding site within 24 h (*150-152*). As a consequence the formation of giant cells is completely suppressed by the *Mi*-mediated hypersensitive responses. The *H1* resistance gene to cyst nematodes in potato also induces a fast hypersensitive response and cell death in the cells surrounding the initial feeding structure, but not in the feeding structure itself (*153*). The 'ring-of-death' induced by the *H1* gene isolates the feeding structure from nearby vascular tissue preventing transfer of nutrients from the flow of assimilates to the feeding nematode. The *Gpa2* resistance gene to cyst nematodes in potato confers a delayed type of hypersensitive response that develops over weeks and allows some nematodes to mature but not to reproduce.

2.5 Immune Modulation by Nematodes in Plants

While embedded in host tissues the sedentary endoparasitic nematodes are continuously exposed to the innate immune system of the plant, yet some of these parasites are able to live inside hundreds of different plant species for long periods. Even amongst biotrophic bacterial and fungal plant pathogens this kind of success in parasitism is exceptional, and it suggests that nematodes are very efficient in governing plant innate immunity. There are three main strategies, which are not mutually exclusive, for a parasite to deal with immunity in a host. The nematode can first avoid being recognized by the immune system. When that fails, it can actively suppress immune

signaling triggered by activated recognition complexes or, as a last resort, it can neutralize antimicrobial compounds that are part of activated defense responses. Immune evasion by cuticular camouflage has been discussed in Section 2.2.1. In this section we will discuss the possible approaches for nematodes to modulate immunity in plants, with a focus on the role of recently discovered effectors.

2.5.1 Detoxification of reactive oxygen species (ROS) and modulation of ROS signaling

One of the earliest responses to pathogen infections in plants is the production of ROS. ROS have two roles in plant defenses: i) as antimicrobial compounds in a 'chemical warfare'; and ii) as a critical messenger in defense signaling. In both susceptible and resistant tomato plants the root-knot nematode *Meloidogyne incognita* induces a fast oxidative burst in the migratory tracks and in the feeding cells. However, in plants resistant to nematodes a second oxidative burst associated with a hypersensitive response occurs, while in susceptible plants the ROS concentration declines in feeding sites after a few hours (*154*). A similar biphasic oxidative burst has been observed in *A. thaliana* infected with the soybean cyst nematode *H. glycines* (*155*). The absence of a biphasic ROS response in susceptible plants suggests that plant-parasitic nematodes may modulate the signaling that leads to the second phase oxidative burst. As this second wave of ROS is specific for resistant plants, it is likely to be part of the highly specific effector-triggered immunity. By the same reasoning, the initial phase of ROS production could then perhaps be produced following non-specific PAMP-triggered immune signaling. So the absence of a biphasic oxidative burst could either be evasion by the nematode of immune responses by avoiding recognition by the ETI receptors or true modulation of the signaling pathways inducing the biphasic oxidative burst downstream of ETI receptors.

A significant outcome of these studies is that even in susceptible plants invading nematodes encounter an oxidative burst. Therefore, protection against ROS seems extremely important for parasite survival in plants, because ROS could probably diffuse through the cuticle of the nematodes and cause significant damage to DNA, proteins and cellular membranes in the underlying tissues. Plant-parasitic nematodes acquire some protection with surface anti-oxidants and ROS scavengers such as secreted glutathione peroxidases and thioredoxin peroxidases (*156, 157*). Thioredoxin peroxidase specifically metabolizes hydrogen peroxide. Although nematode glutathione peroxidases are also capable of converting hydrogen peroxide, they seem to have a higher affinity for the products of lipid peroxidation (see Section 2.4.5). The root-knot nematode, *M. incognita*, produces glutathione-S-transferase as one of the components of its pharyngeal gland secretions, which are probably

injected into the host cells during feeding (*158*). Knocking-down the glutathione-S-transferase with RNAi reduced the egg production by females, but not the number of females in a host. A plausible explanation for this effect on nematode fecundity could be that glutathione-S-transferase supports sustained feeding on host cells rather than host invasion and the establishment of the feeding site. It is tempting to speculate that anti-oxidants at the nematode surface provide protection to apoplastic antimicrobial ROS, while anti-oxidant enzymes in pharyngeal secretions may target hydrogen peroxide in the host cell cytoplasm to intercept ROS-mediated signaling.

2.5.2 Modulation of plant hormone balance and secondary metabolism

Plant hormones are key players in the regulation of plant development and defense responses to biotic and abiotic stress (reviewed in (*159*). Ethylene insensitive Arabidopsis mutants are less susceptible to *H. schachtii*, while ethylene-overproducing mutants are hypersusceptible. The nematode-induced feeding sites in ethylene-overproducing mutants are significantly larger than in wild-type plants. Similarly, auxin-insensitive mutants have shown resistance to *G. rostochiensis* and *H. schachtii*. Resistance in auxin-insensitive plants was characterized by disturbed feeding site formation. So the establishment and maintenance of proper feeding sites are strongly dependent on ethylene and auxin. The dominating theory at the moment assumes that nematodes induce a local increase in auxin levels in feeding site initials, which is followed by the auxin-responsive synthesis of ethylene. The gross phenotype of auxin- and ethylene-insensitive mutants is indeed an increase in nematode resistance, but this may point to a lack of susceptibility rather than to specific immune responses.

While there is some consensus on the importance of plant hormones in feeding site formation, the actual trigger from the nematode driving a rise in auxin levels is not yet known. Chorismate mutase enzymes secreted from the pharyngeal glands of plant-parasitic nematodes have been linked to auxin balances in plant cells (*160-163*). Systemic overexpression of a nematode chorismate mutase in soybean hairy roots gives a disturbed root morphology that can be rescued with the exogenous application of auxin (*163*). It is not clear if these overexpression studies reflect the natural situation correctly but this finding suggests that nematodes injecting chorismate mutase into host cells could reduce local auxin levels, which is not in agreement with earlier mutant analyses. Chorismate mutase is a key enzyme in the Shikimate pathway and catalyzes the conversion of chorismate into prephenate. Chorismate mutase directs the Shikimate pathway away from tryptophane, to favour the production of tyrosine and phenylalanine. Auxin derives from tryptophane, and chorismate mutase could thus affect local auxin levels.

However, auxin is mainly produced in the aerial parts of the plant and then transported to the roots, wherein the nematodes induce their feeding sites. Therefore, nematodes are more likely to raise local auxin levels either by enhancing the influx or reducing the efflux of auxin in feeding site initials. Hence, secreted chorismate mutases may not increase auxin biosynthesis but their deployment in a wide variety of plant-parasitic nematodes suggests that the modulation of secondary metabolism is nonetheless crucial in parasitism. The chorismate mutases could, for example, interfere with the generation of antimicrobial flavanoid derivates of aromatic amino acids.

2.5.3 Modulation of lipid-based defenses

Lipids are important in plant-microbe interactions in the chemical defense against invading pathogens as lipid peroxides, but also as second messengers in defense signaling. Hydrogen peroxides produced in the oxidative burst can react with unsaturated lipids in cellular membranes to produce toxic lipid hydroperoxides and other free radicals (see Section 2.4.5). Similarly, the plant defense responses often involve the up-regulation of lipoxygenases that convert lipids into bioactive lipid hydroperoxides. Besides being highly toxic, lipid hydroperoxides are also rapidly converted in the precursors for lipid-based signaling molecules such as jasmonic acid. A recent paper reports that the lipoxygenase gene *ZmLOX3* is involved in regulating susceptibility to root-knot nematodes in maize (*164*). A comparison of syncytial cells in susceptible and resistant plants also revealed a ten-fold up-regulation of lipoxygenase genes in plants resistant to cyst nematodes (*84*). Furthermore, in nematode resistant pea lipoxygenases were highly induced in cells surrounding the feeding sites and inside feeding sites undergoing a hypersensitive response (*165*). Several studies have shown that the exogenous application of methyl-jasmonate or synthetic jasmonic acid reduces host susceptibility to root-feeding nematodes (*166*). Thus, there is some experimental support for a significant role of lipoxygenase, bioactive lipids, and lipid-based signaling in plant-nematode interactions.

Plant-parasitic nematodes secrete a specific class of fatty-acid and retinol-binding (FAR) proteins that may interfere with lipid-based defenses in the host. Recombinantly produced FAR protein of the cyst nematode *G. pallida* binds to linolenic and linoleic acids (*167*). Further enzyme activity assays showed that the recombinant FAR protein also inhibits the lipoxygenase-mediated conversion of unsaturated linolenic and linoleic acids. The FAR protein is located at the interface of the nematode and the host cells, where it may neutralize toxic lipid hydroxyperoxides or intercept lipid-based defense signaling.

A second group of lipid-binding proteins in nematodes with possible immunomodulatory properties are the so-called annexins. Annexins bind

phospholipids, the main component of cell membranes, in a calcium-dependent manner. Annexins are expressed in the pharyngeal and the amphidial glands of plant-parasitic cyst nematodes (*168, 169*). They have been implicated in vesicle transport during endo- and exocytosis, and in providing a membrane-anchored docking scaffold for other molecules. As such the annexins could have a role in vesicle transport inside nematode glands cells; however, the finding of annexins in the secretory-excretory products of plant–parasitic nematodes suggests that they also may have a role inside host cells.

2.5.4 Modulation of calcium signaling

Ca^{2+} is an important second messenger capable of conveying all sorts of external signals to responsive developmental and cellular processes inside plant cells. A rapid Ca^{2+} influx precedes both basal and specific disease resistance. The exogenous application of a specific Ca^{2+} channel inhibitor (i.e. La_3Cl) also points to a role for Ca^{2+} signaling in potato roots susceptible to nematodes (*170*). Although the authors of this study could not exclude a direct effect of the inhibitor on nematodes, their results suggest that Ca^{2+} signaling is required for successful invasion and feeding site formation by cyst nematodes.

Plant-parasitic nematodes have evolved the means to interfere with Ca^{2+} signaling in host plants. It has been shown that *M. incognita* secretes calreticulin during host invasion and feeding (*171*). The nematode-secreted calreticulin was localized close to the stylet tip *in planta*, and along the cell walls of the giant cells. However, their role in plant-nematode interactions is not well understood at present. Calreticulins are conserved in all multicellular organisms, and carry a sorting signal for secretion and a C-terminal ER retention signal sequence (*172*). They act as molecular chaperones during protein folding in the endoplasmic reticulum. Although the ER is considered the main residence of calreticulins, several studies have reported their presence outside the ER. Cytoplasmic calreticulins have been implicated in modulating Ca^{2+} homeostasis and signaling, gene expression, and cell adhesion (*172*).

2.5.5 Modulation of host protein turnover rate

The addition of small ubiquitin monomers to proteins in eukaryotic cells can target these proteins to the 26S proteasome for degradation (*173*). Ubiquitination of proteins proceeds via a multistep process that is controlled by an E1-E2-E3 enzyme cascade (*174*). First in line are the E1 ubiquitin-activating enzymes involved in the recruitment of free ubiquitin in an ATP-dependent manner. The bound ubiquitin is then rapidly transferred to an E2 ubiquitin-conjugating enzyme. The E2 conjugating enzyme next delivers the

activated ubiquitin monomer to the targeted substrate protein. However, the actual target of an ubiquitination complex is determined by the binding specificity of a third component in the cascade, the so-called E3 ubiquitin ligases. Eukaryotic genomes include hundreds of different E3 ubiquitin ligases each having unique substrate specificity for a particular target protein. Ubiquitination has been implicated in the regulation of a wide variety of processes in plants such as innate immunity, cell death, cell cycle regulation, hormone signaling, circadian rhythms, and many more.

In the last couple of years it has become clear that many plant-pathogens hijack the ubiquitination system of the host to take over control of various cellular processes. For example, the plant pathogenic bacterium *Pseudomonas syringae* delivers an effector protein AvrPtoB with novel E3 ubiquitin ligase activity into the host cell to target the ubiquitination machinery to the protein kinase Fen and suppress innate immunity (*175*). This demonstrates that bacterial plant-pathogens are capable of redirecting the specificity of host ubiquitination complexes so that the plant's innate immunity is no longer effectively controlling pathogen ingress. The recent discovery of several secreted variants of ubiquitination complex components in pharyngeal glands of plant-parasitic nematodes indicates that plant-parasitic nematodes may also exploit the host's ubiquitination system (*176*). Sequencing of pharyngeal gland specific cDNA libraries revealed homologs of SKP1 and RING H2 zinc finger proteins. SKP1 is a subunit of an SKP1-Cullin-F-box (SCF) E3 ubiquitin ligase complex, whereas RING-H2 finger proteins form an E3 ligase complex together with Cullin and a variable substrate recognition subunit. Thus, it is likely that nematodes inject components of E3 ligases into host cells to redirect the ubiquitin-proteasome degradation pathway. However, SKP1 and RING-H2 finger proteins have not been implicated in determining the substrate specificity of the E3 ligase complexes. It is therefore not evident what the roles of parasite secreted SKP1 and RING-H2 finger proteins are, and how these components could redirect E3 ligase complexes. Perhaps other not yet identified components with similarity to F-box proteins or other types of variable substrate recognition subunits are present in nematode secretions that could confer novel substrate recognition specificities of host E3 ligase complexes.

2.5.6 Modulation of host immune receptors

The recently discovered SPRYSEC proteins in the stylet secretions of cyst nematodes could have a role as variable substrate recognition subunits in E3 ligase complexes (*177*). Secreted SPRYSECs have a similar architecture as the GUSTAVUS protein in *Drosophila*, consisting only of SPRY/30.2 domain. GUSTAVUS includes a small C-terminal BC box, and functions as an adaptor subunit in an E3 ubiquitin ligase complex. It is not clear whether the

SPRYSECs also have a functional BC-box like structure. The SPRY/B30.2 domain occurs in many proteins combined with a variety of other domains. It is associated with protein-protein interactions, often involving a receptor molecule.

SPRYSECs have only been found in cyst nematodes of Solanaceous plants so far. In these species of cyst nematode the SPRYSECs occur as large gene families with many highly diverse members. Evolutionary studies on SPRYSECs suggest that they are subjected to strong diversifying selection, which implies that they either tend to evolve new recognition specificities to host targets or that they change to avoid being recognized by host immune receptors (*177*). Recent experimental data have not provided much clarity with regard to these two models. One of the SPRYSECs of *G. rostochiensis* was shown to interact physically with the LRR domain of a classical CC-NB-LRR receptor protein from the SW5 resistance gene cluster in tomato. However, the tomato cultivar harbouring this CC-NB-LRR is fully susceptible to *G. rostochiensis*. Furthermore, co-expression of the SPRYSEC19 with the CC-NB-LRR protein in *Nicotiana benthamiana* did not result in a hypersensitive response. So although there is binding and possible recognition of a nematode effector by an immune receptor in a host plant, this does not lead to activation of ETI and resistance. Because the interaction of the SPRYSEC19 with a CC-NB-LRR protein does not activate plant defenses, it could have the opposite effect on innate immunity through modulation of immune receptors.

However, more recent data on a SPRYSEC homolog in a closely related cyst nematode question this model (*178*). The SPRYSEC homolog RBP-1 in *G. pallida* was found to induce a *Gpa-2*-dependent hypersensitive response in *N. benthamiana* leaves. *Gpa-2* encodes a CC-NB-LRR protein that confers resistance to specific avirulent strains of *G. pallida* in potato. This implies that RBP-1 of *G. pallida* is recognized by Gpa-2, and activates effector-triggered immunity. Obviously, the nematode does not inject RBP-1 proteins into host cells to betray its presence in the host. RBP-1 will have another intrinsic function, which perhaps involves the interaction with other CC-NB-LRR proteins to modulate their activation. Alternatively, SPRYSEC19 may be an evolutionary intermediate that binds to CC-NB-LRRs, but this binding is not yet or no longer sufficient to elicit a defense response in the plant. Further investigations addressing the primary role of the SPRYSECs will clarify the importance of immune receptor modulation in nematode-plant interactions.

2.5.7 Cross kingdom modulation

Animal-parasitic nematodes are renowned for their ability to modulate the innate and adaptive immunity of their host (*179*). A wide range of modulation mechanisms involving the secretory-excretory products of the parasite seem to operate at the animal-parasitic nematode-host interface. The immune

systems in plants and animals are fundamentally different and it is difficult to predict whether plant- and animal-parasitic nematodes use similar entry points to target host immunity. In a review of possible similarities between plant- and animal-parasitic nematodes, Jasmer *et al*. (2003) noted a remarkable conservation in the effector repertoire that could point at overlapping principles of immune modulation. Highly conserved in all parasitic nematodes are the so-called secreted venom-allergen like proteins (VAP or VAL). The VAPs belong to the SCP/TAPS protein family, which is a subclass within the cysteine-rich secretory proteins superfamily (CRISP; (*180*)). The name SCP/TAPS is short for the acronym SCP/Tpx-1/Ag-5/PR-1/Sc7, referring to several of its members such as sperm-coating proteins (SCP), testis-specific extracellular proteins (Tpx), glioma pathogenesis-related proteins, venom-allergen from wasp and ants (Ag), and plant PR-1 proteins.

A rather typical example of a member of SCP/TAPS protein family is the pathogenesis-related protein PR-1. PR-1 is one of the most abundantly expressed secretory proteins following pathogen infection in plants. The PR-1 accumulates locally in the apoplast at the site of infection but sometimes also at a distance from the invading pathogen. Even though PR-1 was identified over 20 years ago, and despite its frequent use as a marker for systemic resistance in plants, the mode of action of PR-1 is not well understood (*117, 181*). It is remarkable that this seems to hold true for many members of the SCP/TAPS protein family, which often appear to have important roles in health and disease but for which the biochemical mode of action is not clear.

The VAPs are amongst the most abundant proteins released by parasitic nematodes, which suggest that these proteins do have an important role in parasitism. Unfortunately, attempts to knock-down VAPs in animal-parasitic nematodes with RNAi have not been successful. Recently, Lozano-Torres (Chapter 4, this thesis) has been able to knock-down VAPs in the potato cyst nematode *G. rostochiensis*. A specific dsRNA treatment of the infective stage juveniles significantly reduced the infectivity of the nematodes on potato plants, which demonstrates that VAPs are indeed required for parasitism. However, the function and the molecular targets of nematode VAPs in host plants remain elusive at present.

The sequence conservation in animal-parasitic and plant-parasitic nematode VAPs is relatively high, so they are expected to have similar biochemical activities and possibly similar effects on host cells. Unfortunately, there is no conclusive data available on the biochemical activities of nematode VAPs. However, the relatively well-characterized VAPs from the animal parasitic hookworms are nonetheless linked to modulation of mammalian immune cells. The VAP-homolog NIF, for example, inhibits neutrophils and blocks the release of reactive-oxygen species from these cells (*182*). Another Ancylostoma SCP/TAPS protein interferes with the extracellular integrin receptors, which inhibits platelet aggregation (*183*). Similarly, a large family

of VAP homologs in the trematode *Schistosoma mansoni* is also associated with modulating immune responses in the host during the infection process (*184*). It is tempting to speculate that VAPs in both animal- and plant-parasitic nematodes are modulators of immunity. However, further investigations into the extracellular targets of VAPs on host cells and a systematic analysis of host defense expression and cytokine profiling following exposure to VAPs are required conclusively to classify them as immune modulators.

2.6 Conclusions and Future Directions

For decades the scientific focus in the field of plant-nematode interactions has centred mainly on host invasion and feeding site formation in susceptible plants. These aspects of parasitism are indeed extremely important for the survival of the nematode inside the host. Currently there is a growing awareness that suppression of host plant immunity may also be essential for a nematode to enable host invasion and feeding. Our field is, therefore, now slowly shifting more towards understanding the role of immune modulation by nematodes in plants. In this chapter we have reviewed current insights in the molecular and cellular aspects of nematode survival in plants, with an emphasis on plant innate immunity. Most of these insights stem from studies with bacterial plant pathogens, but they nonetheless reveal possible entries for nematodes to attack the immune system of the plant. Ongoing investigations on the role of nematode effectors in parasitism will reveal to what extent these parasites indeed have exploited the same vulnerabilities in host innate immunity as other plant microbes. Animal parasitologists have been ahead of plant nematologists by recognizing that immune modulation is a critical issue for the survival of the parasite. We have briefly entered the world of animal parasites in several sections of this chapter to explore potential overlaps in the mechanisms of immune evasion and suppression. We hope that this chapter will contribute to further comparative analyses of immune modulation by animal- and plant-parasitic nematodes. There seems to be sufficient overlap to accelerate the advances in both fields.

2.7 Acknowledgements

2.8 References

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Chapter 3

Dual Disease Resistance Mediated by the Immune Receptor Cf-2 in Tomato Requires a Common Virulence Target of a Fungus and a Nematode

3.1 Abstract

Plants lack the seemingly unlimited receptor diversity of a somatic adaptive immune system as found in vertebrates, and rely on only a relatively small set of innate immune receptors to resist a myriad of pathogens. Here, we show that disease resistant tomato plants use an efficient mechanism to leverage the limited non-self recognition capacity of their innate immune system. We found that the extracellular plant immune receptor protein Cf-2 of the red currant tomato (*Solanum pimpinellifolium*) has acquired dual resistance specificity by sensing perturbations in a common virulence target of two independently evolved effectors of a fungus and a nematode. The Cf-2 protein, originally identified as a monospecific immune receptor for the leaf mold fungus *Cladosporium fulvum*, also mediates disease resistance to the root parasitic nematode *Globodera rostochiensis* pathotype Ro1-Mierenbos. The Cf-2-mediated dual resistance is triggered by effector-induced perturbations of the apoplastic Rcr3^{pim} protein of *S. pimpinellifolium*. Binding of the venom allergen-like effector protein Gr-VAP1 of *G. rostochiensis* to Rcr3^{pim} perturbs the active site of this papain-like cysteine protease. In the absence of the Cf-2 receptor, Rcr3^{pim} increases the susceptibility of tomato plants to *G. rostochiensis*, thus showing its role as a virulence target of these nematodes. Furthermore, both nematode infection and transient expression of *Gr-VAP1* in tomato plants harboring *Cf-2* and *Rcr3^{pim}* trigger a defense-related programmed cell death in plant cells. Our data demonstrate that monitoring host proteins targeted by multiple pathogens broadens the spectrum of disease resistances mediated by single plant immune receptors.

3.2 Introduction

Dominant disease resistance genes encode highly specific immune receptors that offer plants protection against strains of pathogens carrying matching effector genes (*1, 2*). This phenomenon was captured in the classical "gene-for-gene" model of recognition specificity in disease resistance genes sixty years ago, and still forms the basis of disease resistance breeding in major food crops (*3*). Plant pathogens secrete effectors into the apoplastic space and the cytoplasm of host cells to suppress the cell-autonomous defense responses of the plant and to cause disease by targeting specific host cell components (*4*). Plant immune receptors can activate effector-trigged immunity upon direct recognition of unique non-self signatures in these pathogen-derived effector molecules (*5-11*). However, as opposed to directly recognizing effectors, most plant immune receptors are thought to act as sensors of disease-signaling perturbations in the virulence targets of pathogen effectors (*2, 12*).

Unlike most vertebrates, plants lack the seemingly infinite receptor diversity of a somatic adaptive immune system to detect effectors of invasive pathogens (*13*). The molecular mechanisms by which plants are nonetheless able to fend off a myriad of attackers with a limited innate receptor repertoire are largely unknown (*2*). Recent studies show that independently evolving effectors of different plant pathogens interact with common apoplastic and cytoplasmic host proteins involved in defense-related signaling networks (*14, 15*). It is speculated that indirectly perceiving pathogen effectors via their actions on common virulence targets enable plants to leverage the limited recognition capacity of their immune receptor repertoire (*16, 17*). Here we show that the plant immune receptor protein Cf-2 of the currant tomato (*Solanum pimpinellifolium*) has acquired dual resistance specificity by sensing perturbations in a common virulence target of two different plant pathogens.

Tomato cultivars (*Solanum lycopersicum*) harboring the Cf-2 immune receptor originating from currant tomato are resistant to particular strains of *C. fulvum* secreting the effector protein Avr2 (*18, 19*). The *C. fulvum* effector Avr2 interacts with the extracellular papain-like cysteine protease Rcr3 of tomato, which is required for Cf-2-mediated fungal resistance (*18, 19*). It is thought that specific perturbations of Rcr3 by Avr2 activate Cf-2 function in immune signaling cascades resulting in effector-triggered immunity to the fungus. Tomato cells secrete a range of papain-like cysteine proteases into the apoplast, at least two of which are inhibited by Avr2 (i.e. Rcr3 and PIP1; (*20, 21*)). Here, we report on a novel effector (hereafter named Gr-VAP1) of the obligate plant-parasitic nematode *Globodera rostochiensis* that interacts among others with a papain-like cysteine protease highly similar to Rcr3 and PIP1 in a yeast-two hybrid screen of tomato root cDNA. Gr-VAP1 belongs to a class of secreted venom allergen-like proteins that occur in all plant and animal-parasitic nematodes (*22*) and that share no sequence similarity with the effector Avr2 of *C. fulvum*. This prompted us to investigate whether Gr-VAP1-induced perturbations of Rcr3 can nonetheless activate Cf-2-mediated resistance to *G. rostochiensis* in tomato plants. We found that *Cf-2*, which has been used for decades in tomato as a resistance gene against the leaf mold fungus *C. fulvum* (*18*), also confers resistance to the potato cyst nematode *G. rostochiensis*.

3.3 Results and Discussion

***G. rostochiensis* secretes a novel venom-allergen like protein.** The expression of effectors in the potato cyst nematode *G. rostochiensis* is turned on when the obligate dormant period ends and the nematodes begin to hatch from eggs in the soil. To identify novel effectors of *G. rostochiensis*, we conducted a cDNA-AFLP-based transcriptome analysis during this transition in metabolic activity of the nematodes (*23*). Our analysis revealed, among

others, a transcript-derived fragment of 176 base pairs named NC4, which was strongly up-regulated in infective juveniles (Fig. 1A). Rapid amplification of the 5'- and 3'-ends of a matching expressed-sequence tag (GenBank accession AW506232) resulted in a full-length cDNA sequence of 926 base pairs, encoding a predicted secreted protein of 21,900 Dalton. The predicted protein sequence showed a significant similarity with Secreted Cysteine-rich Protein domains (SCP; GenBank accession CD05380 with E-value 2e[-28]), which is found in all members of the CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 proteins) protein superfamily. The NC4 protein is most similar to so-called Venom Allergen-like Proteins (VAPs) from plant- and animal-parasitic nematodes that collectively constitute a monophyletic clade within the CAP superfamily (E-values of 7.6e[-85] and higher in BLASTP). In accordance with a recently proposed nomenclature (22), we formally designate the NC4 protein as Nem-*Gro*-SCP/TAPS-1a, but for the remainder of this paper it is referred to as Gr-VAP1.

Effectors of plant-parasitic nematodes are produced in three single-celled esophageal glands that are connected to a hollow protusible oral stylet. To investigate whether *Gr-VAP1* is expressed in the esophageal glands of *G. rostochiensis*, we used whole mount *in situ* hybridization microscopy on infective juveniles. Anti-sense probes amplified from *Gr-VAP1* cDNA uniquely hybridized to the subventral esophageal glands, which are particularly active during host invasion and the early stages of feeding site formation (Fig. 1B). The complementary sense probe showed no hybridization. Effectors produced in the esophageal glands of *G. rostochiensis* are delivered to the apoplast and cytoplasm of host cells through the stylet. To investigate whether Gr-VAP1 is secreted by the nematodes, we analyzed collected stylet secretions of *G. rostochiensis* using liquid chromatography-mass spectrometry (LC-MS/MS). Two peptides perfectly matching Gr-VAP1 (i.e. SVLACHNNY and YSTSAETTAQNWANGCSMAHSSSSSR) were identified in a tryptic digest of secretions released by nematodes incubated for 24h in potato root exudates. Furthermore, the same tryptic digest included peptides matching plant cell wall-modifying proteins previously identified in stylet secretions of *G. rostochiensis* (Fig. 1C). These plant cell wall-modifying proteins are also produced in the subventral esophageal glands (24-26), and we therefore concluded that Gr-VAP1 is most likely secreted into the apoplast of host cells along with plant cell wall-modifying proteins.

The *G. rostochiensis* effector Gr-VAP1 interacts with the apoplastic cysteine protease Rcr3^pim of tomato. To identify host targets of Gr-VAP1, a tomato root cDNA library (*Solanum lycospersicum* cultivar GCR161) was screened in a yeast two-hybrid analysis using Gr-VAP1 as bait. One of the forty-two tomato cDNA clones interacting with Gr-VAP1 in yeast (SI Fig. 1A) included a partial cDNA sequence encoding a protein with high similarity to the apoplastic
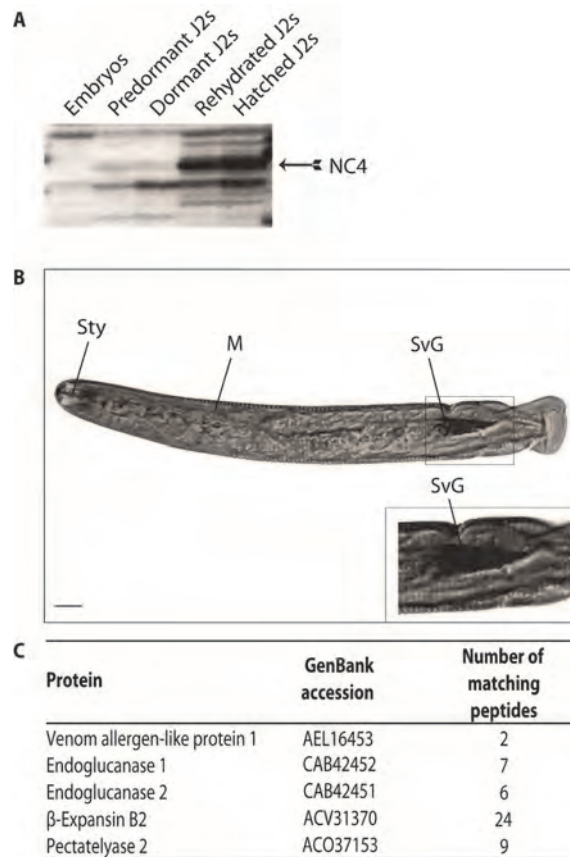
Fig. 1. A novel secreted venom allergen-like protein of *G. rostochiensis*. (A) A differential display of the transcript-derived fragment NC4 in embryos and second stage juveniles (J2s) of *G. rostochiensis* at different phases of dormancy and hatching. (B) In situ localization of transcripts encoding the venom allergen-like protein (Gr-VAP1) in the subventral esophageal gland (SvG) of a pre-parasitic J2 of *G. rostochiensis* (Sty, stylet; M, metacorpus; bar, 10 μm). (C) Numbers of peptides matching Gr-VAP1 and several cell wall-modifying proteins identified with LC-MS/MS in a tryptic digest of collected stylet secretions from preparasitic J2s of *G. rostochiensis*.

papain-like cysteine proteases Rcr3 and Pip1 (*20, 21*). The specificity of this interaction was confirmed by introducing the plasmid containing tomato cDNA into yeast cells together with the bait vector containing human lamin C or the empty bait vector (SI Fig. 1A). Possible autonomous transcriptional activation of reporter genes by Gr-VAP1 in yeast was excluded by co-transforming yeast cells with the bait vector harboring *Gr-VAP1* and the empty prey vector (SI Fig. 1B).

The Cf-2-mediated fungal resistance in tomato specifically requires binding of Avr2 to the protein encoded by the *S. pimpinellifolium* allele of Rcr3 (Rcr3pim; (*18, 19*)). We therefore examined whether affinity-tagged Gr-VAP1 also binds to recombinant Rcr3pim in an in vitro co-immunoprecipitation assay (Fig. 2A). Rcr3pim-His-HA, but not the affinity-tagged product of the Rcr3 allele of *S. lycopersicum* (Rcr3lyc-His-HA; Fig. 2B), was pulled-down by His-FLAG-Gr-VAP1 on agarose beads. By contrast, affinity-tagged Avr2 (His-FLAG-Avr2) pulled-down both Rcr3pim-His-HA (Fig. 2A) and Rcr3lyc-His-HA (Fig. 2B). Notably, the co-immunoprecipitation of Avr2 and Rcr3lyc-His-HA was only detectable with anti-HA serum after an extended exposure of the
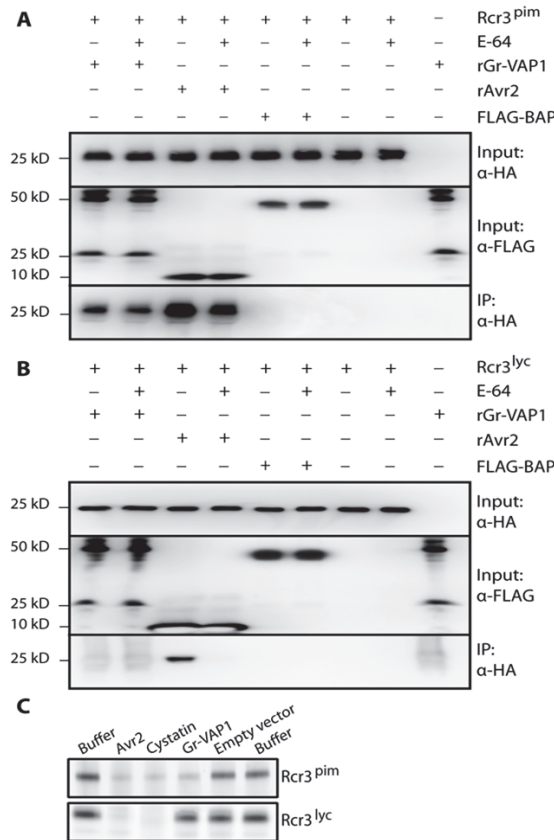
Fig. 2. The *G. rostochiensis* effector Gr-VAP1 interacts with the apoplastic papain-like cysteine protease Rcr3pim of tomato. Co-immunoprecipitation of Rcr3pim (Rcr3pim-His-HA (A)) and Rcr3lyc (Rcr3lyc-His-HA in (B)), with and without prior treatment with the protease inhibitor E-64, by either Gr-VAP1 of *G. rostochiensis* (His-FLAG-Gr-VAP1), Avr2 of *C. fulvum* (His-FLAG-Avr2), and a bacterial alkaline phosphatase (FLAG-BAP). Rcr3pim and Rcr3lyc were detected in the immunoprecipitate (IP) with anti-HA serum using different exposure times (t). (C), Binding of the fluorescent DCG-04 probe to the active site of Rcr3pim and Rcr3lyc following treatment with Gr-VAP1 and Avr2. Treatments with the cystatin, apoplastic fluids from agroinfiltrations with the empty binary expression vector (Empty vector), and with buffer alone (Buffer) were included as controls.

western blot, which suggests a weaker interaction between Avr2 and Rcr3^{lyc}. A similarly tagged bacterial alkaline phosphatase of *Escherichia coli* (FLAG-BAP) did not pull-down Rcr3^{pim} or Rcr3^{lyc} (Fig. 2A and B), demonstrating that the interaction between Gr-VAP1 and Rcr3^{pim} is specific. E-64 is a protease inhibitor that irreversibly binds to the active site of cysteine proteases (*27*). Pre-treatment of Rcr3^{pim} with E-64 in a subset of the samples reduced the binding of Gr-VAP1 to Rcr3^{pim} (Fig. 2A), indicating that this interaction involves the active site of Rcr3^{pim}. To further examine the involvement of the active site of Rcr3^{pim} in the interaction with Gr-VAP1, we used the fluorescent cysteine protease inhibitor DCG-04 for protease activity profiling on apoplastic fluids from agroinfiltrated *Nicotiana benthamiana* leaves either transiently expressing Rcr3^{pim}-His or Rcr3^{lyc}-His (Fig. 2C). Similar to Avr2, pre-incubating Rcr3^{pim} with Gr-VAP1 strongly reduced the access of DCG-04 to the active site of Rcr3^{pim}. Remarkably, DCG-04 labeling of the Rcr3^{lyc}, which differs only in six amino acid residues from Rcr3^{pim} (*18*), was reduced by Avr2 but not by Gr-VAP1. We therefore concluded that Gr-VAP1 specifically interacts with Rcr3^{pim}, and that this interaction perturbs the active site of Rcr3^{pim}.

Both Cf-2 and Rcr3^{pim} are required for defense-related hypersensitive response and resistance to *G. rostochiensis* in tomato. The tomato cultivar MoneyMaker (*Cf-0/Rcr3^{lyc}*), a near-isogenic line of MoneyMaker specifically selected for *Cf-2* resistance (*Cf-2/Rcr3^{pim}*; (*28*)), and an EMS mutant of this near-isogenic line (*Cf-2/rcr3-3*; (*29*)) were challenged with *G. rostochochiensis* pathotype Ro1-Mierenbos to study the effect of Cf-2 and Rcr3^{pim} on susceptibility to nematodes. To assess the effect of Rcr3^{pim} on the susceptibility of tomato plants to *G. rostochiensis* alone, we also included homozygous *Cf-0/Rcr3^{pim}* and *Cf-0/rcr3-3* lines that were generated by crossing *Cf-0/Rcr3^{lyc}* with *Cf-2/Rcr3pim* and *Cf-2/rcr3-3*, respectively. The presence of *Cf-2* and *Rcr3* transcripts in these tomato genotypes was checked with reverse transcription PCR using gene-specific primers (Fig. 3A). As the *Cf-2/Rcr3^{lyc}* genotype shows a constitutive autoimmunity phenotype (*18*), it cannot be tested for susceptibility to nematodes. Compared to the susceptible reference genotype *Cf-0/Rcr3^{lyc}* (*30*) the number of nematodes in *Cf-2/Rcr3^{pim}* plants at three weeks post inoculation was 68 percent lower (Fig. 3B). The nematode susceptibility of the genotypes *Cf-0/rcr3-3* and *Cf-2/rcr3-3* was similar to that of the *Cf-0/Rcr3^{lyc}* reference plants. We concluded that the *C. fulvum* immune receptor Cf-2 also confers resistance to *G. rostochiensis* in tomato, and that Cf-2-mediated resistance to *G. rostochiensis* requires Rcr3^{pim}. Interestingly, the number of nematodes in tomato plants that lack the *Cf-2* gene but harbor the functional *Rcr3^{pim}* allele (*Cf-0/Rcr3^{pim}*; Fig. 3A) was 40 percent higher than that in *Cf-0/Rcr3^{lyc}* and *Cf-0/rcr3-3* plants (Fig. 3B), demonstrating that Rcr3^{pim} from *S. pimpinellifolium* is most likely a virulence target of *G. rostochiensis*.
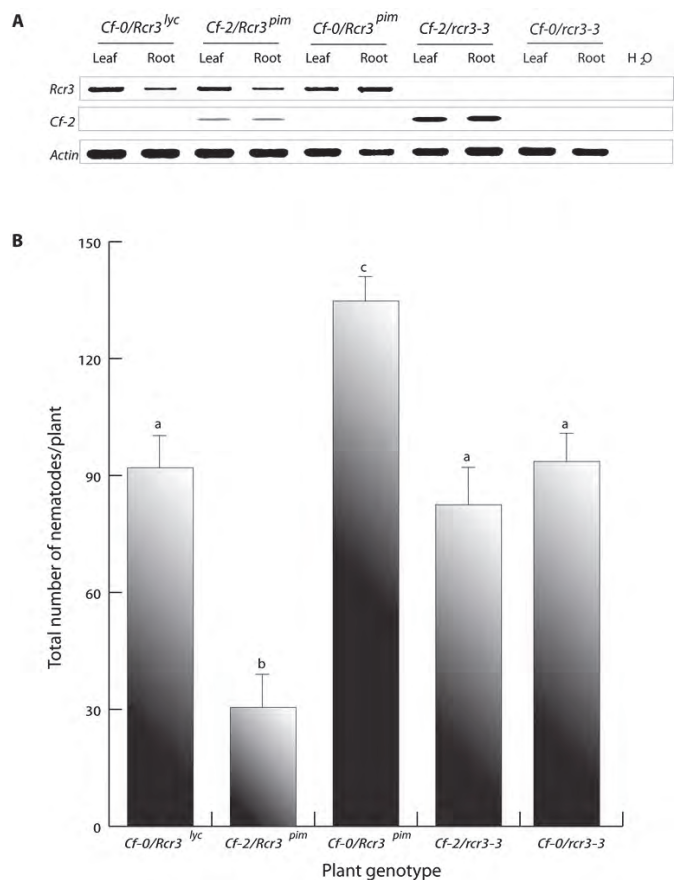
Fig. 3. Both Cf-2 and Rcr3^{pim} are required for resistance to G. rostochiensis in tomato. (A) A reverse transcription PCR of the expression of Cf-2, Rcr3, and actin in nematode-infected roots of five tomato genotypes with (Cf-2) and without the Cf-2 gene (Cf-0), either combined with the Rcr3^{pim} allele of S. pimpinellifolium, the Rrc3^{lyc} allele of S. lycopersicum, or the Rcr3^{pim} null mutant allele rcr3-3. Reactions without template were included as controls (H$_2$0). (B) The mean number of nematodes per tomato plant per genotype at three weeks post inoculation (bars indicate standard deviation). Different characters indicate statistical differences as determined with a one-way ANOVA test (P-values <0.001 with N=10).

Cf-2-mediated resistance to *C. fulvum* in leaves of tomato involves a local programmed cell death, associated with a hypersensitive response, at the infection site of the fungus (*31*). To investigate whether Cf-2/Rcr3^{pim}-mediated nematode resistance also involves a local plant cell death at the infection site of the nematode, we analyzed the subcellular structure of nematode-infected roots of *Cf-0/Rcr3^{lyc}*, *Cf-0/Rcr3^{pim}*, and *Cf-2/Rcr3^{pim}* tomato plants with light and

electron microscopy (Fig. 4; SI Fig. 2). The nematode-infected roots of *Cf-2/Rcr3^pim^* tomato plants revealed a specific cell death response in host cells inside most, but not all, of the infection sites of *G. rostochiensis.* From 7 days post invasion onwards, a progressive local cell death was observed in cells directly surrounding the nematodes and in most of the nematode-induced feeding structures in *Cf-2/Rcr3^pim^* plants. By contrast, the nematode infection sites in *Cf-0/Rcr3^pim^* tomato plants, demonstrating increased susceptibility to *G. rostochiensis*, were structurally similar to those observed in the susceptible reference genotype *Cf-0/Rcr3^lyc^*, and showed no signs of cell death. The cell death response observed in nematode-infected *Cf-2/Rcr3^pim^* plants resembles the response previously described for the nematode resistance genes *H1* and *Hero* (*30, 32*).
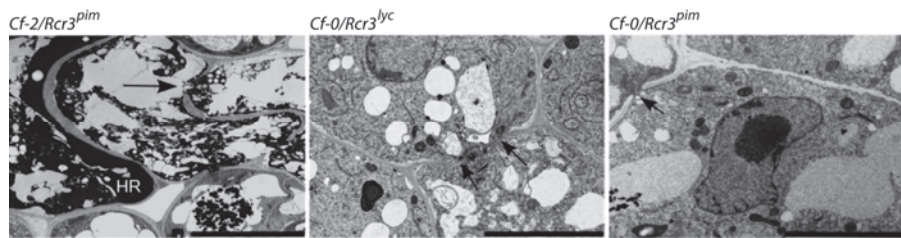
Fig. 4. Cf-2/Rcr3^pim^-mediated nematode resistance involves a defense-related local cell death response in the infection site of *G. rostochiensis* in tomato. Infections with *G. rostochiensis* in the tomato genotype *Cf-2/Rcr3^pim^*, but not in the genotypes *Cf-0/Rcr3^lyc^* and *Cf-0/Rcr3^pim^*, resulted in a local cell death associated with a hypersensitive response (HR) in the nematode-induced feeding structures in roots at the 7 days post inoculation. The arrows in the transmission electron micrographs indicate the typical local cell wall dissolution and subsequent fusion of protoplasts of tomato root cells incorporated into the feeding structure of *G. rostochiensis*. Scale bars: 5 μm.

The *G. rostochiensis* effector Gr-VAP1 triggers a Cf-2- and Rcr3^pim^-dependent programmed cell death in tomato. To test whether the nematode effector Gr-VAP1 is able to trigger a Cf-2/Rcr3^pim^-dependent programmed cell death, we transiently expressed *Gr-VAP1* with its native signal peptide for secretion in leaves of the tomato genotypes *Cf-2/Rcr3^pim^*, *Cf-2/rcr3-3*, *Cf-0/Rcr3^pim^*, and *Cf-0/Rcr3^lyc^* (Fig. 5A). Just like *Avr2* of *C. fulvum*, transiently expressed *Gr-VAP1* only triggered a cell death response in leaves of *Cf-2/Rcr3^pim^* plants. To further confirm that both Cf-2 and Rcr3^pim^ are required for this cell death response, *Gr-VAP1* was transiently co-expressed with and without *Rcr3^pim^* in leaves of the tomato genotypes *Cf-2/rcr3-3* and *Cf-0/rcr3-3* that lack a functional *Rcr3* gene (Fig. 5B). Only the complementation of *Cf-2/rcr3-3* plants with *Rcr3^pim^* resulted in a cell death response to Gr-VAP1. We therefore concluded that both Cf-2 and Rcr3^pim^ are required for the defense-

related cell death response triggered by the nematode effector Gr-VAP1 in tomato.
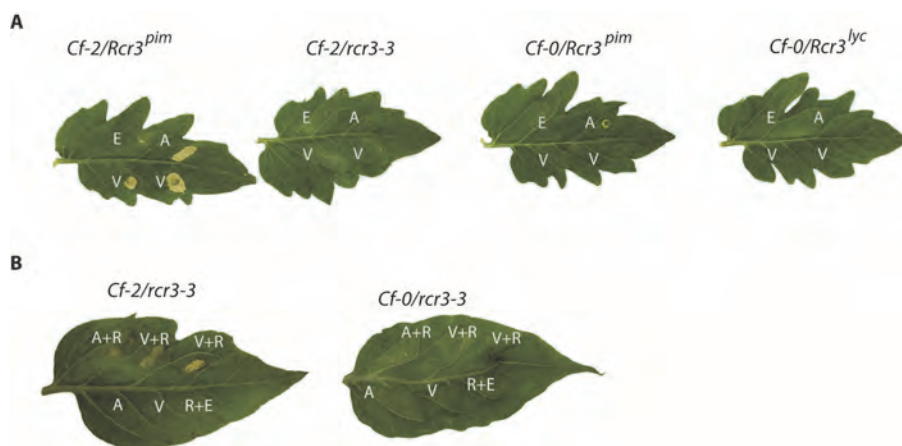
Fig. 5. Gr-VAP1 triggers a Cf-2- and Rcr3pim-dependent defense-related cell death response in tomato. (A) *Agrobacterium tumefaciens* mediated transient expression of *Gr-VAP1* (V), *Avr2* (A), or the corresponding empty binary expression vector (E) in infiltrated leaves of the tomato genotypes *Cf-2/Rcr3pim*, *Cf-2/rcr3-3*, *Cf-0/Rcr3pim*, and *Cf-0/Rcr3lyc*. (B) Transient co-expression of *Gr-VAP1* (V) and *Avr2* (A) with the empty binary plant expression vector (E) or with *Rcr3pim* (R) in leaves of the tomato genotypes *Cf-2/rcr3-3* and *Cf-0/rcr3-3*. Pictures were taken 5 days post infiltration.

Dual Cf-2-mediated resistance requires a common virulence target of two unrelated pathogens. Our data demonstrate that *Cf-2* is a dual resistance gene that offers tomato plants protection against a leaf mold fungus and a root-parasitic nematode. The dual resistance specificity of Cf-2 results from perturbations in the apoplastic papain-like cysteine protease Rcr3pim induced by two structurally different effectors. Recently, it has been shown that the oomycete plant-pathogen *Phytophthora infestans* secretes cystatin-like effectors that also interact with Rcr3pim (*15, 33, 34*). While the perturbations of Rcr3pim induced by Gr-VAP1 of *G. rostochiensis* and by Avr2 of *C. fulvum* may be structurally different, both trigger a Cf-2-mediated programmed cell death and disease resistance. Surprisingly, the perturbations of Rcr3pim brought about by the effectors EPIC1 and EPIC2B of *P. infestans* do not trigger Cf-2-mediated defense responses (*15*), suggesting that they fall outside of the activation window of Cf-2. The constitutive autoimmune phenotype in *Cf-2/Rcr3lyc* plants (*18*), which is not observed in *Cf-2* plants lacking a functional *Rcr3* gene (i.e. *Cf-2/rcr3-3*), further suggests that association with either Rcr3lyc or Gr-VAP-1/Avr2-perturbed Rcr3pim, but not the absence of Rcr3, activates

Cf-2. The next step is to investigate whether the activation of Cf-2-mediated disease resistances indeed involves a physical association between this immune receptor and different effector-Rcr3[pim] complexes.

The dual-specificity of Cf-2 via a common virulence target of a fungus and an invertebrate demonstrates the power of indirect recognition of pathogen-derived molecules for plants. By 'guarding' critical host molecules targeted by multiple and unrelated pathogens, plants can efficiently expand the coverage of their innate immune system with a relatively small set of extracellular and cytoplasmic immune receptors. The recent finding that the *Mi-1.2* gene, originally identified as a root-knot nematode resistance gene, confers resistance to at least four different attackers by an unresolved mechanism suggests that multiple resistance specificities of immune receptors may contribute significantly to the resistance spectrum of plants (*35-39*). The relatively late discoveries of the multiple resistance specificities of *Cf-2* and *Mi-1.2*, which were both introgressed into cultivated tomato in the 1940s, further show how the paradigm of singular specificity of disease resistance genes (*3*) has dominated the screening methods of plant breeders. The notion that single immune receptors conferring multiple resistances to taxonomically unrelated pathogens may not be exceptional, gives breeders a strong incentive to identify and to use common virulence targets as leads to develop broad specificity resistance genes.

3.4 Materials and Methods

Identification of Gr-VAP1 in stylet secretions. A fragment of 176 base pairs derived from the Gr-VAP1 transcript was identified in a cDNA-AFLP analysis of successive developmental stages of *G. rostochiensis* pathotype Ro1 Mierenbos (*23*). The transcript-derived fragment was extended using partially overlapping expressed sequence tags of *G. rostochiensis* in non-redundant database (*40*). The 5'- and 3'-ends of the transcript-derived fragment was cloned by using rapid amplification of cDNA-ends (Invitrogen, Carlsbad, USA). The Gr-VAP1 transcripts were localized in freshly hatched pre-parasitic second stage juveniles of *G. rostochiensis* pathotype Ro1 using whole mount mRNA in situ hybrididization (*26*). Peptides matching the protein sequences of Gr-VAP1 and several cell wall-modifying proteins were detected with LC-MS/MS in collected stylet secretions of preparasitic second stage juveniles of *G. rostochiensis* Ro1 (*41*). Details are given in *SI Materials and Methods*.

Yeast-two-hybrid screening. The Matchmaker System 3 (Clontech, Mountain View, CA, USA) was used to screen a tomato root cDNA library (*S. lycopersicum* cultivar CGR161) in yeast with Gr-VAP1 as bait (*42*). cDNA of *Gr-VAP1* without signal peptide for secretion was amplified with a polymerase chain reaction (PCR) using the primers Gr-VAP1-Y2HFw and Gr-

VAP1-Y2HRv (SI Table 1) and subcloned into the *Eco*RI/*Bam*HI site of pGBKT7-BD for expression in yeast (*Saccharomyces cerevisiae* strain AH109). Details are given in *SI Materials and Methods*.

Co-immunoprecipitation experiments. Recombinantly produced Gr-VAP1, Avr2, and BAP with a FLAG tag were incubated with anti-FLAG serum labeled agarose beads. The beads were subsequently either incubated with recombinant Rcr3^{pim}-His-HA or Rcr3^{lyc}-His-HA in duplicates. In one sample of each duplicate set the active site of Rcr3^{pim}-His-HA or Rcr3^{lyc}-His-HA was blocked with the competitive cysteine protease inhibitor E64 first. The fractions of Rcr3^{pim}-His-HA and Rcr3^{lyc}-His-HA bound to Gr-VAP1, Avr2, and BAP on beads were visualized on western blot with anti-HA serum. Details are given in *SI Materials and Methods*.

Fluorescent protease activity profiling. *Rcr3^{pim} of S. pimpinellifolium* and *Rcr3^{lyc} of S. lycopersicum* were transiently overexpressed in the apoplastic fluids of *N. benthamiana* leaves following agroinfiltration. Apoplastic fluids were incubated with either Avr2, cystatin from chicken egg white, and Gr-VAP1. To label the remaining available active sites in Rcr3^{pim} and Rcr3^{lyc}, the proteins were subsequently incubated with fluorescent DCG-04-TMR. Details are given in *SI Materials and Methods.*

Microscopy of nematode infections. In vitro germinated seedlings of three tomato genotypes *Cf-2/Rcr3^{pim}*, *Cf-0/Rcr3^{lyc}*, and *Cf-0/Rcr3^{pim}* were challenged with infective second stage juveniles of *G. rostochiensis* pathotype Ro1 Mierenbos. At 3, 7, 14, and 21 days post invasion, nematode-infected roots were collected, fixed, sectioned, and stained for transmission electron microscopy. Details are given in *SI Materials and Methods*.

Effector-induced plant cell death response. *Gr-VAP1* and *Avr2* were transiently expressed in the apoplastic space of tomato mesophyll cells following agroinfiltration in leaves of tomato genotypes *Cf-2/Rcr3^{pim}*, *Cf-2/rcr3-3*, *Cf-0/Rcr3^{pim}*, and *Cf-0/Rcr3^{lyc}*. The development of a specific local cell death in the infiltrated leaf area from 3 to 7 days post infiltration was used as indicator of a defense-related hypersensitive response. Details are given in *SI Materials and Methods*.

3.5 Acknowledgements

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3.7 Supporting Information

3.7.1 SI Materials and Methods

Cloning of Gr-VAP1. Messenger RNA was extracted from five stages of *Globodera rostochiensis* pathotype Ro1 for a cDNA-AFLP analysis (*1*). The primary cDNA templates synthesized from five mRNA pools were digested using the restriction enzymes *Nco*I and *Taq*I. Oligonucleotide primers annealing to the *Nco*I and *Taq*I adapter sequences were used to specifically amplify transcript-derived fragments flanked by these restriction enzymes (*2*). Specific transcript-derived-fragments, including transcript-derived fragment NC4, were excised from acrylamide gels. Following reamplification using the original primers of the cDNA-AFLP analysis, NC4 was cloned into the TOPO-pCR4 plasmid (Invitrogen, Carlsbad, CA) and sequenced. Eighteen expressed sequence tags of *G. rostochiensis* partially overlapping the NC4 were identified in the non-redundant database of GenBank with BLASTN.

To resolve the 5'- and 3'-ends of the transcript up- and downstream of the NC4 fragment, we used the rapid amplification of cDNA ends system (Invitrogen). For the 5'-end, first strand cDNA was synthesized using oligo-dT primers from 1.5 mg of total RNA isolated from 0.5 g of grinded *G. rostochiensis* pre-parasitic second stage juveniles using Trizol (Invitrogen). Nested PCR products, generated with the gene specific primer Gr-VAP1-RaceRv (SI Table 1) and adaptor primers from the GeneRacer system, were cloned into the pGEM-T vector (Promega, Fitchburg, WI), and sequenced. For the 3'-end, nested PCR was performed with the gene specific primer Gr-VAP1-RaceFw (SI Table 1) and adaptor primers included in the GeneRacer system (Invitrogen). PCR products were cloned into the pGEM-T vector (Promega), and sequenced. The resulting sequences were assembled into a full-length cDNA sequence, hereafter named Gr-VAP1 (GenBank accession number AJ536826).

***Gr-VAP1* expression in *G. rostochiensis*.** Whole mount in situ hybridization was performed on pre-parasitic second stage juveniles of *G. rostochiensis* pathotype Ro1 to localize *Gr-VAP1* transcription in nematodes (*1, 3*). Briefly, the NC4 fragment was subcloned into pCR2.1-TOPO vector (Invitrogen). Sense and antisense probes were generated by asymmetric PCR in the presence of DIG-dUTP (Roche, Basel, Switzerland) using N+0 and T+0 primers (*1*). The DIG-labelled probes were purified in a G50 Mini Quick Spin DNA column (Roche). Alkaline phosphatase activity was detected by the addition of X-phosphate and NBT (Roche), and hybridization of the probes in infective juveniles was examined with an inverted microscope (Leica Microsystems, Wetzlar, Germany)

Identification of Gr-VAP1 in *G. rostochiensis* **stylet secretions.** To collect stylet secretions of *G. rostochiensis* about 100,000 freshly hatched preparasitic second juveniles were either incubated for 24 h in 2 ml filter sterile potato root diffusates with 4 mM 5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate or in tap water (*4*). Two ml of collected secretions was freeze-dried, dissolved in 50 µl 0.1% (w/v) RapiGest SF Surfactant (Waters, Milford, USA), 5 mM DTT (Sigma) in 0.1 M ammonium bicarbonate, and incubated at 60°C for 1 hr. Alkylation was performed by incubation with 15 mM iodoacetamide (GE Healthcare, UK) for 30 min at room temperature (in the dark). Proteolytic digestion was initiated by adding 1 µl of modified porcine trypsin (0.1 µg/µl; Sequence grade modified; Promega) and incubated overnight at 37°C. After adding trifluoroacetic acid (Fluka,-Buchs, GmBH) to a final concentration of 0.5% (v/v), samples were centrifuged at 15,000 g for 10 min., and the supernatant was applied to a SupelClean™ LC-18 1 ml SPE column (Supelco, Bellefonte USA) equilibrated with 0.1% TFA. Bound peptides were eluted with 84% acetonitrile (HPLC Supra-gradient, Biosolve, Valkenswaard, NL) containing 0.1% formic acid (Merck, Darmstadt, Germany), dried down by vacuum centrifugation, dissolved in 40 µl 0.1% formic acid and further analyzed with mass spectrometry. The tryptic peptide samples were separated using a NanoAcquity UPLC system (Waters Corporation, Manchester, UK) using a BEH C$_{18}$ column (75 µm x 25 cm, Waters, UK) and a 65 min linear gradient from 3 to 40% acetonitrile (in 0.1 % formic acid) at 200 nl/min. The eluting peptides were on-line injected into a Synapt Q-TOF MS instrument (Waters Corporation) using a nanospray device coupled to the second dimension column output. The Synapt MS was operated in positive mode with [Glu^1] fibrinopeptide B (1 pmol/µl; Sigma) as reference (lock mass) and sampled every 30 s. Accurate liquid chromatography-mass spectrometry (LC-MS) data were collected with the Synapt operating in MS/MS mode for data-dependent acquisition. LC-MS/MS was performed by peptide fragmentation on the three most intense multiple charged ions that were detected in the MS survey scan (0.6 s) over a 300-1400 m/z range and a dynamic exclusion window of 60 s with an automatically adjusted collision energy based on the observed precursor m/z and charge state. LC-MS/MS data were processed using ProteinLynx Global Server software (PLGS version 2.4, Waters Corporation, Manchester, UK) and the resulting list of masses containing all the fragment information was analyzed for matching proteins using a custom-build non-redundant database including all currently known (predicted) proteins of *G. rostochiensis*.

Yeast-two-hybrid screening. The Matchmaker System 3 (Clontech, Mountain View, CA, USA) was used to screen a tomato root cDNA library (*S. lycopersicum* cultivar CGR161) in yeast with Gr-VAP1 as bait (*5*). cDNA of *Gr-VAP1* without signal peptide for secretion was amplified with a polymerase

chain reaction (PCR) using the primers Gr-VAP1-Y2HFw and Gr-VAP1-Y2HRv (SI Table 1) and subcloned into the *Eco*RI/*Bam*HI site of pGBKT7-BD for expression in yeast (*Saccharomyces cerevisiae* strain AH109). Yeast cells were co-transformed with the bait vector (pGBKT7-BD::Gr-VAP1) and the tomato root cDNA-library cloned in pACT2 (*5*) and plated on high-stringency medium SD/-Ade/-His/-Leu/-Trp/X-α-gal to select for α-galactosidase activation. After three rounds on selective medium tomato root library plasmids were isolated from α-galactosidase positive yeast colonies and retransformed into yeast cells together with pGBKT7-LaminC to test for transactivation.

Co-immunoprecipitation. cDNA of *Gr-VAP1* was PCR-amplified and cloned into pPIC-9His using *Sma*I and *Eco*RI restriction sites to generate His-FLAG-tagged Gr-VAP1 for heterologous expression in *Pichia pastoris* strain GS115 (Invitrogen)(*6*). Recombinant His-FLAG-Avr2, Rcr3pim-His-HA, and Rcr3lyc-His-HA were produced as previously described (*6*), while bacterial alkaline phosphatase of *Escherichia coli* (FLAG-BAP; Sigma-Aldrich, St. Louis, MO, USA) was used a control. His-FLAG-Gr-VAP1, His-FLAG-Avr2, and FLAG-BAP were each incubated with 40 μl of α-FLAG M2 agarose beads (Sigma-Aldrich) in Co-IP buffer (50 mM sodium acetate, 10 mM L-cysteine, pH 5.0). Next, the agarose beads were incubated overnight at 4°C with either Rcr3pim-His-HA or Rcr3lyc-His-HA in duplicates. One of each set of duplicates was pre-incubated in Co-IP buffer with 40 μM E-64 (Sigma-Aldrich) for 30 min at room temperature to block the active site in Rcr3lyc and Rcr3pim. The agarose beads were subsequently washed three times by incubating in 1 ml Co-IP buffer and centrifugation at 3,000 g. The bound proteins were analyzed on western blot using α-HA(*6*) and α-FLAG serum (Sigma-Aldrich).

Fluorescent protease activity profiling. *Rcr3pim of S. pimpinellifolium* and *Rcr3lyc* of *S. lycopersicum* were transiently overexpressed in the apoplastic fluids of *N. benthamiana* leaves following agroinfiltration (*7*). Twenty-five microliters of these apoplastic fluids were incubated with either 100 nM of *P. pastoris* produced Avr2, 100 nM cystatin from chicken egg-white (Sigma-Aldrich), and 300 nM of *N. benthamiana* produced Gr-VAP1 in 50 mM sodium acetate (pH 5.5) and 100 μM DTT. To label the available active sites in Rcr3pim and Rcr3lyc, the proteins were subsequently incubated for 5h with 1 μM of fluorescent DCG-04-TMR (*8*). Fluorescent proteins separated in 12% Bis-Tris gels (Invitrogen) were detected using a fluorescent imager scanner (Molecular Imager FX, Bio-Rad).

Nematode resistance assays on tomato. The tomato (*S. lycopercium*) cultivar MoneyMaker that carries no resistance to *C. fulvum* ot to *G. rostochiensis* and is homozygous for the Rcr3lyc allele was used as reference genotype for normal

susceptibility to *G. rostochiensis* Cf-0/Rcr3*lyc*). The homozygous near-isogenic line of MoneyMaker that has been used for characterization of Cf-2-mediated and Rcr3*pim* dependent resistance to *C. fulvum* (*6, 9, 10*) was used to test resistance to *G. rostochiensis* pathotype Ro1-Mierenbos. This near-isogenic line derives from a cross between MoneyMaker and the cultivar Vetomold K10, which was repeatedly backcrossed with MoneyMaker and specifically selected for Cf-2 resistance only, carries the *Cf-2* gene and the *Rcr3pim* allele of *Solanum pimpinellifloium* (Cf-2/Rcr3*pim*; (*11*)). The deletion mutant of Rcr3*pim* (Cf-2/rcr3-3) was made by EMS in this near-isogenic line Cf-2/Rcr3*pim* (*10*). The *Cf-0/Rcr3pim* line was made by a cross between *Cf-0/Rcr3lyc* and *Cf-2/Rcr3pim*. Similarly, the Cf-0/rcr3-3 line was made by crossing *Cf-0/Rcr3lyc* and *Cf-2/rcr3-3*. Two homozygous lines were selected from the F2 generation of each cross using gene-specific primers for the absence of *Cf-2* and *Rcr3lyc*, and the presence of *Rcr3pim* and mutant *rcr3-3* genes. Reverse transcription PCR was used to confirm the expression of *Cf-2, Rcr3*, and actin in nematode-infected roots of the tomato genotypes *Cf-2/Rcr3pim*, *Cf-0/Rcr3pim*, *Cf-0/Rcr3lyc*, *Cf-2/rcr3-3*, and *Cf-0/rcr3-3* (*12*) using primer combinations Cf-2Fw and Cf-2Rv (SI Table 1), RC1 and RC4 (*9*), and AC1 and AC2 (*9*), respectively. The PCR products were analysed on agarose gels with ethidium bromide.

Seeds of *Cf-2/Rcr3pim*, *Cf-0/Rcr3pim*, *Cf-0/Rcr3lyc*, *Cf-2/rcr3-3*, and *Cf-0/rcr3-3* were surface sterilized and grown on Gamborg B5 medium including vitamins and minerals (Duchefa, Haarlem, The Netherlands), and 2% sucrose (pH 6.2). After 3 weeks, each seedling was inoculated with 200 surface-sterilized freshly-hatched second stage juveniles of *G. rostochiensis* pathotype Ro1-Mierenbos. After three weeks at 24°C with 16 h light, the number of nematodes inside the roots were counted using a dissection microscope. The means of numbers of nematodes per plant were tested for significant differences in a one-way ANOVA with 10 replicates (= n) per plant genotype, while using a type-1 error level of 0.05. The nematode infection assays were repeated two times.

Light and transmission electron microscopy. Seeds of the tomato genotypes *Cf-0/Rcr3lyc*, *Cf-0/Rcr3pim* and *Cf-2/Rcr3pim* were surface-sterilized in 4% (w/v) sodium hypochlorite for 10 min (*13*). The seeds were subsequently rinsed four times with sterile water and transferred into 1.5% (w/v) water agar. Germinating seeds having 1.0-1.5 cm long roots were transferred to Murashige and Skoog medium supplemented with Gamborg's B5 vitamins, 2% (w/v) sucrose and 0.6% (w/v) agar and incubated for two weeks at 18°C with 16h light. Next, each seedling was inoculated with 200 surface-sterilized freshly-hatched second stage juveniles of *G. rostochiensis* pathotype Ro1. The aerial parts of the plants were removed after inoculation and the plates with roots were incubated in the dark at 18°C. Samples of roots were collected at 3, 7, 14 and 21 days after root invasion, fixed in glutaraldehyde and

paraformaldehyde, and embedded in Epoxy resin. Serial semi-thin (3 μm) and ultrathin (70 nm) sections were examined under an Olympus AX70 "Provis" light microscope (Olympus, Tokyo, Japan) and a FEI M268D "Morgagni" transmission electron microscope (Fei, Hillsboro, OR, USA), respectively.

Effector-induced programmed cell death. *Gr-VAP1* including the native signal peptide for secretion was PCR-amplified from a cDNA clone using primers Gr-VAP1-GWFw and Gr-VAP1_STOP-GWRv (SI Table 1). *Avr2* including the signal peptide for secretion of PR1a (Genbank accession X06930) was PCR-amplified from a cDNA clone using the primers PR1aSP-GWFw and Avr2_STOP-GwRv (SI Table 1). *Rcr3^{pim}* including native signal peptide for secretion was PCR- amplified from a genomic clone using the primers Rcr3^{pim}-GWFw and Rcr3^{pim}_STOP-GWRv (SI Table 1). After cloning the PCR products into pENTR-D-TOPO (Gateway, Invitrogen), the constructs were transferred to the pSOL2086 destination vector. All constructs were introduced into *Agrobacterium tumefaciens* 1D1249 and infiltrated into leaves of 7 week-old plants of the tomato genotypes *Cf-2/rcr3^{pim}* and *Cf-2/Rcr3-3^{pim}*. The agroinfiltrated plants were kept in a growth chamber at 25ºC with 16 h light, and visually inspected for the cell death symptoms in the infiltrated leaf area for 7 days after infiltration.

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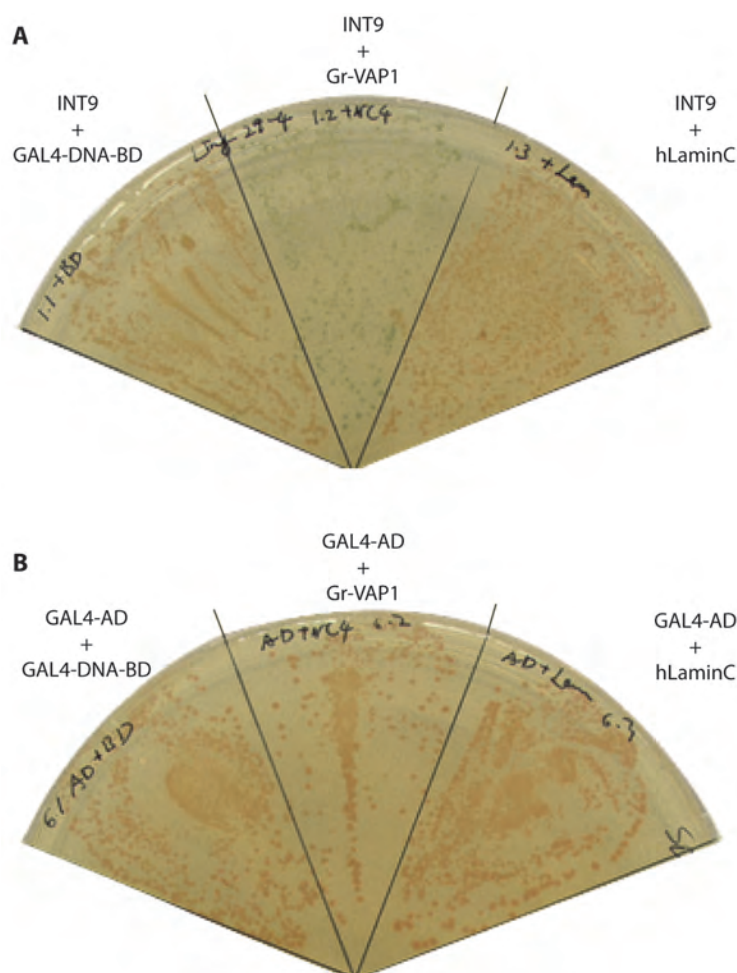
Fig. S1. The effector Gr-VAP1 of *G. rostochiensis* specifically interacts with a partial sequence of an apoplastic cysteine protease of tomato in a yeast-two-hybrid screen. (A) α-Galactosidase (Gal4) active yeast colonies (blue) on selective medium demonstrating the interaction of Gr-VAP1 with the partial sequence of cysteine protease (INT9) similar to Rcr3 and PIP1. Co-transformations of yeast cells with INT9 and either the empty bait plasmid (GAL4-DNA-BD) or human Lamin C (hLaminC) were used to test for autonomous activation of Gal4 by INT9. (B) Co-transformation of yeast cells with Gr-VAP1 and the empty prey plasmid (GAL4+AD) was included to test for autonomous activation of GAL4 by Gr-VAP1.
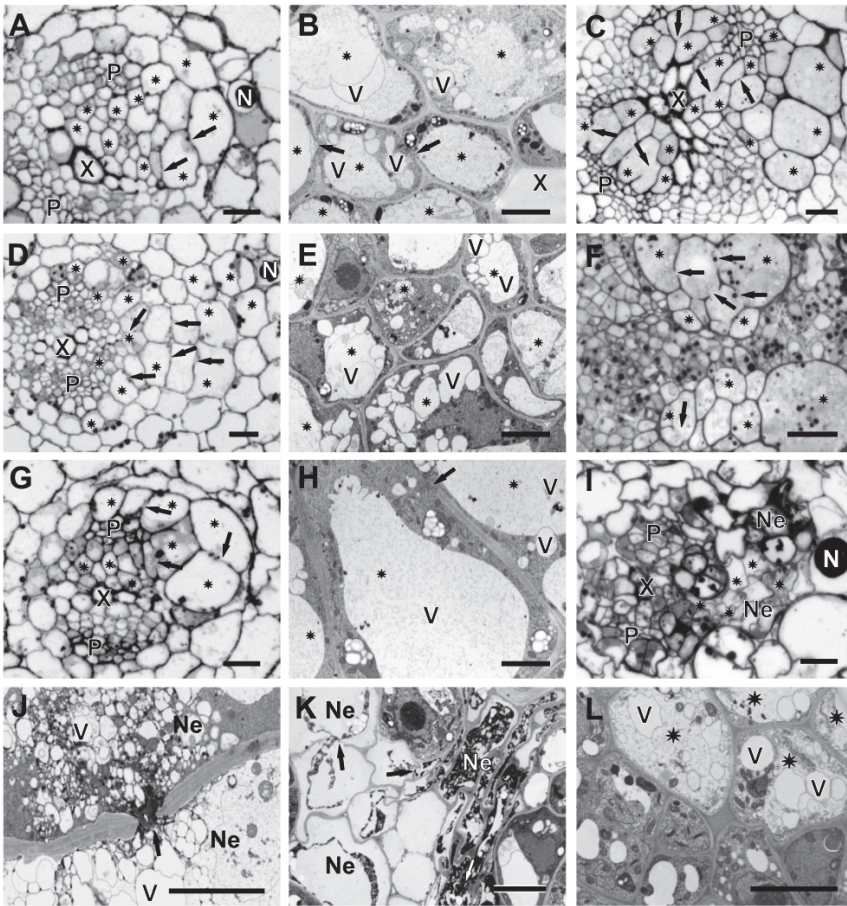
Fig. S2. Resistance to *Globodera rostochiensis* in *Cf-2/Rcr3pim* tomato plants involves a defense-related plant cell death response in the infection site. Microscopic comparison of feeding site development in roots of *Cf-0/Rcr3lyc* (A to C), *Cf-0/Rcr3pim* (D to F) and *Cf-2/Rcr3pim* (G to L) tomato plants. Samples were collected at 3 (A, B, D, E, G and H), 7 (I to L), 14 (C) and 21 (F) days after root invasion. Bright field light microscopy images (A, C, D, F, G and I) and transmission electron microscopy images (B, E, H and J to L) with the following indicators: local cell wall opening (arrow), selected syncytial elements to indicate which cells are included in the feeding structure of the nematode (*), nematode (N), cell death (Ne), phloem (P), xylem (X), and vacuole (V). Scale bars: 20µm (A, C, D, F, G and I) and 5 µm (B, E, H and J to L).

Table S1: Oligonucleotides used for PCR

Name	Oligonucleotide sequence (5'- to 3'-end)
Gr-VAP1-RaceRv	CGTTGAGCGGTAGTTGTTGTGGC
Gr-VAP1-RaceFw	GCCACAACAACTACCGCTCAACG
Gr-VAP1-RTFw	GCATTGGGCATTGGAGTC
Gr-VAP1-RTRv	TTTGTAGACGACCTGGTTC
cAMP-RTFw	ATCAGCCCATTCAAATCTACG
cAMP-RTRv	TTCTTCAGCAAGTCCTTCAAC
Gr-VAP1-Y2HFw	CCGGAATTCCTTTCTGCGTCCAGCC
Gr-VAP1-Y2HRv	CGCGGATCCCCAAAACGCACAGTCCG
Cf-2Fw	GATCTCATTGCGATCCGTATA
Cf-2Rv	ATAGCCCATCAGAGCTGCTTTCC
RC1	TGGCTGTTTAGTTACGGCTTG
RC4	ACGAGCTGTGGATGTCACGTC
AC1	ATGGCAGACGGTGAGGATATTCA
AC2	GCCTTTGCAATCCACATCTGTTG
Gr-VAP1-GWFw	CACCATGGCGTTTGCCCCAACAAT
Gr-VAP1_STOP-GWRv	TTATGGCAAAACGCACAGTCCGCTGG
PR1aSP-GWFw	CACCATGGGATTTGTTCTCTTTTCA
Avr2_STOP-GWRv	TCAACCGCAAAGACCAAAACAGCA
Rcr3^{pim}-GWFw	CACCATGGCTATGAAAGTTGATTTG
Rcr3^{pim}_STOP-GWRv	CTATGCTATGTTTGGATAAGAAGA

Chapter 4

Venom Allergen-like Proteins Secreted by Plant-parasitic Nematodes Modulate Defense Responses Mediated by Extracellular Innate Immune Receptors

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Chapter 4

4.1 Abstract

Plants and animals utilize cell-surface receptors to survey their direct environment for molecular patterns uniquely associated with infections by microbial invaders. Recently, we showed that the venom allergen-like protein Gr-VAP1 from the plant-parasitic nematode *Globodera rostochiensis* targets the papain-like cysteine protease Rcr3^{pim} that functions as a cofactor of the extracellular plant immune receptor Cf-2 in tomato. However, the presence of Rcr3^{pim} alone almost doubles the susceptibility of tomato plants to *G. rostochiensis*, suggesting that Gr-VAP1 modulates the activity of Rcr3^{pim} to enhance the virulence of the nematodes. Although secreted venom allergen-like proteins are highly conserved among plant-parasitic nematodes, their function in parasitism of host plants is largely unknown. Here we demonstrate that venom allergen-like proteins from plant-parasitic nematodes specifically suppress defense responses mediated by extracellular innate immune receptors. Knocking-down the expression of venom allergen-like proteins in *G. rostochiensis* strongly reduced the virulence of the nematodes. Ectopic expression of different venom allergen-like proteins from plant-parasitic nematodes in transgenic Arabidopsis plants significantly reduced their basal defense responses to nematodes, multiple other plant pathogens, and the immunogenic peptide flg22 from bacterial flagella. Remarkably, several venom allergen-like proteins from plant-parasitic nematodes suppressed the defense-related programmed cell death activated by multiple extracellular plant immune receptors in leaves of *Nicotiana benthamiana*, suggesting that they target a conserved mechanism in the activation of extracellular pattern-recognition receptors in plants. The transcriptome of plants expressing recombinant venom allergen-like proteins revealed profound alterations in the extracellular proteome with a strong bias toward innate immunity and plant cell wall associated processes. We therefore propose a model in which venom allergen-like proteins modulate the activation and/or signaling by extracellular immune receptors to suppress defense responses triggered by damage-associated molecular patterns in the host.

4.2 Introduction

Plants employ transmembrane proteins, belonging to the receptor-like kinase (RLK)/Pelle superfamily, to detect extracellular molecular patterns that are either directly derived from microbial invaders or uniquely associated with their actions in the apoplast (*1-10*). The recognition of immunogenic microbe- and host-associated molecular patterns by RLKs activates intracellular immune signaling pathways that result in structural and chemical responses against extracellular invaders. It is thought that signals perceived by the

extracellular domain of the RLKs induce the conformational activation of the cytoplasmic serine-threonine protein kinase domain. This subsequently activates other molecular components in immune signaling pathways further downstream of the receptor. Several members of the RLK/Pelle superfamily lack a cytoplasmic kinase domain, while they are nonetheless able to activate immune responses to pathogens (e.g. Cf-proteins in tomato). The activity of these so-called receptor-like proteins (RLPs) most likely involves mediation by transmembrane RLKs, or other cytoplasmic membrane-associated kinases, that function as co-factors within multimeric receptor complexes. Genome analyses suggest that the RLK/Pelle superfamily has undergone an extraordinary expansion in land plants, most of which has happened through sequence diversification in the extracellular domains that are thought to function as recognition modules in plant immune receptors (*11*).

Recently, we have shown that the extracellular RLP Cf-2 in tomato mediates dual disease resistance by guarding a common virulence target of a nematode and a fungus (*12*). Perturbations of the extracellular papain-like cysteine protease Rcr3[pim] by two unrelated effectors from the leaf mold fungus *Cladosporium fulvum* and from the root parasitic nematode *G. rostochiensis* activate Cf-2-mediated disease resistance. The function of Rcr3[pim] in tomato or any of its close homologs in plants is not known. However, tomato plants harboring Rcr3[pim], but not the receptor Cf-2, are far more susceptible to nematode infections than tomato plants lacking Rcr3[pim]. The Cf-2/Rcr3[pim]-mediated nematode resistance involves a hypersensitive response type of programmed cell death in the feeding structure of *G. rostochiensis*. Like all sedentary plant- parasitic nematodes, *G. rostochiensis* hacks the developmental program of vascular parenchyma cells of host plants to modify them into specialized feeding structures. For several weeks, these modified host cells are the sole source of plant nutrients for sedentary plant-parasitic nematodes during their development. Plants can effectively halt nematode development and thus prevent their reproduction by specifically targeting their defense responses on nematode-induced feeding structures.

Sedentary plant-parasitic nematodes deliver effectors into the apoplast and cytoplasm of host cells to bring about the elaborate molecular and structural changes associated with the formation of feeding structures (*13-15*). In addition to modifying host cell function, sedentary plant-parasitic nematodes most likely also use effectors to modulate the immunocompetence of host cells. However, little is currently understood of the molecular mechanisms underlying the protection of feeding structures by sedentary plant-parasitic nematodes. Recently, we showed that SPRYSEC effectors of *G. rostochiensis* selectively suppress defense-related programmed cell death and disease resistance mediated by cytoplasmic plant immune receptors (*16*). As our earlier work with Cf-2 in tomato demonstrates, sedentary plant-parasitic nematodes are also vulnerable to detection by extracellular plant immune

receptors. Sedentary plant-parasitic nematodes reside in the apoplast of living host cells for several weeks, and it is likely that they also secrete effectors into the apoplast of host cells to suppress the activation of defense responses by extracellular plant immune receptors.

The nematode protein Gr-VAP1 that binds to the active site of Rcr3[pim] and thereby activates Cf-2-mediated resistance in tomato is a venom allergen-like protein (VAP). VAPs constitute a monophyletic group within the SCP/TAPS protein family (*17*). Secreted VAPs are among the very few effectors that are conserved in both animal- and plant-parasitic nematodes (*18*). In fact, in some animal-parasitic nematodes VAPs are the most abundantly secreted proteins during the onset of parasitism, but their role in parasitism is largely unknown (*19-23*). Likewise, VAPs have been identified in all plant-parasitic nematodes studied to date, but how these proteins contribute to nematode survival and reproduction in host plants is not well understood either. Parasitism of animals and plants by nematodes has little in common, except that in both plants and animals, nematodes encounter an innate immune system that is based on extracellular immune receptors. Earlier evidence suggests that VAPs from animal-parasitic hookworms interfere with ligand binding to the extracellular domain of human integrins, which are transmembrane receptors involved in signal transduction from the extracellular matrix into the cell (*24-27*). We reasoned that this may be a conserved feature of secreted VAPs from parasitic nematodes in both plants and animals, and that parasitic nematodes thus use VAPs to interfere with the functioning of extracellular receptors in host plants.

To test this hypothesis, we first knocked-down the expression of venom allergen-like proteins in nematodes to assess their contribution to nematode virulence in host plants. Next, we analyzed the response of transgenic potato and *Arabidopsis thaliana* plants stably overexpressing nematode VAPs to nematodes, other plant pathogens, and a known immunogenic elicitor of the extracellular plant immune receptor FLS2. Transient overexpression by agroinfiltration in leaves of *Nicotiana benthamiana* was subsequently used to test if the venom allergen-like proteins of plant-parasitic nematodes suppressed the defense-related programmed cell death activated by extracellular plant immune receptors. Lastly, RNAseq analysis of Arabidopsis plants overexpressing venom allergen-like proteins provided an insight into the changes in the composition of the extracellular proteome that may explain the loss of immunocompetence in plant cells.

4.3. Results

The venom allergen-like protein Gr-VAP1 is required for virulence of *G. rostochiensis*. To first test whether venom allergen-like proteins are required for virulence of sedentary plant-parasitic nematodes, we soaked infective

juveniles of *G. rostochiensis* in double-stranded RNA, matching 820 base pairs of *Gr-VAP1* transcript sequence. Reverse transcription PCR on nematodes, soaked in *Gr-VAP1*-specific dsRNA, showed a significant reduction in *Gr-VAP1* transcript levels, whereas the control treatment with dsRNA matching the *NAU* gene from *Drosophila melanogaster* did not alter *Gr-VAP1* expression (Fig. 1A). Next, susceptible tomato plants (*Solanum lycopersicum*, cultivar Moneymaker) were challenged with the dsRNA-treated nematodes, and monitored for nematode infections. Treatment with *Gr-VAP1*-specific dsRNA significantly reduced the number of nematodes on tomato roots compared to the treatment with *NAU* dsRNA (P<0.05; Fig. 1B). We therefore conclude that the venom allergen-like protein Gr-VAP1 is required for virulence of *G. rostochiensis* on susceptible host plants.
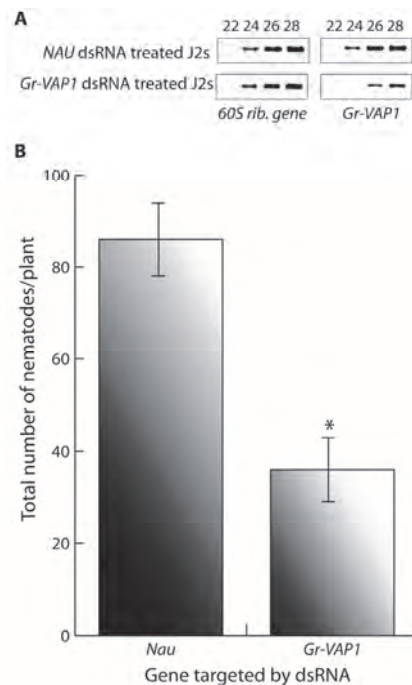
Fig. 1. Gr-VAP1 is required for virulence of *Globodera rostochiensis* in tomato (*Solanum lycopersicum*). (A) Reverse transcription PCR of *Gr-VAP1* and *60S ribosomal gene* (*60S rib. gene*) as control in pre-parasitic second juveniles (J2s) after treatment with either double stranded RNA matching the *Gr-VAP1* transcript or transcripts of the *Nautilus* (*NAU*) gene of *Drosophila melanogaster* as control. Numbers indicate the cycles in the PCR. (B) Number of nematodes inside roots of tomato plant (*S. lycopersicum*) after a challenge with *G. rostochiensis* treated with either double-stranded RNA matching the *Gr-VAP1* transcript or the transcripts of the *NAU* gene of *D. melanogaster* as a control. Bars indicate standard errors (* P<0.05 in Student's t-test with N=10). Two independent experiments were performed and representative results are shown.

Gr-VAP1 selectively inhibits apoplastic cysteine proteases from multiple host plants. To investigate whether Gr-VAP1 targets apoplastic papain-like cysteine proteases other than Rcr3^{pim}, we used DCG-04 activity profiling on apoplastic fluids of agroinfiltrated *N. benthamiana* leaves. These expressed separately the papain-like cysteine proteases Pip1^{lyc}, CatB^{lyc}, C14^{lyc}, and Cyp3^{lyc} from tomato (*Solanum lycopersicum*) and C14^{tub} from potato (*S. tuberosum*) (Fig. 2). Gr-VAP1 did not reduce DCG-04 labeling of any of the other papain-like cysteine proteases from tomato. However, Gr-VAP1 reduced the DCG-04 labeling of C14^{tub} (Fig. 2). We therefore conclude that Gr-VAP1 selectively disturbs the active sites of multiple apoplastic papain-like cysteine proteases from different host plant species of *G. rostochiensis*.
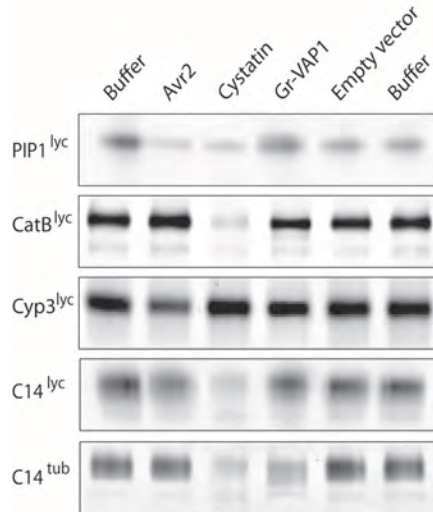
Fig. 2. Gr-VAP1 inhibits a potato apoplastic papain-like cysteine protease. DCG-04 protease activity profiling on apoplastic fluids of agroinfiltrated *Nicotiana benthamiana* leaves expressing four extracellular cysteine proteases of tomato (PIP1^{lyc}, CatB^{lyc}, Cyp3^{lyc}, and C14^{lyc}) and one apoplastic cysteine protease of potato (C14^{tub}). The extracts were pre-incubated for 30 min with either Gr-VAP1 or Avr2 recombinantly produced in agroinfiltrated *N. benthamiana* leaves. Fluorescent DCG-04 was added after pre-incubation to label non-inhibited proteases. Bound DCG-04 was visualized on western blots in a fluorescence scanner. DCG-04 profiling after pre-incubations with egg white cystatin, extracts from agroinfiltrations with the empty binary expression vector (EV), and without inhibitors were included as controls.

Gr-VAP1 expression correlates with nematode migration in host plants. Early parasitic juveniles and adult males of *G. rostochiensis* migrate through host tissues while causing significant damage to host cells. By contrast, intermediate juvenile stages and adult females are immobile, and thus induce little damage. To determine whether *Gr-VAP1* in *G. rostochiensis* correlates

with migration or with sedentarism in host plants, we used reverse transcription PCR on nematodes isolated from infected potato roots at different time points post invasion (Fig. 3). *Gr-VAP1* was highly expressed in infective juveniles just prior to host invasion. Thereafter, the level of *Gr-VAP1* expression declined in successive sedentary parasitic juvenile stages inside host roots to total absence in sedentary adult females. However, the expression of *Gr-VAP1* increased again in juveniles that had differentiated into migratory adult males. We therefore conclude that the expression of *Gr-VAP1* correlates with host invasion and migration of *G. rostochiensis* in host plants.
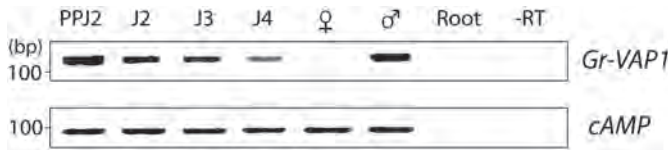
Fig. 3. *Globodera rostochiensis* venom allergen-like protein (*Gr-VAP1*) expression is up-regulated in the motile stages**.** The expression of *Gr-VAP1*, shown by semiquantitative reverse transcription PCR, is highly upregulated in the migratory stages of *G. rostochiensis* (J2 and males (♂)) while its expression decreases in the sedentary stages (J3 and J4) and is absent in females (♀). Expression of *Gr-VAP1* was assessed relative to the expression of the constitutively expressed *CDPK* (*cAMP dependent protein kinase*) gene.

Ectopically expressed venom allergen-like proteins increase susceptibility of host plants to cyst nematodes. To examine whether Gr-VAP1 affects the susceptibility of host plants to *G. rostochiensis*, we generated transgenic potato plants ectopically overexpressing *Gr-VAP1* in the apoplast. Two randomly selected independent transgenic lines without any visible anomalies in shoots and roots were challenged with infective juveniles of *G. rostochiensis*. Six weeks after inoculation the number of cysts in plants expressing *Gr-VAP1* was significantly higher than in the corresponding empty vector control plant (Fig. 4A). To confirm that the altered nematode susceptibility correlates with *Gr-VAP1* expression, we used a real-time quantitative reverse transcription PCR on the two potato lines expressing *Gr-VAP1* (Fig. 4B). Transgenic line Gr-VAP1-A, that showed the highest nematode susceptibility, had a 7.9-fold higher expression of *Gr-VAP1* than transgenic line Gr-VAP1-B. We therefore conclude that Gr-VAP1 enhances the susceptibility of potato plants to *G. rostochiensis.*

    Arabidopsis thaliana is a far better host to study the molecular changes induced by venom allergen-like proteins in plants than either potato or tomato. However, as *A. thaliana* is not a host plant of *G. rostochiensis*, we first cloned two homologous venom allergen-like proteins from the beet cyst

nematode *Heterodera schachtii*, which is able to infect *A. thaliana*. These two venom allergen-like proteins are formally designated as Nem-*Hsc*-SCP/TAPS-1a and Nem-*Hsc*-SCP/TAPS-2A, but for the remainder of this thesis they are referred to as Hs-VAP1 and Hs-VAP2. Hs-VAP1 is 81.4 percent identical to Gr-VAP1, while Hs-VAP2 shows only 34.8 percent sequence identity to Gr-VAP1. In comparison, a previously reported venom allergen-like protein from the root-knot nematode *M. incognita* (hereafter named Mi-VAP1) (*28*) shows 33.9% sequence identity to Hs-VAP2 and on average 28.6% identity to Gr-VAP1 and Hs-VAP1 (Fig. S1).

Line	Fold change
Gr-VAP1-A	32.2±1.5
Gr-VAP1-B	24.3±2.1
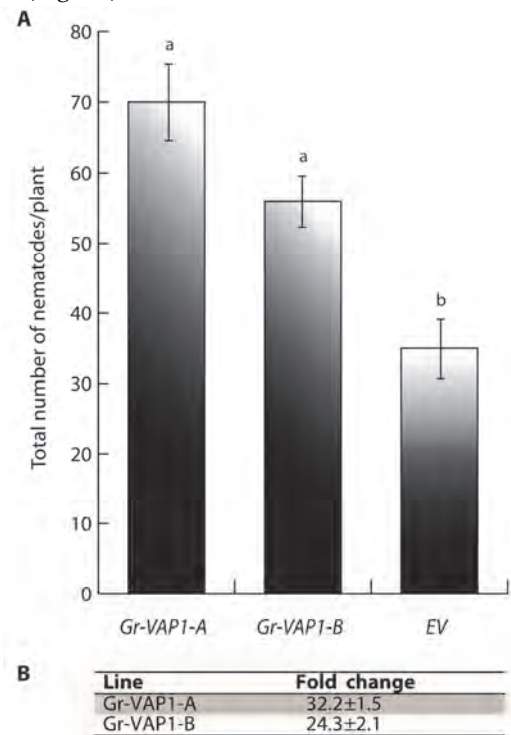
Fig. 4. Potato plants expressing nematode Gr-VAP1 show enhanced susceptibility to the nematode *Globodera rostochiensis*. (A) Nematode susceptibility of potato plants overexpressing *G. rostochiensis VAP1* (*Gr-VAP1*). Two independent transformants were used per construct (-*A* and -*B*), and one empty vector (EV) as control plant. Three-week-old potato plants were inoculated *in vitro* with 200 infective juveniles. The total number of nematodes was determined three weeks after inoculation. Bars represent standard errors of the means. Different characters indicate statistical differences as determined with a one-way ANOVA test (*P*-values <0.05 with N=10). Two independent experiments were performed and representative results are shown. (B) Increased fold change of *Gr-VAP1* expression in the potato transgenic plants. Quantification of *Gr-VAP1* gene expression was determined by qPCR in three-week-old potato plants. *Gr-VAP1* fold change increase was determined for three biological replicates of each independent transformant (-*A* and –*B*).

To test if the venom allergen-like proteins from *H. schachtii* also alter the susceptibility of *A. thaliana* to cyst nematodes, we generated transgenic plants overexpressing *Hs-VAP1*, *Hs-VAP2*, and *Gr-VAP1* including their native signal peptide for secretion. We challenged two independent single insertion lines, without visible anomalies in shoots and roots, of each construct with infective juveniles of *H. schachtii*. Twenty-eight days after inoculation the number of cysts in plants expressing *Hs-VAP1*, *Hs-VAP2*, and *Gr-VAP1* was significantly higher than in the corresponding transgenic empty vector line or in the wild type Col-0 plants (Fig. 5). We therefore conclude that the venom allergen-like proteins from *H. schachtii* alter the susceptibility of *A. thaliana* to cyst nematodes.
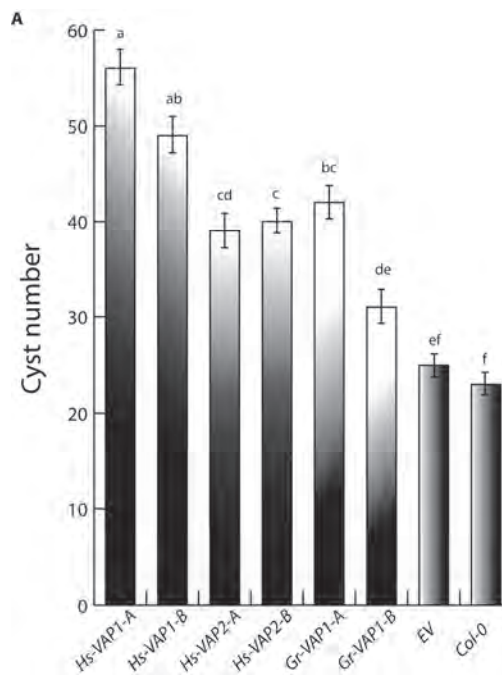
Fig. 5. Arabidopsis plants expressing cyst nematode VAPs show enhanced susceptibility to nematode infections. (A) Nematode susceptibility of Arabidopsis plants overexpressing *Heterodera schachtii VAP1 and VAP2* (*Hs-VAP1* and *Hs-VAP2*), and *Globodera rostochiensis VAP1* (*Gr-VAP1*). Two independent single transformants were used per construct (*-A* and *-B*). Two weeks-old Arabidopsis plants were inoculated *in vitro* with 250 *H. schachtii* infective juveniles. The number of nematodes inside the roots was determined 28 days after inoculation. Nematode numbers on the progenitor Col-0 and empty vector (EV) control line were used as controls. Bars represent standard errors of the means. Different characters indicate statistical differences as determined with a one-way ANOVA test (*P*-values <0.05 with N=12). Two independent experiments were performed and representative results are shown.

Venom allergen-like proteins enhance the susceptibility of Arabidopsis to multiple unrelated plant pathogens. Next, we reasoned that if the VAP-enhanced susceptibility of the Arabidopsis lines to nematodes involves modulation of the innate immunity, these lines might also have altered susceptibility to other unrelated plant pathogens. To test this hypothesis, we analyzed the transgenic Arabidopsis lines overexpressing *Hs-VAP1* and *Hs-VAP2* for enhanced susceptibility towards *Botrytis cinerea*, *Plectosphaerella cucumerina*, a compatible and an incompatible isolate of *Phytophthora brassicae*, *Alternaria brassicicola,* and *Verticillium dahliae*. The overexpression of both *Hs-VAP1* and *Hs-VAP2* resulted in a significant increase in grey mold symptoms caused by the necrotroph *B. cinerea* IMI169558 in *A. thaliana* (Fig. 6; Fig. S2A). Only Arabidopsis plants overexpressing *Hs-VAP1* showed larger necrotic lesions following the inoculation with the necrotrophic fungus *P. cucumerina* (Fig. 6; Fig. S2B). By contrast, the hemibiotrophic oomycete *P. brassicae* (isolate CBS686.95) only caused faster developing and larger lesions on transformants expressing *Hs-VAP2* (Fig. 6; Fig S3A). Surprisingly, the *P. brassicae* isolate HH, which is incompatible with *A. thaliana* accession Col-0, was also able to colonize transgenic *A. thaliana* lines expressing *Hs-VAP2* (Fig. 6; Fig S3B). However, neither *Hs-VAP1* nor *Hs-VAP2* altered the susceptibility of *A. thaliana* towards the necrotrophic fungus *A. brassicicola* or the biotrophic fungus *V. dahliae* (data not shown). Altogether, our data showed that Hs-VAP1 and Hs-VAP2, albeit differently, enhance the disease susceptibility of *A. thaliana* toward multiple, but not all, plant pathogens.
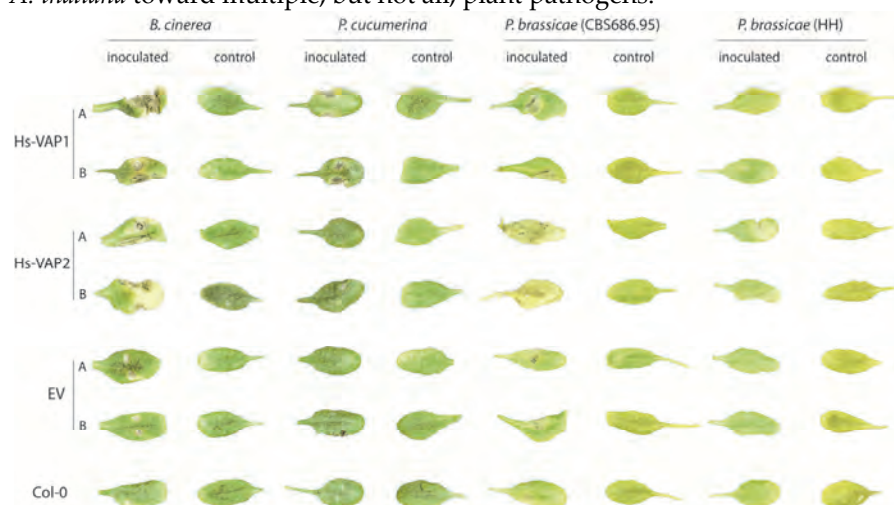
Fig. 6. Arabidopsis plants expressing *Heterodera schachtii* VAPs are more susceptible to fungal and oomycete pathogens. Typical symptoms caused by *Botrytis cinerea*, *Plectosphaerella cucumerina*, and *Phytophthora brassicae* isolates CBS686.95 and HH on four-week-old transgenic plants. Leaves of two independent (-A and -B) *VAPs*-expressing Arabidopsis lines were photographed at 3 days post-inoculation. Disease symptoms on the progenitor Col-0 and empty vector (EV) control lines, and mock inoculations were used as controls.

Venom allergen-like proteins reduce the response of Arabidopsis to the immunogenic peptide flg22. Our challenge assays with diverse plant pathogens suggested that the venom allergen-like proteins suppress broad-spectrum basal defense responses in *Arabidopsis*. To further test this hypothesis we assessed the seedling growth of the transgenic Arabidopsis lines in the continuous presence of the highly conserved peptide flg22, which elicits PAMP-triggered immunity in plants. In both the wild type Col-0 and the transgenic empty vector lines the trade-off between plant defense and plant growth resulted in a strongly reduced seedling growth in the presence of flg22. However, in the Arabidopsis lines overexpressing *Hs-VAP1* and *Hs-VAP2* the seedling growth inhibition by flg22 was largely abrogated (Fig. 7A). Furthermore, the leaves of *Hs-VAP1* and *Hs-VAP2* lines in the presence of flg22 remained greener as compared to the leaves of Col-0 and empty vector control line following the treatment with flg22 (Fig. 7B). Flg22 activates plant defense responses in *A. thaliana* via the extracellular immune receptor FLS2 (*29*). Given that the virulence targets of venom allergen-like proteins are most likely located in the apoplast, we conclude that these nematode effectors might suppress the host defense responses activated by extracellular immune receptors in *A. thaliana*.
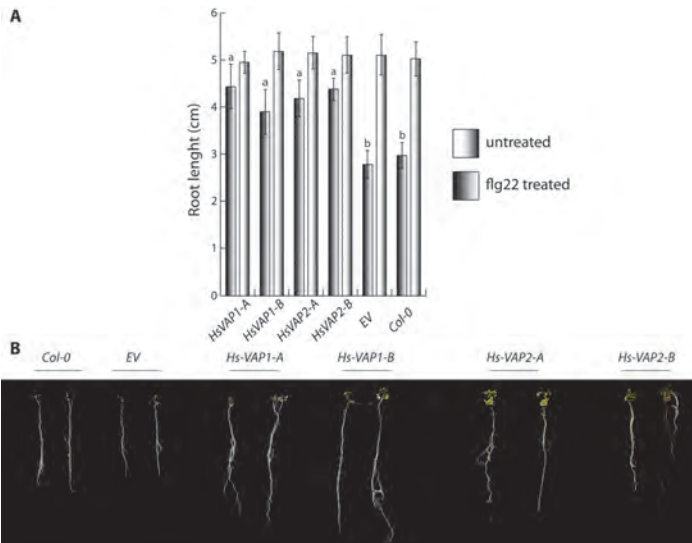
Fig. 7. Arabidopsis plants expressing *Heterodera schachtii* VAPs show altered flg22 perception. (A) Average root length was measured after 10 days of growing *A. thaliana* seedlings in the presence or absence of 10 μM flg22. Two independent single Hs-VAPs transformants were used per construct (-*A* and -*B*). Bars represent standard errors of the means. Different characters indicate statistical differences as determined with a one-way ANOVA test (*P*-values <0.05 with N=10). Two independent experiments were performed and representative results are shown. (B) Photograph of typical phenotype of plants expressing *Hs-VAPs* after 10 days of growing *A. thaliana* seedlings in the presence of 10 μM flg22. *Col-0* and empty vector (*EV*) control lines were used as controls.

Venom allergen-like proteins suppress defense responses mediated by extracellular plant immune receptors. Since seedling growth inhibition is a 'late' response to immunogenic elicitors in plants, the venom allergen-like proteins might still alter this response to flg22 indirectly through their activities on processes unrelated to innate immunity. To test if the venom allergen-like proteins also suppress the defense-related programmed cell death mediated by extracellular plant immune receptors, we transiently co-expressed several cognate pairs of extracellular receptors proteins and elicitors with and without VAPs in leaves of *Nicotiana benthamiana* (Table S3; Fig. 8). Because *N. benthamiana* is not a host plant of *G. rostochiensis* and *H. schachtii*, we also included the homologous venom allergen-like protein Mi-VAP1 from the polyphagous *Meloidogyne incognita* in these cell death suppression assays. Both Mi-VAP1 and Hs-VAP1 consistently suppressed the defense-related programmed cell death induced by the *Phytophthora infestans* effector INF1 in leaves of *N. benthamiana* (Fig. 8A). Both Mi-VAP1 and Hs-VAP1 also suppressed the programmed cell death induced by the transient co-expression of the extracellular receptor protein Cf-4 from tomato and the effector Avr4 from *Cladosporium fulvum* (Fig. 8B). All tested VAPs similarly suppressed the cell death induced by the co-expression of the extracellular receptor protein Cf-9 from tomato with the cognate *C. fulvum* effector Avr9 (Fig. 8C). By contrast, co-infiltration of the venom allergen-like proteins did not suppress the cell death triggered by several autoactive mutants or elicitor-activated intracellular plant immune receptors (i.e. Gpa2, Rx1, Mi1). We therefore conclude that the venom allergen-like proteins from plant-parasitic nematodes selectively suppress the activation of defense-related programmed cell death mediated by extracellular plant immune receptors.

Venom allergen-like proteins alter the immunocompetence of plant cells through their actions on the extracellular proteome. To better understand the molecular mechanisms underlying the suppression of host defenses by venom allergen-like proteins, we analyzed the transcriptome of three biological replicates of Arabidopsis lines expressing *Hs-VAP1*, *Hs-VAP2*, and the corresponding transgenic empty vector control plants with RNAseq. Relative to the empty vector plants, 1,303 genes were significantly down-regulated, while 535 genes were significantly up-regulated in the Arabidopsis lines overexpressing either *Hs-VAP1* or *Hs-VAP2* (FDR<0.05) (Fig. 9A and B). We first queried the gene ontology (GO; (*30*)) and the Arabidopsis SUBcellular localization (SUBA3; (*31*)) databases with all differentially expressed genes to identify the predicted cellular locations of the gene products. Almost 50 percent of the differentially expressed genes in the transgenic Arabidopsis lines with either *Hs-VAP1* or *Hs-VAP2* encode an
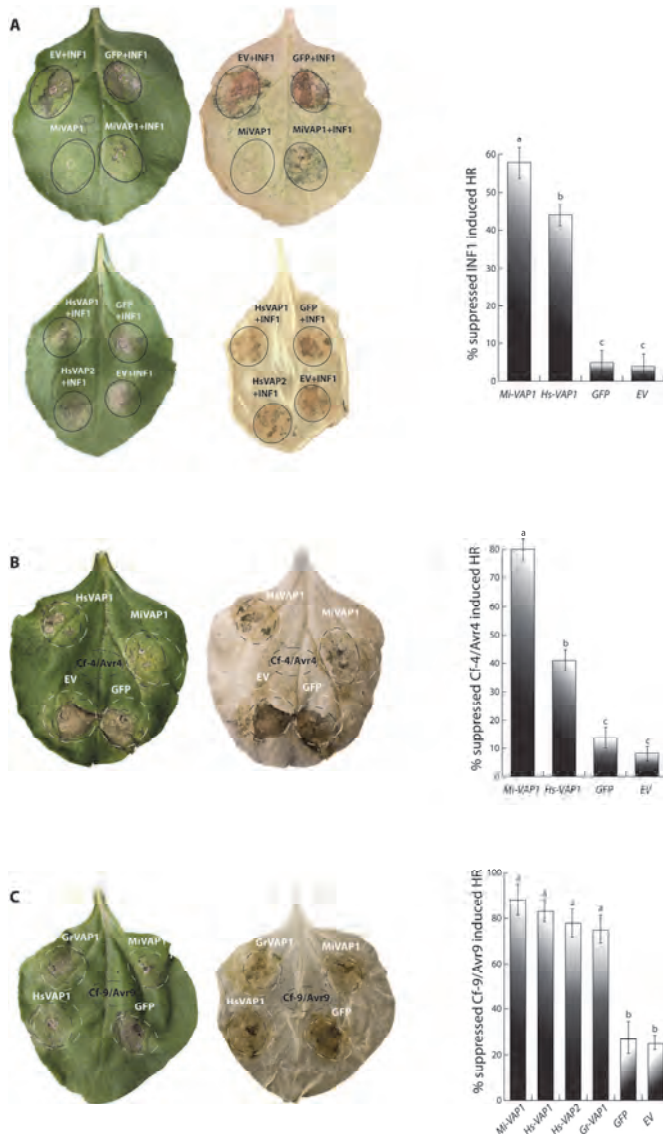
Fig. 8. VAP effectors suppress PRR-triggered immunity. Leaves of *Nicotiana benthamiana* were coinfiltrated with *Agrobacterium tumefaciens* bearing the (A) *INF1*, (B) *Cf-4/Avr4*, and (C) *Cf-9/Avr9* genes, together with *A. tumefaciens* bearing the VAP genes from *Globodera rostochiensis* (*Gr-VAP1*), *Heterodera schachtii* (*Hs-VAP1* and *Hs-VAP2*), and *Meloidogyne incognita* (Mi-VAP1). An empty vector (EV) and a green fluorescent protein (GFP) control were used to compare cell death suppression. All constructs were under a 35S CaMV promoter. The suspensions of the bacteria were infiltrated in a VAP:Receptor/Effector ratio of 6:1 for INF1 and 3:1 for the Cf-4/Avr4 and Cf-9/Avr9 combinations. Photographs of leaves showing cell death were taken 4 d post infiltration for INF1 and 7 d post infiltration for Cf-4/Avr4 and Cf9/Avr9. The number of events in which cell death was suppressed was determined for 12 inoculation spots in 5 replicates. Bars represent standard errors of the means. Different characters indicate statistical differences as determined with a one-way ANOVA test (*P*-values <0.05)

extracellular protein or a protein localized to plasma membrane (Fig. 9C). As only about 3% of the entire proteome of *A. thaliana* is predicted to be extracellular or plasma membrane localized, we conclude that Hs-VAP1 and Hs-VAP2 have a profound effect on the composition of the extracellular and membrane-localized proteome.
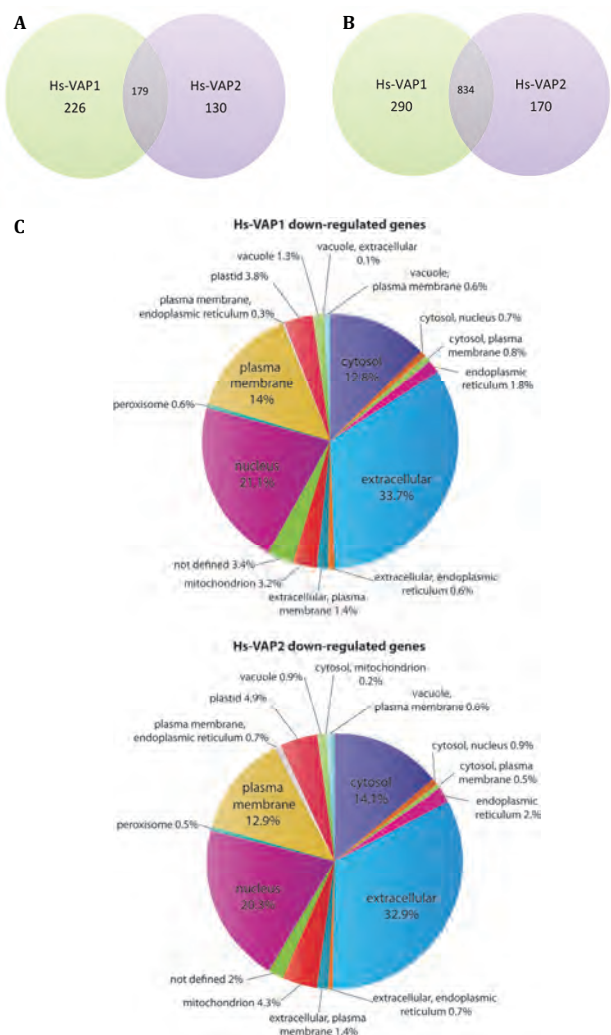
Fig. 9. Arabidopsis plants expressing *Heterodera schachtii* VAPs show altered extracellular proteome gene expression. Global gene expression analysis determined by RNA-seq on two-week-old Arabidopsis plants expressing the *H. schachtii* effectors *Hs-VAP1* and *Hs-VAP2*. Venn's diagrams depict the total number of up- (A) and down-regulated (B) differentially expressed genes (DEG) with a false discovery rate set to 0.05 (FDR <0.05) for the two genotypes compared to empty vector control plants. (C) Pie charts depict the percentage of down-regulated DEG, in both *Hs-VAP1* and *Hs-VAP2* expressing Arabidopsis plants, according to their sub-cellular localization.

Next, to resolve specific pathways affected by the overexpression of *Hs-VAP1* and *Hs-VAP2* in *A. thaliana*, we subjected all differentially expressed genes to a KEGG pathway enrichment analysis (*32, 33*). As compared to the transgenic empty vector control plants, the pathway most altered by both Hs-VAP1 and Hs-VAP2 is named 'plant-pathogen interactions' (KEGG pathway ath04626; Table S4). The vast majority of Arabidopsis genes currently assigned to this pathway are associated with innate immunity to plant pathogens (*34*). We therefore conclude that the overexpression of venom allergen-like proteins in the apoplast of Arabidopsis significantly affects the molecular components in pathways involved in innate immunity.

To further identify specific expression patterns, we focused on the most up- and down-regulated genes in the Arabidopsis lines overexpressing nematode VAPs relative to the empty vector control lines. Overall, there is a strong similarity in most up- and down-regulated genes in the Arabidopsis lines overexpressing *Hs-VAP1* and *Hs-VAP2* (Tables S5 and S6). As expected, several of the most up- or down-regulated genes are involved in the host defense responses to plant pathogens and plant cell wall associated processes in Arabidopsis. However, the most down-regulated expression (~30-fold) was observed in gene locus AT4G21630 in Hs-VAP1-overexpressing plants. AT4G21630 encodes a plant cell wall associated serine protease (i.e. subtilase protein family). Albeit far less, ATG4G21630 was also down-regulated in the Arabidopsis lines overexpressing *Hs-VAP2*. By contrast, the transcript levels of three genes were extremely high in plants either expressing Hs-VAP1 or Hs-VAP2. Two of these loci (AT1G44608 and AT1G44542) have an unknown function, but the product of the third and most up-regulated gene (AT1G44575; NPQ4) is involved in non-photochemical quenching of excited chlorophyll (*35*). Recently, it has been shown that the protein encoded by NPQ4 (i.e. PsbS) also modulates innate immune responses to flg22 (*36*). Altogether, our findings point to a breakdown of immunocompetence in Arabidopsis overexpressing *Hs-VAP1* and *Hs-VAP2*, most likely induced by major alterations in extracellular proteome.

4.4 Discussion

Secreted venom allergen-like proteins are considered critical factors for parasitism of nematodes in plants and animals since their first identification in the hookworm *Ancylostoma caninum*, fifteen years ago (*19*). In spite of their presence in essentially all parasitic nematodes studied to date, their specific expression during the onset of parasitism, and their high relative abundance in nematode secretions, the biological function of venom allergen-like proteins in host-parasite interactions has thus far remained elusive. Here, we show that venom allergen-like proteins of plant-parasitic nematodes suppress host defense responses mediated by extracellular innate immune receptors in

plants. Our data suggests that secreted venom allergen-like proteins from plant-parasitic nematodes most likely exploit a conserved common mechanism in host innate immunity.

The molecular mechanisms underlying the activation and suppression of extracellular immune receptors in plants are still poorly understood. Moreover, little is currently known of the extracellular host targets of venom allergen-like proteins in animals (*17, 37*). We have recently shown that Gr-VAP1 from the plant-parasitic nematode *G. rostochiensis* disturbs the active site of multiple extracellular papain-like cysteine proteases, at least one of which (i.e. Rcr3^{pim}) has a profound effect on the susceptibility of tomato plants to this nematode species (*12*). It is conceivable that the venom allergen-like proteins from both plant- and animal-parasitic nematodes share this ability to inhibit extracellular host proteases and use it to suppress innate immune responses of the host. As multiple unrelated plant pathogens have independently evolved effectors that commonly target extracellular papain-like cysteine proteases, it is thought that they function as key nodes in immune-signaling networks that affect a broad range of pathogens in plants. However, concrete evidence that positions these extracellular proteases within immune signaling pathways leading to basal defenses in plants has thus far not been found.

The alterations in the extracellular proteome induced by the overexpression of venom allergen-like proteins in transgenic Arabidopsis plants also point at an immunomodulatory role for extracellular proteases. One of the most up-regulated genes in the plants overexpressing *Hs-VAP1* encodes a predicted extracellular papain-like cysteine protease (Table S5; AT2G27420.1). By contrast, the most down-regulated gene in Hs-VAP1 overexpressing Arabidopsis plants encodes a plant cell wall associated serine protease of the subtilase protein family (Table S6; AT1G67626.1). Further investigations into allelic variants of extracellular proteases differing in their ability to confer nematode susceptibility in plants (*12*) is required to shed light on a possible mechanistic link between these proteases and innate immune signaling pathways activated by extracellular immune receptors.

Most of the genes differentially regulated by the overexpression of venom allergen-like proteins in Arabidopsis are typically associated with host defenses and plant cell wall-related processes. A notable exception to this is *NPQ4*, which was the most up-regulated gene in both Hs-VAP1 and Hs-VAP2 overexpressing plants. *NPQ4* encodes the PsbS subunit of photosystem II in thylakoid membranes of chloroplasts, which is involved in non-photochemical quenching of excitation energy in chlorophyll (*35*). PsbS dissipates excess excitation energy that may otherwise lead to excessive formation of reactive oxygen species and photooxidative stress inside chloroplasts. However, recent data suggests that PsbS might also constitute a link between photosynthesis and host defense responses to plant pathogens

(*36*), and as such it could regulate the trade-off between abiotic and biotic stresses in Arabidopsis (*38*).

It is thought that PsbS-dependent protection of chloroplasts against damage by reactive oxygen species undermines the ability of plant cells to mount host defense responses that requires the generation of reactive oxygen species by chloroplasts, such as defense-related local programmed cell death (*36, 39*). Our data seems to support this model, as the stable overexpression of VAPs in Arabidopsis induced a more than 30-fold increase in the expression of NPQ4. This resulted in the loss of host defenses to a wide variety pathogens, while transient overexpression of the same venom allergen-like proteins suppress the defense-related programmed cell death in leaves of *N. benthamiana*. The highly up-regulated expression of NPQ4 might thus prevent the build up of reactive oxygen species in chloroplast and thereby inhibit plant cells to undergo local programmed cell death.

By contrast, PsbS-deficient *npq4* mutant Arabidopsis lines seem to be both hypersensitive to treatment with flg22 (*36*), the opposite of what we observed in the transgenic Arabidopsis plant overexpressing venom allergen-like proteins. Furthermore, PsbS-deficient mutants seem to be less attractive to herbivorous insects (*40*), further suggesting that these plants have constitutively elevated levels of defenses. A PsbS-centered model may therefore offer a possible explanation for the loss of host defenses to leaf pathogens and the suppression of cell death that we observed in plants overexpressing venom allergen-like proteins. However, a prominent role for PsbS in determining defenses to parasitic nematodes in chloroplast-devoid roots seems less likely, unless PbsS is also involved in other cellular processes in root cells. Expression data derived from microarray experiments have shown that NPQ4 is expressed in the root cortex and endodermis (*41*), suggesting that this protein might indeed have multiple roles. Alternatively, the virulence targets of venom allergen-like proteins in plants may regulate multiple components of host defense responses, some of which may be specific for aerial parts of plants while others are common to both roots and shoots.

Although our data show that venom allergen-like proteins are capable of suppressing extracellular immune receptors, the recognition specificity of surface receptors targeted by the venom allergen-like proteins in host plants is not clear. In plants, the identities of molecular patterns uniquely associated with nematode infections and the basal defense responses triggered by these elicitors is essentially an unchartered area. However, several leads suggest that the venom allergen-like proteins in plants may be involved in suppressing immune responses triggered by nematode-induced damage to plant cell walls. First, the expression of venom allergen-like proteins in parasitic nematodes is strictly correlated with host invasion and migration inside the host. Second, the venom allergen-like proteins are secreted by

plant-parasitic nematodes along with a large repertoire of plant cell wall degrading enzymes (*12*). Plants monitor the integrity of plant cell wall with specific receptor-like kinases (e.g. WAK1; (*42, 43*)) and fragments of plant cell wall can elicit strong basal defense responses (*44, 45*). Third, many of the differentially regulated genes in Arabidopsis overexpressing venom allergen-like proteins are associated with plant cell wall-related processes. For example, the overexpression of *Hs-VAP1* induces the down-regulation of a proline-rich extensin-like receptor kinase (PERK11). PERK11 belongs to a gene family of fifteen predicted receptor kinases in Arabidopsis (AtPERK) of which the extracellular domain shares similarity with cell wall associated proteins (*46*). The biological function of the AtPERK family members is largely unknown, but the expression of one family member (PERK1) is rapidly induced following wounding and it is therefore thought to mediate early events in defense responses to cell wall damage by invading plant pathogens (*47*). Endoparasitic nematodes cause significant damage to plant cell walls during host invasion and subsequent migration inside host tissues, and the benefits of a system that suppresses early immune responses to plant cell wall-derived elicitors seems evident for these nematodes. We therefore postulate that venom allergen-like proteins of plant-parasitic nematodes act as modulators of extracellular immune receptors that specifically sense damage-associated molecular patterns in plants.

4.5 Materials and methods

Gr-VAP1 silencing by RNA interference. *Gr-VAP1* expression was knocked-down by soaking nematodes in double-stranded (ds) RNA as described elsewhere (*48*). Briefly, an 820 bp region was PCR amplified with the primers Gr-VAP1-RNAiFW and Gr-VAP1-RNAiR and the products were used to *in vitro* generate double-stranded (ds) RNA. The *Nautilus* gene (M68897) from *Drosophila melanogaster* was used as a soaking control as described in the Megascript RNAi kit (Ambion, Cambridgeshire, UK). 15,000 nematodes were soaked in a 1 mg/ml dsRNA solution (50 mM octopamine, 3 mM spermidine, and 0.05% gelatin). Control nematodes were soaked in solutions without dsRNA. Each soaking reaction was performed *in duplo* and 15,000 J2s were used for reverse transcription (RT)-PCR. After dsRNA soaking, nematode viability was examined under a microscope and nematodes were surface sterilized to perform nematode infection assays.

 Solanum lycopersicum cv. Moneymaker seeds were sterilized and grown in square plates (5 seeds per plate) on Gamborg B5 medium. After 2 weeks, the seedlings were infected with 400 surface-sterilized nematodes per plate. The plants were grown at 24 °C and light/dark cycles of 16 h/8 h. Seven days post inoculation, the roots were stained with acid fuchsin, destained using acidified glycerol and the nematodes inside the roots were counted using a

dissection microscope. The results were statistically analyzed in XLSTAT. The soaking and infection tests were repeated three times.

For semi-quantitative RT-PCR, total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). RT-PCR was performed as described in the SuperScript™ III One-Step RT-PCR System (Invitrogen) using the primer combination Gr-VAP1-sRTFw and Gr-VAP1-sRTRv, which was designed outside the region targeted by the dsRNA. As a reference gene the *60S acidic ribosomal protein* was PCR amplified with the primers 60S-RTFw and 60S-RTRv. Reaction aliquots were removed after 26 cycles and visualized on an ethidium bromide stained 1% agarose gel.

Fluorescent protease activity profiling. Fluorescent activity based protease profiling was used to find out whether Gr-VAP1 could inhibit host cysteine proteases. The cysteine proteases $Pip1^{lyc}$, $CatB^{lyc}$, $C14^{lyc}$, and $Cyp3^{lyc}$ of *Solanum lycopersicum*, and $C14^{tub}$ of *S. tuberosum* were transiently overexpressed, as previously described, in the apoplastic fluids of *N. benthamiana* leaves following agroinfiltration (*49*). Twenty-five to fifty microliters of these apoplastic fluids were incubated with either 100 nM of *P. pastoris* produced Avr2, 100 nM cystatin from chicken egg-white (Sigma-Aldrich), and 300 nM of *N. benthamiana* produced Gr-VAP1 in 50 mM sodium acetate (pH 5.5) and 100 µM DTT. To label the available active sites in these cysteine proteases, the proteins were subsequently incubated for 5h with 1 µM of fluorescent DCG-04-TMR (*50*). Fluorescent proteins separated in 12% Bis-Tris gels (Invitrogen) were detected using a fluorescent imager scanner (Molecular Imager FX, Bio-Rad, Hercules, CA, USA).

***Gr-VAP1* expression in *G. rostochiensis*.** Semi-quantitative reverse transcription PCR was used to study the expression of *Gr-VAP1* at different time points post inoculation. Messenger RNA extraction and cDNA synthesis was conducted on parasitic second, third, and fourth stage juveniles and the adult males and females isolated from roots of susceptible potato (*Solanum tuberosum* cultivar Bintje) at 13, 19, 23, 27, and 34 days post inoculation respectively. *Gr-VAP1* expression in each sample was examined with a gene specific fragment of 146 base pairs PCR-amplified in 26 cycles with primers Gr-VAP1-RTFw and Gr-VAP1-RTRv (Table S1). The constitutively expressed cAMP-dependent protein kinase (*Gr-cAMP*; GenBank accession number BM343563) was PCR amplified with the primers cAMP-RTFw and cAMP-RTRv (Table S1) as a reference. We included reactions without reverse transcriptase to test for contaminating genomic DNA of the nematodes, while non-infected potato roots were included to check for non-specific amplification of host-derived cDNA.

Potato transformation. Potato line V (genotype 6487-9) was transformed as described elsewhere (*16*). Briefly, potato stem pieces were incubated for 10 minutes with a suspension of *Agrobacterium tumefaciens* strain AGL1 carrying *Gr-VAP1* in the Gateway© vector pMDC32 (*51*), under the control of a 35S promoter. Transformant callus was selected on ZCVK medium (MS20 medium, 8g/l plant agar, 1mg/l zeatin, 100mg/l kanamycin, 200mg/l cefotaxim, 200mg/l vancomycin; pH 5.8). The introgression of *Gr-VAP1* was checked by PCR on genomic DNA extracted from plant leaves using the DNeasy Plant Mini Kit (Qiagen). The expression of the transgenes was checked by qPCR on RNA extracted using the RNeasy Plant Mini Kit (Qiagen).

Infection assays on potato plants. Dried cysts of *G. rostochiensis* pathotype Ro1-Mierenbos were soaked on a 100-μm sieve in potato root diffusate to collect hatched pre-parasitic J2s (*52*). Freshly hatched pre-parasitic second-stage juveniles in suspension were purified on a sucrose gradient and surface sterilized as previously described (*16*). The nematodes were resuspended in a sterile 0.7% (w/v) solution of Gelrite (Duchefa), and pipetted along the roots of 3-week-old *in vitro*-grown plants. We inoculated ~200 pre-parasitic J2s per potato plant on a plate of 12 cm^2. The adult females per plant were counted 6 to 8 weeks after inoculation. Two independently transformed potato lines were used in these experiments.

  V. dahliae isolate JR2 (*53*) was grown on 4% potato dextrose media (Duchefa) at 28°C for 2 weeks. Fungal spores were transferred to sterile deionized water to a concentration of 1×10^6 spores/ml. The roots of 3-week-old *in vitro*-grown transgenic potato plants were soaked in the fungal spore suspension for 5 min and transferred to pots with soil in a greenhouse. The same two independent transformed lines, as those used for *G. rostochiensis* infections. At 4 weeks post inoculation, pictures were taken, and to determine the fungal biomass in infected plants, stem pieces were cut from the potato plants just above ground level and flash frozen in liquid nitrogen. Total DNA was extracted from plant tissues using DNeasy Plant Mini Kit (Qiagen). A 200-bp fragment of the *ITS* gene of *V. dahliae* was PCR-amplified using primers ITS1-F (*54*) and ST-VE1 (*55*) on DNA samples using FirePol polymerase (Solis BioDyne). As an internal control, potato *actin* was amplified from the same templates using primers StActinF and StActinR (*56*).

Identification of VAPs from *H. schachtii*. We queried expressed sequence tag databases for the presence of venom-allergen like proteins in the beet cyst nematode *H. schachtii*. Four cDNA library clones, from which matching sequence tags derived, were acquired from "The Washington University Nematode EST Project"(*57*). Re-sequencing of these clones, with the primers M13Fw and M13Rv, resulted in the identification of two full-length cDNA

sequence encoding venom allergen-like proteins in *H. schachtii* (Hs-VAP1 and Hs-VAP2). The cDNA sequences encoding the complete open reading frames of Hs-VAP1 and Hs-VAP2, including signal peptides, were PCR amplified with gene specific primers (Table S1) and TOPO cloned into pENTR™∕D-TOPO® (Invitrogen). By using the Gateway© technology, these cDNAs from pENTR™∕D-TOPO® vectors were subsequently cloned into both pMDC32 (*51*) and pGWB411 (*58*) vectors.

Arabidopsis transformation. Transgenic *Arabidopsis thaliana* Col-0 lines expressing *H. schachtii* *Hs-VAP1* and *Hs-VAP2* were generated in this study. The sequences encoding each of the mature venom allergen-like proteins was PCR amplified using primers Hs-VAP1-GWFw, HsVAP1-GwRv, HsVAP2-GWFw, and HsVAP2-GwRv and cloned into the pMDC32 (*51*) vector, which contains the cauliflower mosaic virus 35S promoter for constitutive expression. This vector was introduced into *A. tumefaciens* strain GV3101. Subsequently, *Arabidopsis* transformants were generated using the floral dip method (*59*). First-generation transformants were selected on 50 µg/ml kanamycin after which the plants were transferred to soil. Several independent homozygous single insertion lines were selected, and T3 and T4 lines were used for infection assays (see below). The introgression of *Hs-VAP1* and *Hs-VAP2* was checked by PCR on genomic DNA extracted from seedlings using the DNeasy Plant Mini Kit (Qiagen). The expression of the transgenes was checked by qPCR on RNA extracted from seedlings using the RNeasy Plant Mini Kit (Qiagen).

Infection assays on Arabidopsis plants. Transgenic *Arabidopsis* seeds and wild-type controls (Col-0) were vapor sterilized and planted in 12-well cell culture plates (Greiner bio-one) containing modified Knop's medium (*60*). Plants were grown at 24°C under 16-h-light/8-h-dark conditions. Two-week-old seedlings were inoculated with ~250 surface-sterilized pre-parasitic J2s *H. schachtii* nematodes (*61*). Inoculated plants were maintained under the conditions described above. Two and four weeks after inoculation, the number of *H. schachtii* adult females was counted.

Inoculation of *Arabidopsis* plants with *B. cinerea*, *P. cucumerina*, *A. brassicicola*, and *P. brassicae*, was performed on 4-week-old soil-grown plants, as reported previously (*62-64*). Briefly, for *B. cinerea*, *P. cucumerina*, *A. brassicicola*, plants were drop inoculated by placing two 4-µl drops of conidial suspension (5×10^5 conidia/ml) on each leaf. Plants were incubated at 20°C, 100% RH, and a 16-h/8-h light/dark regime. Inoculation with *P. brassicae* was performed by placing 5-mm-diameter plugs of a 2-week-old *P. brassicae* agar plate culture onto *Arabidopsis* leaves. Subsequently, the plants were incubated at 16°C, 100% RH, and a 16-h/8-h light/dark regime. After two days the mycelial plugs were removed from plants. Disease progression for these

pathogens was scored at regular intervals, and representative pictures were taken at 4 days after inoculation.

For inoculation of *Arabidopsis* with *V. dahliae*, 2-week-old soil-grown plants were uprooted and inoculated by dipping the roots for 2 min in a conidial suspension (10^6 conidia/ml). After replanting in soil, plants were incubated at standard greenhouse conditions of a 16-h/8-h light/dark regime and 60% RH. Disease progression was monitored until 25 days after inoculation. All infection assays were performed at least 2 times.

Growth inhibition assays. Arabidopsis growth inhibition assays were performed as described elsewhere (*65*). Briefly, seedlings were grown for 5 days on Murashige and Skoog (MS) agar plates, supplemented with 1% w/v sucrose and 0.8% agar. Subsequently, seedlings were transferred to liquid MS medium supplied with 10 μM of the flg22 (QRLSTGSRINSAKDDAAGLQIA) synthetic peptide. One seedling was placed on 400 μl of medium in wells of 24-well-plates. The effect of treatment with the flg22 peptide on the growth of transgenic and wild type Arabidopsis (Col-0) seedlings was analyzed after 7 days by measuring root length and fresh weight.

RNA-seq on Arabidopsis plants. Two-weeks-old transgenic *Arabidopsis* plants, grown under the same conditions as for the infection with *H. schachtii*, were collected, flash-frozen in liquid nitrogen and total RNA was extracted with the Maxwell® 16 LEV simplyRNA purification kit (Promega). cDNA synthesis, library preparation (200-bp inserts), and Illumina sequencing (90-bp paired-end reads) was performed at BGI (Hong-Kong). Reads were mapped to the Arabidopsis genome (tair10) using TopHat and transformed into a count per gene per sample by using the BEDTools suite (function coverageBed).

Suppression of defense related programmed cell death in *Nicotiana benthamiana*. The suppression of programmed cell death in leaves of *N. benthamiana* was assessed using the venom allergen-like proteins Gr-VAP1, Hs-VAP1, HsVAP2 and Mi-VAP1 cloned into pGWB411 (*58*). The Mi-VAP1 construct was synthetized at GeneArt® based on the sequence accession AAD01511.1 at NCBI. All the constructs were transferred to *Agrobacterium tumefaciens* GV3101, and used for agroinfiltration in leaves of the *N. benthamiana*. Empty pGWB411 and GFP constructs were used as controls to assess the non-specific suppression of programmed cell death by agroinfiltration. The transient co-expression by agroinfiltration of several pairs of resistance genes and cognate elicitors was used to induce programmed cell death in leaves of *N. benthamiana*. Furthermore transiently expressed mutant CC-NB-LRR proteins were used to trigger an elicitor-independent programmed cell death by agroinfiltration in *N. benthamiana*

leaves (Table S2). *A. tumefaciens* harboring the individual binary vectors was grown at 28°C in yeast extract peptone medium with appropriate antibiotics. The bacteria were spun down and resuspended in infiltration medium (*16*). The bacterial solution was diluted to an optical density at 600 nm (OD600) of 0.1 to 0.3 and infiltrated in the abaxial side of the leaves using a 1 ml syringe. Co-infiltration of different constructs was performed by mixing equal volumes of the bacterial suspensions to a final OD600 of 0.3. Agroinfiltrated leaves were monitored for up to 7 d for visual assessment of cell death.

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4.7 Supporting information

A

B

	Gr–VAP1	Hs–VAP1	Hs–VAP2	Mi–VAP1
Gr–VAP1		82.7%	35.0%	27.9%
Hs–VAP1	82.7%		37.8%	26.6%
Hs–VAP2	35.0%	37.8%		32.2%
Mi–VAP1	27.9%	26.6%	32.2%	
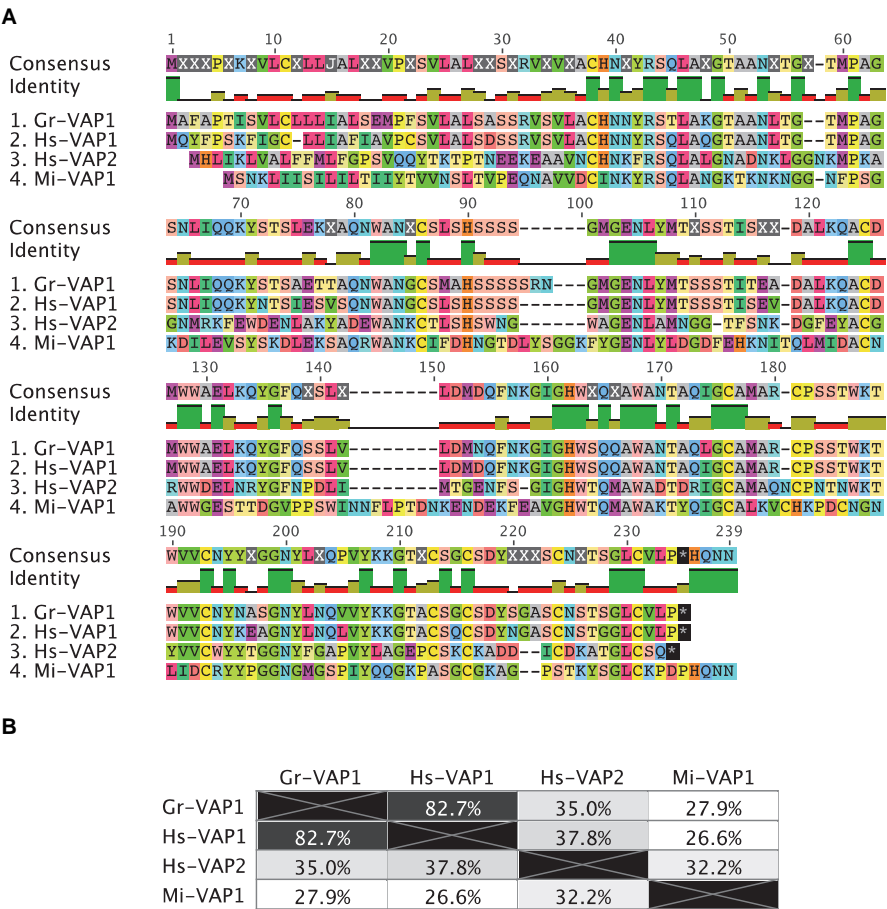
Fig. S1. Venom allergen-like proteins (VAPs) from plant parasitic nematodes vary in identity. (A) Protein sequence alignment of the VAPs from the potato cyst nematode *Globodera rostochiensis* (Gr-VAP1), from the beet cyst nematode *Heterodera schachtii* (Hs-VAP1 and Hs-VAP2), and from the root-knot nematode *Meloidogyne incognita* (Mi-VAP1). (B) Identity matrix for VAPs from the plant-parasitic nematodes *G. rostochiensis* (Gr-VAP1), *H. schachtii* (Hs-VAP1 and Hs-VAP2), and *M. incognita* (Mi-VAP1). Numbers represent the percentage of bases divided by the residues that are identical for any pair of VAPs.
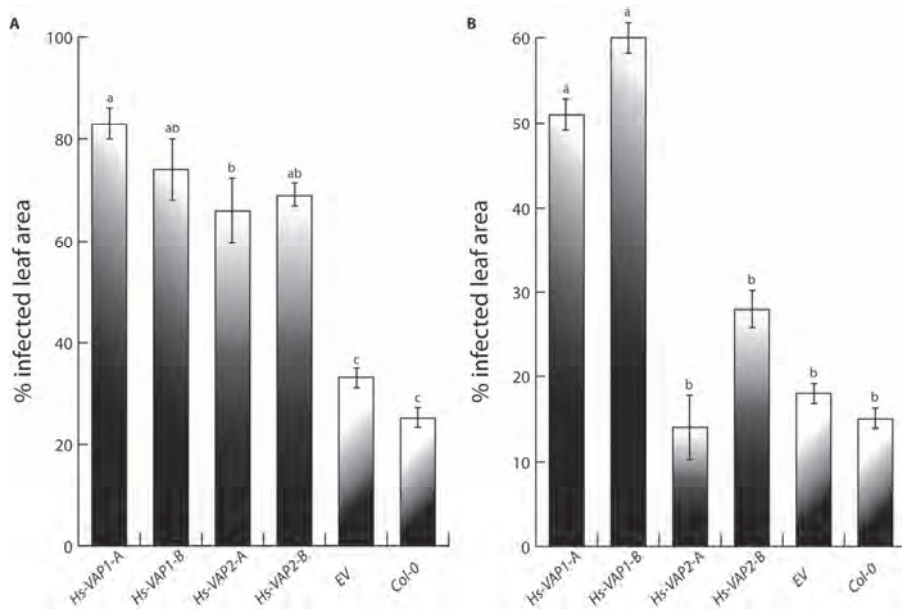
Fig. S2. VAPs-producing Arabidopsis are more susceptible to the fungal pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina*. Fungal susceptibility of Arabidopsis plants overexpressing *Heterodera schachtii VAP1 and VAP2* (*Hs-VAP1* and *Hs-VAP2*). 4-week-old Arabidopsis leaves were drop inoculated with (A) *B. cinerea* and (B) *P. cucumerina*. Two independent VAP-expressing transformants (-A and -B) were tested. Empty vectors (EV) and the progenitor Col-0 were used as controls. The percentage of diseased area was determined 3 days post-inoculation. Bars represent standard errors of the means. Different characters indicate statistical differences as determined with a one-way ANOVA test (*P*-values <0.05 with N=20). Two independent experiments were performed and representative results are shown.
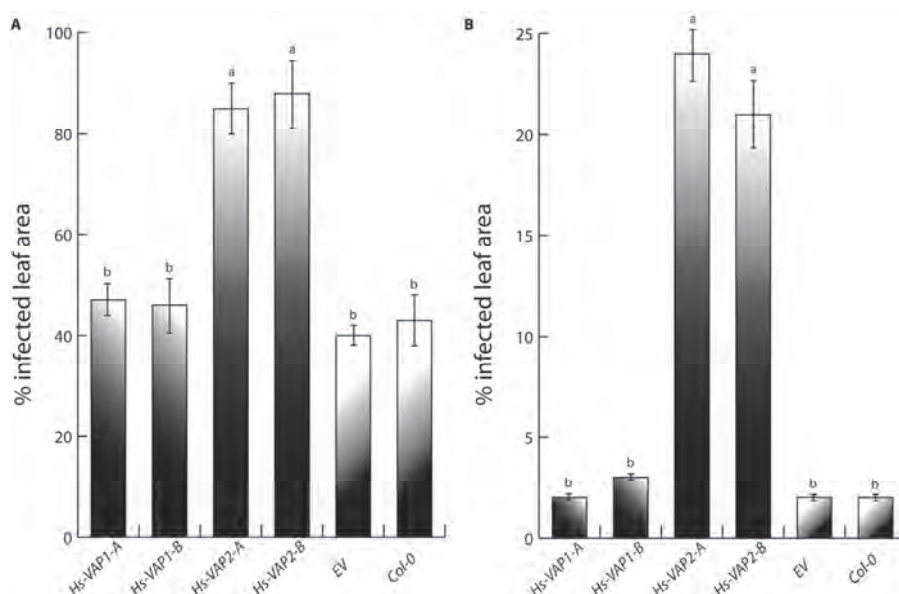
Fig. S3. VAPs-producing Arabidopsis are more susceptible to the oomycete pathogen *Phytophthora brassicae*. Disease susceptibility of Arabidopsis plants overexpressing *Heterodera schachtii VAP1 and VAP2* (*Hs-VAP1* and *Hs-VAP2*). 4-week-old Arabidopsis leaves were plug inoculated with (A) *P. brassicae* isolate CBS686.95 and (B) *P. brassicae* isolate HH. Two independent VAP-expressing transformants (-A and -B) were tested. Empty vectors (EV) and the progenitor Col-0 were used as controls. The percentage of diseased area was determined 3 days post-inoculation. Bars represent standard errors of the means. Different characters indicate statistical differences as determined with a one-way ANOVA test (*P*-values <0.05 with N=20). Two independent experiments were performed and representative results are shown.

Table S1: Oligonucleotides used for PCR.

Name	Oligonucleotide sequence (5'- to 3'-end)
Gr-VAP1-RNAiFw	CCCGGGTGCCACAACAACTACCGCTC
Gr-VAP1-RNAiR	TAATACGACTCACTATAGGGGGGGTCATTAGTGAATATACCG
Gr-VAP1-sRTFw	TGCCACAACAACTACCGCTC
Gr-VAP1-sRTRv	AAGCCGAAAGAATAATTTATC
60S-RTFw	GTGAAATCCGCAAACAACTG
60S-RTRv	AGAGCCTGGAAGAACGAC
Gr-VAP1-RTFw	GCATTGGGCATTGGAGTC
Gr-VAP1-RTRv	TTTGTAGACGACCTGGTTC
cAMP-RTFw	ATCAGCCCATTCAAATCTACG
cAMP-RTRv	TTCTTCAGCAAGTCCTTCAAC
Gr-VAP1-GWFw	CACCATGGCGTTTGCCCCAACAAT
Gr-VAP1-GWRv	TGGCAAAACGCACAGTCCGCTGGT
qGrVAP1-Fw	AGCAGTACGGGTTTCAATCG
qGrVAP1-Rv	AACCCAAGTCTTCCATGTCG
Hs-VAP1-GWFw	CACCATGCAATATTTTCCATCTAAAT
HsVAP1-GwRv	TCATGGCAATACGCACAGTCCGCCGG
qHsVAP1-F	ACGAGCATTGAGTCGGTTTC
qHsVAP1-R	TGTTTCAGCGCATCAACTTC
HsVAP2-GWFw	CACCATGCATTTGATTAAATTAGT
HsVAP2-GwRv	TCATTGAGAGCAAAGTCCCGTGGC
qHsVAP2-F	GCGAGAATTTGGCAATGAAT
qHsVAP2-R	TTTTCCCCGGTCATAATCAG

Table S2: Effectors and cognate resistance genes, or autoactive forms of resistance genes used to determine the immune suppressing abilities of Gr-VAP1, Hs-VAP1, Hs-VAP2, and MiVAP1.

Effector	R Gene	Pathogen effector origin	R Gene species origin
	NRC1**		*Solanum lycopersicum*
	Rx1 **		*Solanum tuberosum*
	RGH10-H1 **		*Solanum tuberosum*
	Gpa2 **		*Solanum tuberosum*
	G13R45**		*Solanum tuberosum*
Inf1*		*Phytophthora infestans*	
Avr4	Cf4	*Cladosporium fulvum*	*Solanum hirsutum*
Avr9	Cf9	*Cladosporium fulvum*	*Solanum pimpinellifolium*
Avr3a	R3a	*Phytophthora infestans*	*Solanum demissum*
Avr-blb1	Rpiblb1	*Phytophthora infestans*	*Solanum bulbocastanum*
Avr-blb2	Rpiblb2	*Phytophthora infestans*	*Solanum bulbocastanum*
CP***	RX	*Potato virus X*	*Solanum tuberosum*

*Effector gives an HR in *Nicotiana benthamiana* plants and the cognate resistance gene is unknown.
** Autoactive resistance gene that induces a hypersensitive response in an effector-independent manner.
*** CP is the coat protein of the Potato virus X.

Table S3. Top significantly enriched gene ontology (GO) terms in differentially expressed genes of *Arabidopsis* plants transformed with the venom-allergen like proteins (Hs-VAP1 and Hs-VAP2) of the beet cyst nematode *Heterodera schachtii*, when compared to empty vector transformed control plants. Calculated P-values went through a Bonferroni correction.

Gene Ontology term	P-value	Transformant
establishment of localization	6.73E-20	Hs-VAP1
localization	4.46E-18	Hs-VAP1
transport	5.15E-18	Hs-VAP1
ion transport	7.31E-17	Hs-VAP1
response to chemical stimulus	8.54E-15	Hs-VAP1
cation transport	1.23E-13	Hs-VAP1
cellular localization	1.10E-12	Hs-VAP1
establishment of localization in cell	2.79E-12	Hs-VAP1
cellular process	1.19E-11	Hs-VAP1
protein targeting to membrane	3.95E-11	Hs-VAP1
response to chitin	2.49E-10	Hs-VAP1
response to organic substance	5.60E-10	Hs-VAP1
intracellular transport	5.97E-10	Hs-VAP1
metal ion transport	2.47E-09	Hs-VAP1
regulation of innate immune response	2.93E-09	Hs-VAP1
regulation of immune system process	4.27E-09	Hs-VAP1
regulation of immune response	4.36E-09	Hs-VAP1
regulation of plant-type hypersensitive response	6.58E-09	Hs-VAP1
regulation of cellular response to stress	8.22E-09	Hs-VAP1
establishment of localization	2.71E-18	Hs-VAP2
transport	1.43E-16	Hs-VAP2
localization	2.18E-16	Hs-VAP2
ion transport	4.04E-15	Hs-VAP2
response to chemical stimulus	2.91E-13	Hs-VAP2
cellular process	1.05E-12	Hs-VAP2
cellular localization	1.80E-12	Hs-VAP2
establishment of localization in cell	4.86E-12	Hs-VAP2
cation transport	2.87E-11	Hs-VAP2
protein targeting to membrane	1.86E-10	Hs-VAP2
response to chitin	3.89E-10	Hs-VAP2
intracellular transport	5.96E-10	Hs-VAP2
intracellular signal transduction	3.96E-09	Hs-VAP2
cellular metabolic process	4.24E-09	Hs-VAP2
regulation of innate immune response	6.91E-09	Hs-VAP2
regulation of immune system process	9.90E-09	Hs-VAP2
regulation of immune response	1.01E-08	Hs-VAP2
small molecule metabolic process	1.30E-08	Hs-VAP2
regulation of plant-type hypersensitive response	1.70E-08	Hs-VAP2
regulation of cellular response to stress	2.09E-08	Hs-VAP2

Table S4. List of top ten (R) KEGG pathway enrichment analysis for differentially expressed genes of *Arabidopsis* plants transformed with the venom-allergen like proteins (Hs-VAP1 and Hs-VAP2) of the beet cyst nematode *Heterodera schachtii*, when compared to empty vector transformed control plants. Calculated P-values derive from hypergeometric tests.

R	Pathway	P-value	Q-value	ID	Transformant
1	Plant-pathogen interaction	6.71E-15	8.59E-13	ko04626	Hs-VAP1
2	ABC transporters	8.28E-07	5.30E-05	ko02010	Hs-VAP1
3	Benzoxazinoid biosynthesis	2.47E-05	1.06E-03	ko00402	Hs-VAP1
4	Oxidative phosphorylation	7.83E-05	2.50E-03	ko00190	Hs-VAP1
5	Flavonoid biosynthesis	0.00025486	6.52E-03	ko00941	Hs-VAP1
6	Ribosome	0.000341943	7.29E-03	ko03010	Hs-VAP1
7	Plant hormone signal transduction	0.001087173	1.99E-02	ko04075	Hs-VAP1
8	Phagosome	0.001343317	2.15E-02	ko04145	Hs-VAP1
9	Amino sugar and nucleotide sugar metabolism	0.004166753	4.99E-02	ko00520	Hs-VAP1
10	Photosynthesis	0.004222945	4.99E-02	ko00195	Hs-VAP1
1	Plant-pathogen interaction	1.79E-14	2.30E-12	ko04626	Hs-VAP2
2	ABC transporters	3.51E-07	2.25E-05	ko02010	Hs-VAP2
3	Benzoxazinoid biosynthesis	4.65E-06	1.98E-04	ko00402	Hs-VAP2
4	Oxidative phosphorylation	7.22E-05	2.31E-03	ko00190	Hs-VAP2
5	Ribosome	0.00022076	5.65E-03	ko03010	Hs-VAP2
6	Flavonoid biosynthesis	0.000656407	1.40E-02	ko00941	Hs-VAP2
7	Phagosome	0.002065439	3.78E-02	ko04145	Hs-VAP2
8	Photosynthesis	0.004059828	6.50E-02	ko00195	Hs-VAP2
9	Plant hormone signal transduction	0.004794847	6.82E-02	ko04075	Hs-VAP2
10	Amino sugar and nucleotide sugar metabolism	0.005813789	7.44E-02	ko00520	Hs-VAP2

Table S5. Gene expression pattern of the top significantly differentially up-regulated (FDR<0.05) genes, in *Arabidopsis* plants transformed with the venom-allergen like proteins (Hs-VAP1 and Hs-VAP2) of the beet cyst nematode *Heterodera schachtii*, when compared to empty vector transformed control plants. Gene expression is given as genewise standardized log2-transformed counts per million (logFC(Hs-VAP1 or Hs-VAP2/EV)).

GeneID	logFC	P-value	FDR	Length	Transformant
AT1G44575.1	36.1	1.9179E-141	2.0076E-137	1059	VAP1
AT1G44608.1	33.2	1.2297E-60	6.43594E-57	661	VAP1
AT1G44542.1	32.6	3.0409E-47	1.06102E-43	1023	VAP1
AT1G44575.2	30.9	1.42748E-20	3.60053E-18	1520	VAP1
AT4G19690.1	5.7	5.63033E-34	7.36693E-31	734	VAP1
AT5G48000.1	3.1	1.80855E-10	1.78594E-08	1792	VAP1
AT3G16430.1	2.2	1.09992E-07	7.6248E-06	1205	VAP1
AT2G36260.1	2.1	2.2514E-09	1.87781E-07	673	VAP1
AT5G36270.1	2.0	2.33035E-05	0.001001764	654	VAP1
AT2G27420.1	2.0	4.78317E-06	0.000246034	1309	VAP1
AT1G44575.1	35.8	1.5741E-125	1.6443E-121	1059	VAP2
AT1G44608.1	33.3	7.93594E-60	4.14494E-56	661	VAP2
AT1G44542.1	32.7	4.47837E-48	1.87124E-44	1023	VAP2
AT1G44575.2	30.7	1.82146E-18	1.9027E-15	1520	VAP2
AT4G19690.1	5.7	5.8572E-32	1.74812E-28	734	VAP2
AT5G48000.1	2.7	3.37794E-07	4.35629E-05	1792	VAP2
AT5G28020.6	2.5	5.14708E-08	8.672E-06	1216	VAP2
AT2G36260.1	2.5	1.35842E-12	7.6703E-10	673	VAP2
AT5G35935.1	2.3	9.60494E-18	9.1212E-15	6504	VAP2
AT5G36270.1	2.2	7.03342E-06	0.000542222	654	VAP2

Table S6. Gene expression pattern of the top significantly differentially down-regulated (FDR<0.05) genes, in *Arabidopsis* plants transformed with the venom-allergen like proteins (Hs-VAP1 and Hs-VAP2) of the beet cyst nematode *Heterodera schachtii*, when compared to empty vector transformed control plants. Gene expression is given as genewise standardized log2-transformed counts per million (logFC(Hs-VAP1 or Hs-VAP2/EV)).

GeneID	logFC	P-value	FDR	Length	Transformant
AT4G21630.1	-32.0	1.75381E-36	4.07956E-33	2319	VAP1
AT3G01345.1	-9.5	7.8436E-215	1.6421E-210	1612	VAP1
AT5G49180.1	-5.7	3.97036E-21	1.1707E-18	1952	VAP1
AT3G14520.1	-4.5	2.74283E-16	4.41702E-14	2031	VAP1
AT2G02490.1	-4.2	5.34408E-19	1.18673E-16	1244	VAP1
AT5G20330.1	-4.0	1.53528E-18	3.18228E-16	1552	VAP1
AT1G10620.1	-4.0	2.14972E-14	2.92237E-12	2157	VAP1
AT2G34870.1	-4.0	1.51571E-48	6.34628E-45	677	VAP1
AT2G36325.1	-4.0	1.25176E-11	1.38654E-09	1144	VAP1
AT5G20390.1	-3.9	1.11178E-27	7.05308E-25	1270	VAP1
AT3G01345.1	-8.8	3.3601E-183	7.0198E-179	1612	VAP2
AT2G02490.1	-5.7	8.47065E-22	1.17979E-18	1244	VAP2
AT5G24240.1	-4.3	5.7319E-68	3.99169E-64	2380	VAP2
AT5G40348.2	-4.0	9.87293E-13	5.75069E-10	746	VAP2
AT1G35310.1	-3.7	1.92458E-23	3.09296E-20	791	VAP2
AT4G12890.1	-3.7	3.43315E-13	2.24142E-10	898	VAP2
AT4G21630.1	-3.6	1.34797E-21	1.76011E-18	2319	VAP2
AT2G34870.1	-3.4	1.07604E-36	3.74676E-33	677	VAP2
AT4G12960.1	-3.1	3.24747E-27	6.16783E-24	949	VAP2
AT1G67626.1	-3.1	1.24615E-09	4.00531E-07	630	VAP2

Chapter 5

Papain-like Cysteine Proteases Affect Nematode Susceptibility of Tomato and Arabidopsis through Plant Cell Wall-associated and Jasmonic Acid-dependent Defense Responses

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5.1 Abstract

Papain-like cysteine proteases (PLCPs) in plants are involved in signaling cascades, developmental processes, and defense responses to diverse pathogens. Previously, we showed that the *Globodera rostochiensis* effector Gr-VAP1 interacts with and inhibits the *Solanum pimpinellifolium* extracellular PLCP Rcr3^{pim}. Furthermore, tomato plants carrying Rcr3^{pim} were almost twice as susceptible to infections by *G. rostochiensis* than plants harboring either the allelic variant Rcr3^{lyc} of S. *lycopersicum* or no functional Rcr3 (*rcr3-3*). Here, we demonstrate that close homologues of Rcr3 and many other PLCPs of *Arabidopsis thaliana* have a profound effect on plant susceptibility to cyst nematode infections. In order to understand the underlying mechanism of the Rcr3^{pim}-dependent increase in plant susceptibility to *G. rostochiensis*, we analyzed with RNA-seq the transcriptome of nematode-infected roots of tomato plants harboring *Rcr3^{pim}*, *Rcr3^{lyc}*, or the null mutant *rcr3-3*. Both allelic Rcr3 variants in tomato have a major impact on the expression of extracellular proteins associated with immune signaling pathways and plant cell wall-related processes in nematode-infected roots. Remarkably, only 28 tomato genes are uniquely regulated in association with *Rcr3^{pim}*. These genes are therefore responsible for the enhanced susceptibility of the tomato plants harboring this allele. The genes expressed in association with Rcr3^{pim} are linked to jasmonic acid-dependent signaling, wounding, and cell wall alterations. Mutant Arabidopsis plants lacking the tomato homologues of the Rcr3^{pim}-regulated genes in tomato show reduced susceptibility to infections by the cyst nematode *H. schachtii*, indicating that there is a conserved protease-activated pathway that needs to be regulated by cyst nematodes for successful parasitism. The integration of the biological processes commonly regulated by both Rcr3 variants and those uniquely associated with Rcr3^{pim} leads to a model in which the apoplastic Rcr3^{pim} modulates innate immune responses involving mechanical wounding, plant cell wall alterations, and jasmonic acid-induced host defenses.

5.2 Introduction

Proteases are broadly defined as enzymes that catalyze the hydrolytic breakdown of proteins into smaller polypeptides and amino acids (*1-3*). Cysteine proteases, also known as thiol proteases, cleave peptide bonds by using a nucleophilic sulfur atom bound to a cysteine residue in their active site (*2*). Cysteine proteases are grouped into 98 families based on sequence similarity, which are further lumped into 12 clans based on common ancestry (MEROPS database release 9.6; (*4*)). Papain-like cysteine proteases (PLCPs; family C1 and clan A1 are produced as inactive precursors that are

posttranslationally activated by removal of an N-terminal autoinhibitory domain (*5*).

PLCPs act in proteolysis-based signaling pathways in diverse cellular processes in plants, including development, cell death, senescence, drought tolerance, and innate immunity (*3, 6*). PLCPs operate both in the extracellular and intracellular branches of the innate immune system of plants. For instance, the extracellular cathepsin B of *Nicotiana benthamiana* is required for a hypersensitive type of programmed cell death and disease resistance to plant-pathogenic bacteria (*7*). RRS1-R-mediated resistance to *Ralstonia solanacearum* of *Arabidopsis thaliana* requires the RD19, which is initially localized in vacuolar vesicles but in the presence of the pathogen effector PopP2 relocates to the nucleus (*8*). Innate immunity of *A. thaliana* to *Botrytis cinerea* depends on the PLCP RD21, which is located in vacuolar and in endoplasmic vesicles (*9*). Lastly, the susceptibility of tomato plants to the tomato yellow leaf curl virus depends on the cytoplasmic PLCP CYP1 (*10*). Thus, PLCPs located in diverse subcellular compartments regulate innate immune responses to a range of plant attackers.

Many plant pathogens have evolved effectors to modulate PLCP-based defense signaling in the apoplast of host plants (*11-13*). For instance, the effector AVRblb2 of *Phytophthora infestans* prevents the secretion of the PLCP C14 into the apoplast of *N. benthamiana*, which may enhance the susceptibility of the leaves to colonization by *P. infestans* (*14*). *In planta* overexpression of the effector Avr2 of *Cladosporium fulvum*, an inhibitor of multiple apoplastic PLCPs in *S. lycopersicum* and *A. thaliana*, also enhances the susceptibility of these plants to multiple fungal plant-pathogens (*15, 16*). Furthermore, a knockout mutant of *Ustilago maydis* deficient in the effector Pit2, an inhibitor of multiple apoplastic PLCPs of maize, shows strong reduction in the virulence of this fungal pathogen (*17*).

Recently, we have shown that the effector Gr-VAP1 of the potato cyst nematode *G. rostochiensis* physically interacts with the apoplastic PLCP Rcr3[pim] of the currant tomato *Solanum pimpinellifolium* (*18*). Gr-VAP1 is a highly conserved venom allergen-like protein that is secreted by infective juveniles along with plant cell wall modifying enzymes. A knockdown of *Gr-VAP1* expression in *G. rostochiensis* strongly reduces the virulence of infective juveniles, whereas the overexpression of venom allergen-like proteins in host plants alters the response of these plants to infections by nematodes, a range of other plant pathogens, and to bacterial flagellin (*Chapter 4, this thesis*). Furthermore, the ectopic expression of venom allergen-like proteins specifically suppresses the programmed cell death activated by extracellular immune receptors in leaves of *N. benthamiana*. Most of the genes differentially regulated in transgenic *A. thaliana* overexpressing venom allergen-like proteins encode extracellular proteins involved in innate immunity and plant cell wall associated processes. Altogether these findings suggest that the

venom allergen-like proteins of sedentary plant-parasitic nematodes function as suppressors of protease-based activation and signaling of host defenses by extracellular plant immune receptors.

Sedentary plant-parasitic nematodes, such as the cyst nematodes (genera *Heterodera* and *Globodera*) and the root-knot nematodes (genus *Meloidogyne*), are biotrophs that engage in an intimate and prolonged relationship with their host plants. During the onset of parasitism, infective juveniles invade a host plant close to the root tip, and subsequently migrate through the cortex towards the vascular tissue. Plant cell wall degrading enzymes in the stylet secretions of the nematodes facilitate the migration of these microscopic worms inside their host plants. Within 24 hours after invasion of a susceptible host plant, the nematodes transform several host cells into permanent feeding cells. These feeding cells are the sole source of the nutrients that the nematodes require for their development and reproduction. For several weeks, the nematodes extract their nutrients from the cytoplasm of the feeding cells with a protrusible oral stylet. As feeding on plant cells coincides with the loss of mobility, the nematodes are confined to a sedentary lifestyle close to the feeding cells. Sedentary plant-parasitic nematodes also use their oral stylet to deliver effectors into the apoplast and cytoplasm of host cells. It is thought that one of the key roles of these effectors is to protect the nematode-induced feeding cells against host defense responses. However, little is currently understood of the molecular mechanisms underlying the modulation of plant innate immunity by nematodes and the role of venom allergen-like proteins of the nematodes and plant PLCPs in this process.

Our previous work on the molecular interactions between Gr-VAP1 and allelic Rcr3 variants revealed a remarkable difference in susceptibility to nematodes in tomato plants carrying the *S. pimpinellifolium* allele of *Rcr3* (*Rcr3^{pim}*), the corresponding *S. lycopersicum* allele (*Rcr3^{lyc}*), or no functional *Rcr3* (*rcr3-3*) at all. Tomato plants carrying Rcr3^{pim} were almost twice as susceptible to infections by *G. rostochiensis* than plants harboring either *Rcr3^{lyc}* or *rcr3-3*. Rc3^{pim} and Rcr3^{lyc} only differ at seven amino acid sites, but these polymorphisms are nonetheless crucial for the physical interaction between allelic Rcr3 variants and Gr-VAP1. To investigate the molecular mechanisms underlying the enhanced susceptibility associated with apoplastic Rcr3^{pim}, we analyzed the genes differentially expressed in nematode-infected roots of each of these tomato genotypes. Here, we show that only 28 tomato genes are uniquely regulated in association with the enhanced nematode susceptibility of the tomato plants harboring *Rcr3^{pim}*, most of which are linked to plant cell wall-associated and jasmonic acid-dependent immune signaling in plants. Furthermore, we demonstrate that Arabidopsis PLCPs, representing six PLCP subfamilies, are involved in cyst nematode susceptibility

5.3 Results

Functional Rcr3 proteins operate in extracellular protease-based immune signaling pathways. To unravel the molecular mechanisms underlying the Rcr3^{pim}-dependent increase in susceptibility to *G. rostochiensis* in tomato, we analyzed the transcriptome of nematode-infected roots of tomato plants harboring *Rcr3^{pim}*, *Rcr3^{lyc}*, or the null mutant *rcr3-3* at 7 dpi with RNA-seq. In total, 382 transcripts were identified as either significantly up- or down-regulated using three biological replicates for each *Rcr3* genotype (Fig. 1A and B; FDR <0.001 and P-value <0.0005).

     Rcr3^{pim} and *Rcr3^{lyc}* encode allelic Rcr3 variants differing in only seven amino acid residues, whereas tomato plants harboring *rcr3-3* lack a functional Rcr3 protein. By comparing gene expression profiles of Rcr3^{pim} versus rcr3-3 plants and Rcr3^{lyc} versus rcr3-3 plants, we first identified 51 similar differentially expressed genes regulated by Rcr3^{pim} and Rcr3^{lyc} (Fig. 1; Table S1). Twenty-five transcripts were significantly down-regulated in tomato plants by both Rcr3^{pim} and Rcr3^{lyc}, while twenty-six transcripts were up-regulated by both Rcr3-variants. Twenty-eight of the 51 differentially expressed transcripts associated with a functional Rcr3 protein derive from loci that so far have not been confidently called as genes in the current draft of the tomato genome. Three differentially expressed transcripts encode small open reading frames with no sequence homology in protein databases.
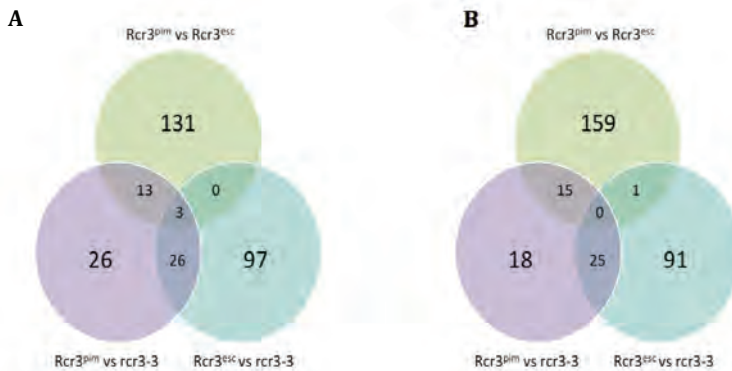
Fig. 1. Tomato plants of the genotype *Cf-0/Rcr3^{pim}* show an altered gene expression pattern, compared to plants of the genotype *Cf-0/Rcr3^{lyc}* or the mutant *Cf-0/rcr3-3*, when infected with the potato cyst nematode *Globodera rostochiensis*. Global gene expression analysis determined by RNA-seq on tomato plant roots of the genotypes *Cf-0/Rcr3^{pim}* (Rcr3^{pim}), *Cf-0/Rcr3^{esc}* (Rcr3^{esc}) and the mutant *Cf0/rcr3-3* (rcr3-3) challenged with the nematode *G. rostochiensis*. Venn's diagrams depict the total number of down- (A) and up-regulated (B) differentially expressed genes (DGE) with a false discovery rate set to 0.001 (FDR <0.001) for three biological replicas per genotype.

To first determine whether the remaining 20 differentially expressed transcripts encoded proteins that can co-localize with Rcr3 in the apoplast, we analyzed their predicted subcellular localization. Seven out of ten significantly down-regulated genes in the presence of a functional Rcr3 protein in nematode-infected tomato roots encode an extracellular protein (Table S2). Three up-regulated genes also encode extracellular proteins in the presence of Rcr3, one of which is an apoplastic papain-like cysteine protease (i.e. Solyc02g076980.2). We therefore conclude that a functional Rcr3 protein in the apoplast significantly impacts the composition of the extracellular proteome.

The differentially expressed genes associated with a functional Rcr3 protein in nematode-infected tomato roots have solely been annotated based on their homology with genes in *A. thaliana*. We have used this homology with Arabidopsis to identify biological processes most likely altered by apoplastic Rcr3 in tomato (Table S3). Homologs of most of the down-regulated proteins are linked to oxidation-reduction processes, plant cell wall reinforcements, and defense responses to pathogens in Arabidopsis. Similarly, homologs of most of the up-regulated proteins in the presence of a functional Rcr3 protein are typically associated with defense responses to pathogens in *A. thaliana*. Assuming that the functions of many of the differentially regulated proteins are conserved among tomato and Arabidopsis, we conclude that Rcr3 operates in the apoplast in extracellular protease-based immune signaling pathways.

Apoplastic Rcr3^{pim} alters JA-mediated defense responses in nematode-infected tomato roots. Tomato plants harboring either *Rcr3^{lyc}* or *rcrc3-3* are equally susceptible to infections by *G. rostochiensis*, while the presence of *Rcr3^{pim}* results in a major increase of susceptibility. To identify differentially expressed transcripts uniquely associated with *Rcr3^{pim}*, we have made pairwise comparisons of expression profiles from nematode-infected roots of *Rcr3^{pim}* versus *Rcr3^{lyc}* plants, and of *Rcr3^{pim}* versus *rcr3-3* plants. We found that only 15 transcripts were significantly up-regulated and 13 transcripts were significantly down-regulated in the presence of *Rcr3^{pim}* (Fig. 1A and B). Seven of these 28 differentially expressed transcripts derive from loci that so far have not been confidently called as genes in the current draft of the tomato genome. Four more differentially expressed transcripts either encode unknown small molecular weight proteins or correspond to partial open reading frames with no match in sequence databases. Nonetheless, we conclude that only a small number of differentially expressed genes are associated with the altered nematode susceptibility of tomato plants harboring Rcr3^{pim}.

The extracellular PLCP Rcr3[pim] is located in the apoplast of tomato cells. To determine if the products of the 17 differentially expressed genes regulated by Rcr3[pim] can co-localize with this PLCP, we analyzed their predicted subcellular localization (Tables 1 and 2). Only one Rcr3[pim]-regulated gene encodes a secreted protein, which has similarity to gamma-thionin containing plant defensin-like proteins. The products of six differentially expressed genes are most likely localized in the plasma membrane. Three of the proteins potentially co-localizing with Rcr3[pim] are known to regulate the activity of serine-type endopeptidases (i.e. Rhomboid-like protein and two proteinase inhibitor II (Pin2)). Two Rcr3[pim]-regulated plasma membrane proteins are associated with the biosynthesis of lipid derivatives and transfer of lipids across the plasma membrane (i.e. PVR-3, patatin-like protein-4). The last membrane-associated protein regulated by Rcr3[pim] has similarity with small arabinogalactan proteins in *A. thaliana*. Remarkably, six out of seven Rcr3[pim]-regulated proteins located either in the apoplast or the plasma membrane have been linked to jasmonic acid-related defense responses. Furthermore, most of the intracellular proteins regulated by Rcr3[pim] and with significant matches to functionally annotated genes in *A. thaliana* have also been linked to defense responses in plants (Tables 1 and 2).

Homologs of Rcr3[pim] and Rcr3[pim]-regulated genes affect susceptibility of Arabidopsis to cyst nematodes. For most of the genes regulated by Rcr3[pim] in nematode-infected roots of tomato no functional information is available and they have therefore been annotated solely based on homology with proteins in *A. thaliana*. To assess whether homologs of Rcr3[pim] itself and Rcr3[pim]-regulated genes also affect the susceptibility of *A. thaliana* to cyst nematodes, we challenged homozygous knockout mutant Arabidopsis lines of several of these genes with the beet cyst nematode *Heterodera schachtii*.

First, we investigated whether apoplastic PLCPs in *A. thaliana* most similar to Rcr3[pim] affect the susceptibility of Arabidopsis plants to nematode infections. For this purpose we challenged six Arabidopsis homozygous mutant lines of the *papain-like cysteine proteases* (*PAP*) *1*, *4*, and *5* genes with infective juveniles of *H. schachtii*. The *PAP*-genes of Arabidopsis belong to the same PLCP subfamily (#6) as *Rcr3* of tomato (*6*). PAP1 and PAP5 are predicted to be apoplastic PLCPs, while the predicted subcellular localization of PAP4 is vacuolar. Fourteen days after inoculation the number of females per plant was almost twice as high in the *pap1-1, pap1-2*, and *pap5-1* mutants than in the corresponding Col-0 wild type plants (Fig. 2). By contrast, the susceptibility of the *pap4-1* mutant to infections by *H. schachtii* was similar to the corresponding wild type Arabidopsis plants.

The fungal effector Avr2 of *C. fulvum* inhibits both Rcr3 of tomato and several homologous apoplastic PLCPs of *A. thaliana* (*16*). Transgenic Arabidopsis lines overexpressing Avr2 show an enhanced susceptibility to

Table 1. Tomato genes that are up-regulated in *Cf-0/Rcr3pim* tomato plants, when compared either to *Cf-0/Rcr3esc* or to the mutant *Cf0/rcr3-3*. Tomato plants were infected with the potato cyst nematode *Globodera rostochiensis* and global gene expression analysis was determined by RNA-seq with a false discovery rate set to 0.001 (FDR <0.001 for differentially expressed genes) on plant roots at 7 days post inoculation.

Gene up-regulated	Description	Cellular component	Homolog in ARATH	Biological Process	Reference
Solyc09g010880	Rhomboid-like family protein	Integral to membrane; Golgi and plasma membrane	AT2G29050	Unknown function; Homolog of Drosophila Rhomboid involved in EGF-receptor activation; Possible serine-type peptidase involved in intramembrane proteolysis	
Solyc01g109390	PVR-3 protein; defective in induced resistance 1-like (DIR-like)	Integral to plasma membrane	AT5G48485	Lipid transfer protein; JA responses	Plant Physiol. 155:1762-1768
Solyc08g065850	Homolog of arabinogalactan peptide-14 (AGP-14)	Integral to plasma membrane	AT5G56540	Protein binding; root hair elongation; ARATH AGP-31 is repressed by JA	
Solyc06g067860	2-Oxoglutarate/iron-dependent dioxygenase	Cytoplasm	AT1G52800	Oxidation-reduction process; JA responses	
Solyc07g049560	Tyrosine phosphatase family protein	Cytoplasm	AT1G05000	Dephosphorylation; Negative regulation of MAP kinase activity	
Solyc12g068070	Filament-like plant protein-3 (FPP)	Nucleus	AT3G05270	Coiled-coiled domain protein with unknown function	
Solyc02g083650	Unknown	Nucleus	AT4G35980	Inactivation of the chloroplast ATP synthase gamma subunit results in high non-photochemical fluorescence quenching and altered nuclear gene expression in Arabidopsis thaliana	Journal Biol. Chem 279: 1060
Solyc06g024300	Unknown LMW protein	-	-	Homologous to small uncharacterized protein in Glycine max and Medicago truncatula	
Solyc00g011160	Unknown LMW protein	-	-	-	

Table 2. Tomato genes that are down-regulated in *Cf-0/Rcr3pim* tomato plants, when compared either to *Cf-0/Rcr3esc* or to the mutant *Cf0/rcr3-3*. Tomato plants were infected with the potato cyst nematode *Globodera rostochiensis* and global gene expression analysis was determined by RNA-seq with a false discovery rate set to 0.001 (FDR <0.001 for differentially expressed genes) on plant roots at 7 days post inoculation.

Gene down-regulated	Description	Cellular component	Homolog in ARATH	Biological Process	Reference
Solyc07g009080	Defensin-like protein (PDF)	Apoplast	AT2G26010	Contains gamma thionin motif; Involved in plant defense response; homologous to ARATH PDF1.4 gene; serine-type protease inhibition; JA responses	
Solyc03g020040	Proteinase inhibitor II (Pin2)	Integral to plasma membrane	-	Wound induced serine-type protease inhibitor; JA responses	
Solyc03g020030	Proteinase inhibitor II (Pin2)	Integral to plasma membrane	-	Wound induced serine-type protease inhibitor; JA responses	
Solyc02g090490	Patatin-like protein 4	Integral to plasma membrane	AT4G37050	Phospolipase A activity; JA responses; Botrytis resistance; auxin responses in development and phosphate deficiencies	
Solyc10g006230	Chlorophyll a-b binding protein 7	Chloroplast	AT3G61470	Photosystem I light harvesting complex	
Solyc02g085950	RUBISCO small subunit 3B	Chloroplast	AT5G38410	Photosynthesis; photorespiration	
Solyc10g086580	RUBISCO activase	Chloroplast	AT2G39730	ATP-binding; Required for the light activation of RUBISCO; JA responses; negative regulation of defense response;	
Solyc07g006500	Trehalose-6-phosphate/synthase	Cytosol; mitochondrion	AT2G18700	Trehalose biosynthesis; Linked to defense against green peach aphid infestations	Plant Sign. Behav. 7: 605
Solyc03g111710	BTB and TAZ domain protein 1; Speckle-type POZ protein	Cytosol; Nucleus	AT5G63160	Include TAZ-type Zinc finger; Short-lived nuclear-cytoplasmic protein targeted for degradation by the 26S proteasome pathway; DNA-dependent regulation of transcription; response to SA and auxin stimuli; response to ROS	
Solyc01g095320	BCL-2 associated anthanogene 6 (BAG6)	Nucleus	AT2G46240	Apoptosis regulator BCL-2 protein, BAG; Plant homologs of mammalian regulators of apoptosis; Knockout mutants exhibited enhanced susceptibility to fungal pathogen Botrytis cinerea	J. Biol. Chem. 281:18793
Solyc01g108710	Unknown LMW protein	-	AT2G15830	Uncharacterized protein	
Solyc01g095340	Unknown LMW protein	-	-	-	
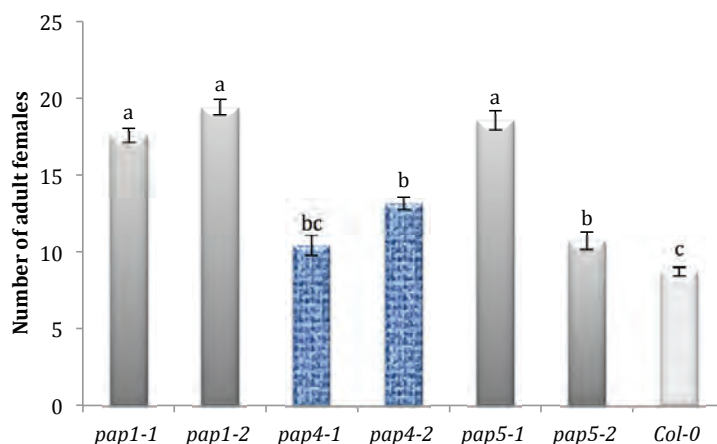
Fig. 2. Arabidopsis mutants lacking the cysteine protease genes *PAP1*, *PAP4* and *PAP5* are more susceptible to the nematode *Heterodera schachtii*. Two-week-old Arabidopsis plants were inoculated *in vitro* with 250 *H. schachtii* infective juveniles. The number of adult females was determined 14 days after inoculation. Number of adult females on the wild type Col-0 (background for all mutants) line was used as a control. Bars represent standard errors of the means. Blue filled columns indicate that the protease is predicted to be vacuolar localized and dark grey indicates that it is predicted to be extracellular. Different characters indicate statistical differences as determined with a one-way ANOVA test (*P*-values <0.05 with N=12). Two independent experiments were performed and representative results are shown.

fungi and oomycetes. We used these transgenic lines to demonstrate that the constitutive inhibition of apoplastic PLCPs homologous to Rcr3 alters the susceptibility of Arabidopsis plants to cyst nematode infections (Fig. 3). We therefore conclude that apoplastic Rcr3-like proteases regulate the susceptibility of tomato and Arabidopsis roots to cyst nematode infections.
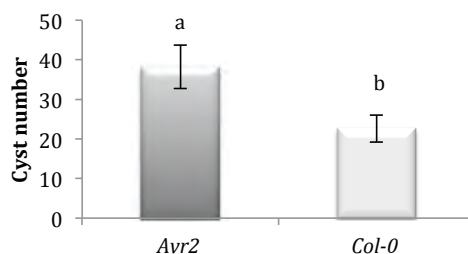
Fig. 3. Arabidopsis plants over-expressing the *Cladosporium fulvum* cysteine protease inhibitor effector protein Avr2 are more susceptible to the nematode *Heterodera schachtii.* Two-week-old Arabidopsis plants were inoculated *in vitro* with 250 *H. schachtii* infective juveniles. The number of cysts was determined 28 days after inoculation. Number of cysts on the wild type Col-0 (background for all mutants) line was used as a control. Bars represent standard errors of the means. Different characters indicate statistical differences as determined with a one-way ANOVA test (*P*-values <0.05 with N=12). Two independent experiments were performed and representative results are shown.

As the role of apoplastic Rcr3-like PLCPs in nematode-plant interactions seems conserved among unrelated plants species, we reasoned that homologs of Rcr3[pim]-associated genes might also affect the susceptibility of Arabidopsis plants to nematode infections. To test this we analyzed the susceptibility of homozygous Arabidopsis knockout lines of several homologs of Rcr3[pim]-regulated tomato genes to infections by *H. schachtii* (Fig. 4). The number of *H. schachtii* females per plant was significantly lower in all knockout mutants as compared to the corresponding wild-type *A. thaliana* Col-0 ecotype. This data demonstrates that the molecular components underlying the Rcr3[pim] phenotype in nematode-infected tomato roots most likely have similar roles in determining the susceptibility of Arabidopsis to *H. schachtii*.
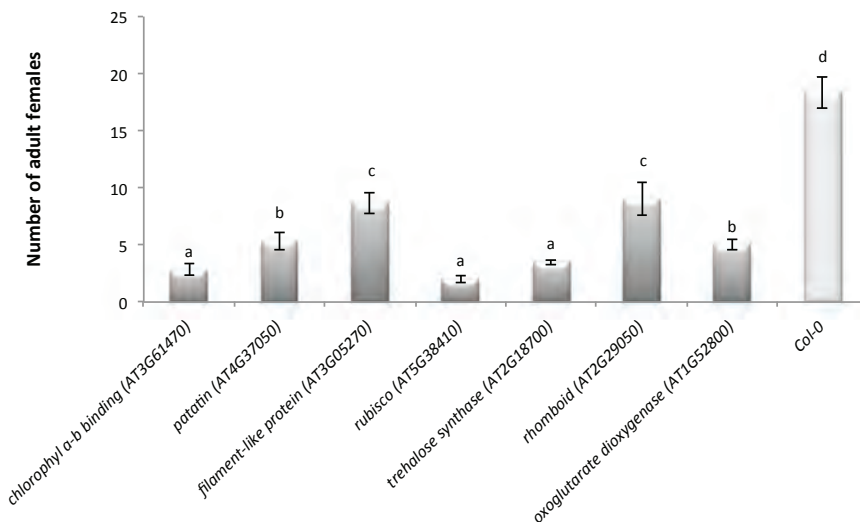
Fig. 4. Arabidopsis mutants lacking several homologs of Rcr3[pim]-regulated tomato genes are less susceptible to the nematode *Heterodera schachtii.* Two-week-old Arabidopsis plants were inoculated *in vitro* with 250 *H. schachtii* infective juveniles. The number of adult females was determined 14 days after inoculation. Number of adult females on the wild type Col-0 (background for all mutants) line was used as a control. Bars represent standard errors of the means. Different characters indicate statistical differences as determined with a one-way ANOVA test (*P*-values <0.05 with N=12). Two independent experiments were performed and representative results are shown.

PLCPs also regulate susceptibility of Arabidopsis to nematode infections in different subcellular compartments. Both Rcr3-variants strongly induced the expression of an apoplastic cathepsin B-like protease, which belongs to a distantly related subfamily of PLCPs in plants (*6*). This finding suggested that other unrelated PLCPs might also be involved in the responses of plants to nematode infections. The genome of *A. thaliana* includes 31 PCLPs, many of

which have unknown functions. We have analyzed the susceptibility of multiple homozygous knockout lines of 21 PCLPs, representatives of six additional PLCP subfamilies in Arabidopsis (*19*), to infections by *H. schachtii* (Fig. 5).

First, we challenged ten homozygous mutant lines of the *Responsive to Dehydration 21* gene (*RD21*) and *Responsive to Dehydration-Like* genes *RDL1, 2, 3, 5*, and *6* with infective juveniles of *H. schachtii*. Fourteen days after inoculation the number of females in the mutants *rd21A*, *rd21B*, *rdl1*, *rdl2*, *rdl3*, *rdl6-1* and *rdl6-2* was significantly higher than the corresponding Col-0 wild type plants (PLCP subfamily 1; Fig. 5A). By contrast, a knockout mutation in the *Cysteine Endopeptidase 2* (*CEP2)* gene (*cep2-1* and *cep2-2*) resulted in a major loss of susceptibility to *H. schachtii* (PLCP subfamily 2; Fig. 5B). Arabidopsis harbors two xylem cysteine proteinase (*XCP1* And *XCP2*) genes, only one of which significantly affects its susceptibility to *H. schachtii* (PLCP subfamily 3; Fig. 5C). Similarly, some mutations in the *Responsive to Dehydration 19A* gene (*rd19a-1* and *rd19a-2*) and the *Aleurain-Like Proteases 1* gene (*aalp1-1* and *aalp1-2*) rendered Arabidopsis plants hypersusceptible to infections by *H. schachtii* (PLCP subfamilies 7 and 8; Fig. 5D and E). Lastly, mutations in *cathepsin B2*-like and *cathepsin B3*-like genes (*catb2-1*, *catb2-2*, *catb3-1* and *catb3-2*) also selectively enhanced the susceptibility of Arabidopsis to *H. schachtii* (PLCP subfamily 9; Fig. 5F). Interestingly, mutants of genes encoding extracellular and vacuolar PLCPs lead to an increase of susceptibility of Arabidopsis to *H. schachtii*. The only mutant line that results in a loss of susceptibility to nematode infections encodes a defective CEP2, which is predicted to be strictly localized in the endoplasmic reticulum. Altogether, our data show that papain-like cysteine proteases located in different subcellular compartments co-determine the susceptibility of Arabidopsis to infections by cyst nematodes.

5.4 Discussion

We have previously shown that allelic variation in the apoplastic papain-like cysteine protease Rcr3 has a major impact on the interaction between tomato plants and *G. rostochiensis* (*18*). Seven polymorphic sites determine the physical association between Rcr3[pim] of tomato and the effector Gr-VAP1, secreted by *G. rostochiensis* and almost double the number of successful nematode infections per tomato plant. Furthermore, the ectopic overexpression of nematode venom allergen-like proteins in plants suppresses defense responses specifically activated by extracellular plant immune receptors (*Chapter 4, this thesis*). To gain more insight into the molecular mechanisms underlying the Rcr3[pim]-enhanced susceptibility in
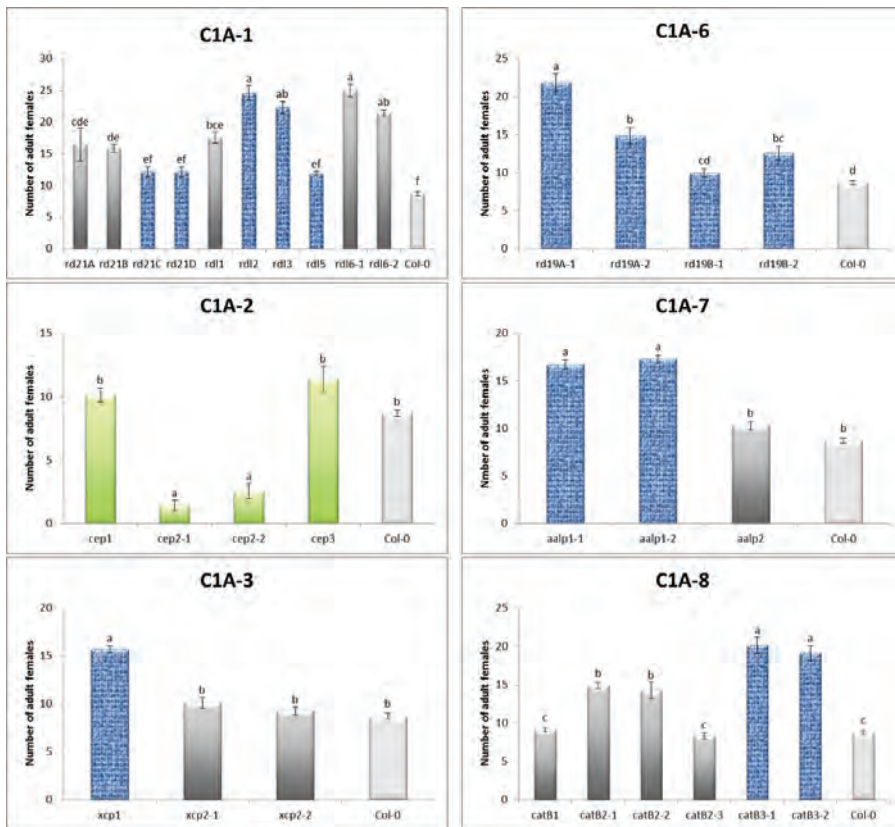
Fig. 5. Arabidopsis mutants lacking papain-like cysteine proteases (PLCPs) show altered susceptibility to the nematode *Heterodera schachtii*. Two-week-old Arabidopsis mutants on 6 PLCPs subfamilies (C1A-1 to -3 and C1A-6 to 8) were inoculated *in vitro* with 250 *H. schachtii* infective juveniles. The number of adult females was determined 14 days after inoculation. Number of adult females on the wild type Col-0 (background for all mutants) line was used as a control. Blue filled columns indicate that the protease is predicted to be vacuolar-, dark grey extracellular-, and green endoplasmic reticulum-localized. Bars represent standard errors of the means. Different characters indicate statistical differences as determined with a one-way ANOVA test (*P*-values <0.05 with N=12). Two independent experiments were performed and representative results are shown.

tomato, we analyzed the global gene expression profiles of nematode-infected tomato roots harboring the *Rcr3*[pim] allele, the *Rcr3*[lyc] allele, or the null-mutant allele *rcr3-3*.

Our analysis revealed that Rcr3 proteins (both Rcr3[pim] and Rcr3[lyc]) in the apoplast operate in protease-based extracellular immune signaling pathways in nematode-infected roots of tomato. The two Rcr3 variants seem to commonly reduce the oxidoreductase capacity, alter the plant cell wall

composition, and selectively suppress components of host defense responses in tomato plants infected by cyst nematodes. Both Rcr3 variants also enhanced the expression of several other defense-related genes (e.g. cathepsin B-like cysteine protease, WRKY and basic helix-loop-helix transcription factors, and major latex-like protein) in nematode-infected roots. Tomato plants with Rcr3^{lyc} and without a functional Rcr3 (i.e. *rcr3-3*) do not differ in susceptibility to *G. rostochiensis*. We therefore conclude that the net effect of the genes commonly regulated by Rcr3^{pim} and Rcr3^{lyc} on the susceptibility of tomato plants to *G. rostochiensis* is not significant.

However, the 28 transcripts uniquely associated with Rcr3^{pim} collectively contribute to a major increase in the susceptibility of tomato plants to *G. rostochiensis*. The biological processes affected by homologs of most of these transcripts in Arabidopsis suggests that Rcr3^{pim} most likely alters wound-inducible and jasmonic acid-related defense responses in nematode-infected roots of tomato. Three of the Rcr3^{pim}-regulated genes have no specific links to jasmonic acid responses, but are nonetheless involved in regulating defense-related apoptosis (i.e. BCL-2-associated anthanogene 6 (*20*)), tolerance to abiotic and biotic stress (i.e. trehalose-6-phopshate synthase (*21-23*)), and suppression of intracellular immune signaling (i.e. tyrosine phosphatase (*24*)). The integration of the biological processes commonly regulated by both Rcr3 variants and those uniquely associated with Rcr3^{pim} leads to a model in which apoplastic Rcr3^{pim} modulates innate immune responses involving mechanical wounding, plant cell wall alterations, and jasmonic acid-induced host defenses.

A role for Rcr3^{pim} as a modulator of wound-inducible / jasmonic acid-dependent host defenses would also fit earlier findings. First, Rcr3^{pim} is a virulence target of the venom allergen-like protein Gr-VAP1, which is secreted along with plant cell wall modifying enzymes by migratory infective juveniles of *G. rostochiensis* during the onset of parasitism (*18*). Like necrotrophic plant pathogens and herbivorous insects, migratory juveniles of cyst nematodes cause extensive damage to plant cell walls. During the onset of parasitism it seems likely that cyst nematodes liberate damage-associated molecular patterns capable of triggering innate immune responses, which may depend on Rcr3^{pim}. Second, the presence of Rcr3^{pim} does not alter the susceptibility of tomato plants to fungal pathogens that are strictly biotrophic (*25*), while it affects the colonization by the hemibiotrophic *P. infestans* (*26*). Similarly, tomato plants harboring Rcr3^{pim} do not show an enhanced susceptibility to infections by the root-knot nematode *Meloidogyne incognita*, which has evolved stealthy invasion strategies (unpublished data). Thus, Rcr3^{pim} function seems to be linked to the degree of damage done to host tissues during infections by plant parasites and pathogens. Third, the overexpression of venom allergen-like proteins in Arabidopsis induces down-regulation of a proline-rich extension-like receptor kinase (PERK11) (*Chapter*

4, this thesis). PERK11 belongs to a gene family of fifteen receptor kinases in Arabidopsis (AtPERK) of which the extracellular domain shares similarity with cell wall associated proteins (*27*). The biological function of the AtPERK family members is largely unknown, but the expression of one member, i.e. PERK1, is rapidly induced following wounding. It is therefore thought that PERK1 mediates early events in defense responses to cell wall damage by invading plant pathogens (*28*). In conclusion, further research is needed to test whether Rcr3pim operates in an extracellular signaling pathway associated with immune receptors specific for damage-associated molecular patterns.

The most up-regulated gene associated with Rcr3pim in nematode infected roots is highly homologous to the intramembrane Rhomboid-like protein 1 in Arabidopsis (Solyc09g010880.2). Although the function of Rhomboid-like proteins in plants is not clear, their serine-type of endopeptidase activity may be involved in intramembrane proteolytic regulation of other membrane proteins (*29*). Interestingly, the up-regulation of Rhomboid-like protein 1 by Rcr3pim coincides with the down-regulation of two membrane-bound serine protease inhibitors (i.e. PIN2 variants). The predicted subcellular localizations of Rhomboid-like protein 1 of Arabidopsis are the plasma membrane and Golgi apparatus, and in that sense it resembles Rhomboid in *Drosophila melanogaster*. Drosophila Rhomboid promotes the release of the extracellular domain of the epidermal growth factor (EGF)-like growth factor Spitz allowing it to function as an activating ligand of the EGF receptor (*30, 31*). Whether such receptor activation by regulatory intramembrane proteolysis occurs in plants remains to be investigated. However, the Arabidopsis genome harbors 26 receptor-like kinases with an extracellular EGF domain that might be the targets of such a mechanism (*32*). At present, the only EGF-type receptor-like kinases with a known function in plants are the so-called wall-associated kinases (WAK), which are thought to survey and to act upon changes in the integrity of plant cell walls. Although it would fit our model of Rcr3pim as modulator of plant cell wall-associated host defenses, further research is needed to investigate if Rcr3pim-regulated Rhomboid-like proteins are involved in the activation of EGF-type of receptor-like kinases associated with cell walls in plants.

More puzzling is the Rcr3pim-dependent down-regulation of components of the photosystems I and II in chloroplast (Solyc10g006230, Solyc02g085950, Soly10g0806580, and Solyc05g013160) of nematode-infected roots. The occurrence of photosynthetic activity in roots in the first place may be explained by the fact that the nematode-infected roots were collected from *in vitro* cultured plants that grow on translucent media in the light. However, it is not clear whether Rcr3pim in nematode-infected roots modulates the photosynthetic capacity directly, or whether the photosystem components are antagonized by jasmonic acid-dependent host defense responses. One of the components down-regulated by Rcr3pim (i.e. RUBISCO activase [encoded by

Solyc10g086580]) has been linked to jasmonic acid signaling before and may mediate a trade off between photosynthetic capacity and jasmonic acid-dependent host defenses in nematode-infected roots.

The presence of a functional Rcr3 protein in the apoplast of tomato strongly increased the expression of another extracellular papain-like cysteine protease, while it down-regulated the expression of matching protease inhibitors. This finding suggests that Rcr3 may operate in extracellular signaling pathways that involve multiple apoplastic papain-like cysteine proteases. Our investigations of homozygous mutant *Arabidopsis* lines showed that besides the direct Rcr3 homologs (*PAP1* and *PAP5)* several other apoplastic PLCPs affect the susceptibility of Arabidopsis to infections by *H. schachtii*. Based on the analysis of these mutants alone it is not possible to conclude whether multiple PLCPs form an extracellular proteolytic cascade or complex, or whether they regulate host defense in parallel but interdependent signaling pathways. More insight in the native substrates of defense-related apoplastic PLCPs in plants is needed to assess whether they act in a cascade, in a complex, or alone.

Our Arabidopsis mutant screen further showed that PCLPs, independent of their cellular localization, affect the susceptibility to infections by *H. schachtii*. Some of these PLCPs have been linked to host defense responses to plant pathogens (i.e. At1g47128 (*9*), At4g39090 (*8*), and At1g02300, At1g02305, and At4g01610 (*33*)), but the function of most PLCPs in Arabidopsis is unknown. It is likely that besides their involvement in host defense and cell death, PLCPs also regulate developmental processes in plants that affect their susceptibility to infections by cyst nematodes. For instance, mutations in *CEP2* (At3g48350) result in a major loss of susceptibility of Arabidopsis to *H. schachtii*. *CEP2* promoter activity is especially high in very young root tips and in the root elongation zone of Arabidopsis (*34*), which is the preferential site of invasion of plants by cyst nematodes. Endoplasmic CEPs have been shown to breakdown hydroproline-rich proteins such as plant cell wall-derived extensins that serve as scaffolds for depositions of carbohydrate polymers in plant cell wall formation (*34*). Local plant cell wall breakdown and synthesis is a crucial process during the transformation of host cells into feeding cells by cyst nematodes in plants (*35-39*). It is therefore conceivable that Arabidopsis CEP knockout mutants are almost resistant to *H. schachtii*, because cyst nematodes require this plant PLCP to remodel host cell walls during feeding cell formation.

5.5 Materials and methods

Nematode infection assays. Dried cysts of *G. rostochiensis* pathotype Ro1-Mierenbos were soaked on a 100-μm sieve in potato root diffusate to collect hatched pre-parasitic J2s (*40*). Freshly hatched pre-parasitic second-stage

juveniles in suspension were purified on a sucrose gradient and surface sterilized as previously described (*41*). The nematodes were resuspended in a sterile 0.7% (w/v) solution of Gelrite (Duchefa), and pipetted along the roots of two-week-old *in vitro*-grown tomato plants.

Pre-parasitic J2s of *H. schachtii* were extracted from the soil of *Brassica oleraceae* infected plants and surface sterilized as previously described (*42*). The nematodes were resuspended in a sterile 0.7% (w/v) solution of Gelrite (Duchefa), and pipetted near the base of two-week-old *in vitro*-grown Arabidopsis plants.

RNA-seq on tomato plants. *Solanum lycopersicum* seeds of the genotypes *Cf-0/Rcr3pim*, *Cf-0/Rcr3esc* and the mutant *Cf0/rcr3-3*, were sterilized and grown in square plates (1 seed per plate) on Gamborg B5 medium. After two weeks, the seedlings were infected with ~250 sterilized *G. rostochiensis* infective juveniles. The plants were grown at 24°C and light/dark cycles of 16 h/8 h. Seven days post inoculation, the roots were collected and flash-frozen in liquid nitrogen. Total RNA was extracted with the RNesy plant minikit (Qiagen) according to the manufacturer instructions. A total of 15 tomato roots were pooled together per biological replicate to obtain ~20 µg of total RNA. Three biological replicates were collected for each genotype. cDNA synthesis, library preparation (200-bp inserts) and Illumina sequencing (90-bp paired-end reads) was performed at BGI (Hong-Kong). Reads were mapped to the tomato genome (ITAG 2.3) using TopHat (v. 1.4.0 (*43*)). Transcript assembly and transcript abundance estimations were performed with Cufflinks (v. 0.9.3 (*44*)).

Infection assays on Arabidopsis plants. Seeds of Arabidopsis mutants (Table S4) and wild-type controls (Col-0) were vapor sterilized and planted in 12-well cell culture plates (Greiner bio-one) containing modified Knop's medium (*45*). Plants were grown at 24°C under 16-h-light/8-h-dark conditions. Two-week-old seedlings were inoculated with ~250 surface-sterilized pre-parasitic J2s *H. schachtii* nematodes (*42*). Inoculated plants were maintained under the conditions described above. Two and four weeks after inoculation, the number of *H. schachtii* adult females was counted. Average numbers of J4 females were calculated, and statistically significant differences between plant lines and the corresponding wild-type control were determined by a one-way analysis of variance (ANOVA) with Tukey's multiple comparison for pairwise comparison. Statistical significance was set at $P < 0.05$.

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5.7 Supporting information

Table S1. Tomato genes that are similarly up- and down-regulated (Reg.) in *Cf-0/Rcr3pim* and *Cf-0/Rcr3lyc* plants when compared to the mutant *Cf0/rcr3-3*. Tomato plants were infected with the potato cyst nematode *Globodera rostochiensis* and global gene expression analysis was determined by RNA-seq on plant roots at 7 days post inoculation. Genes are organized according to the p (p_value) and q (q_value) values. Signal peptides were predicted by both the Solgenomics prediction program (SigP_1) and SignalP v. 3.0 (SigP_2). Transmembrane domains (T.m.) were predicted in TMHMM v. 2.0.

XLOC_ID	Gene	p_value	q_value	Reg.	Description	Sig.P_1	Sig.P_2	T.m.
XLOC_006414	Solyc02g076980.2	0.0000	0.0000	Up	Cathepsin B-like cysteine proteinase (AHRD V1 **-* CYSP_SCHMA); contains Interpro domain(s) IPR013128 Peptidase C1A, papain IPR000169 Peptidase, cysteine peptidase active site	SP (0.12)	SP (0.90)	T
XLOC_023015	Solyc07g049660.2	0.0000	0.0000	Up	Acetyl coenzyme A cis-3-hexen-1-ol acetyl transferase (AHRD V1 **-* Q9SRQ2_ARATH); contains Interpro domain(s) IPR003480 Transferase	N	N	N
XLOC_006463	Solyc02g077940.1	0.0000	0.0000	Up	Unknown Protein (AHRD V1)			
XLOC_030773	-	0.0000	0.0000	Down	-			
XLOC_018956	-	0.0000	0.0000	Up	-			
XLOC_009225	-	0.0000	0.0000	Up	-			
XLOC_009221	-	0.0000	0.0000	Up	-			
XLOC_022029	-	0.0000	0.0000	Down	-			
XLOC_014137	Solyc04g072280.2	0.0000	0.0000	Down	Laccase (AHRD V1 ***- Q38757_ACEPS); contains Interpro domain(s) IPR017761 Laccase	SP (0.1)	SP (0.99)	T
XLOC_021233	Solyc06g061210.2	0.0000	0.0000	Down	Fucosyltransferase 7 (AHRD V1 **** B6SRP0_MAIZE); contains Interpro domain(s) IPR004938 Xyloglucan fucosyltransferase	SP (0.32)	SP (0.88)	T
XLOC_009220	Solyc02g077940.1	0.0000	0.0000	Up	Unknown Protein (AHRD V1)	N	N	N
XLOC_034249	-	0.0000	0.0000	Down	-			
XLOC_003342	-	0.0000	0.0000	Up	-			
XLOC_001007	-	0.0000	0.0000	Up	-			
XLOC_035182	Solyc11g011210.1	0.0000	0.0000	Down	Gibberellin regulated protein (AHRD V1 **- A2Q374_MEDTR); contains Interpro domain(s) IPR003854 Gibberellin regulated protein	SP (0.046)	SP (0.97)	T
XLOC_039252	-	0.0000	0.0000	Down	-			
XLOC_022017	-	0.0000	0.0000	Down	-			

XLOC_ID	Gene	p_value	q_value	Reg.	Description	Sig.P_1	Sig.P_2	T.m.
XLOC_009592	Solyc03g020010.1	0.0000	0.0001	Down	Kunitz-type trypsin inhibitor alpha chain (AHRD V1 **** ID5A_PROJU); contains Interpro domain(s) IPR002160 Proteinase inhibitor I3, Kunitz legume	SP (0.31)	SP (0.99)	
XLOC_022016	-	0.0000	0.0001	Down	-			
XLOC_038991	-	0.0000	0.0003	Down	-			
XLOC_023557	Solyc07g005950.2	0.0000	0.0006	Up	Pentatricopeptide repeat-containing protein (AHRD V1 ***- D7MBL7_ARALY); contains Interpro domain(s) IPR002885 Pentatricopeptide repeat	N	N	N
XLOC_021327	Solyc06g065060.1	0.0000	0.0004	Down	FAD-binding domain-containing protein (AHRD V1 **-- D7MFI0_ARALY); contains Interpro domain(s) IPR006094 FAD linked oxidase, N-terminal	SP (0.085)	SP (1.00)	
XLOC_036776	Solyc12g015970.1	0.0000	0.0006	Up	GPI-anchored protein (AHRD V1 ***- A2PZD8_IPONI)	SP (0.082)	SP (0.99)	T
XLOC_028218	Solyc09g011550.2	0.0000	0.0006	Up	Glutathione S-transferase-like protein (AHRD V1 **** A8DUB0_SOLLC); contains Interpro domain(s) IPR004046 Glutathione S-transferase, C-terminal	N	N	N
XLOC_029143	Solyc09g097950.1	0.0000	0.0002	Up	Aldo/keto reductase (AHRD V1 *-*- A2Q580_MEDTR); contains Interpro domain(s) IPR001395 Aldo/keto reductase	N	N	N
XLOC_016134	-	0.0000	0.0030	Up	-			
XLOC_035429	-	0.0000	0.0021	Down	-			
XLOC_035432	Solyc11g027710.1	0.0000	0.0048	Up	Unknown Protein (AHRD V1)	N	N	N
XLOC_028525	-	0.0000	0.0000	Up	-			
XLOC_025810	Solyc08g067360.2	0.0000	0.0090	Up	WRKY transcription factor 9 (AHRD V1 **** C9DHZ8_9ROSI); contains Interpro domain(s) IPR003657 DNA-binding WRKY	N	N	N
XLOC_016003	-	0.0000	0.0016	Up	-			
XLOC_035339	Solyc11g018800.1	0.0000	0.0052	Down	Peroxidase 2 (AHRD V1 ***- Q9SSZ8_SCUBA); contains Interpro domain(s) IPR002016 Haem peroxidase, plant/fungal/bacterial	SP (0.056)	SP (0.999)	
XLOC_027667	-	0.0000	0.0057	Down	-			
XLOC_025049	-	0.0000	0.0067	Down	-			
XLOC_025254	Solyc08g007460.2	0.0000	0.0000	Up	Non-specific lipid-transfer protein (AHRD V1 -*** B9RGU5_RICCO); contains Interpro domain(s) IPR003612 Plant lipid transfer protein/seed storage/trypsin-alpha amylase inhibitor	N	SP (0.99)	N

XLOC_ID	Gene	p_value	q_value	Reg.	Description	Sig.P_1	Sig.P_2	T.m.
XLOC_034989	Solyc11g006270.1	0.0000	0.0073	Down	3-oxo-5-alpha-steroid 4-dehydrogenase family protein (AHRD V1 ***- D7M7I5_ARALY); contains Interpro domain(s) IPR001104 3- oxo-5-alpha-steroid 4-dehydrogenase, C-terminal	N	N	T
XLOC_021948	-	0.0000	0.0069	Down	-			
XLOC_012981	-	0.0001	0.0084	Down	-			
XLOC_016321	-	0.0001	0.0182	Up	-			
XLOC_009328	-	0.0001	0.0107	Down	-			
XLOC_020849	-	0.0001	0.0127	Down	-			
XLOC_012121	Solyc03g098730.1	0.0001	0.0134	Down	Kunitz trypsin inhibitor (AHRD V1 ***- B8Y888_TOBAC); contains Interpro domain(s) IPR011065 Kunitz inhibitor ST1-like	SP (0.19)	SP (0.99)	
XLOC_008979	-	0.0001	0.0282	Up	-			
XLOC_012987	-	0.0001	0.0142	Down	-			
XLOC_009381	-	0.0001	0.0161	Down	-			
XLOC_006465	Solyc02g078130.2	0.0001	0.0110	Up	Basic helix-loop-helix family protein (AHRD V1 ***- D7MFG5_ARALY); contains Interpro domain(s) IPR011598 Helix-loop-helix DNA-binding	N	N	N
XLOC_025050	-	0.0002	0.0431	Up	-			
XLOC_019058	Solyc05g046160.1	0.0002	0.0309	Up	Major latex-like protein (AHRD V1 **-- B5THI3_PANGI); contains Interpro domain(s) IPR000916 Bet v l allergen	N	N	N
XLOC_018826	Solyc05g013160.2	0.0003	0.0317	Down	Set domain protein (AHRD V1 ***- C1EFC8_9CHLO); contains Interpro domain(s) IPR011192 Rubisco methyltransferase	N	N	N
XLOC_009226	-	0.0003	0.0405	Up	-			
XLOC_009757	Solyc03g036470.1	0.0004	0.0419	Down	Phenylalanine ammonia-lyase (AHRD V1 ***- B5LAW0_CAPAN); contains Interpro domain(s) IPR001106 Phenylalanine/histidine ammonia-lyase	N	N	N

Table S2. Arabidopsis homologues of the tomato genes that are similarly up- and down-regulated (Reg.) in $Cf-0/Rcr3^{pim}$ and $Cf-0/Rcr3^{lyc}$ plants when compared to the mutant $Cf0/rcr3-3$. Tomato plants were infected with the potato cyst nematode *Globodera rostochiensis* and global gene expression analysis was determined by RNA-seq on plant roots at 7 days post inoculation.

Gene	Reg.	Description	Homolog in ARATH	Cellular component	Biological Process
Solyc02g076980	Up	Cathepsin B-like cysteine proteinase	AT2G34080.1	Endomembrane system	Cysteine-type endopeptidase
Solyc07g049660	Up	Acetyl coenzyme A cis-3-hexen-1-ol acetyl transferase	AT5G17540.1	Cytoplasm	Transferase activity
Solyc04g072280	Down	Laccase	AT5G09360.1	Apoplast	Lignin catabolic process; oxidation-reduction process
Solyc06g061210	Down	Fucosyltransferase 7	AT2G03220.1	Golgi apparatus; membrane	Cell wall biogenesis; response to stress
Solyc11g011210	Down	Gibberellin regulated protein	AT1G74670.1	Apoplast	Gibberellic acid mediated signaling pathway; response to stimulus
Solyc03g020010	Down	Kunitz-type trypsin inhibitor alpha chain	AT1G17860.1	Apoplast; cell wall	Nitrate transport; protein folding; response to stress
Solyc07g005950	Up	Pentatricopeptide repeat-containing protein	AT4G30825.1	Mitochondrion; nucleus	Uncharacterized protein
Solyc06g065060	Down	FAD-binding domain-containing protein	AT5G44440.1	Endomembrane system	Electron carrier activity; oxidoreductase activity; catalytic activity
Solyc12g015970	Up	GPI-anchored protein	AT5G56170.1	Plasma membrane	Arabidopsis thaliana protein match is: lorelei
Solyc09g011550	Up	Glutathione S-transferase-like protein	AT3G09270.1	Cytoplasm	Fatty acid beta-oxidation; proteasomal catabolic process; response to stress
Solyc09g097950	Up	Aldo/keto reductase	AT1G60730.2	Cytoplasm	Oxidoreductase activity
Solyc08g067360	Up	WRKY transcription factor 9	AT1G80840.1	Nucleus	Pathogen-induced transcription factor; coexpression with WRKY18 or WRKY60 made plants more susceptible to both *P. syringae* and *B. Cinerea*
Solyc11g018800	Down	Peroxidase 2	AT1G14550.1	Endomembrane system	Peroxidase activity; heme binding
Solyc08g007460	Up	Non-specific lipid-transfer protein	AT1G70250.1	Plasma membrane	Protein phosphorylation

Gene	Reg.	Description	Homolog in ARATH	Cellular component	Biological Process
Solyc11g006270	Down	3-oxo-5-alpha-steroid 4-dehydrogenase family protein	AT5G16010.1	Chloroplast; integral to membrane	Oxidoreductase activity
Solyc03g098730	Down	Kunitz trypsin inhibitor	AT1G17860.1	Apoplast; cell wall	Endopeptidase inhibitor activity
Solyc02g078130	Up	Basic helix-loop-helix family protein	AT4G20970.1	Nucleus	Sequence-specific DNA binding transcription factor activity
Solyc05g046160	Up	Major latex-like protein	AT1G70840.1	Chloroplast	Defense response
Solyc05g013160	Down	Set domain protein	AT1G14030.1	Chloroplast	Aromatic amino acid family biosynthetic process; embryo development ending in seed dormancy
Solyc03g036470	Down	Phenylalanine ammonia-lyase	AT2G37040.1	Cytoplasm	L-phenylalanine catabolic process; biosynthetic process, defense response

Table S3. GO biological process terms identified with a reporter *P*-value cut-off of < 1 for differentially expressed genes in *Cf-0/Rcr3^pim^* tomato plants, either compared to *Cf0/rcr3-3* (C 1) or *Cf-0/Rcr3^esc^* (C 2), infected with the potato cyst nematode *Globodera rostochiensis*.

Gene Ontology term	Cluster frequency	Genome frequency of use	P-value	C
polysaccharide binding	3 out of 65 genes, 4.6%	29 out of 15104 genes, 0.2%	0.01436	1
pattern binding	3 out of 65 genes, 4.6%	34 out of 15104 genes, 0.2%	0.02317	1
S-adenosylmethionine-dependent methyltransferase activity	3 out of 65 genes, 4.6%	61 out of 15104 genes, 0.4%	0.12831	1
solute:cation antiporter activity	2 out of 65 genes, 3.1%	27 out of 15104 genes, 0.2%	0.33443	1
carbohydrate binding	3 out of 65 genes, 4.6%	93 out of 15104 genes, 0.6%	0.41966	1
antiporter activity	2 out of 65 genes, 3.1%	44 out of 15104 genes, 0.3%	0.86007	1
solute:solute antiporter activity	2 out of 65 genes, 3.1%	44 out of 15104 genes, 0.3%	0.86007	1
peptidase inhibitor activity	2 out of 65 genes, 3.1%	46 out of 15104 genes, 0.3%	0.93582	1
tetrapyrrole binding (view genes)	11 out of 247 genes, 4.5%	68 out of 15104 genes, 0.5%	1.40E-06	2
iron ion binding (view genes)	15 out of 247 genes, 6.1%	339 out of 15104 genes, 2.2%	0.05532	2
aldehyde-lyase activity (view genes)	3 out of 247 genes, 1.2%	19 out of 15104 genes, 0.1%	0.40009	2
ammonia-lyase activity (view genes)	2 out of 247 genes, 0.8%	7 out of 15104 genes, 0.0%	0.61461	2
poly-pyrimidine tract binding (view genes)	2 out of 247 genes, 0.8%	8 out of 15104 genes, 0.1%	0.81068	2

Table S4. Arabidopsis mutants on papain-like cysteine proteases as reported in this study. Common name of the papain-like cysteine proteases (Published PLCP) and associated genes (AGI codes). Single homozygote T-DNA collection codes as given by the Arabidopsis Biological Resource Center (ABRC code) or by the European Arabidopsis Stock Centre (NASC ID).

Published PLCP	AGI Code	ABRC code	NASC ID
RD21A	At1g47128	SALK_090550C	N653357
		SALK_065256C	N662768
RD21B	At5g43060	Absent	Absent
RDL1	At4g36880	SALK_085378C	N659002
		SALK_051510C	N660866
RD21C	At3g19390	WiscDsLoxHs026_06D	N902444
RDL2	At3g19400	SAIL_598_A10	N825487
RDL3	At3g43960	SALK_135674C	N680320
RDL4	At4g11310	Absent	Absent
RDL5	At4g11320	SALK_057171C	N668323
RDL6	At4g23520	SALK_097102C	N659173
		SALK_015458C	N659174
RD21D	At1g09850	SALK_138483	N638483
CEP2	At3g48350	SALK_024692C	N656284
		SALK_016791C	N679360
CEP3	At3g48340	SALK_079519C	N669864
CEP1	At5g50260	SALK_137016C	N667437
		SALK_013036	N513036
XCP2	At1g20850	SALK_111997C	N653435
		SALK_057921C	N653229
XCP1	At4g35350	SALK_084789C	N666585
THM	At1g06260	Absent	Absent
SAG12	At5g45890	SALK_203275C	
		SALK_095721	N595721
PAP1	At2g34080	SALK_134303C	N659567
		SALK_083394C	N675996
PAP2	At1g29090	Absent	Absent
PAP3	At1g29080	Absent	Absent
PAP4	At2g27420	SALK_085797C	N663178
		SALK_024861C	N680743
PAP5	At3g49340	SALK_131226C	N656984
		SALK_039844C	N660392
RD19A	At4g39090	SALK_053577C	N656500
		SALK_082491C	N675959
RD19B	At2g21430	SAIL_18_D04	N871507
RD19C	At4g16190	SAIL_355_D07C	
RD19D	At3g54940	SALK_069903C	N668458

Published PLCP	AGI Code	ABRC code	NASC ID
AALP	At5g60360	SALK_033293C	N674361
		SALK_139239C	N682976
ALP2	At3g45310	SALK_088620C	N660022
		SALK_079981C	N668536
CatB3	At1g02305	SALK_089030C	N658326
		SALK_111776C	N658372
CatB1	At4g01610	WiscDsLox336C03	N851669
CatB2	At1g02300	SALK_063455C	N670827
		SALK_007675C	N671116

Chapter 6

General Discussion

José L. Lozano-Torres

6.1 Introduction

Parasitic nematodes are a major threat to plant, animal, and human health. A common characteristic of plant- and animal-parasitic nematodes is their phenomenal persistence inside the host, presumably resulting from their ability to suppress host immunity. However, the mechanisms of immunomodulation by parasitic nematodes in plants and animals are not well understood. It is thought that secretions released by parasitic nematodes are instrumental in the persistence of infections in both plants and animals. These secretions are complex mixtures of molecules, most of which have unknown molecular targets in the host. The composition of the secretions of animal-and plant-parasitic nematodes has little in common, except for one group of proteins that are referred to as venom-allergen like proteins (VAPs or VALs (*1*)). In fact, the venom allergen-like proteins are the most abundant proteins in secretions of animal-parasitic nematodes, and all plant-parasitic nematodes studied to date produce venom allergen-like proteins. Due to their conservation in animal- and plant-parasitic nematodes and their extraordinary abundance in nematode secretions during the onset of parasitism, venom allergen-like proteins are believed to be crucial for parasitism. However, the exact role of the VAPs in host-parasite interactions remains poorly understood. The objective of this thesis was to gain a better understanding of the function of VAPs during nematode parasitism.

6.2 The omnipresence of the SCP/TAPS

The VAPs form a monophyletic group within the SCP/TAPS-protein superfamily, members of which occur in many eukaryotes. All members of the SCP/TAPS family possess at least one cysteine-rich SCP domain. The biological functions of most SCP/TAPS proteins are not known, but their expression and release by eukaryotic cells in association with stress, led to their reputation as stress-related factors in animals and plants. For instance, some mammalian genes coding for SCP/TAPS show elevated expression levels in sickness, i.e. cancer, nerve damage, pancreatitis, and heart failure (*2*), while others are detected in several cell types of the adaptive immune system (e.g. pre-B-cells, neutrophils, and eosinophils; (*3, 4*)). In plants, the SCP/TAPS protein pathogenesis-related protein 1 (PR-1) is abundantly produced in response to pathogen attack and it is used as a marker for the salicylic acid-mediated stress responses (*5*). *PR-1* expression also increases in non-diseased plant parts, which implicates its involvement in systemic acquired resistance in plants (*6, 7*).

Free-living non-parasitic nematodes, such as *Caenorhabditis elegans*, also have multiple SCP/TAPS genes, some of which may be involved in the innate immune system of the nematodes. However, many SCP/TAPS genes in *C.*

elegans seem to be involved in entirely different cellular processes. Ce*VAP-1* of *C. elegans* encodes two isoforms of double SCP-domain proteins with a classical signal peptide for secretion. Silencing of this gene results in altered nematode locomotion (*8*). The *lon-1* gene of *C. elegans* encodes a SCP/TAPS protein that regulates polyploidization, body size and is a target of TGF-beta signaling (*9, 10*). The so-called *SCP-like (scl)* genes in *C. elegans* may function as anti-microbial factors for *Microbacterium nematophilum* infection (*11*). Transcript levels of *scl-2*, *scl-20* and *scl-27* increase when the nematode is challenged by diverse pathogens (*11, 12*). In contrast, *scl-1* positively regulates longevity and heat, starvation, and UV stress resistance, and its expression appears to be under the control of the DAF-2/insulin-like signaling pathway (*13*). The products of *scl-19* and *scl-20* are involved in immunity and may have a pro-apoptotic activity mediated by an increase in the production of reactive oxygen species (*14, 15*). Furthermore, during aging several *scl*-genes in *C. elegans* are strongly up-regulated (*16, 17*). From all these different studies, it seems that SCP/TAPS proteins must be important in the regulation of a common fundamental process that functionally connects development, ageing, and immune responses. Such as, for instance, programmed cell death.

Plant- and animal-parasitic nematodes secrete VAPs during their transition from free-living or dormant juveniles to infective juveniles. In this thesis, we show that the potato cyst nematode *Globodera rostochiensis* secretes venom allergen-like proteins along with plant cell wall degrading enzymes during the onset of parasitism (**Chapter 3**), and that the expression of these proteins is associated with host invasion and migration inside host plants (**Chapter 4**). In hookworms, VAPs are amongst the most abundantly secreted proteins by infective juveniles (*1, 18*). From the 105 proteins identified in excretory/secretory products of the blood-feeding stage of *Ancylostoma caninum* 28% are VAPs. Furthermore, during the transition of this hookworm to parasitic L3 about 60% of the most up-regulated mRNAs encode VAPs (*19*). Similar observations have been done on L3 stages of the human hookworm *Necator americanus* (*20, 21*). Venom allergen-like proteins are also the dominant secretory proteins in infective juveniles of *Heligmosomoides polygyrus*, a parasitic nematode of mice (*22*). The picture that arises from all these observations is that secretion and/or expression of secreted venom allergen-like proteins by parasitic helminths is strongly linked to active invasion of host tissues (*18, 19, 23*).

If VAPs are indeed specifically required for active migration through host tissues, it is expected that they have evolved differently in migratory plant-parasitic nematodes than in sedentary plant-parasitic nematodes. To test this hypothesis, we queried the genomes of both the sedentary root-knot nematode *Meloidogyne incognita* (*24*) and the migratory nematode *Bursaphelencus xylophilus* (*25*) for SCP/TAPS signature sequences (i.e. Interpro accessions IPR14044, IPR001283, and PF00188). About half of the predicted

proteins with SCP/VAPs signatures had a predicted N-terminal signal peptide for secretion. To further investigate the evolutionary history of VAPs within the *B. xylophilus* and *M. incognita*, sequence alignment of the predicted secreted SCP/TAPS domains were analyzed with maximum likelihood methods (i.e. PhyML). In the analysis representative metazoan (*Homo sapiens* GliPR, *Conus textile* TEX31, *C. elegans* SCL22 and SCL10, *N. americanus* ASP2, and *B. malayi* VAL1) and plant (*N. tabacum* PR1a, PR1b, and PR1c, *S. lycopersicum* P14, *S. tuberosum* PR1b, and *Oryza sativa* PR1a and Pr1b) SCP/TAPS proteins were also included. The derived phylogeny shows that the VAPs from *B. xylophilus* (Fig. 1) are more distantly related to each other than to some of the VAPs from *M. incognita*. While most of the VAPs from all plant parasitic nematodes analyzed converged in one major clade, there seems to be two other major lineages for the VAPs present in *B. xylophilus*. Furthermore, some of the VAPs from *B. xylophilus* (i.e. BUX_s00658.8, and BUX_s00713.380) are more closely related to plant SCP/TAPs proteins than to any nematode VAP identified to date, indicating that the VAPs have undergone an evolutionary expansion in the genome of *B. xylophilus*. All together our data supports the hypothesis that VAPs diverged as an adaptation for active migration of plant parasitic nematodes through the host.

6.3 All for one and one for all

It has been shown that a limited set of highly connected "cellular hubs" is targeted by different pathogen effectors (*26*) and that pathogens converge onto similar processes, such as the control of cell death and signaling, to cause disease (*27*). Host proteins targeted by multiple pathogen effectors could make ideal guardees for plant immune receptors. The cysteine protease Rcr3[pim], which is guarded by the extracellular receptor-like protein Cf-2 in tomato plants, is targeted by the effectors Avr2 from the fungal pathogen *Cladosporium fulvum* (*28-30*), EPIC1/2B from the oomycete plant-pathogen *Phytophthora infestans* (*31*), and Gr-VAP1 from the parasitic nematode *G. rostochiensis* (*32*). Therefore, Rcr3[pim] seems to function as a "cellular hub" targeted by multiple unrelated pathogens.

From an evolutionary perspective, if different pathogens converge on a limited set of cellular hubs, these host proteins might be under the selective pressure exerted by multiple effectors. Recent efforts to understand the selection forces shaping the evolution of the *Rcr3* locus in Solanaceae, suggest that the different *Rcr3* alleles in the wild tomato species *Solanum peruvianum* are maintained within the gene pool (*33*). The mutations that occur in *Rcr3* cause frequent amino acid substitutions, driven by the need of improving pathogen recognition while preventing the auto-activation of immune responses by extracellular plant immune receptors. Therefore,
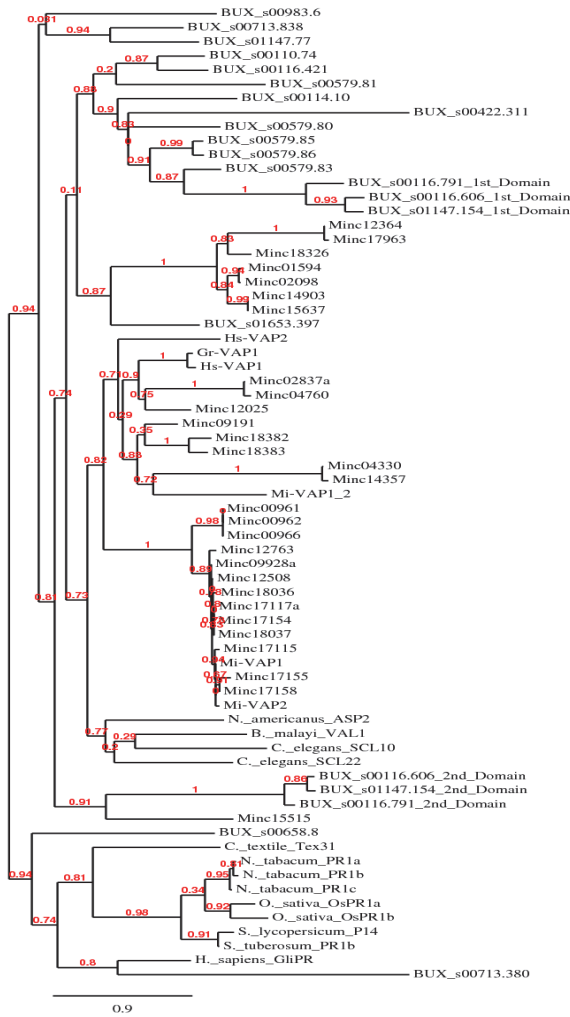
Fig. 1. Phylogenetic analysis of *Bursaphelencus xylophilus* (BUX)*, Meloidogyne incognita* (Minc or Mi), and other representative eukaryotic SCP/TAPS proteins. The phylogenetic tree was inferred from a COBALT alignment using PhyML and rendered by TreeDyn. The maximum allowed fraction of mismatched bases in the aligned region between any pair of sequences is 0.85. The non-metazoan protein sequences include plant SCP/TAPS proteins from *Nicotiana tabacum*, *Oryza sativa*, and *Solanum lycopersicum*. Metazoan SCP/TAPS protein sequences include proteins from the plant parasitic nematodes *Globodera rostochiensis*, and *Heterodera schachtii*, the human-parasitic nematodes *Brugia malayi*, and *Necator americanus* and the free-living nematode *Caenorhabditis elegans.*

persistent mutations in the gene pool are thought to be the result of 1) coevolution between allelic variants of pathogen effectors targeting Rcr3 and Rcr3 variants or 2) coevolution between Rcr3 and other interacting host molecules. The majority of Rcr3 alleles inhibited by the effector Avr2 from *C. fulvum* also activate a defense response in tomato plants carrying the *Cf-2* resistance gene. However, one amino acid substitution in Rcr3 (N195D) uncouples the inhibition by Avr2 and Cf-2-mediated defense responses (*29, 33*). Likewise, we showed that the Rcr3^{pim} from *S. pimpinellifolium* is inhibited by Gr-VAP1, while the Rcr3^{lyc} allele of *S. lycopersicum*, which differs at seven amino acid sites when compared to Rcr3^{pim}, does not interact with Gr-VAP1 (**Chapter 3**). Thus, indeed pathogen effectors could drive the evolution of Rcr3. Alternatively, Cf-2 or an unknown host protein interacting with Rcr3 may also drive its evolution.

As cellular hubs can be targeted by multiple unrelated effectors from entirely different pathogens, monitoring the 'health' status of these molecules with immune receptors appears to be an efficient defense strategy for plants to deal with multiple pathogens with a limited set of plant immune receptors. The strategy of monitoring host molecules to perceive non-self has been first captured by animal scientists in a model called the "Danger-signal model" (*34*) and later on by plant scientist in the "Guard hypothesis" (*35, 36*). In the so-called "danger-signal" pathway of *Drosophila melanogaster*, fungi and gram-positive bacteria release proteases that can be detected by the serine protease Persephone (*37-39*). Persephone activation leads to a proteolytic cascade that ultimately cleaves off the cytokine Spätzle, releasing a ligand that activates the Toll receptor-mediated responses (*40*). In plants, perturbations of Rcr3^{pim} induced by Gr-VAP1 of *G. rostochiensis* (Chapter 3, (*32*)) and by Avr2 of *C. fulvum* (*28*), but not by EPIC1/2B of *P. infestans*, trigger the receptor-like protein Cf-2 to activate programmed cell death and disease resistance (*41*). It is not clear why Cf-2 activation occurs only upon recognition of some, but not all, effectors targeting Rcr3^{pim}. It would be interesting to know if Cf-2-mediated disease resistance involves a physical association between the immune receptor and effector-Rcr3^{pim} complexes or if there is a third component which is cleaved by Rcr3^{pim} in the presence of specific effectors to activate Cf-2 signaling. The dual-specificities of Toll in insects (*37, 39, 42*) and of Cf-2 in plants (*28, 32*) suggests a conservation of the activation of extracellular innate immune receptors in different eukaryotic organisms. Plants and insects lack the seemingly unlimited receptor diversity of a somatic adaptive immune system, as found in vertebrates, and rely on only a relatively small set of innate immune receptors to resist a myriad of pathogens. However, by "guarding" critical proteases plants and insects use an efficient mechanism to leverage the limited nonself recognition capacity of their innate immune system in order to limit pathogen attack.

6.4 Tango down

All complex systems follow some simple rules. In the case of pathogens, one rule seems to be the need to regulate host immune responses for survival. In this thesis, we show that VAPs are required for nematode virulence and that they alter basal plant immunity (**Chapter 4**). Silencing of *Gr-VAP1* resulted in a loss of nematode virulence, while overexpression of *VAP*s in potato and Arabidopsis, altered their susceptibility to multiple unrelated pathogens. Several nematode effectors have been shown to alter host defense responses in plants. When overexpressed in plants, the effector *Mi-CRT* from *Meloidogyne incognita*, which is delivered by nematodes into the apoplast of host cells, enhances *Arabidopsis* susceptibility to oomycete pathogens and alters the response to the pathogen-associated molecular pattern elf18 (*43*). The effector 10A06 of *Heterodera schachtii* enhances *Arabidopsis* susceptibility to nematodes, bacteria and viruses (*44*). The effectors 4F01 and 30C02 also of *H. schachtii* enhance *Arabidopsis* susceptibility to nematodes (*45, 46*). SPRYSEC19, an effector of *G. rostochiensis*, enhances potato susceptibility to nematodes, fungi and viruses (*47*). Similarly, the ectopic expression of the effector GrUBCEP12, also of *G. rostochiensis*, enhances potato susceptibility to nematodes. Once processed into the small peptide GrCEP12 it suppresses cell death mediated by cytoplasmic resistance proteins (*48*). It is thus reasonable to assume that suppression of immunity by parasitic nematodes is a requirement for the establishment of permanent nematode-induced feeding structures. This assumption derives from the fact that root-knot and cyst nematodes deliver multiple effectors both in the apoplast and cytoplasm of host cells to regulate host immune responses.

In **Chapter 4**, we showed that nematode VAPs suppress the activation of defense responses mediated by the membrane-bound receptor-like kinase (RLK) FLS2 and by the receptor-like proteins (RLP) Cf-4 and Cf-9. The mechanisms by which plant pathogens suppress extracellular immune receptors are best studied in bacteria-plant interactions, where at least four different effectors from *Pseudomonas syringae* and *Xanthomonas campestris* target RLK complexes after their delivery into host cells (*27*). For example, the bacterial effector AvrPto from *P. syringae* targets directly the receptor-like kinases FLS2 and EFR in Arabidopsis to block PTI signaling, probably by inhibiting the kinase activity (*49-51*). Other *P. syringae* effectors (e.g. HopAI1 and HopF2), target proteins involved in MAPK cascades, which are downstream of RLKs, in order to inhibit PTI signaling (*52-54*). In contrast to these bacterial effectors, nematode VAPs regulate the extracellular proteome of host cells. By doing so, VAPs most likely suppress defense responses mediated by extracellular plant immune receptors. The exact mechanisms of activation/deactivation of extracellular plant immune receptors via other extracellular host proteins remain obscure and require further research.

Plant and animal parasitic nematodes target host papain-like cysteine proteases during infection. In **Chapter 3 and 4** we demonstrated that Gr-VAP1 interacts with the tomato cysteine protease Rcr3^{pim} and the potato cysteine protease C14^{tub}. Furthermore, in **Chapter 4** we showed that one of the most up-regulated genes in the plants overexpressing Hs-VAP1 encodes for an extracellular papain-like cysteine protease (AT2G27420), while the most down-regulated gene in VAPs overexpressing Arabidopsis plants encodes a plant cell wall associated serine protease (AT1G67626). Although little is known of other plant parasitic nematode effectors targeting host proteases, several studies have demonstrated that cystatins secreted by animal-parasitic nematodes are capable of modulating host innate immunity (*55*, *56*). For example, recombinant cystatin from the murine nematode parasite *Heligmosomoides polygyrus* drives dendritic cell differentiation, phenotype, and function (*57*). The cystatin homolog Bm-CPI-2 from the human parasite *Brugia malayi* and Nippocystatin from the rodent parasite *Nippostrongylus brasiliensis* interfere with antigen presentation and processing (*58*, *59*), while Onchocystatin from the human parasite *Onchocerca volvulus* inhibits T-cell proliferation by stimulating the production of the anti-inflammatory cytokine Interleukin-10 (*60*). The immunomodulatory properties of parasitic-derived protease inhibitors are evident i*n vivo* models, where the cystatin Av17 from the rodent filarial nematode *Acanthocheilonema viteae* reduces allergic lung inflammation and colitis (*61*). Although the exact mechanisms of how pathogens benefit from the modulation of host proteases and how this has an effect on host immunity are largely unknown, in the following section we propose a working model in this direction.

When trying to understand the immunomodulatory roles of VAPs in the apoplast of host plants, we studied in great detail which genes were up- or down-regulated in Arabidopsis plants overexpressing nematode VAPs (**Chapter 4**). Zooming into these genes, we found that the most down-regulated gene is a *subtilase* (AT4G21630), which is involved in proteolysis. Recently it has also been shown that this subtilase acts as a molecular switch in the priming of Arabidopsis defense responses (*62*). So VAPs apparently might contribute to reduce the priming of host defenses. Furthermore the top 10 most down-regulated genes also include *cell wall-degrading enzymes* (AT5G49180) and proteins typically involved in defense responses, i.e. *Pathogenesis related* (*PR*) *proteins* (AT5G20330 and AT5G20390) and an extracellular *receptor-like kinase protein* (AT1G10620). Strikingly, two of these top 10 most down-regulated genes are jasmonic acid (JA)-regulated genes, i.e. the *terpenoid cyclase* (AT3G14520) (*63*) and the *maternal effect embryo arrest 26* (AT2G34870) (*64*). Among the top 10 most up-regulated genes by VAPs are two transcripts encoded by the *NPQ4* gene (AT1G44575). This gene is involved in non-photochemical quenching capacity (*65*). Meaning, *NPQ4* dissipates the excess of excitation energy in the photosystem and in the

absence of this protein there would be an increase in ROS production. Recently it has also been shown that the protein encoded by *NPQ4* (i.e. PsbS) also modulates innate immune responses to flg22, whereby overexpression of *NPQ4* results in suppression of cell death, most likely by a reduced ROS production (*66*). Another interesting observation is a strong trade-off between JA biosynthesis and the expression of *NPQ4* (*67*). The expression of the *iron-regulated transporter 1* (AT4G19690) is also suppressed by JA (*68*) and up-regulated by VAPs. Altogether, we conclude that VAPs from plant parasitic nematodes affect JA-regulated, plant wall-associated and protease-dependent immunity mediated by extracellular immune receptors.

6.5 Lights, Camera, Action...Cut!

Papain-like cysteine proteases (PLCPs) play diverse roles in signaling, development and immunity in plants and animals. These roles depend not only on the conformation of the protease itself but also on the proteolytic processing of the substrate targets of these enzymes. The substrates of plant PLCPs are largely unknown and therefore their mechanisms of action remain poorly understood. In **Chapter 3**, we showed that Gr-VAP1 interacts with the PLCP Rcr3pim but not with Rcr3lyc. Although these two allelic variants differ only at seven amino acid positions, the presence of one or the other in the same tomato background results in a remarkable difference in susceptibility to nematodes. Tomato plants carrying the *S. pimpinellifolium* allele (*Rcr3pim*) were almost twice as susceptible to infections by *G. rostochiensis* than plants harboring either the corresponding *S. lycopersicum* allele (*Rcr3lyc*) or no functional *Rcr3* (*rcr3-3*). In **Chapter 5**, we investigated the role of Rcr3pim in cyst nematode infections and found that 28 tomato genes uniquely associate with *Rcr3pim* and are therefore accountable for a major increase in the susceptibility of tomato plants to *G. rostochiensis*. From these 28 genes, 17 have ascribed functions. The majority of these functions are linked to plant protease-based and jasmonic acid-dependent immune signaling pathways (Fig. 2).

Further analysis of the genes differentially expressed in nematode-infected tomato roots harboring *Rcr3pim* suggests that this PLCP might act as an apoplastic regulator in JA-dependent signaling. More specifically, 11 of the 17 Rcr3pim-regulated genes in tomato plants, or their close homologs in other plant species, have been linked to JA signaling and/or plant responses to wounding in different pathosystems. For instance, the expression of *defensin-like proteins*, *tomato proteinase inhibitors II*, *oxoglutarate- and iron-dependent oxygenases*, *speckle-type poxvirus and zinc finger* (*POZ*) *domain proteins*, *gamma thionins, chlorophyll A- and B-binding* proteins, and the small unit of *Rubisco* are all regulated by wounding and/or by the exogenous application of the JA derivative methyl jasmonate (MeJA) (*69-78*).
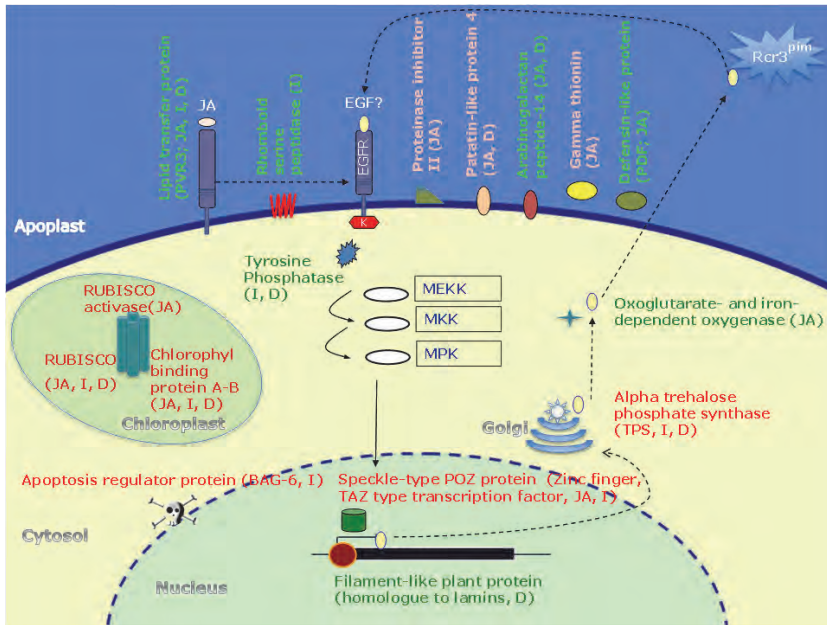
Fig. 2. A model of Rcr3pim-regulated JA-dependent host defenses in nematode-infected tomato roots. Rcr3pim modulates innate immune responses involving mechanical wounding, plant cell wall alterations, and jasmonic acid (JA)-induced host defenses in nematode-infected tomato roots. Genes are shown as either down- (red) or up-regulated (green). Parentheses indicate whether these genes or homologs in plants and/or animals are linked to JA signaling (JA), immune signaling cascades (I), and immune defenses (D). A short description of the model can be found in the text.

Similarly, wounding and MeJA application repress the expression of an *arabinogalactan peptide AGP31* in Arabidopsis, which is a close homolog of the down-regulated *arabinogalactan peptide-14* in nematode-infected *Rcr3pim* tomato plants (*79*). Furthermore, the phospholipase A activity of patatin-like proteins, one of which is down-regulated by Rcr3pim, might contribute to the production of JA in *Arabidopsis* plants by cleavage of fatty acids precursors from lipid membranes (*80*). It has also been shown that the exogenous application of lipid transfer protein and jasmonic acid complexes enhances resistance to *Phytophthora parasitica* in tobacco (*81*) and to *Botrytis cinerea* in grapevines (*82*). Lastly, Arabidopsis *Rubisco activases* are down-regulated both in transcript and protein abundance by JA and have been identified as molecular keys in the induction of leaf senescence by JA (*83, 84*). All together, these data support the hypothesis that Rcr3pim is an apoplastic regulator of JA-dependent signaling.

Besides regulating JA-dependent signaling, Rcr3pim also seems to regulate host immune signaling cascades. Homologues of six Rcr3pim-

regulated genes in nematode-infected tomato roots have been directly linked with immune signaling. The Arabidopsis homolog of the *lipid transfer protein PVR3* (i.e. DIR1) has been shown to mediate systemic resistance signaling and strongly bind to phospholipids (*85, 86*). Furthermore, it has been hypothesized that the DIR1 signals through an uncharacterized lipid transport mechanism involving the interaction with a putative receptor protein (*86*). Since very little is known about how DIR1 signals in plant immunity, we took a look at how lipid transfer proteins signal in animal systems. In animals, lipid transfer proteins mediate the recognition of, among others, bacterial cell wall lipopolysaccharides (LPS) (*87, 88*). Only upon incorporation of LPS by the lipid transfer protein (LBP), cluster of differentiation 14 (CD14) binds LPS and dimerize with the toll-like receptor 4 (TLR4) and the immune response receptor MD-2 to activate a protein kinase cascade (i.e. MEKK, MKK, and MPK) (*87, 88*). Interestingly, a mouse tyrosine phosphatase (*MKP-1*) is induced in macrophages upon stimulation with LPS and it is able to inactivate c-Jun N-terminal kinases (JNK), presumably preventing an excess in inflammation responses (*89, 90*). Although less is understood in plants, ectopic overexpression of *tyrosine phosphatases* increases host susceptibility to *Magnaporthe griseae* in rice and to *Pseudomonas syringae* pv. *tomato* DC3000 in Arabidopsis. Altogether, these data suggest that apoplastic Rrc3[pim] might affect the immunocompetence of tomato cells, by manipulating key immune signaling components.

Most likely further downstream in the immune signaling pathways in tomato plants, Rcr3[pim] down-regulates the expression of a homologue of the *apoptosis regulator protein BCL-2-associated anthanogene 6* of Arabidopsis (*AtBAG6*). In contrast, a homolog of *AtBAG6* is the most up-regulated gene in association with resistance to soybean cyst nematodes in soybean cultivars harboring the *Rhg1* resistance locus (*91*). The apoptosis regulator protein BCL-2-associated anthanogene 6 can modulate Ca^{2+}-signaling by binding to the Ca^{2+}-receptor protein calmodulin (*92, 93*). Additionally, the expression of *AtBAG6* in yeast and in plants induces programmed cell death phenotypes (*92*), while Arabidopsis knockout mutants show enhanced susceptibility to the fungal pathogen *Botrytis cinerea* (*94*). Whether or not *AtBAG6* is being modulated by cyst nematodes in order to avoid the induction of host programmed cell death remains to be investigated. Since cell death is better understood in animals, we checked the role of BAG proteins in relation to apoptosis and immune signaling. For example, the murine BAG-6 plays a crucial role in apoptosis and cell proliferation (*95*). BAG-6 deficient mice show developmental defects in lungs, kidneys and brains. These defects associate with the alteration of apoptosis and cellular proliferation (*95*). Besides, the BCL-2-associated anthanogene, *BAG3* expression in humans is induced by LPS and blocked by JNK or nuclear factor kappa-B (NF-κB) inhibition (*96*), meaning that BAG3 acts downstream of toll-like receptor 4 signaling. These

data shows that there seems to be a link between the apoptosis regulator BAG proteins and toll-like receptor perception in animals. Altogether, we conclude that in nematode-infected Rcr3[pim] tomato plants, the ability to undergo an apoptosis like defense-related programmed cell death is repressed.

Rcr3[pim] also down-regulates a *putative transcription factor* that might be linked to immunity in plants through its speckle-type poxvirus and zinc finger (POZ) domain. There are 80 BTB/POZ proteins identified in the genome of Arabidopsis with diverse functions in stress response and development (*77, 97*). From all of these BTB/POZ proteins, the best studied is the Nonexpresser of *PR* genes 1 (NPR-1). NPR-1 is a master regulator of a type of broad-spectrum systemic acquired resistance to biotrophic pathogens in plants. Furthermore, NPR-1 plays a central role as a modulator of the cross talk between salicylic acid- (SA-) and JA-mediated defense responses (*7, 98-100*). Therefore the ability to induce systemic acquired resistance, in Rcr3[pim] tomato plants, could also be compromised by cyst nematodes.

Last but not least, Rcr3[pim] also regulates r*homboid* gene expression. Although in plants rhomboids have not been linked to immune signaling, looking at the animal literature a very different picture arises. In *Drosophila*, for example, rhomboid-1 is a key activator of the epidermal growth factor receptor (EGF) pathway (*101, 102*). This pathway controls several developmental and innate immune responses in animals (*103-108*). In mice, the rhomboid protease Rhbdd3 negatively regulates TLR3-triggered natural killer cell activation (*109*), while knockout mutants in the rhomboid protease *iRhom2* have a reduced TNFα response to LPS and showed increased susceptibility to the bacterial pathogen *Listeria monocytogenes* (*110*). Altogether, we conclude that Rcr3[pim] regulate the activation of immune receptors by proteolytic cascades that are still not well understood in plant signaling pathways.

Rcr3[pim] also seems to regulate some genes for which others have shown to be modulated during plant-biotic interactions and immune defense. A total of seven Rcr3[pim]-regulated homolog genes seem to be regulated by different organisms. Mutants of a close homolog of the *Alpha trehalose phosphatase* (*ATP*) *synthase γ subunit* in Arabidopsis show high non-photochemical quenching (*111*). Non-photochemical quenching is required for mounting a proper immune response to the immunogenic peptide flg22 by the plant Toll-like receptor FLS2 (*66*). A homolog of the *arabinogalactan peptide-14*, the Arabidopsis *AtAGP17* mutant is resistant to *Agrobacterium tumefaciens* root transformation (*112*). In *Medicago truncatula* a root-specific *lipid transfer protein* is required by the symbiont *Sinorhizobium meliloti* and up-regulated by the pathogen *Fusarium semitectum* during infection (*113*). In plants, Rubisco consists of a large and a small protein subunit. Silencing of the *Rubisco* small subunit of *Nicotiana benthamiana* (*NbRbCS*) enhances local virulence of the *Tomato mosaic tobamovirus* (ToMV) in susceptible plants and compromised

resistance in ToMV-resistant plants (*114*). Both fungi and viruses have been shown to down-regulate *chlorophyll a-b binding proteins* during infection (*115, 116*). *Trehalose 6-phosphate* is involved in plant growth, development, defense, and senescence (*117, 118*). In Arabidopsis plants, when the alpha trehalose-phosphate synthase gene *TPS11*, which synthesizes trehalose 6-phosphate, is knocked out, the plants show an attenuated defense against green peach aphid infestations (*119*). Although little is known about the role of filament-like plant proteins in plant-biotic interactions, the closest animal homologs of the filament-like proteins are involved in several diseases associated with defects in the nuclear envelope and play key roles in nuclear organization and function (*120-122*). In summary, Rcr3^{pim} modulates several genes, which also appear to be regulated by diverse microorganisms during plant-biotic interactions.

VAPs from plant-parasitic nematodes affect jasmonic acid-regulated, plant wall-associated, and protease-dependent immunity mediated by extracellular immune receptors. While the host protease Rcr3^{pim} regulates innate immune responses involving JA-induced defenses. Coupling these two conclusions together, we can state that nematode VAPs, through interaction with host proteases in the apoplast, affect the host immune responses that involve plant cell wall alterations and JA-induced defenses mediated by extracellular receptors. In nematode-infected tomato roots, VAPs are released together with cell-wall degrading enzymes during nematode migration. Plant cell wall damage, associated with cyst nematode infections, most likely releases JA from membrane phospholipids. Seemingly, VAPs interfere with the modulation of JA responses mediated by plant cysteine proteases. At the same time diverse nematode effectors could also modulate JA biosynthesis, e.g. the *Globodera pallida* retinol- and fatty-acid-binding protein (Gp-FAR-1) binds linolenic and linoleic acids, which are precursors of the JA signaling pathway (*123*). The transport of phospholipids and fatty acids from the apoplast to the cytoplasm and *vice versa* can be influenced by lipid transfer proteins (LTPs). LTPs might interact with receptor-like proteins e.g. the epidermal growth factor receptor (EGFR) to initiate a protein kinase cascade (i.e. MEKK, MKK, and MPK). The signal transduction in this cascade is suppressed by elevated level of tyrosine phosphatases (Tyr_Phos). Downstream of this cascade, several immune-related genes are also being regulated in Rcr3^{pim} tomato roots by cyst-nematodes. These genes include the BCL-2-associated anthanogene 6 (BAG6) and the speckle-type POZ protein (SPOZ) that can modulate the transcriptional repression immune responses in plants. Furthermore, in the chloroplast, components of the photosystems I and II (i.e. Rubisco, rubisco activase and Chlorophyll A-B) are antagonized by JA-dependent host defense responses, which suggests a trade-off between photosynthetic capacity and JA host defenses.

Our analysis further identified genes of unknown function as being up- or down-regulated by Rcr3^{pim}, some of which are predicted to encode small secretory proteins. We hypothesize that one of these secretory proteins may be further processed by the Rcr3^{pim}-mediated proteolytic cleavage, the product of which might be recognized by membrane-bound extracellular receptor-like kinase. Alternatively Rcr3^{pim} may directly cleave a toll-like receptor kinase to exert its function. Although not well defined in plants, the rhomboid-like protein through its serine-type endopeptidase activity may be involved in intramembrane proteolytic regulation of membrane-bound proteins, e.g. lipid transfer proteins and receptor-like proteins. The up-regulation of the *rhomboid-like protein 1* by Rcr3^{pim} coincides with the down-regulation of two membrane-bound serine protease inhibitors (i.e. *PIN2*), which may directly regulate the serine-proteolytic activity of rhomboid.

Besides the mechanistic pattern of genes regulated by the presence of Rcr3^{pim} or VAPs, which is related to lipid or JA signaling, another common denominator of the pathways regulated by Rcr3 and VAPs is host proteolysis. As mentioned before, the expression of VAPs in Arabidopsis plants results in the regulation of, among others, a *papain-like cysteine protease* (AT2G27420) and a plant cell wall associated *serine protease* (AT1G67626). Furthermore, the presence of either allelic variant of the Rcr3 protein in the apoplast of tomato plants strongly increased the expression of the extracellular *Cathepsin B-like cysteine protease* (Gene Solyc02g076980.2), while it down-regulated the expression of several *cysteine* and *serine protease inhibitors*. This finding suggests that Gr-VAP1 and its protease host targets, like Rcr3, may operate in extracellular signaling pathways that involve multiple apoplastic proteases. Our investigations of homozygous mutant *Arabidopsis* lines showed that, besides the closest Rcr3 homologs (i.e. *PAP1*, *PAP4*, and *PAP5)*, several other apoplastic PLCPs affect the susceptibility of Arabidopsis to infections by *H. schachtii*. Based on the analysis of these mutants alone it is not possible to conclude whether multiple PLCPs and other type of proteases act in proteolytic cascades to activate immune responses, or whether each protease cleaves different substrates to activate host defense. It is worth mentioning once again that Rcr3^{pim} alone seems to modulate the expression of r*homboid* in nematode infected tomato plants. rhomboid proteins are intramembrane serine proteases that control diverse biological processes by cutting the transmembrane domain of membrane-anchored proteins (*124-126*). There are 22 *rhomboid-like* genes predicted in the genome of Arabidopsis, from which only 13 sequences encode active proteases (*127, 128*). The roles of plant rhomboids remain largely elusive and experimental evidence on the majority of them is lacking. However the Arabidopsis rhomboid protein AtRBL2 has been shown to cleave the *Drosophila* ligands Spitz and Keren. These ligands activate epidermal growth factor receptors, demonstrating that plant rhomboids are able to regulate intramembrane proteolysis to release proteins

from cell membranes (*129*). Therefore, we propose that the rhomboid-like protein regulated by cyst nematodes in Rcr3^pim tomato plants, may directly or indirectly regulate the activity of e.g. lipid transfer proteins and extracellular receptors proteins. Directly, rhomboid-like proteins could cleave membrane-bound receptors, while indirectly rhomboids-like could cleave membrane-bound ligands which activate extracellular receptors. The contribution of rhomboids and other host proteases in plant and animal defense against parasitic nematodes remains largely unexplored. Finding the substrates of defense-related host proteases in plants will be fundamental in our understanding on how proteases regulate host immunity.

Our Arabidopsis mutant screening further showed that PCLPs not only localized in the apoplast, but also those predicted to be either in the vacuole or in the endoplasmic reticulum, affect the susceptibility to infections by *H. schachtii*. Some of these PLCPs have been linked to defense responses to plant pathogens before (i.e. AT1G47128 (*130*), AT4G39090 (*131*), and AT1G02300, AT1G02305, and AT4G01610 (*132*)), but the function of most PLCPs in Arabidopsis is unknown. It is likely that besides their involvement in host defense and cell death, PLCPs also regulate developmental processes in plants. Both processes may influence host susceptibility to infections by cyst nematodes. For instance, mutations in *CEP2* (AT3G48350) result in a major loss of susceptibility of Arabidopsis to *H. schachtii*. *CEP2* promoter activity is especially high in very young root tips and in the root elongation zone of Arabidopsis (*133*), which is the preferential site of invasion of plants by cyst nematodes. Endoplasmic CEPs can break down hydroproline-rich proteins such as plant cell wall-derived extensins that serve as scaffolds for deposition of carbohydrate polymers in plant cell wall formation (*133*). Local plant cell wall breakdown and synthesis is a crucial process during the transformation of host cells into feeding cells by cyst nematodes in host plants (*134-138*). It is therefore conceivable that Arabidopsis CEP knockout mutants are less susceptible to *H. schachtii*, because cyst nematodes require this plant PLCP to remodel host cell walls during feeding cell formation.

When I started this thesis I wanted to contribute to science by better understanding the function of one of the most abundant, but least understood proteins secreted by parasitic nematodes, the venom allergen-like proteins "VAPs". After all these years of work and although there is still a long way to go before we can fully understand what the VAPs and the VAPs-like are doing, I can finalize this thesis by stating that: "Plant-parasitic nematode VAPs, as well as some of the VAP-targeted host proteases, function to modulate innate immune responses involving mechanical wounding, plant cell wall alterations, and jasmonic acid-induced defenses in host roots".

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Summary

Parasitic nematodes are highly specialized microscopic worms that threaten human, animal and plant health in essentially all parts of the world. A shared characteristic of plant- and animal-parasitic nematodes is their phenomenal capacity to persist inside a host, presumably resulting from their ability to suppress host immunity. Nevertheless, the mechanisms of immunomodulation by parasitic nematodes in plants and animals are not well understood. In recent years, research on effectors secreted by nematodes into the host during the infection process has led to the discovery of a diverse array of molecules that might affect the immunocompetence of host cells. Many of these effectors are currently being investigated for the development of vaccines, the treatment of immune disorders in humans, and for the improvement of disease resistance breeding programs for food crops.

Among the most abundantly secreted effectors by parasitic nematodes of both animals and plants are the so-called venom allergen-like proteins (VAPs). The expression of VAPs in both animal- and plant-parasitic nematodes is strongly up-regulated during the onset of parasitism. Therefore, these conserved proteins are thought to be important for the virulence of the nematodes inside a host. However, the actual role and the host targets of VAPs remain largely obscure. Earlier work on VAPs of animal-parasitic nematodes demonstrated that they might function as modulators of immunity in animals, but conclusive evidence to substantiate this hypothesis is still lacking. The aim of this thesis is the functional characterization of VAPs of parasitic nematodes, with a special emphasis on their role in nematode-plant interactions.

To introduce the reader into the context of the topic of this thesis, chapters 1 and 2 first offer short descriptions on cyst nematodes and their effectors, VAPs, the plant innate immune system, direct *vs.* indirect recognition of pathogen effectors by plant immune receptors, and papain-like cysteine proteases in plants. Next, we review in greater detail the morphological, molecular and physiological adaptations that plant-parasitic nematodes have evolved to deal with the host defenses that are regulated by the plant immune system. At the start of this thesis, not much was known of the molecular mechanisms underlying the activation and suppression of plant innate immunity by plant-parasitic nematodes. But, in the past five years this research area has changed from an underexplored territory to the main focus of the research community. In the approaches of their research on immunomodulatory effectors of parasitic nematodes, there is a marked difference between animal and plant nematologists. While the former group focuses its attention primarily on the use of effectors for vaccine and/or drug development, the latter tries to understand effector functions with the hope of applying this fundamental knowledge to improve natural nematode

resistance in crops. Since the main focus of our research group is mainly on nematode-plant interactions in Solanaceous crops, the objective of this thesis was to better understand the function of a venom allergen-like protein (Gr-VAP1) from the potato cyst nematode *Globodera rostochiensis* in tomato and potato.

By first focusing on the identification of the molecular targets of VAPs in host plants, we show that Gr-VAP1 interacts with several immune-related tomato proteins (Chapter 3). We found that Gr-VAP1 specifically interacts with Rcr3[pim], a papain-like cysteine protease from the currant tomato *Solanum pimpinellifolium*. It was previously shown that Rcr3[pim] is required for mounting Cf-2-mediated resistance against the leaf mold fungus *Cladosporium fulvum*. In chapter 3, we demonstrate that the Cf-2 protein, originally identified as a monospecific extracellular immune receptor for the leaf mold fungus *C. fulvum* carrying the avirulence gene *Avr2*, also mediates disease resistance to the cyst nematode *G. rostochiensis* pathotype Ro1-Mierenbos. The Cf-2-mediated dual resistance is triggered by Avr2- and Gr-VAP1-induced perturbations of the Rcr3[pim]. Gr-VAP1 and Avr2 have no structural similarity, but the binding of both perturbs the active site of Rcr3[pim] in the apoplast of tomato cells. Furthermore, both nematode infection and transient expression of *Gr-VAP1* in tomato plants harboring both *Cf-2* and *Rcr3[pim]* trigger a defense-related programmed cell death in plant cells. Based on our findings, we concluded that monitoring host proteins targeted by multiple pathogens broadens the spectrum of disease resistances mediated by the limited innate immune receptor repertoire of plants.

Although Gr-VAP1 can trigger a Cf-2 mediated resistance response in tomato plants, its intrinsic function for parasitic nematodes in host plants is to promote their virulence. To study the virulence function of VAPs of plant parasitic nematodes, we performed a series of experiments of which the main findings are summarized here (Chapter 4). First, knocking-down the expression of VAPs in *G. rostochiensis* strongly reduced the virulence of the nematodes in tomato plants. Ectopic expression of different VAPs from cyst nematodes in the apoplast of Arabidopsis plants significantly reduced their basal defense responses to nematodes, multiple other plant pathogens, and the immunogenic peptide flg22 from bacterial flagella. Furthermore, transient expression of VAPs from different plant-parasitic nematodes selectively suppressed the defense-related programmed cell death activated by multiple extracellular plant immune receptors in leaves of *Nicotiana benthamiana*. These findings suggest that the VAPs target a conserved mechanism in the activation of host defense responses mediated by extracellular immune receptors in plants. The transcriptome of plants ectopically expressing VAPs further revealed profound alterations in the extracellular proteome with a strong bias toward innate immunity and plant cell wall associated processes. As the VAPs are secreted by plant-parasitic nematodes along with plant cell

wall degrading enzymes, we conclude that VAPs of plant-parasitic nematodes most likely modulate the protease-based activation and/or signaling by extracellular immune receptors to suppress defense responses triggered by plant cell wall derived damage-associated molecular patterns.

Plants and animals utilize cell-surface receptors to survey their direct environment for molecular patterns uniquely associated with infections by microbial invaders. When investigating the role of VAPs we found that Gr-VAP1 targets the papain-like cysteine protease Rcr3^{pim} that functions as a cofactor of the extracellular plant immune receptor Cf-2 in tomato (Chapter 3). However, tomato plants carrying Rcr3^{pim} in absence of Cf-2 were almost twice as susceptible to infections by *G. rostochiensis* than plants harboring either the allelic variant Rcr3^{lyc} of S. *lycopersicum* or no functional Rcr3 (*rcr3-3*). Since the presence of Rcr3^{pim} alone almost doubled the susceptibility of tomato plants to *G. rostochiensis*, we hypothesized that VAPs modulate the activity of Rcr3^{pim} and other papain-like cysteine proteases to enhance the virulence of parasitic nematodes (Chapter 5). Through a series of experiments we showed that close homologues of Rcr3 and many other papain-like cysteine proteases of *Arabidopsis thaliana* indeed have a profound effect on plant susceptibility to cyst nematode infections. To understand the underlying mechanism of the Rcr3^{pim}-dependent increase in susceptibility to *G. rostochiensis* in tomato, we analyzed the transcriptome of nematode-infected roots of tomato plants harboring *Rcr3^{pim}*, *Rcr3^{lyc}*, or the null mutant *rcr3-3* with RNA-seq. Both allelic Rcr3 variants in tomato had a major impact on the expression of extracellular proteins associated with immune signaling pathways and plant cell wall-related processes in nematode-infected roots. Remarkably, only 28 tomato genes were uniquely regulated in association with *Rcr3^{pim}*, and we therefore conclude that these genes are responsible for the enhanced susceptibility of the tomato plants harboring this allele. The majority of the genes expressed in association with Rcr3^{pim} are linked to jasmonic acid-dependent signaling and plant innate immunity. Mutant Arabidopsis plants lacking the homologues of the Rcr3^{pim}-regulated genes in tomato showed reduced susceptibility to infections by the cyst nematode *H. schachtii*, indicating that there is a conserved protease-activated pathway that needs to be regulated by cyst nematodes for successful parasitism. The integration of the biological processes commonly regulated by both Rcr3 variants and those uniquely associated with Rcr3^{pim} led us to conclude that the apoplastic Rcr3^{pim} modulates innate immune responses involving mechanical wounding, plant cell wall alterations, and jasmonic acid-induced host defenses.

In the final chapter of this thesis (Chapter 6), we integrate our findings on the virulence function of VAPs during host invasion in plants and the role of apoplastic proteases in regulating jasmonic acid-mediated defense signaling induced by wounding. First, we present evidence that supports the

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hypothesis that VAPs diverge as an adaptation for active migration of endoparasitic plant-parasitic nematodes through host tissues. To finalize this thesis, we elaborate a testable model in which VAPs of plant-parasitic nematodes target extracellular papain-like cysteine proteases, such as Rcr3$^{\text{pim}}$, of host plants to modulate innate immune responses involving mechanical wounding, plant cell wall alterations, and jasmonic acid-induced host defenses.

Samenvatting

Parasitaire nematoden zijn heel gespecialiseerde microscopisch kleine organismen die een gevaar vormen voor de gezondheid van mensen, dieren en planten over de gehele wereld. Een gemeenschappelijke eigenschap van zowel plant- en dier-parasitaire nematoden is de extreme persistentie van hun infecties in een gastheer, vermoedelijk door hun vermogen om het immuunsysteem van de gastheer te onderdrukken. De mechanismen die ten grondslag liggen aan de modulatie van het immuunsysteem van planten en dieren door parasitaire nematoden zijn nog onduidelijk. Recent onderzoek naar effectoren – speekseleiwitten die worden uitgescheiden door nematoden in de gastheer ter bevordering van het infectieproces - heeft geleid tot de ontdekking van een reeks eiwitten die de immuuncompetentie van de gastheer mogelijk aantasten. Veel van deze effectoren worden daarom momenteel nader onderzocht als uitgangspunt voor de ontwikkeling van vaccins, de behandeling van immuunstoornissen in de mens, en de verbetering van nematode resistenties in voedselgewassen.

Eén groep van de meest abundante effectoren van parasitaire nematoden in zowel plant als dier, zijn de zogenaamde *venom allergen-like proteins* (VAPs). Omdat de expressie van VAPs sterk toe neemt tijdens de beginfase van de parasitaire interactie met de gastheer, neemt men aan dat deze geconserveerde eiwitten belangrijk zijn voor het vermogen van nematoden om de gastheer succesvol te infecteren (virulentie). Echter, van de exacte rol en de doelwitten in de gastheer van de VAPs was weinig tot voor kort bekend. Uit eerder onderzoek naar VAPs van dierlijke parasitaire nematoden bleek dat ze mogelijk als modulatoren van innate immuniteit functioneren, maar overtuigend bewijs voor deze hypothese ontbreekt nog. Het doel van dit proefschrift is het toetsen van deze hypothese door de functionele karakterisering van VAPs van parasitaire nematoden, met een bijzondere focus op hun rol in nematode-plant interacties.

Om de lezer te introduceren in de context van het onderwerp van dit proefschrift, bieden hoofdstuk 1 en 2 eerst korte beschrijvingen van cystenaaltjes en hun effectoren, de VAPs, het innate immuunsysteem van planten, directe *vs.* indirecte herkenning van effectoren door immuunreceptoren van planten, en papaïne-achtige cysteïne proteases in planten. Vervolgens bespreken we in meer detail de morfologische, moleculaire en fysiologische aanpassingen van plant-parasitaire nematoden aan het immuunsysteem van hun gastheren.

Aan het begin van dit proefschrift was niet veel bekend over de moleculaire mechanismen die ten grondslag liggen aan de activering en onderdrukking van innate immuniteit door parasitaire nematoden in planten. Echter, in de afgelopen vijf jaar heeft dit onderzoeksgebied zich ontwikkeld van een onderbelicht terrein naar een van de belangrijkste aandachtsgebieden

binnen de nematologie. In de aanpak bij het bestuderen van immunomodulerende effectoren van nematoden is er een duidelijk verschil tussen de dier- en plantenwetenschappers. Terwijl de eerste groep de aandacht vooral richt op het gebruik van effectoren voor de ontwikkeling van vaccins en/of geneesmiddelen, probeert de laatste groep effectorfuncties te begrijpen met de hoop deze fundamentele kennis toe te passen om natuurlijke nematoderesistentie in gewassen te verbeteren. Omdat de focus van onze onderzoeksgroep vooral gericht is op nematode-plant interacties in Solanaceae, was het doel van dit proefschrift het beter begrijpen van de functie van een *venom allergen-like protein* (Gr-VAP1) van het aardappelcystenaaltje *Globodera rostochiensis* in het totstandbrengen van een parasitaire interactie met tomaat en aardappel.

Door ons eerst te richten op de identificatie van moleculaire doelwitten van VAPs in gastheerplanten, hebben we ontdekt dat Gr-VAP1 associeert met verschillende immuun-gerelateerde eiwitten uit tomaat (Hoofdstuk 3). Wij vonden dat Gr-VAP1 specifiek bindt aan Rcr3^{pim}, een papaïne-achtige cysteïne protease van de cherrytomaat *Solanum pimpinellifolium*. Eerder was al aangetoond dat Rcr3^{pim} nodig is voor Cf-2-afhankelijke resistentie tegen de bladschimmel *Cladosporium fulvum* in tomaat. In hoofdstuk 3 demonstreren we dat het Cf-2 eiwit, oorspronkelijk geïdentificeerd als een monospecifieke extracellulaire immuunreceptor voor *C. fulvum*, ook bescherming biedt tegen het aardappelcystenaaltje *G. rostochiensis* pathotype Ro1-Mierenbos. De Cf-2-gemedieerde meervoudige resistentie wordt veroorzaakt door Avr2- en Gr-VAP1-geïnduceerde verstoringen van Rcr3^{pim}, die door Cf-2 wordt bewaakt. Gr-VAP1 en Avr2 hebben geen structurele gelijkenis, maar de binding van beide verstoort de enzymatische activiteit van Rcr3^{pim}, waardoor het waarschijnlijk niet meer in staat is om het natuurlijke substraat in de apoplast van tomaatcellen te verwerken. Infecties met nematoden en de transiënte expressie van Gr-VAP1 in tomatenplanten met zowel Cf-2 als Rcr3^{pim}, induceren een lokale geprogrammeerde celdood in de plant. Op basis van onze bevindingen concluderen we dat het bewaken van andere eiwitten, die het gemeenschappelijke doelwit zijn van meerdere pathogenen, het spectrum verbreedt van resistenties op basis van het beperkte repertoire van innate immuunreceptoren in planten.

Om de virulentiefunctie van VAPs van parasitaire nematoden te bestuderen, hebben we een serie experimenten uitgevoerd waarvan de belangrijkste bevindingen hieronder zijn samengevat (Hoofdstuk 4). Ten eerste, het uitschakelen van de expressie van VAPs in *G. rostochiensis* reduceerde de virulentie van nematoden in tomatenplanten sterk. Heterologe expressie van verschillende VAPs van cystenaaltjes in de apoplast van Arabidopsis verminderde de basale afweer van deze planten tegen nematoden, verschillende andere ziekteverwekkers, en het immunogene peptide flg22. Bovendien onderdrukte de transiënte expressie van VAPs uit

verschillende plant-parasitaire nematoden selectief de lokale geprogrammeerde celdood, geactiveerd door meerdere extracellulaire immuunreceptoren in de bladeren van *Nicotiana benthamiana*. Deze bevindingen suggereren dat de VAPs een geconserveerd mechanisme in de activatie van de afweer van planten als doelwit hebben. De overexpressie van VAPs in transgene Arabidopsis planten veranderde vooral de samenstelling van het extracellulaire proteoom, en dan met name eiwitten die betrokken zijn bij de innate immuniteit en de celwand. Aangezien parasitaire nematoden de VAPs samen met plantencelwand-afbrekende enzymen uitscheiden, moduleren deze effectoren hoogstwaarschijnlijk de activatie van innate immuniteit als gevolg van celwand afbraak.

Planten en dieren maken gebruik van extracellulaire receptoren om hun directe omgeving te inspecteren op de aanwezigheid van moleculaire patronen die uniek geassocieerd zijn met infecties door microbiële indringers. Bij het onderzoek naar de functie van VAPs vonden we dat Gr-VAP1 de papaïne-achtige cysteïneprotease Rcr3^{pim} bindt. Rcr3^{pim} fungeert als cofactor van de extracellulaire immuunreceptor Cf-2 in tomaat (Hoofdstuk 3). Echter, tomatenplanten met Rcr3^{pim} maar zonder Cf-2 waren bijna tweemaal zo vatbaar voor *G. rostochiensis* dan planten met de allelische variant Rcr3^{lyc} van *S. lycopersicum* of zonder een functioneel Rcr3 eiwit (*rcr3-3*). Op basis van deze waarnemingen vooronderstellen we dat VAPs de activiteit van Rcr3^{pim} en andere papaïne-achtige cysteïneproteasen moduleren om de virulentie van nematoden te versterken (Hoofdstuk 5). Door een reeks experimenten hebben we aangetoond dat nauw verwante homologen van Rcr3 en andere papaïne-achtige cysteïne proteasen in *Arabidopsis* van invloed zijn op de vatbaarheid van planten voor infecties met cystenaaltjes. Om het onderliggende mechanisme van de Rcr3^{pim}-afhankelijke toename in vatbaarheid voor *G. rostochiensis* in tomaat te begrijpen, hebben we het transcriptoom geanalyseerd van nematode-geïnfecteerde wortels van tomatenplanten met Rcr3^{pim}, Rcr3^{lyc}, of de mutant *rcr3-3*. Beide allelische Rcr3-varianten in tomaat hebben een grote invloed op de expressie van andere extracellulaire eiwitten, die betrokken zijn bij innate immuniteit en celwand-gerelateerde processen. De expressie van slechts 28 genen is uniek geassocieerd met het Rcr3^{pim} allel en daarom concluderen we dat deze genen verantwoordelijk zijn voor de verhoogde vatbaarheid van tomaat voor *G. rostochiensis*. De meeste van deze 28 genen zijn al eerder met jasmonzuur-afhankelijke immuniteit in verband gebracht. Arabidopsis mutanten waarin een groot aantal van deze 28 genen zijn uitgeschakeld bleken verminderd vatbaar voor cystenaaltjes. Op basis van deze data concluderen wij dat Rcr3^{pim} fungeert als een extracellulaire regulator van afweerreacties, geïnduceerd door mechanische verwonding en celwandafbraak en wordt gereguleerd door jasmonzuur.

In het laatste hoofdstuk van dit proefschrift (Hoofdstuk 6) komen we tot een integratie van onze data. Als eerste maken we een analyse van de

hypothese dat de VAPs een aanpassing zijn voor actieve migratie van endoparasitaire plantparasitaire nematoden in weefsels van waardplanten. Vervolgens presenteren we een toetsbaar model waarin VAPs van plant-parasitaire nematoden extracellulaire papaïne-achtige cysteïne proteasen als doelwit gebruiken om de innate immuniteit van waardplanten te onderdrukken.

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The writing of this part of my thesis is one of nicest moments that I have had so far in my life. Soon I will reach one of my biggest dreams. I assume everyone who is reading this knows how nice it feels when you reach, or are about to reach a dream. I am siting in my office, which is on the third floor of the Radix building and I can see the beautiful campus of Wageningen University, it is truly a magnificent view. Looking out through the window the first building I see is the Forum building and then it occurs to me that I can compare the realization of this particular dream of mine to the construction of this architectonic piece. Many people have contributed in one way or another to construct it and to make it all happen. Therefore, I would like to thank each one of the persons that with their help, knowledge, support and friendship have made this dream come true.

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Besides rooms and the structural work of my dream, a lot of external experts helped me throughout the years to make sure that the building would be functional. I have to start here by giving credits to the people of the Laboratory of Phytopathology, who have shared with me knowledge, materials, advice and sometimes drinks and dancing. Pierre, the collaboration with you resulted in a very nice paper. Bart, Francine and Matthieu your doors were always open for sharing knowledge and all kinds of materials. Peter, John, Ronnie, Patrick, Harrold, Klaas, Emilie, and Thomas you all have contributed with your expertise and work when my students or me had to perform several of the experiments presented or not in this book. Peter, it is a pity you left Wageningen, I enjoyed a lot talking with you about science. Managing all the plant experiments without the help of the people from Unifarm would be impossible. Bert Essenstam, thank you so much for all the material you had ready for me whenever I requested it. Here I also acknowledge Jan Cordewener and Antoine America, from Plant Research International, for their contribution in our PNAS publication and their continuous collaboration and suggestions in all our proteomics work. Renier van der Hoorn and Muhammad Ilyas, from the Plant Chemetics Group of the Max Planck Institute for Plant Breeding Research, having worked with you was a pleasure. You two have contributed a great deal to the end result of the

third chapter of my thesis and gave me a lot of ideas. The same holds true for Łukasz Baranowski and Mirosław Sobczak, from the Department of Botany of Warsaw University of Life Sciences, who provided the microscopy of tomato roots infected with nematodes for the same chapter of this thesis. Norbert de Ruijter, from the Laboratory of Cell Biology, I appreciate all your help with fluorescent microscopy. John Jones, from the Scottish Crop Research Institute, thanks for hosting me in your lab at the beginning of my PhD, I have learned a lot and had a very good time there. My blessings to you all, together you have made my "Forum" dream functional, you gave to it electricity, water, heating, Internet and all what was necessary to have it running.

The whole "Wageningen Campus of Dreams" needed some administrators to run behind the stage all of the things that needed to be coordinated. Lisette, your work is amazing and because of you all of us here in Nematology can work without worrying about all the paper work, which I find particularly difficult to handle. I appreciate all your help, advice and your nice way of reminding me that I should speak more Dutch. Liesbeth, thanks for watching over me when I was working on the Bioexploit project, for all the nice conversations and for taking good care of part of the social cohesion in the lab. Douwe Zuidema and Ria Fonteyn, from the graduate school Experimental Plant Sciences, and Ton Bisseling, from the Laboratory of Molecular Biology, thank you so much for all the help and guidance when I was involved in the PhD council, your work in the graduate school make our studies so much nicer. Heartfelt thanks to all of you for helping all the architects, workers and external experts to come together and perform their best in this campus of dreams.

Not only administrators were needed to have all the things working in my Forum dream. Many, many roads were built before my dream took place, but I cannot talk about them all. The N781, the way that connects the highway with my dream was of fundamental importance, before everything started working. Clemencia Avila de Moreno and Martha Isabel Cáceres, from the Biological Management Systems Group in Colombia, you ignited in me the flame of nematology long time ago, working with such fine researchers and teachers was a joy. Ariena van Bruggen and Aad Termorshuizen, from the previous Biological Farming Systems Group, because of your help and support I chose Wageningen as the place where I should do my Master studies and that was a turning point in my life. Aad and Ariena, I know that your recommendations were crucial for finding a PhD. Geert, thanks again for your advice and for arranging my second thesis at the INRA in France. Marie-Noëlle and Marie-Pierre, thanks for holding my hand while I was taking baby steps into the world of molecular biology. You are all a gift to my life for shaping the way long before I started working on my dream.

In the completion of my dream several people, besides Geert and Aska, have checked that this book as a whole is well written and have little

mistakes. To all of you thanks for the input, the positive energy and for reminding me that no matter how hard a person works, we always make mistakes. I will forever be indebted to you for being the inspectors that make sure that my building is working fine.

A building like the Forum would be nothing if there were no people walking around, talking, studying, teaching, learning, laughing, loving and even partying. If I mentioned at this point all the friends that played an important role in my life during the past years of my life this book would look more like a dictionary than a PhD thesis. My previous housemates of Dijkstraat 1, we shared wonderful times together, you are a true treasure to me. My salsa co-instructors, my students, my friends, you make a huge difference to my life. My football, futsal and FIFA mates, much love and I hope we can keep on winning. My fellow ISOW members, your generosity, kindness, and charisma will remain in my memory. My Colombian compatriots in Wageningen, you bring the joy of my land to my life every day. My dearest friends Dieguín, Ferdiecito, van der Voort, Cesi, Timo, Sannita, the two Naticas, and Amalita, people like you are a rare gift, you have been like my family in this town, thanks for all what you have done for me. My Kleinekes, thank you for all your love and support, for bringing me a smile every day, for the help all these years and for just being who you are and how you are, you are my all in all, I love you. I am eternally grateful for having in my life every one of you who beautifies my dream giving to it the soul on the inside and also shaping the landscape with trees, lakes, flowers, sunshine and a perfect clear sky.

I cannot conclude without thanking the people to whom I owe the basis where all my dreams have been constructed, my family. Mamita linda, you are the most important person in my world, your love and lessons have been always the support for everything in my life. You made me fall in love with biology and plants. I remember when I was a child and you took me to the lab to see the heart of an amphibian working and later on when you started with the project of an orchard in the farm, I enjoyed so much to grow by your side. On the first year of my university you suggested me to join a research group and introduce me to Clemencia Avila. Thank you for seeing in me something, only you know what it was, that led me on the path to do what I love: science and teaching. I hope I can make you very proud. Cami, Chana, Xime y Granadilla, hermanitos, without your love, trust, encouragement and company none of this would have been possible. To my family, the basis of my construction, all my love and thanks a million.

It is Wednesday 30th of April of 2014, it is 15:40 in the afternoon and I am siting in my office again, looking at the beautiful campus of Wageningen. The Forum building looks very quiet at the moment, it is really nice and I cannot help but thinking what will the next project be?

Curriculum vitae

José Luis Lozano Torres was born on July 2nd 1979 in Tunja, Colombia. He graduated from high school in the *Colegio de Boyacá*, Tunja, in 1997. After a year in an exchange study program at Permian High School, in Odessa, U.S.A, he began his University studies at the *Universidad Pedagógica y Tecnológica de Colombia* (U.P.T.C.). He graduated *cum laude* in 2002 as Agronomic Engineer. During the five years of his Agronomy studies he joined, as a young researcher, the Biological Crop Management Systems Group (*Grupo Manejo Biológico de Cultivos*) and under the supervision of dr. Clemencia Avila de Moreno he completed his thesis entitled "Biological management of the stem nematode *Ditylenchus dipsaci*". For two years, he was part of the national coordination in the Colombian Network for Undergraduate Researchers (RedCOLSI). In 2003 he won a scholarship of the Netherlands organization for international cooperation in higher education (Nuffic) to do an M.Sc. in Wageningen University. Under the supervision of drs. Aad Termorshuizen and Ariena van Bruggen he completed his M.Sc. thesis on the relationship between compost particle size and disease suppression. In 2005 he graduated *cum laude* from his M.Sc. in Organic Agriculture, specialization in farm and rural environment. While doing his first M.Sc. in Wageningen, he enrolled in an M.Sc. in Plant Sciences. For the thesis on this master he joined the, Unité Interactions Plantes-Microorganismes et Santé Végétale at INRA in France. Under the supervision of Marie-Noëlle Rosso and Geert Smant he completed his thesis entitled "TRV mediated *in planta* production of dsRNA for the induction of RNAi in the root knot nematode *Meloidogyne incognita*". In 2006 he graduated from his M.Sc. in Plant Sciences, specialization in Plant Pathology and Entomology. During his Master studies he became president of the International Student Organization of Wageningen (ISOW) and member of the International Student Panel (ISP). In February 2006 he started his PhD in the Laboratory of Nematology, part of the Plant Sciences Group at Wageningen University focusing on the modulation of innate immunity by nematode effectors in host plants and animals. During his PhD studies, he worked as president of the Experimental Plant Sciences PhD council. He acts as a volunteer teacher for Salsa, Merengue and Bachata dancing at the ISOW. At the moment he works as a postdoctoral fellow in the Nematology department of Wageningen University.

List of publications

Lozano-Torres J.L., Wilbers R.H.P., Gawronski P., Boshoven J.C., Finkers-Tomczak A., Cordewener J.H.G., America A.H.P., Overmars H.A., Van 't Klooster J.W., Baranowski L., Sobczak M., Ilyas M., Van Der Hoorn R.A.L., Schots A., De Wit P.J.G.M., Bakker J., Goverse A., Smant G. (2012) **Dual disease resistance mediated by the immune receptor Cf-2 in tomato requires a common virulence target of a fungus and a nematode**. Proc. Natl. Acad. Sci. U. S. A. 109:10119-10124.

Postma, W.J., Slootweg, E.J., Rehman, S., Finkers-Tomczak, A.M., Tytgat, T.O.G., Gelderen, K. van, Lozano-Torres, J.L., Roosien, J., Pomp, H., Schaik, C.C. van, Bakker, J., Goverse, A., Smant, G. (2012) **The effector SPRYSEC-19 of *Globodera rostochiensis* suppresses CC-NB-LRR-mediated disease resistance in plants**. Plant Physiol. 160: 944-954.

Lozano J., Smant G. (2011) **Survival of plant-parasitic nematodes inside the host**, in: R. N. Perry and D. A. Wharton (Eds.), CAB International, Wallingford.

Lozano, J., Blok, W.J., Termorshuizen, A.J. (2009) **Effect of compost particle size on suppression of plant diseases**. Environ. Eng. Sci. 26: 601 - 607.

Dubreuil, G., Magliano, M., Dubrana, M.P., Lozano, J., Lecomte, P., Favery, B., Abad, P., Rosso, M.N. (2009) **Tobacco rattle virus mediates gene silencing in a plant parasitic root-knot nematode**. J.Exp. Bot. 60: 4041 - 4050.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School
EXPERIMENTAL PLANT SCIENCES
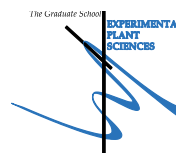
Issued to:	**José Luis Lozano Torres**
Date:	**28 May 2014**
Group:	**Nematology, Wageningen University & Research Centre**

1) Start-up phase	*date*
▶ **First presentation of your project**	
Venom allergens from Globodera rostochiensis	Jan 10, 2006
▶ **Writing or rewriting a project proposal**	
Immunomodulation of plant defense responses by venom allergens from Globodera rostochiensis	Jan-Mar 2006
▶ **Writing a review or book chapter**	
Survival of plant-parasitic nematodes inside the host. IN: Molecular and Physiological Basis in Nematode Survival, eds Perry RN & Wharton DA (CAB International, Wallingford, 2011, pp 28-65)	2011
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13,5 credits**

2) Scientific Exposure	*date*
▶ **EPS PhD student days**	
SDV PhD Student Day 2006, Paris (France)	Jun 06, 2006
EPS PhD Student Day 2006, Wageningen University	Sep 19, 2006
EPS PhD Student Day 2007, Wageningen University	Sep 13, 2007
1st Joint Retreat of PhD Students in Experimental Plant Sciences, Wageninen	Oct 02-03, 2008
EPS PhD Student Day 2010, Utrecht University	Jun 01, 2010
▶ **EPS theme symposia**	
EPS theme 2 symposium 'Interactions between Plant and Biotic Agents', Amsterdam University	Feb 02, 2007
EPS theme 2 symposium 'Interactions between Plant and Biotic Agents', Utrecht University	Jan 22, 2009
EPS theme 2 symposium 'Interactions between Plant and Biotic Agents', Amsterdam University	Feb 03, 2011
EPS theme 2 symposium 'Interactions between Plant and Biotic Agents', Wageningen University	Feb 10, 2012
EPS theme 2 symposium 'Interactions between Plant and Biotic Agents', Utrecht University	Jan 24, 2013
EPS theme 2 symposium 'Interactions between Plant and Biotic Agents', Amsterdam University	Feb 25, 2014
▶ **NWO Lunteren days and other National Platforms**	
NWO-ALW Experimental Plant Sciences	Apr 02-03, 2007
NWO-ALW Experimental Plant Sciences	Apr 19-20, 2010
NWO-ALW Experimental Plant Sciences	Apr 04-05, 2011
NWO-ALW Experimental Plant Sciences	Apr 02-03, 2012
NWO-ALW meeting Experimental Plant Sciences, Lunteren, The Netherlands	Apr 22-23, 2013
▶ **Seminars (series), workshops and symposia**	
BioExploit Science Meeting. Exploitation of natural plant biodiversity for the pesticide-free production of food	Mar 31-Apr 01, 2009
CBSG Proteomics Hotel Meeting	Dec 13, 2010
CBSG Workshop on Open Source Biotech with Richard Jefferson (CAMBIA-BiOS)	Oct 04, 2011
Plant Science Seminar by Young PSG: Speek, Present and Debate	Jul 24, 2012
▶ **Seminar plus**	
▶ **International symposia and congresses**	
COST 872 Nemagenics annual meeting, La Colle-sur-Loup, France	May 09-11, 2007
International congress on molecular plant microbe interactions, Sorrento, Italy	Jul 21-27, 2007
Keystone Symposya on Plant Innate immunity, Keystone, USA	Feb 10-15, 2008
COST 872 Nemagenics annual meeting, Postojna, Slovenia	May 26-29, 2008
Pathogenesis and Immune Regulation in Helminth Infections, Tahoe City, USA	Feb 01-05, 2009
COST 872 Nemagenics annual meeting, Toledo, Spain	May 25-28, 2009
International congress on molecular plant microbe interactions, Quebec, Canada	Jul 19-23, 2009
Molecular and cellular Biology of helminth parasites VI, Hydra, Greece	Sep 05-10, 2010
SPIT Meeting, Cornell, USA	May 18-20, 2011
SPIT Meeting, Ghent, Belgium	May 23-24, 2012
Molecular and cellular Biology of helminth parasites VII, Hydra, Greece	Sep 02-09, 2012
Immunomodulation by plant-associated organisms, Fallen Leaf Lake, USA	Sep 16-19, 2012
▶ **Presentations**	
International congress on molecular plant microbe interactions, Sorrento, Italy (poster)	Jul 21-27, 2007
NWO-ALW Experimental Plant Sciences, Lunteren (poster)	Apr 02-03, 2007
Keystone Symposya on Plant Innate immunity, Keystone, USA (poster)	Feb 10-15, 2008
COST 872 Nemagenics Annual Meeting, Postojna, Slovenia (poster)	May 26-29, 2008
Bioexploit PhD Summer School, Wageningen (oral)	Jun 18-20, 2008
1st Joint Retreat of PhD Students in Experimental Plant Sciences, Wageninen (oral)	Oct 02-03, 2008
Pathogenesis and Immune Regulation in Helminth Infections, Tahoe City, USA	Feb 01-05, 2009
BioExploit Meeting. Exploitation of natural plant biodiversity for the pesticide-free production of food (oral)	Mar 31-Apr 04, 2009
COST 872 Nemagenics annual meeting, Toledo, Spain (oral)	May 25-28, 2009
International congress on molecular plant microbe interactions, Quebec, Canada (poster)	Jul 19-23, 2009

	Molecular and cellular Biology of helminth parasites VI (oral)	Sep 5-10, 2010
	SPIT Meeting, Cornell, USA (oral)	May 18-20, 2011
	SPIT Meeting, Ghent, Belgium (oral)	May 23-24, 2012
	Molecular and cellular Biology of helminth parasites VII, Hydra, Greece (oral)	Sep 02-09, 2012
	Immunomodulation by plant-associated organisms, Fallen Leaf Lake, USA (poster)	Sep 16-19, 2012
	NWO-ALW meeting Experimental Plant Sciences, Lunteren, The Netherlands	Apr 22-23, 2013
	COST FA 1208 Annual meeting, Birnam, Scotland (poster)	Oct 09-11, 2013
	Mini-workshop Warsaw University of Life Sciences-SGGW, Warsaw, Poland (oral)	Nov 22, 2013
▶	**IAB interview**	Dec 05, 2008
▶	**Excursions**	
	Subtotal Scientific Exposure	*42.9 credits*

3) In-Depth Studies		*date*
▶	**EPS courses or other PhD courses**	
	Summer School "Signaling in Plant Development and Defence: towards systems biology"	Jun 19-21, 2006
	Gateway to gateway technology	Nov 20-24, 2006
	Nematode identification	Feb 12-23, 2007
	Bioinformatics "a user's approach"	Mar 13-16, 2007
	Summer School "On the evolution of plant-pathogen interaction: from principles to Practise"	Jun 18-20, 2008
	Spring School "RNAi & the World of Small RNA Molecules"	Apr 14-16, 2010
▶	**Journal club**	
	Member of literature discussion group at Nematology, once every two weeks	2006-2008
▶	**Individual research training**	
	Short Term Scientific Mission COST872, SCRI, Dundee, Scotland (2 weeks)	Sep 23-Oct 06, 2007
	Subtotal In-Depth Studies	*14,1 credits**

4) Personal development		*date*
▶	**Skill training courses**	
	EndNote Advanced	Feb 16, 2006
	Information Literacy	Feb 27-28, 2007
▶	**Organisation of PhD students day, course or conference**	
	EPS PhD Student Day 2006, Wageningen University	Sep 19, 2006
	Wageningen PhD party, Wageningen University	Oct 24, 2008
▶	**Membership of Board, Committee or PhD council**	
	President PhD council	2007-2010
	Member EPS Research Committee	2007-2010
	Member EPS Educational Committee	2007-2010
	Member Wageningen Graduate Schools Council	2007-2010
	Subtotal Personal Development	*6,6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**77,1**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by

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