Molecular contest between potato and the potato cyst nematode *Globodera pallida*: modulation of *Gpa2*-mediated resistance

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“Niewazne skad pochodzisz- wazne dokad zmierzasz”
Brian Tracy

Contents

Chapter 1

General Introduction

Plant-parasitism by cyst nematodes

The phylum Nematoda is characterized by a remarkable high biological diversity. Based on their feeding behavior, members in this phylum can be divided into fungal and bacterial feeders, predators and omnivores as well as a variety of plant-, animal- and human parasites. Most of the plant-parasitic species are obligatory biotrophs highly adapted to feeding from living host cells. They have evolved different strategies to infect their host plants, and to acquire their nutrients from the host. For example, migratory ectoparasitic nematodes live in the rhizosphere and feed from outside the plant on epidermal root cells or root hairs. At the other end of the spectrum are the sedentary endoparasitic species that migrate into the host roots to establish a permanent feeding site. The group of the sedentary endoparasites represents the most sophisticated form of plant parasitism and includes cyst nematodes (*Heterodera* spp. and *Globodera* spp.) and root-knot nematodes (*Meloidogyne* spp).

The level of sophistication in plant parasitism reflects in the survival strategies deployed by cyst nematodes in and outside host plants. For instance, at the end of each growing season, fertilized nematode eggs remain in the soil inside so-called cysts, which are protective containers formed by hardened dead female bodies. The eggs can survive in the soil for up to 30 years in a dormant anabiotic state (reviewed in Williamson et al., 1996). Juveniles of the potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) exit their dormancy and hatch from the eggs only in the presence of root diffusates from a proper host. This phenomenon essentially synchronizes the lifecycles of host and parasite. The invigorated, freshly-hatched juveniles move towards a root of a host plant. They penetrate this root to migrate further intracellularly through the root tissues while searching for a proper location to induce a feeding site.

The feeding site induced by cyst nematodes is a so-called syncytium, which is initiated in the cortex or pericycle cell layers but later expands into the vascular tissues of an infected root (reviewed by Gheysen and Mitchum, 2008; Castelli et al., 2006; Sobczak and Golinowski, 2008). A syncytium comprises of a multinucleate structure made through the coalescence of neighboring cells close the nematode's head; even up to 200 cells can be incorporated (Jones 1981). Cells included into the syncytium undergo drastic changes in morphology and metabolic activity. This is apparent by the enormous proliferation of subcellular organelles involved in energy production and protein synthesis such as mitochondria, endoplasmic reticulum, and ribosomes. Another hallmark feature of syncytia are elaborate cell wall ingrowths in the cells bordering phloem and xylem cells. The cell wall ingrowths greatly enlarge the surface area of the cell membrane, and thereby enhance the possible transport of solutes from the vascular tissue of the plant into the syncytium. In fact, these nematode-induced changes elevate the social status of the feeding sites in the plant's household to that of a nutrient sink. Cyst nematodes are completely depending on the syncytium for their development and reproduction, because once the syncytium formation sets off the parasitic second stage juveniles become immobile. In the course of several weeks they molt further into third and fourth juvenile stages and finally the adult stage. Adult males regain their mobility and leave the root to mate with females.

Cyst nematode secretions

In nematodes, the adaptation into a parasitic lifestyle on plants is always associated with the presence of an oral stylet, which is formed as a cuticular structure in the oral cavity and the esophagus. Plant-parasites use their stylet to puncture the rigid plant cell wall of host cells during host invasion and during the ingestion of nutrients from the cytoplasmic contents of host cells. The stylet is also used as an injection needle to release esophageal glands secretions into the host and those are believed to be important for host invasion, feeding, and modulation of host innate immunity.

Stylet secretions are produced in one dorsal and two subvental esophageal glands of the nematode. The activity of the subventral esophageal glands predominates in the early stages of parasitism during host invasion and feeding site initiation, whereas the dorsal gland cell is most active in the subsequent sedentary stages during feeding (Hussey 1989). The primary function of the glandular secretions is either direct or indirect modification of host gene expression (Davis et al., 2009), which ultimately leads to morphological, physiological, and molecular changes associated with feeding sites (for more details see review by Davis et al., 2008). When the nematodes are removed at any point during parasitism the feeding site will degenerate, demonstrating that there is a continuous stimulation from the nematode needed to maintain a functional syncytium (Hussey 1989).

Recent progress in molecular biology and functional genomics has allowed the discoveries of hundreds of secreted proteins from both plant- and human/animal-parasitic nematodes (Curtis 2007; Davis et al., 2009; Bird and Opperman, 2009). The majority of these so-called secretome members have no similarity with known proteins in the databases. However, those that do have a match with functionally annotated proteins from other organisms fall into a various functional classes. For example, a suite of cell wall modifying enzymes, such as endoglucanases and expansins, has been identified in the subventral gland secretions of cyst nematodes (Qin et al., 2004). These cell wall modifying proteins facilitate the enzymatic degradation of the plant cell wall during host invasion.

Other secreted nematode proteins seem to be involved in the modulation of a diverse range of cellular processes in the host, including feeding cell formation, altered cellular metabolism, cell-cycle regulation, and protein degradation (Davis et al., 2004). A remarkable category is formed by the small nematode-secreted proteins, interfering with peptide signaling in host cells. Ten years ago, secretions collected from hatched juveniles of the potato cyst nematode *G. rostochiensis* were found to contain small peptides (3 kDa) that can induce mitogenic activity in tobacco protoplasts (Goverse et al., 1999). More recently, it has been demonstrated that this nematode species deploys a whole series of genes coding for small hypervariable peptides, which are able to interfere in plant developmental processes (van Bers 2008). Other exciting studies suggest that cyst nematodes use ligand mimicry to redirect developmental processes in host cells, because nematode-secreted peptides with similarity to CLAVATA/ESR-like peptides from plants are able to rescue CLAVATA3 mutants in Arabidopsis (Wang et al., 2005).

Until now most of the genes potentially involved in nematodes parasitism were identified in whole nematode ESTs libraries, libraries from aspirated esophageal glands, and various applications of differential display techniques (discussed in Davis et al., 2009). The recent completion of the genome sequences of two root-knot nematodes (*Meloidogyne incognita* and *M. hapla*) and the nearly completed genome sequence of the cyst nematode *G. pallida* will open up new avenues towards understanding the whole repertoire of genes involved in nematode parasitism (Abad et al., 2009; Opperman et al., 2009; Trust Sanger Institute; http://www.sanger.ac.uk/sequencing/Globodera/pallida). A major bottleneck though, which has also troubled the functional analysis of nematode genes in this thesis, is the persistent lack of methods to transform plant-parasitic nematodes and to use these methods in a workable reverse genetics approach.

Potato cyst nematodes

Potato cyst nematodes (PCN) originate from the Andes region in South America and were introduced into Europe in the 19th century. They subsequently spread throughout Europe together with *Solanum tuberosum* (potato) seed material. There are 13 known *Globodera* species that are primarily important parasites of members of the *Solanaceae* family, such as potato, tomato, eggplant, and tobacco. Two different species of European PCN have been identified, the yellow *Globodera rostochiensis* and the white *Globodera pallida*. In Europe *G. rostochiensis* is further classified into five pathotypes (Ro1–Ro5), while within the white species three pathotypes (Pa1–Pa3) have been described (Moxnes and Hausken, 2007).

The damage that PCN causes in Europe became more widely recognized during the first half of the 20th century. For example, *G. pallida* is one the most important pathogens in the United Kingdom and forms an increasing problem for potato growers. It has been estimated that in the United Kingdom PCN along cause yield losses of 9% of the annual potato production. Within the European Union the total monetary losses caused by PCN are estimated to be 300 million euro (Moxnes and Hausken, 2007). The United States have tried to contain PCN in small area on the east coast by strict quarantine measurements. However, in 2006 *G. pallida* also appeared in a few potato-growing locations in the state of Idaho (Idaho State Department 2006, http://www.idahoag.us).

Infection with cyst nematodes causes severe crop losses because of the damage caused by intracellular migration of J2s and their feeding behavior. Plants infected with PCN generally have reduced concentrations of nitrogen, phosphorus and potassium in the foliage. Infected plants are also more susceptible to other parasites and show wilting symptoms. Yield loss following nematode infection is associated with reduced light interception due to a reduced leaf area. It has been suggested that a nematode-disturbed hormone balance in the plant causes this reduction, but nutrient deficiency due to nematode feeding likely also affects the photosynthetic rate (DeRuijter and Haverkort, 1999).

Because potato constitutes one of the major food crops in the world there is a strong need for developing sustainable methods to control potato cyst nematodes. The availability of chemical control is limited and not widely accepted due to the environmental concerns. Many of the most effective nematicides (methyl bromide or aldicarb) have already been withdrawn from the market. Likely, this trend will continue in the future especially with new European Union legislation that will prohibit most of the known active compounds used for controlling nematodes (Rosso et al., 2009). Next to chemicals, biological control and crop rotation are used in agricultural practice. However, the sources of natural enemies for nematodes are limited and crop rotation leads to yield reduction because of suboptimal use of farmland. Therefore, many research programs all over the world have focused on the exploitation of natural resistance to PCN.

Resistance against potato cyst nematodes

Several major resistance genes (*R* genes) and quantitative trait loci (QTLs) against cyst nematodes have been identified in wild germplasms and were subsequently introduced into cultivars through conventional breeding (Dale et al., 1998; reviewed by Bakker 2002; Tomczak et al., 2009). Breeding for cyst nematode resistance with major *R* genes is more durable than it is for fungi and bacteria, as they have often only one generation per year and migration is limited to small distances in the soil resulting in slowly expanding infection foci in the field. For example, a single dominant gene *H1* was introduced into potato cultivars in the 1950s and it is still functional in restricting *G. rostochiensis* (Dale and Phillips, 1984).

Resistance to potato cyst nematodes can be classified in two types of responses (reviewed by Bakker et al., 2006). The first type is characterized by a classical hypersensitive response in the cell layers surrounding the young feeding cell, which results in the

encapsulation of the developing syncytium by a ring of dead cells as described for the *H1* gene (Rice et al., 1985). Consequently, the connection with the vascular tissue is disrupted resulting in limited food uptake by the feeding nematode. For cyst nematodes, sex is determined epigenetically depending on the amount of food available in early stages of the infection (Trudgill 1967). Therefore, a shift towards male development is observed for this type of resistance, as they require less food than females. The second type of response, however, is characterized by an arrest in female development due to a late resistance response as observed for the *Gpa2* gene (van der Vossen et al., 2000), which results in the degradation of the syncytium after sex is determined for the potato cyst nematode.

To date, thirteen QTLs conferring resistance to potato cyst nematodes have been mapped on different chromosomes in potato. They confer either resistance to different population of *G. pallida* (*Gpa, Gpa4, Gpa5, Gpa6, GpaVSspl, GpaXISspl, GpaM1, M2, M3*) or *G. rostochiensis* (*Gro1.2; Gro1.3* and *Gro1.4*) or both species as shown for *Grp1* (reviewed by Tomczak et al., 2009). In potato, five single dominant *R* genes are known against both *G. pallida* (*Gpa2, Gpa3*) and *G. rostochiensis* (*Gro1, H1, GroVI*), of which two genes have been cloned. These include the genes *Gpa2* (Van der Vossen et al., 2000) and *Gro1* (Paal et al., 2004), and the cloning of the *H1* gene is in progress (Anna Tomczak pers. comm.). In tomato, a close relative of potato, the *Hero* gene was isolated, which confers broad-spectrum resistance against *G. rostochiensis* (Ernst et al., 2002). Details of the structure and the function of the genes are described in Chapter 2 (this thesis).

The *Gpa2* gene, introgressed from *S. tuberosum* ssp. *andigena* into cultivated potato, is a typical single-dominant *R* gene that encodes for a protein with a coiled-coil, nucleotide binding and leucine-rich repeat domain (CC-NB-LRR) (Van der Vossen et al. 2000). *Gpa2* is highly homologous (88% identity at the amino-acid sequence) to *Rx1*, which confers resistance to potato virus X (PVX). Both genes are tightly linked on chromosome XII of potato (Bendahmane et al., 1999) in a small *R* gene cluster. Interestingly, *Gpa2* confers a mild nematode resistance response in the roots of potato, whereas *Rx1* confers an extreme resistance response to PVX in the areal parts of the plant. Having two highly similar genes with different pathogen specificity is a valuable tool to unravel the role of protein domains and single residues in nematode recognition and the activation of a disease resistance response as described in this thesis.

Thesis outline

This thesis describes different aspects of the molecular mechanisms underlying resistance to the potato cyst nematode *Globodera pallida* mediated by the *Gpa2* gene in potato (*Solanum tuberosum* ssp. *andigena*).

Chapter two presents an overview on how a resistant plant responds to an infection by endoparasitic nematodes. A compendium of known nematode resistance genes is described in the chapter. We have also discussed the consequences of defense responses and their underlying mechanisms that are triggered upon nematodes recognition, which often leads to feeding site degradation and therefore nematode starvation.

In **chapter three**, the activity and recognition specificity of the *Gpa2* gene was analysed in more detail using chimeras between *Gpa2* and the homologous resistance gene *Rx1*. Structure-function analyses demonstrated that the LRR domain is the main specificity determinant of *Gpa2* involved in recognition of *G. pallida*. Furthermore, we found that the CC-NB domains of Gpa2 are able to confer extreme resistance to the potato virus X in potato

when fused to the LRR domain of *Rx1*. This finding implicates that disease resistance signaling likely follows the same pathways for *Gpa2* and *Rx1*, even though the signaling generates an entirely different outcome – the slow hypersensitive response induced by *Gpa2* versus the ultra-fast extreme resistance by *Rx1*. Furthermore, we gained new insights in the activation threshold of *Gpa2* and *Rx1* by modulating the expression levels of the wild type and mutant genes.

Chapter four starts with a detailed description of the *Gpa2* resistance response. In a histological study we showed that the nematode feeding cell becomes disconnected from the conductive tissue by a layer of necrotic cells in *Gpa2* resistant potato roots challenged with the avirulent *G. pallida* population D383. This finding suggests that resistance conditioned by *Gpa2* is the result of inadequate flow of plant nutrients from the vascular tissues to the feeding nematode. To further investigate the spatial and temporal regulation of the *Gpa2* gene in potato, a histological GUS assay was performed as well. It was observed that the *Gpa2* promoter activity is specifically down-regulated by the virulent nematode population Rookmaker inside the feeding structure and in cells in close proximity to syncytium.

In **chapter five**, we addressed the question which effector protein from *G. pallida* is recognized by *Gpa2*. Recently, it was shown that Gpa2 interacts with RanGAP from potato (Sacco et al., 2007; Tameling et al., 2007) and Blanchard et al., (2005) showed juveniles of *G. pallida* express a RanBPM like protein (RBP-1). Because both RanGAP and the RanBPM are associated with the Ran cycle in eukaryotic cells and RanGAP is required for resistance conditioned by Rx1 and possibly Gpa2, RBP-1 was a likely candidate for being the nematode effector recognised by the Gpa2 resistance gene. Ten additional homologous of RBP-1 were identified from cDNA of virulent and avirulent juveniles of *G. pallida*. RBP-1 variants from both the avirulent population D383 and 8 out 10 from virulent population Rookmaker were able to trigger a specific *Gpa2* mediated HR in an agroinfiltration assay on *N. benthamiana* leaves. Loss of *Gpa2* activation observed only for two RBP-1 variants from the virulent nematode population was correlated with a single amino acid substitution (S166P).

In **chapter six**, a structure-function analysis of RBP-1 recognition by Gpa2 is presented showing that RBP-1 is recognized by the most C-terminal part of the LRR domain of Gpa2. Furthermore, we could show that RBP-1 recognition in an agroinfiltration assay correlates with *Gpa2* mediated nematode resistance in potato. Structural modeling of the S166P mutation in RBP-1 predicts a significant change in the likely protein-protein interaction surface of the RBP-1 protein. In addition, we were able to demonstrate that two non-eliciting RBP-1 variants from the virulent Rookmaker population suppress the HR induction by Gpa2 activating forms of RBP-1.

In **chapter seven**, we give an outlook on the potential role of effector proteins in plant resistance to cyst and root-knot nematodes, including the activation and suppression of effector-triggered immunity by RBP in potato plants harboring the *Gpa2* gene.

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Chapter 2

Plant defense induced by parasitic nematodes

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** equal contributions*

Plants are constantly under attack from a wide range of pathogens and pests including bacteria, viruses, fungi, oomycetes, insects and nematodes. Fortunately, the majority of plant-pathogen interactions are incompatible. Co-evolution between plants and pathogens resulted in the development of an immune system which, in contrast to animals that have both an adaptive and an innate immune system, is completely innate (Zipfel and Felix, 2005). This defense system is composed of overlapping layers including non-host immunity (*e.g.* specific recognition of non-specific pathogen-derived components), host resistance, which is only effective against a specific pathogen race or population, and induced systemic resistance (ISR).

The development of molecular techniques has made it possible to gradually uncover the mechanisms underlying the different layers of disease resistance in plants. In the last decade an increasing number of defense-related genes involved in resistance to various pathogens have been isolated from different plant species, and several elicitors of defense responses were identified from a wide range of pathogens (reviewed by Bonas and Lahaye, 2002). However, the molecular mechanisms underlying resistant plant responses to parasitic nematodes are still largely unknown.

Cyst and root-knot nematodes are major pathogens of a number of agronomically important crops such as cereals, soybean, potato, tomato and sugar beet. The lack of natural enemies and the shortage of adequate resistance genes in crop plants are factors that underlie the very substantial damage caused by these organisms. The estimated worldwide losses caused by plant-parasitic nematodes are about US $125 billion annually (Chitwood 2003). One way to control them is the use of nematode resistant cultivars. To that end a broad range of resistances to either cyst or root knot nematode species has been identified over the years in several crop species in order to develop durable crop protection strategies Williamson 1998; Jung and Wyss, 1999; Williamson 1999; Bakker 2003).

To date, six genes conferring resistance to cyst and root-knot nematodes have been isolated from beet (Cai et al., 1997), potato (Van der Vossen et al., 2000; Paal et al., 2004), tomato (Milligan et al., 1998; Vos 1998; Ernst et al., 2002), and pepper (Chen et al., 2006), which allow structural and functional analyses to unravel their role in nematode recognition and the activation of a disease resistance response. Many aspects of disease resistance signaling in response to plant-parasitic nematodes are thought to resemble the mechanisms underlying the defense responses to other plant pathogens, which are often better characterized.

Pre-Infectional Resistance

Before a pre-parasitic juvenile from cyst and root-knot nematodes is able to parasitize a plant species, it has to hatch from the egg, become attracted to the plant roots and penetrate the plant tissue. If the infective juvenile is blocked at any of these stages, the plant apparently is not a suitable host for this particular nematode and therefore de facto resistant. Because this resistance occurs before the pre-parasitic nematode has had a chance to enter the plant, we will refer to this type of resistance as pre-infectional resistance.

Resistant Plant Responses

Eggs of sedentary endoparasitic nematodes hatch under the influence of plant components released by the plant into the soil. In 1953, Jones and Winslow noticed that pre-parasitic juveniles from beet, potato, and carrot cyst nematodes hatch when soaked in root diffusates of their respective hosts but not when soaked in root diffusates of nonhosts. However, sometimes infective juveniles do hatch in the presence of certain plant species that cannot be successfully parasitized by the nematode. Apparently, nematode infection is then blocked at a later stage of the infection process. Such phenomena can be of great agronomical

value because these plants can be used as so-called trap crops to reduce the number of cysts in the soil. For instance, the density of cysts from the soybean cyst nematode *Heterodera glycines* was reduced between 70 and 90% after cultivation of the nonhost species *Crotalaria juncea* and *C. spectabilis* when compared to fallow (Kushida et al., 2003). Interestingly, it was also noticed that the number of juveniles entering the *C. juncea* and *C. spectabilis* roots did not differ significantly from those entering the susceptible soybean roots. Plant cells are protected by the presence of a rigid cell wall to ward off foreign invaders. However, based on several studies regarding nematode infection of resistant and susceptible cultivars as well as host and nonhost plants (reviewed by Kaplan and Keen, 1980) it can be concluded that nematodes freely penetrate roots of host and nonhost alike and that these mechanical barriers rarely appear to be effective against plant-parasitic nematodes. The presence of a typical robust hollow spear (stylet) located in the head region of the nematode enables the nematode to overcome this major barrier. The action of stylet thrusting combined with the release of cell-wall degrading enzymes via this stylet facilitate the penetration of the root and subsequent migration of endoparasitic nematodes to the appropriate feeding site (for details see the review by Davis et al., 2004).

Quantitative and qualitative disease resistance

In contrast to non-host immunity, host resistance is only effective against particular subpopulations of the pathogen, usually within a species. Host-specific resistance is called gene-for-gene resistance if it requires the presence of both a race-specific avirulence **(***Avr***)** gene in the pathogen and a corresponding cultivar-specific single dominant resistance **(***R***)** gene in the host plant (Flor 1946). The biochemical interpretation of this concept is a receptor-ligand model in which plants activate a defense mechanism upon R protein-mediated recognition of a pathogen-derived *Avr* product (Van Der Biezen and Jones, 1998). Although several pairs of cognate *R* and *Avr* genes are identified, direct interaction could only be proven in four cases (Jia 2000; Deslandes 2003; Dodds et al., 2006; Ueda et al., 2006). Alternatively, other plant components were shown to interact with avirulence proteins (reviewed in Bogdanove 2002) supporting the so-called guard model (Dangl and Jones, 2001) in which *Avr* protein-induced modifications of host products are recognized by R proteins that 'guard' these host products.

R gene-mediated resistance has several attractive features for disease control. In many cases, a single *R* gene can provide complete resistance to a particular population, strain or certain species of pathogen when present in an otherwise susceptible plant. Monogenic resistances are desirable for breeding purposes because of their simplicity in being introgressed. The plant response is also usually very fast and local, which restricts the collateral damages in the plant caused by a pathogen infection. Unfortunately, in many plant-pathogen interactions, this type of resistance can be broken down relatively fast due to alterations in the coevolving pathogen.

Plant breeders have used disease resistance genes to control plant disease long before they were identified and analyzed. Over the years, various nematode resistances have been mapped and some of the underlying *R* genes have now been cloned. This work has been extensively reviewed (Williamson 1999; Bakker 2006; Williamson and Kumar, 2006).

Identification and characterization of nematode *R* genes

R genes encode proteins with a modular structure and they can be classified in different groups based on the specific combination of functional domains of which they are composed. The majority of *R* genes belong to the super family of nucleotide-binding (NB) – leucine-rich repeat (LRR) genes (Ellis and Jones, 1998; Meyers et al., 1999). This class of genes is very abundantly present in plant species and encodes large proteins ranging from 860

to about 1900 amino-acids. In *Arabidopsis*, it is estimated that at least 200 different NB-LRR genes exist compromising up to 1% of the genome (Meyers et al., 1999). Based on the N-terminal part, the NB-LRR proteins can be further subdivided into two classes containing either a coiled-coil (CC) domain or a Toll-Interleukin receptor (TIR) homology domain. The CC-NB-LRR proteins are present in both monocots and dicots, whereas monocots are lacking the TIR-NB-LRR proteins. A current overview of plant R protein structure and function can be found in the review written by Van Ooijen et al., 2007 and by Takken and Tameling, 2009.

In 1997, the first nematode resistance gene was cloned from sugar beet by Cai et al. The gene $Hs1^{pro-1}$ confers resistance to the beet cyst nematode *Heterodera schachtii*. Sequence comparison revealed that it encodes an R protein, which shows no homology with any known *R* gene although it contains an atypical LRR domain of only 146 amino acids and a putative transmembrane domain. Investigation of gene expression patterns under biotic and abiotic stresses by means of a promoter: reporter gene fusion showed that $Hs1^{pro-1}$ is up-regulated only during the incompatible plant- nematode interaction and its promoter activates a feeding site-specific gene expression pattern (Thurau et al., 2003).

The identification and characterization of four other nematode resistance genes (*Mi-1, Gpa2, Hero, Gro1*) from potato and tomato showed that they do belong to the super family of NB-ARC-LRR resistance genes. *Gro1* distinguishes itself from the other three by having a TIR domain, whereas the others have a CC domain. Despite the structural similarities, some differences in function do occur. The two genes identified in potato, *Gpa2* (Van der Vossen et al., 2000) and *Gro1* (Paal et al., 2004), confer resistance against specific populations of the potato cyst nematodes *G. pallida* and *G. rostochiensis*, respectively: whereas the tomato gene *Hero* (Ernst et al., 2002) recognizes a broad spectrum of potato cyst nematode species and populations. It confers resistance to all economically important pathotypes of both *G. rostochiensis* and *G. pallida* (Sobczak et al., 2005). A similar broad spectrum resistance is mediated by the tomato gene *Mi-1* (Milligan et al., 1998; Vos 1998), which is effective against the three major root-knot nematode species: *M. incognita, M. javanica* and *M. arenaria*. Additionally, the *Mi-1* gene confers resistance to piercing/sucking insects i.e. the potato aphid *Macrosiphum euphorbiae* (Vos 1998) and the whitefly *Bemisia tabaci* (Nombela et al., 2003), which suggests multiple recognition specificities.

Interestingly, both the Mi-1 protein and the HERO protein harbor an unusual N-terminal domain. Although in both proteins two CC regions are predicted (Williamson et al., 2000), the two domains do not show any significant sequence or structural similarity (Ernst et al., 2002). The N-terminal domain of Mi-1 resembles that of the late blight resistance protein Rpi-blb2 (Van der Vossen et al., 2005), which is positioned in a homologues region in potato and has an overall amino acid identity of 82%. Apart from that, like the N-terminal domain of HERO, it has no significant similarity to other sequences. Interestingly, preliminary data from sequence analysis of the *H1* locus in potato, conferring resistance to the potato cyst nematode *G. rostochiensis*, revealed the presence of resistance gene candidates with a similar N-terminal domain (amino acid homology with both *Mi-1* and *Rpi-blb2* is around 50%) (Tomczak et al., unpublished data).

Recently, an *R* gene candidate gene was identified in soybean at the *rhg1* locus, which is involved in resistance against the soybean cyst nematode *Heterodera glycines* (Ruben 2006). Sequence comparison revealed that this putative resistance gene belongs to a distinct class of *R* genes consisting of three functional domains including an LRR domain composed of 12 extracellular repeats, a trans-membrane domain and a kinase domain. The encoding protein shows high homology to the bacterial resistance gene *Xa21* from rice and an *Arabidopsis* receptor-like kinase gene family.

Activation of *R* gene-mediated nematode resistance

Mapping and cloning of *R* genes conferring resistance to endoparasitic nematodes is a major contribution to the elucidation of the genetic and molecular mechanisms underlying nematode resistance. Resistance to cyst and root-knot nematodes is characterized by an arrest in feeding cell induction and development often as the result of a local hypersensitive response (HR) at the infection site. The HR is a form of programmed cell death (Greenberg 1997; Morel and Dangl, 1997; Pontier et al., 1998), and shares several features with apoptosis in mammalian cells (Lamb and Dixon, 1997; Mittler et al., 1997). A number of nematode resistance phenotypes have been described for both cyst and root-knot nematodes (Bakker 2006). Responses range from the complete abolishment of nematode development when the establishment of the feeding site is arrested in an early stage of infection, to a significant reduction of the number of fully developed adult females and cysts when feeding cell development is blocked in a later stage.

The modular structure of the encoding R proteins allows the study of their separate roles in nematode recognition and the induction of a defense response that leads to nematode resistance. Unfortunately, these structure-function studies are seriously hampered by the fact that the corresponding elicitors from the nematodes are still unknown. However, an elicitor-independent hypersensitive response for the *Mi-1* gene was obtained in an agroinfiltration assay in *Nicotiana benthamiana* leaves upon expression of a chimeric construct that consisted of the N- terminal domain from *Mi-1.1*, a nonfunctional homologue that is 91% identical to *Mi-1.2*, the functional *Mi-1* gene. For the same chimeric *R* gene, no transgenic tomato plants could be recovered (Hwang et al., 2000), suggesting that an HR reaction takes place in cells that express this gene. In this paper, it was shown that a six-amino acid region in the LRR in *Mi-1* is required but not sufficient for resistance. In a follow-up study, the amino acids that are essential for nematode resistance were determined by introducing each of the 40 amino acid differences between the LRR of *Mi-1.2* and *Mi-1.1* into *Mi-1.2*. They found 24 amino acids that appeared to be required for signaling and three consecutive amino acids that may be involved in nematode recognition. Apparently, the N-terminal part 1 (NT-1), which consists of the first 161 amino acids, was able to repress the transmission of a signal by the LRR domain that leads to an HR and a model was proposed in which this negative regulation was compromised in the presence of a root-knot nematode elicitor (Hwang and Williamson, 2003).

Similar results were obtained in a structure-function study of the potato resistance gene *Rx1*, which is a close relative (93% nucleotide identity) of the nematode resistance gene *Gpa2*. Physical interactions were observed between the N-terminal CC domain and the NB-LRR domains or between the CC-NBS domains and the LRR domain in the absence of the elicitor, the coat protein from the potato virus X. However, those interactions were disrupted in the presence of the avirulent coat protein, suggesting the activation of Rx-mediated signaling by relieving the negative intramolecular regulation of the NBS domain (Moffett et al., 2002). This domain, also called the NB-ARC (nucleotide binding adaptor shared by NOD-LRR proteins, APAF1, R proteins and CED4) domain (McHale et al., 2006), seems to be involved in specific binding and hydrolysis of ATP as was shown for the two tomato resistance genes *Mi-1* and *I2* (Tameling et al., 2002). ATP hydrolysis is thought to result in conformational changes that regulate downstream signaling. High sequence homology between the closely-related viral resistance gene *Rx1* and the nematode resistance gene *Gpa2* suggests that a similar model might be applicable for the activation of a resistance response to the potato cyst nematode.

R gene-mediated defense responses to nematodes

Plant responses to pathogens are associated with massive changes in gene expression. For example, in *Arabidopsis thaliana*, a change in the gene expression levels of more than 2000 genes has been observed within nine hours upon inoculation with the bacterial pathogen *Pseudomonas syringae* (Tao et al., 2003). It is thought that early activation of genes involved in phytohormone biosynthesis modifies the hormonal balance of the host plant, leading to the appropriate transcriptome changes. Gene expression studies of several plant-nematode interactions showed that different defense-related genes are upregulated upon infection of both susceptible and resistant plants, including genes encoding peroxidase, chitinase, lipoxygenase, extensin and proteinase inhibitors (reviewed in Williamson and Hussey, 1996; Gheysen and Fenoll, 2002). Furthermore, genes encoding enzymes involved in biosynthetic pathways are induced early during infection. For example, glyceollin in soybean appears to be involved in phytoalexin biosynthesis after *M. incognita* infection (Kaplan et al., 1980) and chalcone synthase is produced in white clover upon infection with *M. javanica* (Hutangura et al., 1999).

Expression of defense-related genes in both the compatible and the incompatible interaction suggests their role in basal resistance. However, it is hypothesized that the defense response is only strong and quick enough to prevent successful nematode infection in the presence of a functional R protein that can recognize the appropriate AVR protein from the nematode. The induction of toxins, pathogenesis-related (PR) genes and the hairpin-induced *hin1*-like gene during the compatible interaction between root-knot nematodes and tomato suggests that the nematodes are identified as pathogens (Bar-Or et al., 2005). However, in the absence of components necessary for a host-specific defense reaction (like a functional *R* gene), no HR is elicited and the defense response is not fully effective.

There is now a significant amount of evidence pointing to specific MAPKs as fundamental components of defense pathways that play a role in both basal defense and in more specific interactions involving *R* gene-mediated resistance (Pedley and Martin, 2005). They are involved in the generation of reactive oxygen species (ROS) (Kovtun et al., 2000; Ren et al., 2002), the induction of PR proteins and in gene transcription (Ahlfors et al., 2004; Kim and Zhang, 2004; Lee et al., 2004). Although there is no direct evidence yet for the role of MAPKs in nematode resistance, it was recently shown that *Mi-1*-mediated aphid resistance was abolished in tomato when *Le*MKK2, *Le*MPK2 , *Le*MPK1 or *Le*MPK3 were silenced (Li et al., 2006). It will be interesting to see whether MAPKs play a similar role in *Mi-1*-mediated nematode resistance.

An oxidative burst, Ca^{2+} uptake, and phosphorylation changes are among the earliest responses associated with a host-specific resistance response. Rapid production of ROS, some of which may be generated by a multi-subunit NADPH oxidase complex in the plasma membrane (Xing et al., 1997), is often associated with cell death. Recent research has implicated nitric oxide (NO), together with ROS, in the induction of a HR during plant-pathogen interactions (Shapiro 2005). Generation of elevated levels of NO was shown in tomato plants in response to avirulent root knot nematodes (Melillo 2006b).

Two key components in *R* gene-mediated resistance signaling are SGT1 and RAR1 (Austin et al., 2002; Azevedo et al., 2002; Liu et al., 2002; Peart et al., 2002). In yeast, SGT1 is a component of the SCF (SKP cullin F-box) complex, which is an integral part of protein ubiquitination (Kitagawa et al., 1999). This suggests that protein degradation is implicated in resistance signaling, which is supported by the observation that the *Arabidopsis thaliana* R protein RPM1 is degraded when the elicitor (AvrRpm1 or AvrB) is present (Boyes et al., 1998). Another important protein involved in *R* gene-mediated signaling is HSP90 (heat shock protein 90) (Liu et al., 2004) which directly interacts with SGT1 and RAR1 (Holt et al., 2003; Shirasu and Schulze-Lefert, 2003). RAR1, SGT1 and HSP90 are suggested to form a

chaperone complex mediating the folding of R proteins and their incorporation into functional complexes (Shirasu and Schulze-Lefert, 2003). For *Mi-1*-mediated resistance to aphids and nematodes, it appears that HSP90 is required while RAR-1 is not for either resistance (Kaloshian unpublished results). It was reported that different R proteins varied in their requirement for SGT1 and RAR1 to function. Whether SGT-1 is involved in *Mi-1* mediated resistance remains to be seen.

Another gene specifically required for *Mi-1*-mediated resistance is *Rme-1*, which is unlinked to *Mi-1* and not required for the functioning of other resistance genes like *Pto*. *Rme-1* acts early in the *Mi-1* pathway, either at the same step as the *Mi-1* product or upstream of *Mi-1* (De Ilarduya et al., 2004). Interestingly, the *rme-1* mutant also compromised the *Mi-1* mediated aphid resistance (De Ilarduya et al., 2001). The structure and function of *Rme-1* has to be investigated, including the possibility that Rme-1 is a potential virulence target for nematodes and aphids, guarded by the Mi-1 protein.

Considering the practical applications for isolated resistance genes, it is important to know if they can be transferred to a range of economically important crops where similar resistance is not available. So far, there has been limited success in transferring functional R genes to other species (Williamson and Kumar, 2006). For example, the *Mi-1* gene confers effective resistance against root-knot nematodes and the potato aphid when transferred into susceptible tomato. When introduced into tobacco or *Arabidopsis*, however, it does not confer any of these resistance specificities (Williamson et al., unpublished). On the other hand, a heterologous expression of the *Mi-1* gene in eggplant caused resistance to root-knot nematodes but no resistance anymore to the potato aphid (Goggin et al., 2006). The tomato gene *Hero*, which confers resistance to potato cyst nematodes (PCN), was not effective in potato according to another report (Sobczak et al., 2005).

Thus, the influence of the plant's genetic background can be proposed as a factor determining the heterologous gene functionality, probably through the presence of other gene components necessary for the resistance response (Williamson and Kumar, 2006). It was shown that even within cultivated tomato; genotype differences were influencing the efficacy of *Mi-1* resistance (Jacquet et al., 2005). Understanding this phenomenon will be a challenge but it seems to be necessary for a successful transfer of nematode resistance to a new species. It might also provide insight into host factors that mediate specificity of recognition and signaling (Williamson and Kumar, 2006).

Genomic organization and molecular evolution of nematode *R* gene clusters

Genome-wide sequence analysis and genetic mapping of *R* gene candidates have shown that disease resistance genes are often located in clusters of homologous *R* genes spread throughout the plant genome (reviewed by Gebhardt and Valkonen, 2001). *R* gene clusters from different genotypes and even related species are often located in the same chromosomal region. These regions are therefore called "hot-spots of resistance". Remarkably, in potato, QTL conferring resistance to the potato cyst nematode often co-localize with hot-spots of single dominant resistance genes, suggesting that they may contribute to partial resistance to nematodes. Another option suggests that quantitative resistance is mediated by an *R* gene but that the potato cyst nematode populations used to screen for resistance consist of a mixture of virulent and avirulent genotypes. Most of the cyst nematodes reproduce by obligate outcrossing, and there is generally great variation in host range and response to specific resistance genes between and within field populations (Bakker et al., 1993).

The multigenic nature of most resistance loci may facilitate meiotic instability in a heterozygous state. Unequal crossing-over and gene conversion have been suggested to play a role in the generation of new *R* gene specificities (Hammond-Kosack and Jones, 1997;

Hulbert 1997; Parniske et al., 1997). However, since these processes tend to homogenize the paralogs, divergent evolution must be strong enough to counteract the homogenization process.

With the exception of the beet cyst nematode resistance gene *Hs1*$^{pro-1}$ (Cai et al., 1997), all nematode resistance genes cloned to date reside in complex loci harboring tandemly-repeated *R* gene homologues. The root-knot nematode gene *Mi-1* (Milligan et al., 1998; Vos 1998) is located in a cluster of seven homologous *R* genes on chromosome VI of tomato, whereas the potato cyst nematode resistance gene *Hero* (Ernst et al., 2002) is located in a genomic region containing at least 14 homologous genes on chromosome IV of tomato. The gene *Gro1* (Paal et al., 2004) is also member of a large cluster containing 13 *R* gene homologues located on chromosome VII of potato, whereas the potato gene *Gpa2* (Van der Vossen et al., 2000) is present in a relatively small cluster of four highly homologous genes on chromosome XII.

The specificities of the other members of these nematode *R* gene clusters are unknown except for the *Gpa2* locus in potato. For bacterial and fungal resistance loci, members of an *R* gene cluster often confer resistance to different isolates or strains from the same pathogen species. Interestingly, the *Gpa2* cluster also harbors the resistance gene *Rx1* (Bendahmane et al., 1999), which confers extreme disease resistance to a completely unrelated pathogen, namely the potato virus X. The fact that these two highly homologous resistance genes (88% amino acid identity) reside in the same cluster and on the same haplotype of the diploid potato clone SH83-92-488 (Van Der Voort et al., 1997) strongly suggest that unequal crossing-over and gene conversion play a role in the evolution of the two specificities. The other two members of this cluster are a pseudogene and a putative resistance gene of the unknown specificity.

In contrast to the *Gpa2/Rx1* cluster, sequence analysis of the *Mi-1* locus in tomato did not point at a role for unequal crossing-over and gene conversion (Seah et al., 2004). The *Mi-1* gene is introgressed from the wild relative and supposedly ancestral progenitor Ly*copersicon peruvianum.* Although evidence has been found for an inversion of this locus between the two species, the copy numbers of the homologues in each of the two clusters is conserved.

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Chapter 3

Domain exchange between *Rx1* and *Gpa2* in potato reveals flexibility of CC-NB-LRR genes to switch between virus and nematode resistance

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**equal contribution*

To be submitted

ABSTRACT

Plants are challenged by a myriad of pathogens and to defend themselves they have evolved numerous disease-resistance (*R*) genes, of which most encode a nucleotide binding domain (NB) and a specificity determining leucine-rich repeat region (LRR). NB-LRR proteins are often rapidly evolving molecules and it has been shown that only few changes in the LRR domain are required to alter the resistance specificity towards novel variants of a pathogen. However, little is known about the ability of NB-LRR genes to generate resistance to phylogenetically unrelated pathogens. Here we exchanged the LRR domains of the paralogs Gpa2 and Rx1, which mediate resistance to the cyst nematode *Globodera pallida* and *Potato virus X* (PVX), respectively, in potato (*Solanum tuberosum*). Both *R* genes have a predicted coiled-coil domain (CC) at their amino terminus. The genetic fusion of the CC-NB of *Gpa2* with the LRR of *Rx1* ($Gpa2_{CN}/Rx1_{L}$) showed autoactivation, but lowering the expression levels resulted in extreme resistance to PVX as observed in wild type potato plants. In contrast, transgenic potato expressing the reciprocal construct ($Rx1_{CN}/Gpa2_{L}$) showed a loss-of-function phenotype. Reintroduction of the first 5 LRRs of Rx1 resulted in a gain of resistance, and a mild inhibition of nematode development was obtained similar to wild type resistance to *G. pallida*. Our results show that the CC, NB, and ARC domains are non-pathogen specific modules and support the hypothesis that changing the recognition specificities of LRR domains is sufficient to switch the resistance specificities of NB-LRR genes towards taxonomically unrelated pathogens, irrespective the route of invasion or mode of parasitism.

INTRODUCTION

Plants are constantly exposed to a diverse array of pathogens and parasites that attempt to invade leafs, stems or roots by various mechanisms. To sense foreign invaders, plants have evolved a sophisticated immune system consisting of receptor-like resistance (R) proteins and a more generic microbe-associated molecular pattern (MAMP) recognition system (Holt et al., 2003; Jones and Takemoto, 2004). The dominant *R* genes operate in a gene-for-gene system in which the R proteins limit the growth of viruses, bacteria, fungi and invertebrate pests by triggering a host defence response upon recognition of pathogen-derived elicitors. This recognition may involve a direct interaction between the R protein and its cognate elicitor, or an indirect interaction by sensing elicitor-dependent modifications of host proteins. The subsequent host defence response may include the production of anti-pathogenic compounds, the induction of a reactive oxygen burst and a local programmed cell death, or a so-called hypersensitive response (HR) (Lam et al., 2001). Most known *R* genes encode a nucleotide binding site (NB) and a leucine-rich repeat domain responsible for the direct or indirect recognition of the pathogen. Within the NB-LRR class of disease resistance genes two large families can be distinguished: CC-NB-LRR proteins that have a N-terminal coiled-coil (CC) domain and TIR-NB-LRR proteins with a N-terminal Toll/ Interleukin-1 receptor (TIR) domain (Meyers et al., 2003).

Plant resistance genes of the NB-LRR class are highly polymorphic and are among the most rapidly evolving genes in the genome (Mondragon-Palomino et al., 2002; Cork and Purugganan, 2005). Although mutations are a major source of variation, much of the diversity within resistance gene families arises from intra- and intergenic sequence exchanges that shuffle polymorphic sites between individual genes. While several simple *R* gene loci do

exist, most *R* genes belong to gene families located at complex loci harbouring several tandemly repeated NB-LRR homologs. The occurrence of *R* gene homologs in clusters is thought to promote sequence exchange by gene conversion and unequal crossing-over. A detailed study of the *Rp1* rust resistance complex of maize showed that reshuffling of sequences played a central role in the creation of genetic diversity and even lead to new specificities (Hulbert 1997; Sun et al., 2001; Smith and Hulbert, 2005). Extensive work with the *L, M, N,* and *P* loci in flax demonstrated the role of recombination in the evolution of new recognition specificities to *Melampsora lini* strains (Ellis et al., 1999; Luck et al., 2000; Doddset al., 2001; Dodds et al., 2001).

A key issue in plant pathology is the capacity of plants to generate novel resistance specificities. It has clearly been shown that the LRR domain plays a crucial role in recognizing foreign invaders and only few amino acid changes may alter the recognition specificity towards different variants of a pathogen (Farnham and Baulcombe, 2006; Takken et al., 2006). At complex loci, but also at simple loci, highly similar *R* genes have been found that recognize series of variants of a single pathogen (Hayes et al., 2004). However, the ability of NB-LRR genes to switch resistance specificities between taxonomically unrelated pathogens is largely unknown. Molecular studies to address this issue are hampered, because the vast majority of the *R* gene specificities have not been identified yet. Of all presently known NB-LRR sequences, 149 in the *Arabidopsis* genome, about 400 in poplar and over 500 in rice, relatively few can be coupled to a cognate pathogen (The Arabidopsis Genome Initiative 2000; Meyers et al., 2003; Zhou et al., 2004; Tuskan et al., 2006). Even in clusters containing *R* genes of known specificities, the functions of adjacent paralogs are often unknown. An indication that altering resistance specificities towards widely different pathogens involves relatively few molecular changes, comes from the observation that resistance genes for downy mildew (*RPP8*) and for Turnip Crinckle virus (*HRT*) in *Arabidopsis* are highly homologous and are found at the same genomic position in different accessions (Cooley et al., 2000). Also random *in vitro* mutagenesis in the LRR domain of the *Rx1* gene conferring resistance to *Potato virus X* showed that extending the recognition spectrum to poplar mosaic virus, required only single amino acid changes in the LRR to recognize the related coat protein (Farnham and Baulcombe, 2006).

The *R* genes of the CC-NB-LRR and TIR-NB-LRR families have a well defined modular structure and confer disease resistance through a multistage activation process initiated by the LRR domain in the presence of the elicitor (Takken et al., 2006). Activation of the N-terminal domains leads to the transduction of a yet unknown signal that initiates the defence response. Hence, the flexibility of NB-LRR genes to generate resistance specificities to phylogenetically unrelated pathogens will not only depend on the ability to develop novel recognition specificities by the LRR domain, but also on the ability of the CC, TIR and NB domains to transduce signals that arrest the development of entirely different pathogens. The R proteins Gpa2 and Rx1 are highly homologous and located in the same *R* gene cluster of potato, *Solanum tuberosum,* but confer resistance to two different types of pathogen, the potato cyst nematode *Globodera pallida* and to *Potato virus X* (PVX), respectively (van der Vossen et al., 2000). Potato cyst nematodes penetrate the vascular tissue of the roots and fuse plant cells into multinucleate feeding cells. In resistant *Gpa2* plants the syncytium is surrounded by necrotic cells and the reduced flow of nutrients delays the growth, and finally blocks the development of fertile adult females. PVX, however, is a single stranded RNA virus that is transmitted above ground by insects and other forms of mechanical injury, resulting in systemic infection of the aerial parts of the plant. A striking feature of Rx1 mediated resistance is the rapid arrest of PVX accumulation in the initial infected cells, resulting in symptomless resistance, so-called extreme resistance. Gpa2 and

Rx1 therefore provide an excellent test system to investigate the exchangeability of recognition and signaling domains and explore the evolutionary flexibility of R proteins.

Here, we provide evidence for the hypothesis, that, via intergenic sequence exchanges and various types of mutations, NB-LRR proteins have the potential to alter resistance specificities towards taxonomically unrelated pathogens in relatively short evolutionary time periods. Both the regulatory sequences and CC-NB domains of the paralogs Gpa2 and Rx1 are non-pathogen specific and exchangeable. Remarkably, the genetic fusions of the CC-NB of *Rx1* with the LRR of *Gpa2* ($Rx1_{CN}$/$Gpa2_{L}$) and the reciprocal domain swap ($Gpa2_{CN}$/$Rx1_{L}$) were not functional when driven by the endogenous promoters or 35S promoter. Gain of wild type resistance was obtained by re-introducing the first five LRRs of Rx1 in $Rx1_{CN}$/Gpa2, restoring the compatibility between the N-terminal part of the LRR and the ARC2 domain. Decreasing the expression levels for $Gpa2_{CN}$/$Rx1_{L}$ resulted in extreme resistance against PVX, indistinguishable from wild type plants. Our results indicate that not only coding sequences, but that also optimizing the expression levels may play a role in generating novel resistances.

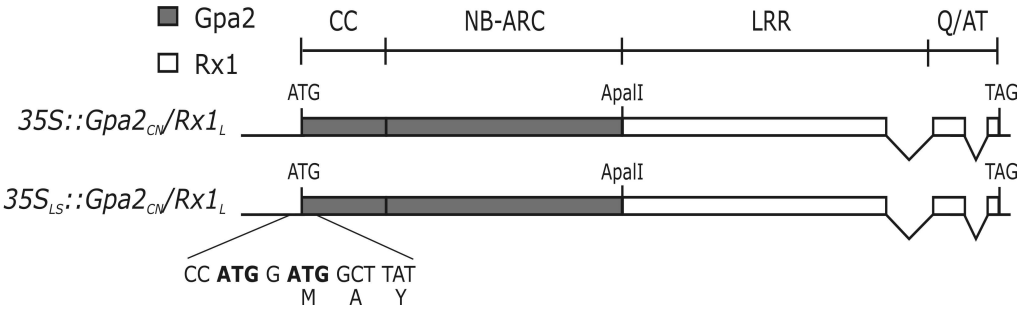
RESULTS

The CC-NB domain of Gpa2 signals extreme resistance to PVX

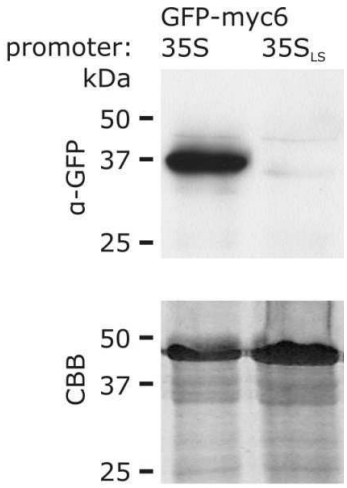
To test the versatility of the various domains of Gpa2 and Rx1 in triggering defense responses to PVX and potato cyst nematodes, a chimeric gene encoding the CC-NB-ARC domain of *Gpa2* and the LRR domain of *Rx1* was created (Fig. 1A). However, under control of the double enhanced CaMV 35S promoter this construct results in a constitutive cell death response in an agroinfiltration assay on leaves of *Nicotiana benthamiana* (Rairdan and Moffett, 2006). To see if attenuating the expression level would dicrease the autoactive response, we introducted an out of frame start codon upstream of the original translation initiation site (Fig. 1A). Hence, translation of the *R* gene reading frame becomes dependent on leaky scanning (LS) by the ribosome (Kozak 1995; Kozak 1999), resulting in a strong reduction of the level of correctly translated protein. The effect of the leaky scanning ($35S_{LS}$) promoter was evaluated by the expression of GFP construct under control of the 35S promoter and the $35S_{LS}$ promoter and comparing the protein levels by Western blots. As expected the $35S_{LS}$ promoter showed a strong reduction of the expression of GFP (Fig. 1B). Expression of the recombinant protein $Gpa2_{CN}$/Rx_{L} in an agroinfiltration assay on leaves of *N. benthamiana* under control of the $35S_{LS}$ promoter showed that the protein levels were now below the autoactivation threshold (Fig. 1C**)**. The construct, however, is at these protein levels able to induce a specific HR in the presence of the avirulent coat protein of PVX (Fig. 1D).

Transgenic potato plants harboring the $35S_{LS}$::$Gpa2_{CN}$/$Rx1_{L}$ were created to test for PVX resistance. The potato clone SH containing the endogenous *Rx1* gene and a transgenic line containing the *Rx1* gene under control of the 35S promoter were included as resistant controls. Plants were inoculated with either the avirulent strain PVX_{UK3} or the virulent strain PVX_{HB} and three weeks after inoculation the compound leaves near the shoot apex were harvested for virus detection using ELISA. Figure 1E shows that no detectable amounts of the avirulent PVX strain could be observed in the transgenic plants expressing the recombinant gene $35S_{LS}$::$Gpa2_{CN}$/$Rx1_{L}$, as was the case for the resistant control plants. In the susceptible control plants, however, large amounts of PVX_{UK3} could be detected, indicating that the inoculation with the avirulent strain was successful. Infection of the plants with the *Rx1*-

resistance breaking strain resulted in systemic spreading of the virus in all plants, although reduced in the plants expressing *Rx1* from the 35S promoter. These results show that $Gpa2_{CN}/Rx1_{L}$ confers extreme resistance to PVX in shoots of potato in a gene-for-gene specific manner like the original *Rx1* gene. These data support earlier findings that the recognition specificity of *Rx1* is determined by the LRR domain (34), but more interestingly, that the CC-NB-ARC domain of the nematode resistance gene *Gpa2* is able to activate an extreme resistance response against potato virus X.



A



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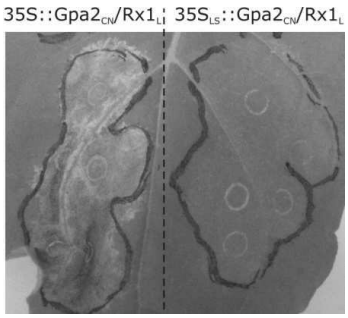


C

	CP106	**CP105**	**YFP**
35S::Rx1	HR	-	-
35S::Gpa2	-	-	-
35S:: $Gpa2_{CN}/Rx1_{L}$	HR	HR	HR
$35S_{LS}$::$Gpa2_{CN}/Rx1_{L}$	HR	-	-

D

	PVX-UK3	PVX-HB
Rx1 (SH)	0.038 ± 0.020	2.2 ± 0.066
35S::Rx1	0.042 ± 0.019	0.52 ± 0.090
$35S_{LS}$::$Gpa2_{CN}/Rx1_{L}$	0.031 ± 0.011	1.3 ± 0.6
rx1 (lineV)	2.9 ± 0.072	2.0 ± 0.47

E

Figure 1
A. The reciprocal domain swap construct $Gpa2_{CN}/Rx1_{L}$ was obtained by exchanging the LRR domain of *Gpa2* with the corresponding domain of *Rx1* using the *Apal*I restriction site in the context of a CaMV 35S promoter cassette for expression in plants. A second translation initiation site was introduced in p$35S_{LS}$::$Gpa2_{CN}/Rx1_{L}$ to obtain leaky scanning of ribosomes (39, 40) and a subsequent reduction of the expression levels of the protein.

B. Comparison of the expression levels of the green fluorescent protein GFP-myc6 under control of the CaMV 35S and the leaky scanning $35S_{LS}$ promoter in an agroinfiltration assay. Leaf protein extracts were separated on SDS-PAGE followed by western blotting and detection of the protein by a polyclonal anti-GFP peroxidase-conjugated antibody (α-GFP) or Coomassie Brilliant Blue staining (CBB). The GFP specific band is indicated by an arrow.

C. Agroinfiltration of *Nicotiana benthamiana* leafs with p35S::$Gpa2_{CN}/Rx1_{L}$ results in a constitutive cell death response in the absence of the PVX elicitor, whereas no such autoactivition response was observed for $35S_{LS}$::$Gpa2_{CN}/Rx1_{L}$.

D. Agroinfiltration of *Nicotiana benthamiana* leafs with 35S::$Gpa2_{CN}/Rx1_{L}$ and $35S_{LS}$::$Gpa2_{CN}/Rx1_{L}$ in the presence and absence of the virulent and avirulent PVX elicitor CP105 and CP106, respectively. Expression of the wild type *R* genes *Rx1* and *Gpa2* under control of the normal CaMV 35S promoter were included as a positive and negative control. HR = hypersensitive response.

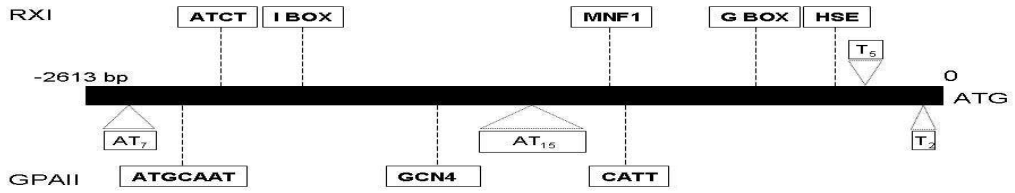
E. A greenhouse virus resistance assay was performed on transgenic potato plants expressing the wild type *Rx1* gene under control of the CaMV 35S promoter and the domain swap construct $Gpa2_{CN}/Rx1_{L}$ under control of the leaky scanning $35S_{LS}$ promoter. The diploid potato clone SH, which contains the endogenous *Rx1* gene, was used as the resistant control and the diploid potato clone lineV, which was used for the transformation of the constructs, was used as susceptible control. Leaf material was collected from secondary infected leafs of the plant apex three weeks after infection with the avirulent strain PVX_{UK3} or the virulent strain PVX_{HB} and systemic spreading of the virus in the plants was detected by ELISA.

Both the endogenous promoters of Rx1 and Gpa2 are able to drive virus and nematode resistance

Comparison of the flanking sequences of Gpa2 and Rx1 showed that the sequences upstream of the start codon share more similarity than the sequences downstream of the ORF. Analyses of an approximately 2.6 kb DNA fragment upstream of the start codon revealed two extra TA-rich regions in the Gpa2 sequence at -2458bp (TA_7) and at -1329 (TA_{15}), which are predicted to function as an enhancer, and two small indels just upstream of the start codons (Fig. 2A). The remaining sequences show a similarity of about 97% and only differ in a number of single base pair substitutions. For this region, a number of *cis* acting regulatory elements (CARE) was predicted including an AT-rich element (binding site for AT rich DNA binding protein ATBP-1) and AT-rich sequence (for maximal elicitor-mediated activation), an ethylene (ERE), auxin (TGA) and wound (WUN) responsive element. Analysing the genomic sequence +298 bp downstream of the stop codon revealed that the 3'UTR regions of Rx1 and Gpa2 were identical until +160 followed by a more variable region containing 8 single base pair substitutions and two small indels of 1 and 2 nucleotides in case of Gpa2 and one indel of 3 nucleotides for Rx1 (Fig. 2B).

The homologous DNA fragment upstream of the start codon of *Rx1* (2571 bp) and *Gpa2* (2613 bp) were tested for promoter activity in an agroinfiltration assay in leaves of *N. benthamiana*. Expression of *Rx*1 under control of either the *Rx1* promoter region (p*RXI*) or the *Gpa*2 promoter region (p*GPAII*) in combination with the corresponding terminator sequences (298 bp) resulted in the detection of an HR within 2 dpi in the presence of the avirulent elicitor CP106. A similar response was observed for the original genomic BAC clone harboring *Rx1* (Fig. 3A). Subsequently transgenic potato plants harbouring pGPAII*::Rx*1 and pRXI::*Rx*1 were tested in a virus resistance assay. No systemic spreading of the avirulent strain PVX_{UK3} was detected, whereas an accumulation of the virulent strain PVX_{HB} was observed using ELISA (Fig. 3B). From this experiment, it was concluded that the original *Rx1* promoter activity was retained in the selected DNA fragment and that the *Gpa2* promoter is able to drive *Rx1*-mediated extreme resistance against PVX in the shoots of potato.

The activity of the putative *Gpa2* promoter was tested in transgenic potato plants harbouring pGPAII::*Gpa2* upon nematode infection in the greenhouse. This resulted in almost a complete reduction of the number of females on roots of transgenic plants infected with the avirulent nematode population D383 compared to plants infected with the virulent nematode population Rookmaker **(**Fig. 3C). A more mild resistance response was obtained for the potato clone SH containing the endogenous *Gpa2* gene, which shows that the original *Gpa2* promoter activity is retained in the selected DNA fragment. In addition, transgenic plants harbouring pRXI::*Gpa*2 were included in the nematode resistance test, to see whether the *Rx1* promoter is able to drive *Gpa2*-mediated nematode resistance. This resulted in a similar reduction in the number of cysts on plants infected with the avirulent nematode population D383, whereas normal nematode development was observed on roots infected with the virulent population Rookmaker**.** These data show that the *Rx1* and *Gpa2* promoter and terminator sequence are interchangeable and able to drive either nematode resistance in the roots as well as virus resistance in the shoot of potato. Apparently, transcriptional regulation of separate members from a single R gene cluster can be highly conserved and independent of their recognition specificity (virus vs. nematode) or target tissue (shoots vs. roots).



A

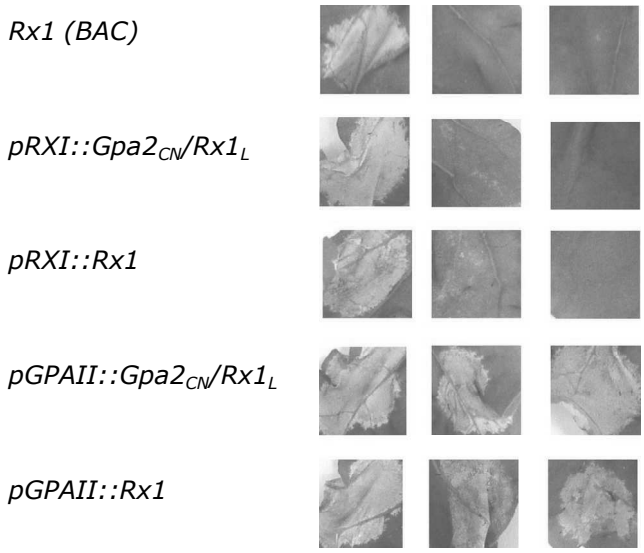
Cis acting regulatory elements	*Rx1*	*Gpa2*	Function
AT-rich element	- 658	- 672	Binding site for AT rich DNA binding protein ATBP-1
AT-rich sequence	- 1219	- 1234	Element for maximal elicitor-mediated activation
ERE	- 1333	- 1378	Ethylene-responsive element
TC-rich repeats	- 2488	- 2528	Involved in stress and defense responsiveness
TGA element	- 766	- 780	Auxin-responsive element
WUN motif	- 1944	- 1989	Wound responsive element

B

Figure. 4
A. Schematic representation of the Rx1 and Gpa2 promoter region, which are highly homologous in a ~2600 bp region upstream of the ATG start codon (97.3 % identity). Two extra TA-rich regions are present in the Gpa2 promoter (AT_7 at – 2458 bp and AT_{15} at -1329 bp) and two small indels (T_5 and T_2) are located just upstream of the start codon (-207 bp and -6 bp, respectively). Various single base pair substitutions are distributed over the promoter region, resulting in the prediction of several additional *cis* acting regulatory elements (PlantCARE) for either the Rx1 or Gpa2 promoter. Most elements have a function in light responsiveness, except for HSE, and are therefore most likely not directly involved in the regulation of Gpa2 and Rx mediated resistance.
B. PlantCARE prediction of several *cis* acting regulatory elements involved in plant defense and stress in the *Rx* and *Gpa2* promoter regions (-2573 bp and -2613 bp, respectively).

To test whether the nematode resistance responses mediated by the transgenic lines harboring the *GPAII::Gpa2* and *35S::Gpa2* were indistinguishable from the wild type, roots of *in vitro* grown transgenic plants were infected with avirulent pre-parasitic second stage juveniles of *G. pallida* D383 for microscopic observations (Fig. 4). As a control, roots were infected with the virulent population Rookmaker resulting in normal nematode development on all roots (Fig. 4A). In the wild type resistant roots of SH, however, a variable and mild resistance response was observed resulting in the arrest of nematode development also in later stages of their life cycle, although occasionally some avirulent nematodes were able to develop on resistant roots (Fig. 4B). The encapsulation of the induced feeding cell by a layer of necrotic cells resulted in the starvation of the developing nematodes and subsequently, the

appearance of a small number of translucent undeveloped adult females (Fig. 4C). The majority of the infective juveniles, however, were arrested by a hypersensitive-like response at the feeding site (Fig. 4D). This response explains also the detection of a low number of adult females on the roots of SH in the greenhouse resistance assay, whereas hardly any females were detected on roots of the transgenic resistant plants. Normal development of adult females was observed on the roots of the susceptible potato Line V, which was used for transformation (Fig. 4E). It was noticed that nematode development was completely inhibited by a local cell death response at the feeding sites in transgenic roots when Gpa2 was expressed under control of the native GPAII promoter (Fig. 4F) or the 35S promoter (Fig. 4G and H). These data suggest that expression of Gpa2 in the background of the potato genotype line V is more effective than in the potato clone SH.



A

construct	**PVX$_{UK3}$** value A_{405} ± SD	**PVX$_{HB}$** value A_{405} ± SD
Rx1$^{\#}$ (BAC)	0.041 ± 0.045	1.7 ± 0.57
pRXI::Rx1	0.052 ± 0.035	1.3 ± 0.39
pGPAII::Rx1	0.025 ± 0.024	0.46 ± 0.32

B

Construct	**D383** No. cysts ± SD	**Rookmaker** No. cysts ± SD
Gpa2 (SH)	8 ± 4	316 ± 130
pGPAII::Gpa2	2 ± 1.9	439 ± 235
pRXI::Gpa2	0.2 ± 0.4	435 ±302
pGPAII::Rx1$_{CN}$/Gpa2$_{L}$	261 ± 162	482 ± 150
pRXI::Rx1$_{CN}$/Gpa2$_{L}$	307 ± 156	665 ± 295
empty (Line V)	729 ± 378	305 ± 0

C

Figure 3
A. Agroinfiltrationassay on *N. benthamiana* leafs of *Rx1*, $Gpa2_{CN}/Rx1_{L}$ and $Rx1_{CN}/Gpa2_{L}$ when coexpressed with the Rx1 elicitor CP106, the virulent control CP105 and YFP as negative control. The chimeric constructs were expressed from the endogenous RXI and GPAII promoters. The original BAC clone harboring *Rx1* was used as a positive control. Images were taken 7 days post infiltration.
B. Greenhouse virus resistance assay: mean absorbance values (A405) are shown of homogenate of secondary compound leaves in ELISA of transgenic potato plants. Genes were expressed from the 2.8 kb of 5'-UTR sequence of the wt *Rx1* gene (pRXI) or 2.8 kb of 5'-UTR sequence and 0.5 kb of 3'-UTR sequence of the wt *Gpa2* gene (pGPAII). Leaves were harvested three weeks after primary leaf inoculation with PVX_{UK3} or PVX_{HB}. Four to tvelwe plants from 2 to 4 independent lines were assayed per construct.
C. Greenhouse nematode resistance assay on transgenic potato plants harboring the *Gpa2* gene and the domain swap construct $Rx_{CN}/Gpa2_{L}$ under control of the endogenous *Rx1* or *Gpa2* promoter and terminator. Plants were tested with the avirulent Pa_2-D383 population and the virulent population Pa_3-Rookmaker of the potato cyst nematode *Globodera pallida*. Three independent transgenic lines were assayed in multiple replicates for each transgene. Cysts were counted on these plants at 16 weeks post inoculation and the average number ± SD are shown. Plants were scored resistant when the number of cysts found on the roots of the plants was < 20.

The CC-NB-ARC domain of Rx1 signals mild nematode resistance to *G. pallida*

For the chimeric $Gpa2_{CN}/Rx1_{L}$ construct we showed that it is autoactive when driven by the 35S promoter, but regains its wild-type phenotype when lowering the expression level by reducing its translation efficiency. To study the effect of expression levels on the exchangeability of the Gpa2 and Rx1 CC-NB and LRR domains in more detail, the domain swap construct was expressed under control of its endogenous promoter and terminator regions. When expressed under control of the native regulatory sequences of both Gpa2 and Rx1 the $Gpa2_{CN}/Rx1_{L}$ construct exhibited a consitutive cell death response in a transient assay on leaves of *N. benthamiana* (Fig. 3A). Consequently, no stable transgenic potato plants could be generated for this construct in an *Agrobacterium*-mediated plant transformation assay. Apparently, the expression level of this domain swap construct under control of its native promoter sequence was still above the activation treshold.

To test the hypothesis if the CC-NB domain of Rx1 was able to mediate nematode resistance to the potato cyst nematode *G. pallida*, a reciprocal construct consisting of the CC-NB region of Rx1 and the LRR region of Gpa2 was constructed (*GPAII::*$Rx_{CN}/Gpa2_{L}$). As the cognate elicitor of *Gpa2* is unknown, the functionality of the recombinant gene product was tested in a nematode resistance assay. When $Rx1_{CN}/Gpa2_{L}$ was expressed from the native Gpa2 and Rx1 promoter and terminator sequences, the chimeric gene lost its ability to mediate nematode resistance in transgenic potato plants (Fig. 3C). Proper expression of the transgene was confirmed by RT-PCR (data not shown). Real time RT-PCR was performed, showing that expression from the GPAII promoter leads to 64 times lower transcript levels when compared with expression from the 35S promoter (Fig. 5A). This was confirmed by the detection of a 50 to 100 times lower expression level of the 27-kD green fluorescent protein (GFP) on anti-GFP Western blots (Fig. 5B). Furthermore, the discrepancy between the functionality of the wild-type Gpa2 protein and the chimeric $Rx1_{CN}/Gpa2_{L}$ protein could not be explained by a difference in protein stability. Western blot analyses of GFP-Gpa2 and GFP-$Rx1_{CN}/Gpa2_{L}$ showed that both proteins accumulated to similar levels (Fig. 5C). These data show that the endogenous regulatory sequences are not able to drive functional expression of this chimeric protein.

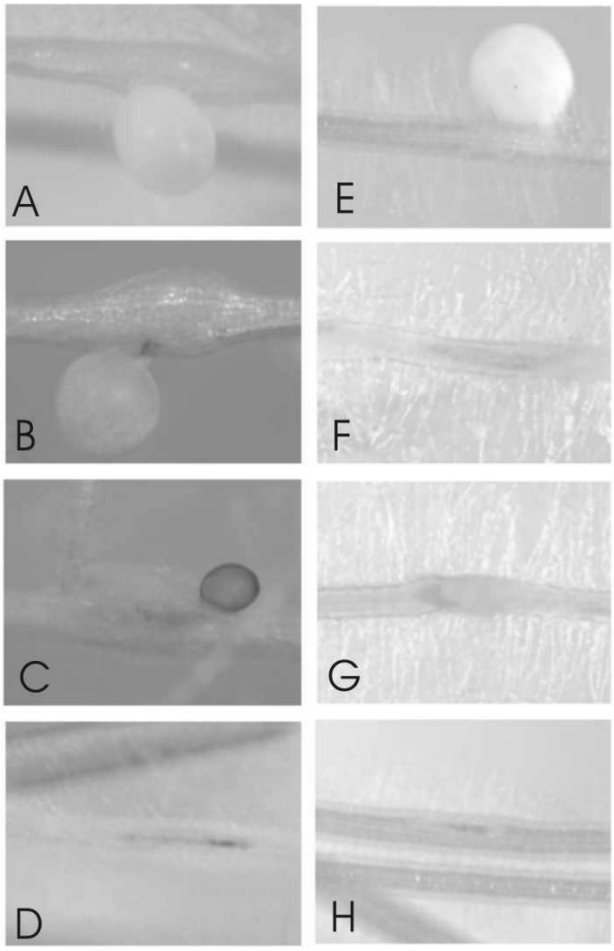


Figure 4

A. Normal development of adult females of the virulent *Globodera pallida* population Rookmaker on in vitro grown plants of the diploid potato cone SH harboring the endogenous *Gpa2* gene.

B. A small number of the infective nematodes from the avirulent *G. pallida* population D383 develop into normal adult females on resistant roots of SH.

C. The mild *Gpa2* resistance response in SH results in an arrest in nematode development resulting in typical undeveloped translucent females for D383.

D. The majority of the infective nematodes of D383 is blocked in SH by a local cell death response at the onset of parasitism.

E. Infective nematodes from D383 develop into normal adult females on susceptible roots of transgenic control plants (line V) harboring an empty vector.

F. On transgenic potato roots harboring either the GPAII::Gpa2 (F) or 35S::Gpa2 (G and H) constructs, nematode development was also inhibited by a local hypersensitive response at the feeding site.

To investigate whether we could regain wild type nematode resistance by increasing the expression level of the chimeric protein $Rx1_{CN}/Gpa2_{L}$, its function was tested under control of the stronger CaMV 35S promoter and the Tnos terminator (Fig. 6A). Greenhouse experiments showed that transgenic plants expressing the *Gpa2* gene under control of the CaMV 35S promoter and the Tnos terminator of were resistant to the avirulent population D383 of the

potato cyst nematode *Globodera pallida*, but susceptible to the virulent *G. pallida* population Rookmaker. Similar results were obtained for the wild-type resistant potato clone SH (*Solanum tuberosum* ssp. *andigena*) harbouring the *Gpa2* gene (Fig. 6B). The potato line V, a susceptible diploid potato clone used to create the transgenic resistant plants, was susceptible in all cases as expected. For transgenic potato plants harboring Gpa2 under control of the 35S and native GPAII promoter, a similar wild type resistance response was observed when tested under *in vitro* conditions (Fig. 6C) However, transgenic plants harboring an N-terminal GFP fusion with $Rx1_{CN}/Gpa2_{L}$ under control of both the constitutive CaMV 35S and GPAII promoter resulted in a similar loss of function phenotype as observed for this chimera under control of the endogenous regulatory sequences. Apparently, enhancing the expression levels could not compensate for the lack of functionality of this chimera.

Previously, it was demonstrated for an autoactive construct identical to $Gpa2_{CN}/Rx1_{L}$ that restoration of the compatibility between the N-terminal end of the LRR of Gpa2 and its ARC2 domain was essential for proper gene function (Rairdan et al., 2006). Therefore, the first five LRRs of Rx1 (L5) were re-introduced to see if we could restore wild type nematode resistance. Infection of transgenic *in vitro* plants harboring the construct $Rx_{CNL5}Gpa2_{L6\text{-}15}$ under control of the 35S promoter and Tnos terminator resulted in wild type resistance to the avirulent *G. pallida* population D383 (Fig. 6D). A similar phenotype was obtained for Gpa2 under the same experimental conditions. These data demonstrate that the compatibility between the N-terminal end of the LRR of Rx1 with its ARC2 domain is required for functionality of the chimera. Furthermore, it is concluded that the remaining part of the LRR region of Gpa2 is the sole determinant of nematode recognition. Fusion of this moiety to the CC-NB-ARC-L5 region of Rx1, normally involved in the activation of extreme resistance against PVX, results in a functional recombinant R protein conferring pathotype-specific nematode resistance.

DISCUSSION

Exchanging the recognition specificity determining LRR domain of Gpa2 and Rx1 showed that the CC and NB domains could both mediate extreme virus resistance in the shoots and a mild nematode resistance response in the roots of potato. To our knowledge, this is the first example of the formation of functional bidirectional chimeric R proteins between two members of a single *R* gene cluster that confer resistance to two completely unrelated pathogens with distinct modes of parasitism and different routes of invasion. Most *R* genes are located in clusters in the plant genome and evolve via single base substitutions, small deletions/insertions, and intra- and intergenic sequence exchanges (Baumgarten et al., 2003; Kuang et al., 2004; Leister 2004). The exchange of functional domains between two R genes without disturbing pathogen recognition and disease signaling as shown in this paper provides experimental evidence for the hypothesis that divergent selection at complex *R* gene loci may result in resistance specificities to radically different pathogens, irrespective the recognition specificity of the parental *R* genes. Apparently, the structural backbone of these modular proteins forms a framework in which intergenic sequence exchange is allowed, but our experiments also point out the functional constraints that act on the generation of effective R proteins by intergenic recombination. The observation that the CC and NB-ARC domains of Gpa2 and Rx1 are versatile modules that can mediate resistance to widely different pathogens is corroborated by the recent finding that the *Arabidopsis* resistance gene *RPP13* is able to confer resistance to transgenic strains of *Pseudomonas syringae* and turnip mosaic virus carrying the cognate effector *ATR13* from the oomycete *Hyaloperonospora parasitica* (Rentel et al., 2008).

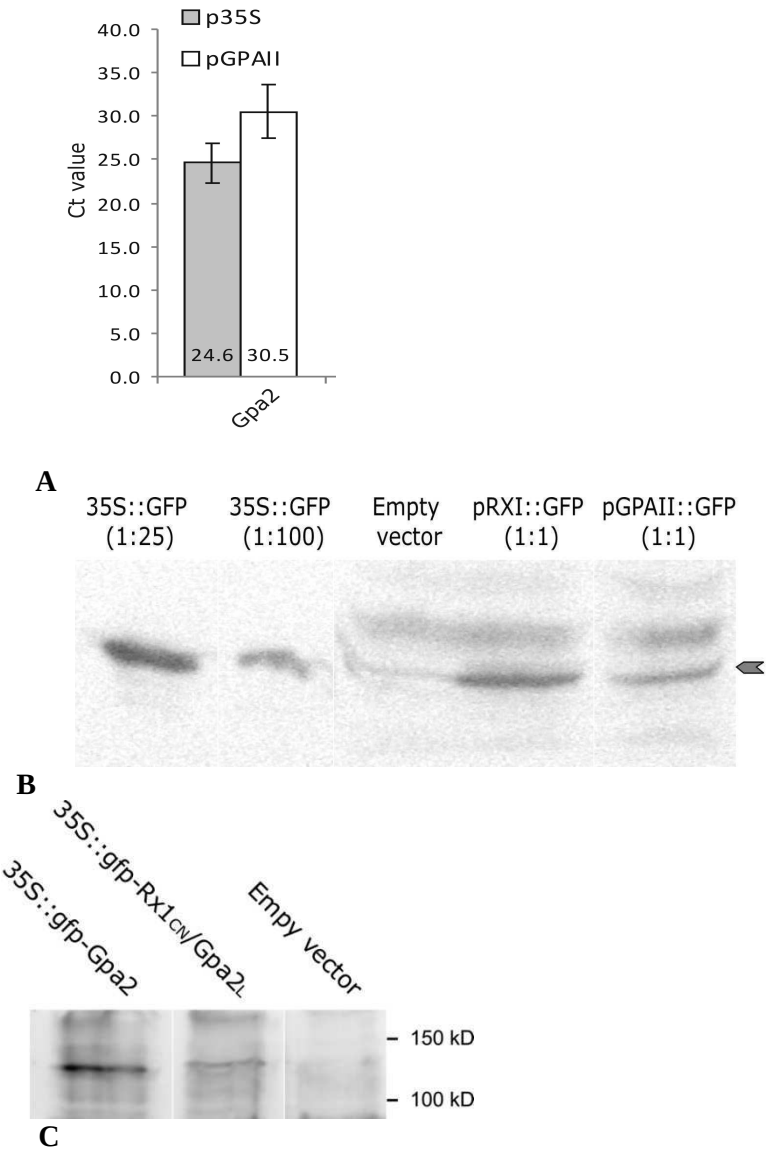


Figure 5

A. Real-time RT-PCR was performed to compare transgene expression under control of the CaMV 35S promoter or the endogenous *Gpa2* promoter upon agroinfiltration in *N. benthamiana* leafs resulted in a ΔCt of about 6. No significant differences were observed between the wild type *Gpa2* gene and the chimeric constructs $Rx1_{CN}/Gpa2_L$. Results were obtained in two independent experiments.

B. Comparison of protein production under control of the 35S promoter and the GPAII promoter on Westernblot shows that the amount of protein is significant lower (about 100 fold) for constructs driven by the endogenous promoter.

C. Detection of the chimeric protein GFP-$Rx1_{CN}/Gpa2_L$ on Westernblot ith anti-GFP antibody after agroinfiltration shows that it is produced in similar amounts as the wild type GFP-Gpa2 protein *in planta*

A

Potato line	**D383** (No. cysts ± SD)	**Rookmaker** (No. cysts ± SD)
SH (resistant)	8 ± 4	316 ± 130
35S::Gpa2	0.1 ± 0.2	599 ± 293
Line V (susceptible)	729 ± 378	305 ± 0

B

Potato line	**D383** (No. cysts ± SD)
GPAII::Gpa2	0
35S::Gpa2	0
GPAII::Rx_{CN}/$Gpa2_L$	43 ± 15
GPAII:: GFP-Rx_{CN}/$Gpa2_L$	49 ± 29
35S::GFP-Rx_{CN}/$Gpa2_L$	18 ± 20
Line V (susceptible)	31 ± 21

C

Potato line	**D383** (No. cysts ± SD)
GPAII::Gpa2	0
35S::Gpa2	0
35S::$Rx_{CNL5}Gpa2_{L6\text{-}15}$	0.9 ± 1.1
Line V (susceptible)	22 ± 6.5

D

Figure 6
A. The domain swap construct *$Rx1_{CN}$/$Gpa2_L$* was obtained by exchanging the LRR domain of *Rx1* with the corresponding domain of *Gpa2* via the *Apal*I restriction site.
B. Greenhouse nematode resistance assay on transgenic potato plants harboring the *Gpa2* gene and the domain swap construct *$Rx1_{CN}$/$Gpa2_L$* under control of the CaMV 35S promoter and Tnos terminator. The diploid potato clone SH, which contains the wild type *Gpa2* gene, was used as a resistant control plant. The diploid potato clone line V, which was used to create the transgenic plants, was used as a susceptible control. Plants were tested with the avirulent Pa_2-D383 population and the virulent population Pa_3-Rookmaker of the potato cyst nematode *Globodera pallida*. Three to five independent transgenic lines were assayed in multiple replicates for each transgene. Cysts were counted on these plants at 16 weeks post inoculation and the average number ± SD are shown. Plants were scored resistant when the number of cysts found on the roots of the plants was < 20.

Both CC-NB-ARC domains of Rx1 and Gpa2 are able to facilitate an extreme and a mild resistance response. This striking difference between the two resistance phenotypes suggests

that the pathogen determines to some extend the outcome of the resistance response. The effectiveness of *Globodera pallida's* secreted effectors in suppressing plant immunity provides a plausible explanation. Pathogens like bacteria, fungi, oomycetes and nematodes secrete an impressive array of proteins of which many are thought to be involved in suppressing plant defenses (Gurlebeck et al., 2005; Ridout et al., 2006; Thomas 2006; Truman et al., 2006; da Cunha et al., 2007; He et al., 2007). However, not all resistance responses to feeding cell-inducing nematodes are mild. The resistance proteins Hero and Mi-1.2 respond with a fast HR upon nematode infection (Sobczak et al., 2005; Williamson and Kumar, 2006). Another explanation for the milder Gpa2 response is that both the concentration of the elicitor and the efficiency of the recognition by the LRR domains play a role in eventual response levels.

Our data indicate that no pathogen-specific barriers on the level of pathogen-specific responses may exist within *R* gene clusters and that *R* gene clusters may generate resistances to novel pathogens in relatively short evolutionary time scales.

Assays on transgenic potato plants showed that the endogenous promoters of *Gpa2* and *Rx1* are exchangeable and that the resistance phenotypes were indistinguishable from their wild types. This means that the regulatory sequences for both genes allow for proper expression in under- and aboveground plant tissues, and pose no limitation to the formation of new specificities against pathogens with diverse lifestyles. This could also explain why most *R* genes are constitutively expressed at low levels throughout the plant, even in tissues that are normally not invaded by the cognate pathogens. Exchangeability of regulatory sequences of R gene homologs in one cluster provides additional versatility to adapt quickly to a wide range of pathogens. For example, the *R* gene *Mi-1.2* is expressed constitutively at low levels in all plant parts and confers resistance against nematodes and aphids and whitefly.

Recently, it was shown that RanGAP2 binds to the CC domains of both Rx1 and Gpa2 (Sacco et al., 2007). Its presence is necessary for full Rx1-mediated PVX resistance (Tameling and Baulcombe, 2007), and its overexpression has an activating effect on Rx1 (Sacco et al., 2007). The N-terminal domain of several R proteins has been shown to be the binding place of a guarded host protein, a role that links it to pathogen recognition (Mackey et al., 2003; Mucyn et al., 2006; Ade et al., 2007). If Rx1 and Gpa2 are actually guarding RanGAP2, this would imply that exchanging specificities by exchanging the LRRs was only possible because they guard the same host protein which is targeted by both pathogens. Although a role for RanGAP2 in *Gpa2*-mediated resistance has not been shown yet, it could be a virulence target for *G. pallida*. Both PVX and potato cyst nematodes recruit the plant cell machinery for their own benefit and reproduction. In that case the specific recognition by the LRR could be triggered via a pathogen specific modification of the guarded protein or a specific interaction with the elicitor-guardee complex.

It is likely that the principles we observed in this study play a prominent role during the evolution of R proteins. The constitutively active phenotypes we observed for several chimeric constructs show that sequence divergence and coevolution between domains constrain the possibilities for reshuffling sequences within R gene clusters. The autoactivity presents a strong selection factor as was illustrated in this study by the inability to regenerate transgenic potato plants with the constitutively active R gene constructs. However, regulation of transcript levels, translation efficiency or protein stability may assuage the effects of domain incompatibility in newly formed chimeras as demonstrated in this study. This presents us with a model of R gene evolution wherein recognition specificity, activation sensitivity, and protein concentration together determine the eventual resistance response.

MATERIALS AND METHODS

DNA constructs

For expression under the control of the double enhanced CaMV 35S promoter and Tnos terminator, Rx1 was amplified from the binary plasmid pBINRx1 (van der Vossen et al., 2000) using the primers 5GpRxbn and Rxrev (Table 1) and cloned into the NcoI-SalI sites of pUCAP (van Engelen, Molthoff et al., 1995), resulting in pUCAPRx1. For *Gpa2*, the proximal end was amplified from pBINRGC2 (van der Vossen et al., 2000) with the primers 5GpRxbn and GpRxSturev (Table 1) to generate a NcoI-AvrII fragment, which was cloned together with an AvrII-PstI fragment from pBINRGC2 into the NcoI-PstI digested pUCAPRx1.

The Rx1 3'UTR (transcription termination) region was amplified from pBINRx1 using the primers 5UTRkp and 3UTRrev (Table 1) and cloned into the KpnI-PacI sites of the reporter plasmid pUCAPYFP, replacing Tnos. Next, the promoter region of *Rx1* (2805 bp between the XbaI site and ATG startcodon) was cloned in two steps. First, the region between the DraIII site (-1429 bp) and the startcodon was amplified from pBINRx1 using the primers bRxAdeIf and RxbnREV (Table 1) and second, the DraIII-NcoI fragment was cloned together with the 1431 bp AscI-DraIII fragment of pBINRx1 into pUCAPYFP, replacing p35S (AscI-NcoI). The *Gpa2* 3'UTR region was amplified from pBINRGC2 using the primers 5UTRkp and 3UTRrev (Table 1) for cloning in the KpnI-PacI sites of pUCAPYFP, replacing Tnos. The *Gpa2* promoter region was constructed in two steps. First, the region between the BstZ17I (SnaI) site (-2744 bp) and the startcodon was amplified from pBINRGC2 using the primers bGpaSnaIf and GPbnREV (Table 1). This BstZ17I-NcoI fragment was cloned alongside the 720 bp PacI-BstZ17I fragment of pBINRx1, fused to a PacI-AscI adapter consisting of AD1 and AD2 (Table 1), into the AscI-NcoI digested pUCAPYFP with Gpa2 3'UTR after digestion with AscI-NcoI. Thereafter, the YFP sequence was subsequently replaced by the coding sequence of *Rx1* and *Gpa2* via the NcoI and KpnI restriction sites.

The domain swap constructs $Gpa2_{CN}/Rx1_{L}$ and $Rx1_{CN}/Gpa2_{L}$ were made by exchanging the LRR fragments of *Gpa2* and *Rx1* using the unique ApaLI and PstI site, which are conserved and situated in the beginning and the end of the LRR encoding region of the genes, respectively.

The N-terminal GFP fusion constructs were created by first providing GFP with NcoI and SstI–KpnI sites and cloning of this fragment in pUCAP. Then the AscI-SstI (35S::GFP) was cloned with a 12 amino acids encoding linker (-GGGSGGGSGGGS-) into the pGPAII driven Rgene constructs.

The leaky scan construct $35S_{LS}$::$Gpa2_{CN}/Rx1_{L}$ was created following the same procedure as for 35S::$Gpa2_{CN}/Rx1_{L}$, but in this case the Gpa2 sequence was amplified with Gpa2LSFor instead of 5GpRxbn as forward primer. For the leaky scan GFPmyc6 construct $35S_{LS}$::GFPmyc6, GFP was amplified with the primer pair 5nGFP and 3CFP. The PCR fragments were transferred as NcoI-SstI fragments into pRAPmyc6, pGPAIImyc6 and pRXImyc6. The 6 fold myc-tag, present in these vectors was built from 3 tandem repeats generated by triple fusion of the NheI-SpeI fragments of the annealed oligos mMYC1 and mMYC2 (Table 1).

The PVX coat proteins CP106 and CP105 were amplified from the PVX amplicons pGR106 (Jones, Hamilton et al., 1999) containing cDNA of the *Rx1*-avirulent PVX strain UK3 and pGR105 containing cDNA of the *Rx1*-resistance breaking strain HB (Goulden, Kohm et al., 1993), respectively, using the primers 5UK3cp and 3UK3CP (Table 1) for CP106 and 5HBcp and 3HBCP (Table 1) for CP105. The products were cloned into the NcoI-KpnI sites of pUCAP between the CaMV 35S promoter and the Tnos terminator.

For agro-infiltration assays and *Agrobacterium tumefaciens* – mediated plant transformation, the expression cassettes containing the constructs were cloned into the AscI and PacI sites of the binary vector pBINPLUS (van Engelen et al., 1995) and transformed to *A. tumefaciens* (pMOG101).

Agroinfiltration assays

Agrobacterium tumefaciens strain pMOG101 were cultured for agroinfiltration as described earlier (Van der Hoorn, Laurent et al., 2000). For co-infiltration experiments, cultures were mixed prior to infiltration. Leaves were infiltrated of 6 weeks old *Nicotiana benthamiana* plants grown in the

greenhouse at 20°C and 16 hours of light. Each combination was tested at least *in duplo* on two different plants in at least two independent experiments.

Plant transformation
The susceptible diploid potato line V was used for *Agrobacterium*–mediated plant transformation as described (van Engelen et al., 1994). Genomic DNA was extracted using the Dneasy plant mini kit (Qiagen) for PCR to analyse the incorporation of the transgene in the plant genome. RNA was extracted using Trizol LS Reagent (Life Technologies) for RT-PCR using the Superscript TM First strand synthesis system (Life Technologies) to test expression of the transgene with gene specific primers.

Virus resistance test
To obtain infectious virus particles, leaves of *Nicotiana benthamiana* were agroinfiltrated with the PVX amplicons pGR106 and pGR105. Systemically infected leaf material was homogenized in 10 ml of 50 mM NaPO4 buffer pH 7 and 20 µl was used for inoculation by rubbing four leaves per plant of 4 weeks old transgenic potato plants with carborundum powder. At least 3 plants per construct were used. As a control for each construct one plant was mock inoculated. Infected plants were grown in the greenhouse at 23°C and 16 hours of light. Three weeks after infection 10 leaf discs were taken from compound leaves of the apex and homogenized as described above. The relative virus concentration was determined using DAS-ELISA (Maki-Valkama et al., 2000). ELISA plates were coated with a 1:1000 dilution of a polyclonal antibody against PVX to bind the antigen and an alkaline phosphatase conjugated version of this antibody against PVX conjugated with alkaline phosphatase was used for detection (a kind gift of J. Saaijer).

Nematode resistance test
For the nematode resistant tests, the avirulent *Globodera pallida* population D383 and the virulent population Pa3-Rookmaker were used for infection of transgenic potato lines. The resistant diploid potato clone SH harboring the Gpa2 gene (van der Vossen et al., 2000) was used as a control. Stem cuttings of *in vitro* potato plants were grown on agar plates and after three weeks, roots were infected with approximately 300 surface sterilized second stage juveniles per plate as described (Goverse et al., 2000). For each construct three independent transformed lines were used. After 21 days and 8 weeks nematode development was monitored by microscopic inspection. For the resistance test in soil, transgenic potato plants were transferred from *in vitro* cultures and grown under greenhouse condition for two months and then inoculated with 10.000 eggs per pot of *G. pallida* Rookmaker or D383. Three and a half month after inoculation cysts were isolated from the roots and counted.

Real time RT-PCR
Leaves of *N. benthamiana* were infiltrated with *Agrobacterium tumefaciens* (pMOG101) carrying constructs of interest. At 48 hours after inoculation leaves were collected and frozen in liquid nitrogen. For RNA extraction, 60 mg of leaf tissue was used for the isolation of total RNA with the RNeasy Plant Mini Kit from Qiagen, including extra DNAse treatment. The total RNA concentration was measured using a NanoDrop spectrophotometer (Isogen) and all samples were adjusted to the same concentration. For cDNA synthesis, Super Script III (Invitrogen) and random hexamer primers were used. For real-time PCR reactions, primers were designed for Gpa2 and Rx1 using the Beacon 4.0 software. Actin was used as a reference gene. The following primers were used: Nb.actinF, Nb.actinR, Gpa.LRR-F A, Gpa.LRR-R B, Rx.C-F, and Rx.D-R (Table 1). The iQ SYBR Green Supermix (Bio-Rad) was used in a reaction volume of 25 µl (7.5 µl water, 2x 1 µl primer (5 mM), 3 µl template, 12.5 µl Supermix). The annealing temperature for the actin and Gpa.LRR primers was 64 °C and for the Rx primers 63 °C. The applied PCR program was 98 °C for 3 minutes followed by 50 cycles of 95 °C for 10 sec and 63°C for 20 sec and 70 °C for 30 sec.

Protein analysis
Total protein extract of *A. tumefaciens* transformed *N. benthamiana* leafs was made by grinding leaf material in protein extraction buffer (50 mM Tris, 10% glycerol, 150 mM NaCl, 1 mM EDTA,

20mg/ml polyclar-AT PVPP, 1 mg/ml PEFA bloc+, 5 mM DTT) on ice. The soluble fraction was analyzed by SDS-PAGE and subsequent visualisation by Coomassie Brilliant Blue staining or Western blotting and protein detection with 1:5000 diluted HRP conjugated Rabbit polyclonal anti-GFP (Novus Biologicals). HRP activity was visualised using the Pierce ECL substrate.

Table 1 Primer, adapter and linker sequences

	Primer / adapter /linker sequence 5' > 3'
Fh1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTGTTCATTTTCATACTGAGAG
Fh2	GGGGACCACTTTGTACAAGAAAGCTGGGTGGCTAGTCCTCAGACCAAC
5GpRxbn	TTTTTGGATCCATGGCTTATGCTGCTGTTACTTCCC
Rxrev	GATAGCGTCGACCACCTTAACTACTCGCTGCA
GpRxSturev	CAAAGAAAGAAGGCCTAGGAGTAC
3CCNot	GTGGTACCTTAAGCGGCCGCACCAACCATTATATTCTCGGGCTGC
5CFPsbn	TCGACGGATCCATGGTGAGCAAGGGCGAGGAGCTGTTC
3CFP	AGGTACCTTAGCTCATGACTGACTTGTAGAGCTCGTCCATGCCGAGAG
5nGFP	CGGATCCATGGATGGTGAGCAAGGGCGAGGAG
5UTRkp	TGGTACCTTCTGCAGCGAGTAGTTAAGGTGTTCTGAGGAC
3UTRrev	CTTAATTAACCCGGGAGATTGAGGACTCCCAAGAAAGG
bRxAdeIf	GAGATTCACTATGTGCATCACCCAC
RxbnREV	AGCATAAGCCATGGATCCAAAAAATAGAAATATCTCT
bGpaSnaIf	CAATTGTATACTTTCTTGCC
GPbnREV	AGCATAAGCCATGGATCCAAAAAAAATAGAAATATCTCT
AD1	CGCGCCACCGGTTCTAGAT
AD2	CTAGAACCGGTGG
Gpa2LSFor	TACGACCATGGATGGCTTATGCTGCTGTTAC
NBSeRev	TGGTACCTTAAGAATTCATGTTTCGAGCTTCCCTCAAACAG
For-LRRrx-1	CTCGACATTATTGCGGCAAGAAGC
Rev-LRRrx-1	ATGAATTTTGTGAATGTTATCAGAGG
5UK3cp	TCCATGGGCGGTGGAGTCATGAGCGCACCAGCTAGCACAACACAGCC
3UK3CP	AGGTACCTGCGGTTATGGTGGTGGTAGAGTGACAACAGC
5HBcp	TCCATGGGCGGTGGAGTCATGACTACGCCAGCCAACACCACTC
3HBCP	AGGTACCTGCGGTTATGGTGGGGGTAGTGAGATAACAGC
L12for	AGCTCTACAAGGGCGGCGGAAGTGGAGGCGGATCCGGGGGAGGCAGCATG
L12rev	CTGCCTCCCCCGGATCCGCCTCCACTTCCGCCGCCCTTGTAG
Nb.actinF	CCAGGTATTGCCGATAGAATG
Nb.actinR	GAGGGAAGCCAAGATAGAGC
Gpa.LRR-F A	GGTCCATACTCGTTATCTTTATCG
Gpa.LRR-R B	TCATCTTCATCTTCATCTGTTGTC
Rx.C-F	GACAACAGATGAAGATGATGATG
Rx.D-R	CCTCAGAACACCTTAACTACTCC
mMYC1	GGCCGCTAGCGAGCAAAAGCTCATTAGTGAGGAAGACTTAGGTGAACAGAAGCTAATCTCT GAAGAGGATCTTACTAGTTAAT
mMYC2	CTAGATTAACTAGTAAGATCCTCTTCAGAGATTAGCTTCTGTTCACCTAAGTCTTCCTCACTA ATGAGCTTTTGCTCGCTAGC

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Chapter 4

Gpa2 resistance disconnects the nematode feeding site from nutrition source, but virulent nematodes seem to overcome this by local suppression of the *Gpa2* gene

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Abstract

The resistance gene *Gpa2* leads to a slow but highly specific nematode resistance in potato. Cross sections of resistant potato roots challenged with avirulent *Globodera pallida* showed that the *Gpa2*-triggered response manifests in the degeneration of cells surrounding the nematode feeding site – the syncytium. Due to this ring of death cells, the syncytium is no longer able to connect to the conductive tissue in the vascular cylinder. As a result the syncytium slowly degenerates rendering an inadequate flow of nutrients to the nematode. In later stages of the infection, the remnants of the syncytium seem to be relocated outwards to the root cortex by local hyperplasia in between the vascular cylinder and the syncytium. A histological GUS assay further suggests specific down-regulation of *Gpa2* promoter activity in and around syncytia by juveniles from a resistance-breaking population, which may contribute to the evasion or delay of the defense response. Furthermore, functional analysis of truncated *Gpa2* transcripts indicated that both introns and 3'UTR are required for full R gene function.

Introduction

Obligate endoparasitic nematodes are perfectly adapted to a long life inside a host plant. The second stage juveniles (J2) of *Globodera* species hatch from eggs to invade roots of a host plant. Inside root tissue, the nematodes migrate intracellularly until they select an initial syncytial cell (ISC) (Endo 1964; Endo 1965). The ISC incorporates neighboring cells and develops into a syncytium, which is a multi-cellular complex produced through partial cell wall dissolution and subsequent protoplast fusion. The nematodes gain access to the nutritional resources from the plant via the syncytium (Endo 1978). Specific cellular features of the feeding site such as a dense cytoplasm, an increase in abundance of mitochondria and rough ER suggest a high metabolic rate. Syncytial cell walls abutting the xylem vessels are thickened and covered with cell-wall ingrowths indicating the intense fluid exchange between the vascular system and the syncytium. Once a nematode has established a syncytium, it becomes immobile and completely dependent on the living syncytium as its sole food source.

Plant innate immunity consists of two levels of defense responses, the basal (PAMP-triggered) and host specific (effector-triggered) immune response (Jones and Dangl, 2006). Both types of immunity are thought to be involved in plant responses to parasitic nematodes, but there is currently little experimental data on plant immunity to nematodes available. Nematodes are individuals that have adapted to long life inside a host, and which may have evolved sophisticated means to suppress host immunity. To date, six genes conferring resistance to cyst and root-knot nematodes have been isolated, i.e. $Hs1^{pro-1}$ from beet (Cai et al., 1997), *Gpa2* and *Gro1-4* from potato (van der Vossen et al., 2000); Paal et al., 2004), *Mi1* and *Hero A* from tomato (Milligan et al., 1998), (Vos 1998; Ernst et al., 2002, and CaMi from pepper (Rugang Chen 2007). Despite significant recent progress in cloning nematode resistance genes, many aspects of disease resistance signaling in response to plant-parasitic nematodes have remained elusive.

In most nematode-host plant combinations, the feeding site induction proceeds in a similar way in both susceptible and resistant host plants. Host resistance is often manifested in a local cell death in cells at the periphery of the initial feeding cell, which inhibits further expansion of the syncytium (Cabrera Poch 2006). As a result, a syncytium induced in a resistant plant does not support the nematode to complete its life cycle. However, the timing, the strength, and the localization of the defense response varies among nematode resistance genes ranging

from a very rapid hypersensitive response in the initial feeding cell mediated by *Mi-1* (Williamson 1998) to a mild and late response like in *Hero A* resistance (Sobczak et al., 2005). In sugar beet plants with the $Hs1^{pro-1}$ gene avirulent nematodes induce a syncytium, but it develops abnormally and does not support nematode development beyond the J2 stage (Cai et al., 1997). For *Hero A*, the *Rhg1* and *Rhg4* genes abnormal syncytium development is correlated with the degeneration of surrounding cells (Sobczak et al., 2005; Concibido et al., 2004; Lightfoot 2000). In cyst nematodes sex determination is epigenetically controlled and males are formed under food limiting conditions. Some nematode resistances therefore lead to an increase in the number of males, while development of females appears reduced or completely inhibited.

The resistance gene *Gpa2* originates from the potato *Solanum tuberosum* ssp. *andigena* and conditions a highly specific resistance to the avirulent population of *G. pallida* D383, but not to the resistance-breaking population Rookmaker (van der Voort et al., 1997). The *Gpa2* gene is highly similar to the *Rx1* resistance gene against Potato Virus X (Bendahmane et al., 1999). Both genes are located in a cluster along with additional R gene homologues of unknown specificity, in a region of approximately 115-kb at the distal end of the short arm of chromosome XII (Bakker et al., 2003). *Gpa2* encodes a protein of 912 amino acids containing a coiled-coil domain at the N-terminus followed by a nucleotide-binding domain (NB-ARC) and a leucine-rich repeat domain (LRR) (van der Vossen et al., 2000). *Rx1* confers an extremely fast and effective resistance to avirulent PVX strains, while the resistance response activated by *Gpa2* to the avirulent nematodes develops over weeks.

The objective of this study was to gain more insight into the molecular and cellular mechanism of the nematode resistance response mediated by the *Gpa2* gene in potato. To address this, we conducted a detailed histological analysis of nematode-induced changes in *Gpa2* resistant plants upon infection with avirulent and resistance-breaking populations of *G. pallida*. A promoter GUS assay was performed to study the *Gpa2* promoter in potato roots following a challenge with both avirulent and virulent nematode populations. Lastly, the importance of introns and 3'UTR in the *Gpa2* transcript mediated resistance was investigated.

Results

Gpa2 resistance is based on disconnection of the feeding site from the vascular tissue

To study the resistance response of the *Gpa2* gene we have monitored the establishment of a feeding site by the avirulent population D383 in comparison to the virulent resistance-breaking population Rookmaker in the plant genotype SH harboring the *Gpa2* gene. Microscopic observations showed that pre-parasitic secondary juveniles of both avirulent and virulent populations were able to recognize, penetrate the roots of resistant potato plants, and initiate a syncytium in a similar way. However, avirulent juveniles induced syncytia mainly in cortical parenchyma cells, whereas virulent individuals also induced syncytia in other cell types such as endodermis, pericycle, procambium, or cambium cells.

Cross sections of nematode-infected roots at 5 days post inoculation (dpi) showed that at this stage the syncytia had incorporated cortical parenchyma, endodermis and pericycle cells. Only a few J2s had initiated a syncytium inside the vascular bundle, but these syncytia were relatively small and consisted of only pericycle and (pro)cambium cells. Detailed observations, using transmission electron microscopy, showed essentially the same morphological changes inside the initial syncytial elements induced by virulent and avirulent

nematodes. As the vacuolar system in the syncytial cells was rearranged, the numerous plastids contained starch grains, and the nuclei and nucleoli were enlarged and had irregular shapes. Thin cell wall remnants between syncytial elements were perforated. The intercellular spaces included deposits of osmophilic material.

At seven days after inoculation the syncytia induced by avirulent nematodes were still largely composed of cortical parenchyma and endodermal cells. However, a small proportion of syncytia that had infiltrated the vascular cylinder also included cells derived from the pericycle and (pro)cambium. Syncytia originating from an initial syncytial cell inside the vascular cylinder included only a few small cells with partly degenerated cytoplasm, which were insulated by necrotic cells (data not shown).

Ten days after inoculation, syncytia established by the avirulent nematodes had grown further toward the vascular cylinder in an attempt to connect the vascular bundles (Fig. 1B). However, in most of the samples, a layer of necrotic cells had formed in between the syncytium and vascular bundles**.** Furthermore, in the direct surrounding of the syncytium a high rate of pro-cambial cell divisions resulted in an outward movement of the syncytium. No necrotic cells were observed in material collected at this time point from roots infected with the resistance-breaking virulent population (Fig. 1A). All the syncytia of the virulent individuals had a well-established interface with the xylem and the phloem bundles.

At fourteen days after inoculation the sequence of events in the *Gpa2*-mediated defense responses resulted in two distinct phenotypes. In the first type, the syncytium had infiltrated the vascular cylinder, but never incorporated procambial cells in contact with phloem and xylem bundles. In the second type, the syncytium was physically isolated from vascular tissues by a layer of necrotic endodermal and pericycle cells. At this stage, parenchyma cells in close proximity to the deteriorating feeding site exhibit hyperplasia, which induces an outward movement of the syncytium (data not shown). Closer inspection of the cellular changes with transmission electron microscopy revealed the disintegration of cytoplasm and organelles in plant material typically associated with cell death.

At the same time point, the syncytia of resistance-breaking nematodes had further expanded inside the vascular bundle, including extensive cell wall ingrowths at the interfaces with vascular tissues. The syncytial elements exhibited extensive hypertrophy. The vacuoles inside syncytium appeared to be replaced by many small vacuoles of different sizes. The cytoplasm, mitochondria, the ER and the Golgi apparatuses had proliferated, and most of the plastids contained starch grains. The nuclei and the nucleoli were strongly enlarged and acquired irregular shapes (data not shown).

At 21 and 28 days post inoculation, most of the avirulent nematodes were arrested in second or third juvenile stage, whereas the virulent nematodes had already molted into J4 or adult males and females. Further disintegration of syncytia was observed in roots infected with avirulent nematodes, with a line of necrotic cells separating syncytia from the vascular cylinder (Fig. 1C and D).

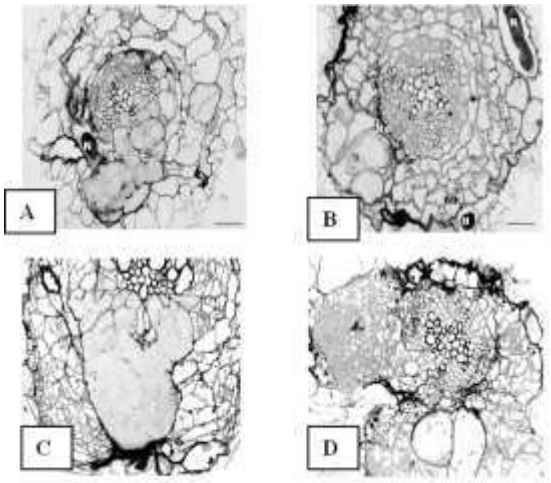

Figure 1.
Ultrastructural response of potato roots possessing Gpa2 gene to cyst nematode *Globodera pallida*.
A,C- Cross section through syncytium initiated by juveniles from Rookmaker population 10 (A) and 28 (C) days post inoculation. Typical syncytium development for compatible potato *G.pallida* interaction.
B,D- Cross section through syncytium initiated by juveniles from D383 population 10 dpi (B) where the first layer of necrotic cells is visible outside the syncytium from the vascular bundle side. 28 (D) days post inoculation fast dividing hyperplastic cells were pushing degradated syncytium towards the outside of the root.

Table 2.

Construct	**Stably transgenic potato**		**Hairy roots of potato**	
	D383	Rookmaker	D383	Rookmaker
35S::Gpa2	0.11 ± 0.58	480 ± 212	0 ± 0	48.5 ± 29
GPAII::Gpa2	2 ± 1.9	439 ± 235	0.1 ± 0.01	15.3 ± 11.29
SH	8 ± 4	316 ± 130	1 ± 0.6	20.5 ± 4.9
Line V	729 ± 378	305 ± 55	42.6 ± 4.5	23.25 ± 5.61

Results of nematode resistance assay on transgenic potato hairy roots in vitro and under the greenhouse conditions. Numbers indicated females which developed on plant roots.

GUS expression driven by the Gpa2-promoter is inhibited by virulent nematodes

A genomic DNA fragment of 2.8 kb upstream of the start codon of the *Gpa2* was cloned to study whether virulent nematodes break resistance by regulation *Gpa2* gene expression. To first prove that the cloned promoter fragment is sufficient to regulate *Gpa2* expression, we created two constructs in which the *Gpa2* coding sequence with introns was either expressed from the CaMV 35S promoter (35S::Gpa2) or the putative *Gpa2* promoter fragment (GPAII::Gpa2). Transgenic potato plants were generated with both constructs and subsequently challenged with avirulent nematodes to test for nematode resistance. Results from stable transgenic plants in the greenhouse and in *Agrobacterium rhizogenes* induced hairy roots expressing both constructs showed that the cloned fragment is sufficient for functional expression of *Gpa2*. The GPAII::Gpa2 expressing transgenic potato and hairy-roots of potato both restricted development of avirulent *G. pallida* D383 population in a similar fashion as the wild type plants harboring the *Gpa2* gene (SH) (Table 2). Next, the GPAII promoter was fused to the GUS reporter gene and tested for GUS expression in agro-infiltrated leaves of *Nicotiana benthamiana*. The cloned 2.8 kb promoter fragment constitutively drove GUS expression following agroinfiltration (data not shown).

To study the spatial and temporal expression pattern of the *Gpa2* gene in challenged and non-challenged plants, we have tested potato hairy root cultures expressing the GUS reporter gene regulated by the *Gpa2* promoter (GPAII::GUS). The activity of the *Gpa2* promoter was assessed at 7 different time points (0, 3, 5, 7, 14, 21, and 28 days post infection) following infection with the avirulent *G. pallida* population D383, the virulent *G. pallida* population *R*ookmaker, and the virulent *G. rostochiensis* line 19.

In non-challenged roots the *Gpa2* promoter was constitutively active in the stem and leaves of potato. Roots of these plants showed a tissue-specific basal level of promoter activity. Strong GUS expression was visible in young parts of the root, especially in the region of the meristematic tissue, just behind the root cap (Fig. 3A). Expression was also present in the vascular cylinder of young roots and in the lateral root primordia. Cross sections of roots expressing GPAII::GUS showed that the strongest *Gpa2* promoter activity occurred in cortical parenchyma cells, whereas lower levels were observed in pericyclic and pro/cambial cells (Fig. 3B). No GUS expression occurred in the root parts being in the secondary state of growth.

Syncytial elements in young syncytia induced by juveniles from the avirulent nematode population D383 (3 dpi) revealed a strong GUS activity as compared to cells directly surrounding the syncytia. However, in root samples collected 5 and 7 days after inoculation the level of GUS activity inside syncytia had decreased again, similar to that in cells surrounding the syncytia (Fig. 3C). This weaker GUS activity in- and directly outside the syncytium sustained until the last time point in our series (21 dpi). Thus, infection with avirulent nematodes of potato plants harboring the *Gpa2* promoter fused to GUS induces a transient up-regulation of the *Gpa2* promoter.

In similar time series we only observed GUS activity in cortical parenchyma cells in the direct vicinity of nematodes from the resistance-breaking *G. pallida* population Rookmaker and from the virulent sister species *G. rostochiensis* at the onset of parasitism. GUS activity was only detected close to individuals that had not yet begun feeding. For later stages we have not observed any GUS activity in or around syncytia induced by the virulent Rookmaker population or *G. rostochiensis* (Fig. 3D). So, while avirulent nematodes induce a transient up-regulation of the *Gpa2* promoter fused to GUS in potato, virulent nematodes induce a local suppression of the activity of the *Gpa2* promoter.

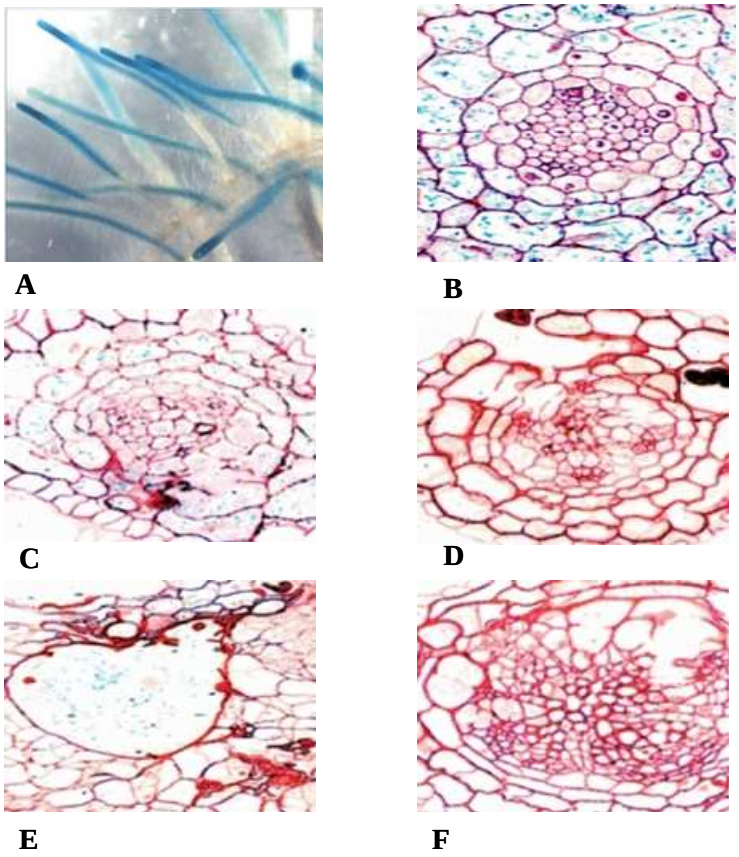

Figure 3.
Ultrastructural response of potato roots possessing the endogenous Gpa2 gene to infection with the cyst nematode *G. pallida*.
A,C - Cross section through a syncytium initiated by juveniles from the virulent Rookmaker population at 10 (A) and 28 (C) days post inoculation.
B,D - Cross section through a syncytium initiated by juveniles from the avirulent G. pallida population D383 at 10 dpi (B), in which the first layer of necrotic cells is visible disconnecting the syncytium from the vascular tissue. At 28 days post inoculation (D) fast dividing hyperplastic cells were pushing the degenerated syncytium towards the outside of the root.

The tissue-specific expression of *Gpa2* indicates a tight regulation of its promoter activity. To unravel the possible regulatory mechanisms of this promoter activity, we scanned the *Gpa2* and the homologous *Rx1* promoter for known regulatory elements. Several cis-acting elements were identified in the promoter and in the 3'UTR sequence. Regulatory sequences and motifs recognized in the Gpa2 promoter point among others at an involvement of plant hormones in this regulation. Firstly, the ASF-1 binding site (TGACG) was identified at position -2695. This motif is implicated in transcriptional gene activation by auxin or salicylic acid. Furthermore, a TGA element (AACGC), which is also an auxin-responsive element, was found at position -1857. At position -2693 of the Gpa2 promoter there is a GADOWNAT motif, which was identified from the promoters of gibberellin down-regulated genes (Ogawa et al., 2003). Next, the WUN motif (TCATTACGAA), a wound-responsive element was identified at position -679 and TC-rich repeats (GTTTTCTTAC) at position -141, which is implicated in defense and stress responses. A W-box (TTGAC), which is also present in the Gpa2 promoter (position -2320 and -494), is identical with the one from the promoter of the *A. thaliana NPR1* gene. This box is specifically recognized by salicylic acid-induced WRKY DNA binding proteins. A WB box sequence (TTTGATC) found in parsley *WRKY1* gene

promoter is also present in the *Gpa2* promoter at position -527. This motif is a WRKY1 protein binding site.

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  Gpa2-cDNA  (2661) CATTCAAGACAACTATGGAAGCTCTATCGAGGTCCATACTCGTTATCTTTA------------------
   Gpa2gDNA  (2661) CATTCAAGACAACTATGGAAGCTCTATCGAGGTCCATACTCGTTATCTTTAGTAAGACATCTTCTTCCTT
 Gpa2-noUTR  (2661) CATTCAAGACAACTATGGAAGCTCTATCGAGGTCCATACTCGTTATCTTTAGTAAGACATCTTCTTCCTT

                                                                        Intron I
  Gpa2-cDNA  (2712) ------------------------------------------------------------------------
   Gpa2gDNA  (2731) GATTTACAACAATATTTAACTCATCATCATAGTAAACTCGATAATAATCTGGATAATAGCTTTAGTAAGT
 Gpa2-noUTR  (2731) GATTTACAACAATATTTAACTCATCATCATAGTAAACTCGATAATAATCTGGATAATAGCTTTAGTAAGT

                                                Intron I
  Gpa2-cDNA  (2712) ------------------------------------------------------------------------
   Gpa2gDNA  (2801) CAAATTGCACCAATTCAACAAAAGTTCTTGATGCTGTCATTGTGATTGATTCGAATCCTTCCAATATTGT
 Gpa2-noUTR  (2801) CAAATTGCACCAATTCAACAAAAGTTCTTGATGCTGTCATTGTGATTGATTCGAATCCTTCCAATATTGT

                                                Intron I

 Gpa2-cDNA  (2712) ------------------------------------------------------------------------
   Gpa2gDNA  (2871) GTAACTTGTTATACTTGCATGTTCATTCTTGATTTTGGGAAGTGTAACATTTCCATTTTTCATCTTGATT
 Gpa2-noUTR  (2871) GTAACTTGTTATACTTGCATGTTCATTCTTGATTTTGGGAAGTGTAACATTTCCATTTTTCATCTTGATT

                                                Intron I
  Gpa2-cDNA  (2712) --------TCGAAATGGAGCATTTTTGGTAGTGTGA----------------------------------
   Gpa2gDNA  (2941) TTGGGAAGTCGAAATGGAGCATTTTTGGTAGTGTGACAACAGATGAAGATGATGATGATAGTGTGACAAC
 Gpa2-noUTR  (2941) TTGGGAAGTCGAAATGGAGCATTTTTGGTAGTGTGA----------------------------------

                                                          3'Untranslated region
   Gpa2gDNA  (3011) AGATGAAGATGAAGATGAAGACTTTGAGAAAGAAGTTGCTTCTTGCGGCAATAATGTGTAAGTTCTTATA
   Gpa2gDNA  (3081) CCTGCATGCTCATTCTTGCTATAATGTTCTCTTGTTCCTTAATTATGGGACATCTAACATATTATTTTCC
   Gpa2gDNA  (3151) ATTTTTTGCATCTTTTTTTTTTCCTGCAGCGAGTAGTTAAGGTGTTCTGAGGACTAGCCAGTTCTCTGAA
   Gpa2gDNA  (3221) ATAAATGTCAAATCAGAAGCCAAATGTGTGAGTGTTTGTTTTGTTCGTTTTCATTTTTTCTGCATAAGGT
   Gpa2gDNA  (3291) GGCAGGATGATTGCAAATGGCTTGTAATTTAATTGTATATGATATTTCGTATAGCCATTTGCCAGTGGTT
   Gpa2gDNA  (3361) TTTTAGATACTCCAAATTTTATGTACATACATAATGGTATAGGCCAGAACAGGCTCCATATATAACGTGT
   Gpa2gDNA  (3431) GTTTCCTTTCTTGGGAGTCCTCAATCTCCCGGGTTAATTAACAATTCACTGGCCGTCGTTTTACAACGTC
   Gpa2gDNA  (3501) GTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCG
   Gpa2gDNA  (3571) TAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTG
   Gpa2gDNA  (3641) ATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCT
   Gpa2gDNA  (3711) GCTCTGA
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Figure 4.
Sequence alignment of the 3' ends of the DNA constructs derived from the *Gpa2* genomic sequence and the cDNA sequence as described by Van der Vossen in 2000.

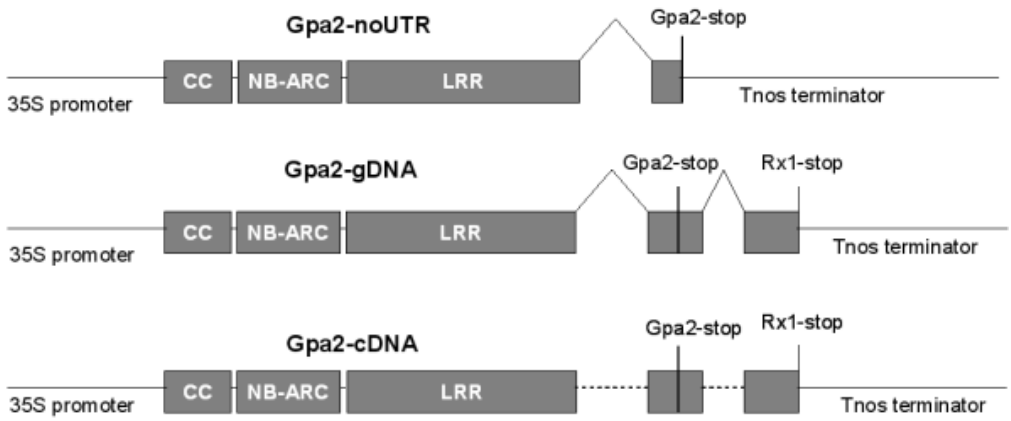

Figure 5.
Schematic overview of *Gpa2* constructs used for potato transformation. All coding sequences are introduced between the CaMV 35S promoter and T_{nos} termination sequence.

3' Un-translated region of *Gpa2* and both introns are required for full Gpa2 functionality

To test whether the mature Gpa2 transcript lacking two previously predicted introns is sufficient for full nematode resistance both the cDNA sequence and corresponding genomic DNA fragment of *Gpa2* were cloned in the CaMV 35S expression cassette of pBIN+ (Van der Vossen et al., 2000); (Fig. 4). We further removed the 3'-UTR from the *Gpa2* gDNA construct (junction directly after the predicted *Gpa2* stop-codon) to study its effect on nematode resistance (Fig. 5). The transgenic plants were challenged with the avirulent population of *G. pallida* D383 and the virulent population Rookmaker. Remarkably, transgenic plants expressing the *Gpa2* cDNA construct including its 3'UTR showed an intermediate degree of nematode resistance (6-10 adult females per plant for two independent transgenic lines), while plants expressing the genomic DNA fragment of Gpa2 without its 3'UTR were as susceptible to the nematodes from the avirulent population as plants transformed with the empty pBIN expression vector (16-33 adult females per plant). Plants expressing the full genomic *Gpa2* fragments were used as resistant controls (no adult females scored).

To investigate whether the current prediction of the Gpa2 transcript is not the only transcript required for complete resistance against nematodes, we scanned the *Gpa2* sequence for possible other splicing variants. We used an HMM gene predictor to search for alternatively spliced variants using the learning sets of *S. lycopersicum*, *A. thaliana* and *N. tabaccum* (Stanke and Waack, 2003). One of the predicted (alternative) transcripts based on tomato was identical to the *Gpa2* potato transcript as published by van der Vossen et al., (2000). Furthermore, a second possible transcript using the *N. tabacum* training set showed a read-through in the first intron, which resulted in an early stop codon and a nine amino acids shorter protein. The variants of *Gpa2* transcripts found with the *A. thaliana* training set suggest an alternative exon within the intron I, which causes a frame shift that leads to the addition of an acidic tail similar to the *Rx1* gene (Fig.7).

	Phenotype	Mean of adult D383 females
Gpa2gDNA	Resistant	0 ± 0
Gpa2cDNA cl.2	Semi-resistant	9.87 ± 2.9 **
Gpa2cDNA cl.3	Semi-resistant	6.125 ± 1.64 **
Untransformed LV	Susceptible	29 ± 5.958

Figure 4.
Results of nematode resistance assay in transgenic potato. Numbers indicated with the double asterisks indicate that those numbers were significantly different from the controls (T-test).

```
Gpa2cDNA  (841) LERLFLSDCFYLDSIPRDFADITTLALIDIFRCQQSVGNSAKQIQQDIQDNYGSSIEVHT
 A. tha 1 (841) LERLFLSDCFYLDSIPRDFADITTLALIDIFRCQQSVGNSAKQIQQDIQDNYGSSIE---
A. tha 4  (841) LERLFLSDCFYLDSIPRDFADITTLALIDIFRCQQSVGNSAKQIQQDIQDNYGSSIECNI
 tobacco  (841) LERLFLSDCFYLDSIPRDFADITTLALIDIFRCQQSVGNSAKQIQQDIQDNYGSSIEVHT


 Gpa2cDNA (901) RYLYRNGAFLVV-------------------------------------
A. tha 1  (898) ---------SKWSIFGSVTTDEDDDDSVTTDEDEDEDFEKEVASCGNNV-
A. tha 4  (901) SIFHLDFGKSKWSIFGSVTTDEDDDDSVTTDEDEDEDFEKEVASCGNNV-
Tobacco   (901) RYL---------------------------------------------
```

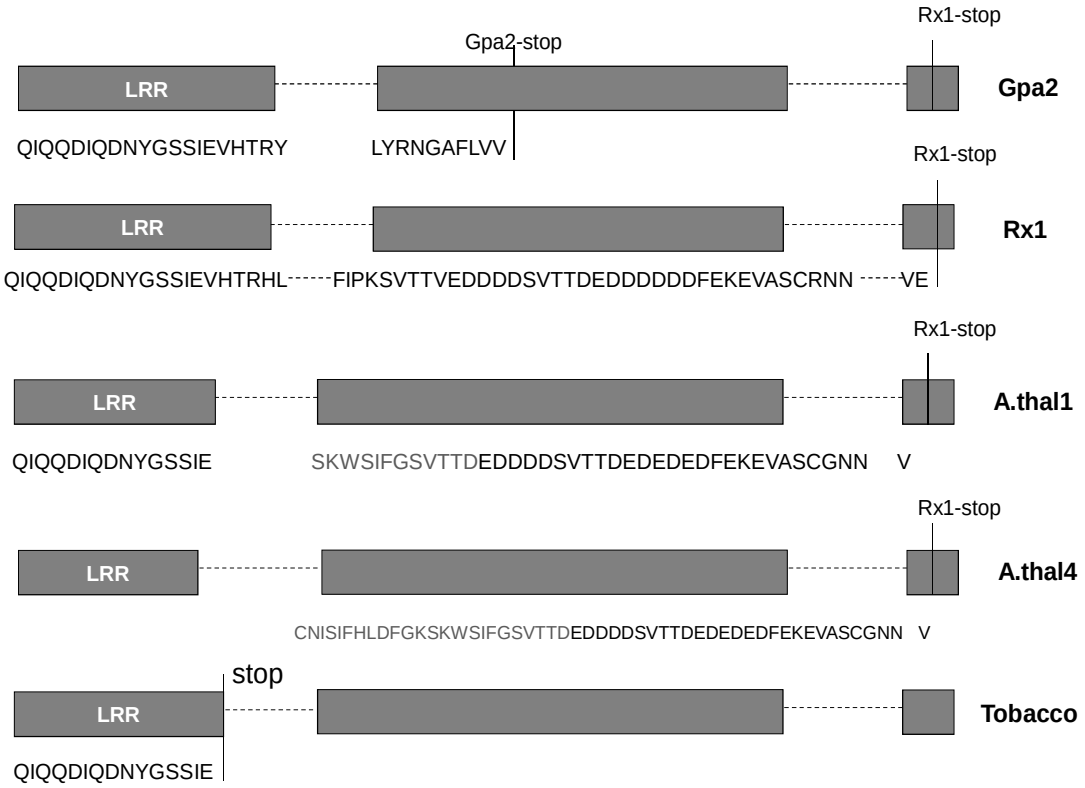

Figure 5.
Alignments and schematic illustration of predicted alternatively spliced Gpa2 variants obtained from the gene predictor HMM with learning set in *Arabidopsis* (A. thal1 and 4) and in *N. tobacum* (tobacco) with the Gpa2 cDNA.

Discussion

This chapter presents a detailed description of the defense responses in roots of potato plants expressing the *Gpa2* resistance gene to the invasion of avirulent *G. pallida* juveniles. Of particular importance in the *Gpa2*-mediated cellular resistant reaction seems to be a layer of necrotic cells that separates the growing syncytium from the nutrient flow in the vascular cylinder. This slow, but specific, response becomes first notable around one week after nematode inoculation despite the constitutive activity of the *Gpa2* promoter, as was shown in un-infected plants. At a later stage (three weeks post infection) hyperplastic cells between the syncytium and the vascular bundle pushed the syncytium away from the vascular cylinder. This is a unique rejection reaction, which has to our knowledge not been observed before. It could be a part of the *Gpa2*-mediated defense response or a secondary reaction to the presence

of necrotic, dead cells in order to remove them from important parts of the root. Remarkably, the *Gpa2* promoter activity appears to be responsive to presence of avirulent individuals during the first days of feeding from the syncytium, to return to basal levels a few days after inoculation. In contrast, the *Gpa2* promoter appears to be down-regulated (inside and in close proximity of the syncytium) by nematodes from the virulent resistance-breaking Rookmaker population, and by the virulent individuals from the sister species *G. rostochiensis*.

Gpa2 is a single dominant R gene which confers a race specific, resistance to *G. pallida* in potato plants. Juveniles of the resistance-breaking Rookmaker population are able to avoid recognition by the Gpa2 protein and undergo the same development as is observed in susceptible roots (Melillo 1990; Castelli et al., 2005). Histological analysis of infected potato roots harboring the *Gpa2* gene revealed that juveniles from the avirulent population of *G. pallida* (D383) are able to enter, migrate inside the roots and initiate a feeding site like the virulent nematodes. This suggests that *Gpa2*-mediated resistance is not caused by physical changes in root morphology, which would make roots impenetrable or unattractive for juveniles. The same phenomenon was found in studies with tomato plants carrying the *Hero* resistance gene (Sobczak et al., 2005).

The first ultrastructural differences between cells transformed into a syncytium between roots of a susceptible and a resistant plant are visible only after 5-7 days post inoculation. It is much later than it is known for other nematode R genes. For example, the defense response conditioned by the *Hero* gene is observable already 2 days after root invasion and another 2 days later the syncytia were completely surrounded by necrotic cells (Sobczak et al., 2005). In roots carrying the $Hs1^{pro-1}$ gene, the syncytia are also completely necrotized at 4 dpi (Holtmann et al., 2000). This difference in response time suggests that there are at least two distinct resistance mechanisms to parasitic nematode operating in plants. The first type is associated with a typical fast hypersensitive response demonstrated by a rapid cell death inside young syncytia (Bleve-Zacheo et al., 1998; Paulson 1972). The second type, mostly described for cyst nematode infections, is often referred to as a “hypersensitive-like” or a “delayed hypersensitive” response, because it appears when the syncytium is already well established and it results in slow deterioration or abnormal development of the feeding site (Grymaszewska and Golinowski, 1998). To our surprise, we observed that even for avirulent nematodes a few syncytia were able to avoid or to resist the necrotic insulation, enabling their development into a normal feeding site. A similar phenomenon was observed for nematodes developing on tomato plants with *Hero* gene (Sobczak et al., 2005). Because we used field populations we cannot exclude that the D383 population includes a small proportion of virulent genotypes.

For cyst nematodes, sex of juveniles is determined epigenetically by the amount of food that is available. Well-developed syncytia support the development of adult females, whereas poor developed syncytia result in nutrition limitations still sufficient to support the development of adult males. Hence, a bias towards male development is observed. In case of a rapid defense response, a complete reduction in the amount of females is obtained, and a large number of males will develop on the resistant roots. This so called „male-based resistance” is commonly observed against cyst nematodes (Acedo 1984; Rice 1985). In a case of the *Gpa2* response, the syncytium development is arrested in a later stage, after the nematode sex is determined. As a result, we observed no increase in number of males in comparison to susceptible plants (data not shown), but aberrant translucent females appeared on the roots of Gpa2 resistant potato roots either due to starvation or to lack of fertilization. Some females, however, were able to develop normally on roots of resistant potato. Apparently, in these cases the *Gpa2*-

mediated response is too slow to mount an effective response. It will be interesting to see whether this phenotype can be explained by a correlation between the site of syncytium induction and the efficacy of the resistance response.

R genes are believed to be constitutively expressed in plants in the absence of the pathogen, for example the *Mi-1* gene (Milligan et al., 1998). The *Gpa2* promoter was active in the root tips and in the cortex, what corresponds with the root invasion strategy of *G. pallida*. The pre-parasitic juveniles penetrate the roots preferentially near the tips in the region of the elongation and differentiation zone. As described above, execution of the defense response mediated by the *Gpa2* gene depends on the location of the initial syncytial cell selected by the parasitic juveniles. A similar correlation was also shown for the tomato *I-2* gene, which site of activity corresponds with the site of fungal containment (Mes 2000). R gene expression is often triggered upon pathogen infection as shown for *Xa1*, *Xa21*, *Pib* and *Pi-ta* in rice (Yoshimura et al., 1998; Century 1999; Wang et al., 2001). For example, a basal level of *N* gene transcript was detected in uninfected tomato plants and this level increased significantly after plants were challenged with tobacco mosaic virus (Levy et al., 2004). A similar response was observed for beet cyst nematodes, which induce about a 4-fold increase in the $Hs1^{pro-1}$ transcript levels one day after nematode inoculation (Thurau et al., 2003). Similarly, the up-regulation of tomato *Hero* gene during *G. rostochiensis* infection was quantified in roots of resistant tomato (Sobczak et al., 2005). For *Gpa2*, we have found a local, transient increase of GUS staining inside young syncytia (less than 3 days) initiated by individuals from the avirulent *G. pallida* population suggesting a similar early increase in transcriptional activity of the *Gpa2* gene.

Remarkably, no such increase in *Gpa2* promoter activity was observed in case of infection with virulent nematodes. This suggests that virulent nematode have an ability to down-regulate the activity of the *Gpa2* promoter in syncytia and their surroundings between the first 5 days (in case of Rookmaker) and 7-14 days (in case of *G. rostochiensis*). This ability is apparently missing or less effective for the avirulent population as the promoter is still active in samples collected 14 days post infection. We were unable to quantify this effect using real time RT-PCR on RNA extracted from root segments probably due to the fact that this suppression effect was restricted to syncytial elements and therefore undetectable when root segments were used for RNA extraction. Furthermore, this phenomenon was detected in susceptible transgenic plants harboring the Gpa2 promoter GUS fusion construct and it will be interesting to study changes in promoter activity in a resistant background in the presence of the *Gpa2* gene.

Very little is known about the transcriptional regulation of nematode resistance genes. However, the tissue-specific expression and a local increase upon infection with avirulent nematodes indicate that *Gpa2* promoter activity is tightly regulated in plants. Various cis-acting elements identified in the promoter point at the involvement of transcription factors and plant hormones in the expression of *Gpa2*. A role for transcription factors in nematode resistance is reported for the WRKY transcription factors (6, 61 and 72-like) that are specifically up-regulated during the incompatible interaction between root knot nematodes and tomato, whereas others (3, 23, 33-like) are up-regulated in both compatible and incompatible situation (Bhattarai et al., 2008). There is ample support for a role of plant hormones in feeding site development in compatible interactions (Wubben et al., 2008), (Engler et al., 2005). Possibly, plants have used this feature of nematode-plant interactions to regulate the expression of nematode R genes. As it was shown that a local increase of auxin in the feeding site is indispensable for syncytium establishment and development (Goverse et al.,

2000; Engler et al., 2005), auxin regulated R gene expression would provide a specific means to regulate the plant's defense system upon invasion by endoparasitic nematodes. Recently, salicylic acid signaling was found to be important in *Mi-1*-mediated resistance to root knot nematodes and aphids (Branch et al., 2004) in *A. rhizogenes* induced tomato hairy roots. Whether plant hormones are involved in transcriptional activation of the *Gpa2* gene needs further investigation.

The occurrence of alternatively spliced and truncated transcripts was reported for a few NB-LRR genes. However, their requirement in plant defense is not fully resolved (reviewed recently in Gassmann 2008). For example, the *N* gene encodes two transcripts, N_S and $N_{L,}$ via alternative splicing of the alternative exon present in intron III and presence of both variants only conferred a full TMV resistance (Dinesh-Kumar and Baker, 2000). Also, for the *Arabidopsis RPS4* gene the removal of just one of the introns abolished R gene functionality, but the function of an intron-deprived transgene was complemented by the presence of a second, differently truncated *RPS4* transgene (Zhang and Gassmann, 2003). Surprisingly, when cDNA of *RPS4* gene was overexpressed in tobacco, the hypersensitive response was induced in the presence of avrRPS4 (Zhang et al., 2004), what can point at the quantitative nature of R gene mediated defense. In contrast to above examples, intronless *L6* showed a full resistance (Ayliffe et al., 1999). Interestingly, for the *RPS4* gene apparently the expression level is an additional determinant of transcript functionality. The same splicing variant can be efficient in recognizing AvrRPS4 when overexpressed in tobacco leaves, but not in stable transformants of *Arabidopsis* (Zhang et al., 2004).

Until now, we have collected several lines of evidence indirectly supporting the occurrence of alternative splicing for the *Gpa2* gene. First of all, the intronless *Gpa2* cDNA construct conferred an intermediate resistance to nematodes in potato, which may point that the predicted *Gpa2* transcript is not the only one required in nematode resistance. Not only removing the intron sequence abolished the *Gpa2* functionality, but also the construct with the first intron present but lacking a native Gpa2 3'UTR region (after the predicted stop codon) was not able to confer a nematode resistance in transgenic potato. Additional indications for alternative splicing at the 3' end of *Gpa2* came from our unsuccessful immunodetection of HA, STREP, MYC or GFP tag fusions at the carboxy-terminus (data not shown). To obtain additional support, we have predicted *in silico* variants of the Gpa2 transcripts and future work should confirm whether these putative variants are functional. However, the previously done RACE experiments on potato root and leave samples did not result in the finding of any alternatively spliced transcript (van der Vossen et al., 2000). These studies were made on unchallenged plants what might explain negative results. On the top of that the Gpa2 transcript is present at the low level in potato roots restricted to root tips and cortex and the other variants may remain undetectable. Taking into account that the nematode infection is difficult to synchronize and is restricted to a single feeding site the future work on dynamics of *Gpa2* expression remains a challenge.

Materials & Methods

DNA constructs

The *Gpa2* promoter region was constructed in two steps. First, the region between the BstZ17I (SnaI) site (-2744 bp) and the startcodon was amplified from pBINRGC2 using the primers bGpaSnaIf (5'-CAA TTG TAT ACT TTC TTG CC-3') and GPbnREV (5'-AGC ATA AGC CAT GGA TCC AAA AAA AAT AGA AAT ATC TCT-3'). In a second step, this BstZ17I-NcoI fragment was cloned alongside the 720 bp PacI-BstZ17I fragment of pBINRx1, fused to a PacI-AscI adapter consisting of AD1 (5'-CGC GCC ACC GGT TCT AGA T-3') and AD2 (5'-CTA GAA CCG GTG G-3'), into the pUCAPYFP with Gpa2 3'UTR after digestion with AscI-NcoI.

For *Gpa2* expression under the control of the double enhanced CaMV 35S promoter and Tnos terminator, the proximal end was amplified from pBINRGC2 (van der Vossen, van der Voort et al., 2000) with the primers 5GpRxbn and GpRxSturev (5'-CAA AGA AAG AAG GCC TAG GAG TAC) to generate a NcoI-AvrII fragment, which was cloned together with an AvrII-PstI fragment from pBINRGC2 into the NcoI-PstI digested pUCAPRx1. For expression under control of the endogenous promoter, first the YFP reporter gene was amplified by PCR using the primers 5CFPsbn (5'- TCG ACG GAT CCA TGG TGA GCA AGG GCG AGG AGC TGT TC-3') and 3CFPsrk (5'-AGG TAC CTT AGC TCA TGA CTG ACT TGT AGA GCT CGT CCA TGC CGA GAG-3') and cloned as NcoI-KpnI fragment in the vector pUCAP, resulting in the vector pUCAPYFP. The *Gpa2* 3'UTR (transcription termination) region was amplified from pBINRGC2 using the primers 5UTRkp and 3UTRrev for cloning in the KpnI-PacI sites of pUCAPYFP, replacing Tnos.

Total RNA was extracted from *Nicotiana benthamiana* leaves infiltrated with GPAII::Gpa2gDNA. cDNA was prepared using Superscript III (Invitrogen) and use as a template for PCR with 5'GpEx (CCTCCAACTGATGTTCCAACTTTC) forward and GpUNI (5'CCTGAGGTACCTAGCTAGCCAGGACCAGCGGCCGCTCCCACTACCAAAAATGCTCCAT TTCGA) reverse primer. The reverse primer was designed to introduce adapter sequences for future affinity tag fusions. The PCR product which covers the C-terminal part of the *Gpa2* transcript was cloned into Topo 2.1 vector (Invitrogen) and sequenced. The correct clone was digested with AccIII and KpnI restriction enzymes and ligated together with N terminal *Gpa2* transcript fragment (as NcoI-BspEI restriction) into the pGPAII (containing *Gpa2* promoter sequence) vector open with NcoI and KpnI enzymes, what result in pGPAII::Gpa2cDNA construct. Fragment covering *Gpa2* promoter and transcript sequence was transferred into the pBIN+ binary vector as AscI-PacI fragment.

The β-glucuronidase reporter gene (GUS) was amplified with specially designed primers to introduce NcoI and PstI restriction sites at the 5' and 3' ends. The GUS gene was introduced as NcoI-PstI fragment into the pRAP vector, under control of CaMV 35S promoter and Tnos terminator, to check if the GUS gene is functional. Afterwards the same fragment was used for cloning into the pGPAII vector, which contains the endogenous promoter of the Gpa2 gene, and its 3'UTR (*Gpa2* termination). The insert was digested from the plasmid using the unique restriction sites AscI and PacI, and cloned into the binary vector pBIN+.

Agrobacterium transient transfection assay (ATTA)

To test the function of created constructs, we used a transient expression system via *A. tumefaciens* in *N. benthamiana* leaves (Hood et al., 1993). *Agrobacterium* cells were inoculated into 5ml YEB medium with rifampicin (25 mg/l) and kanamycin (50 mg/l) and incubated 20 h at 28ºC and 250 rpm in a shaker. Part of the overnight culture was inoculated into YEBi (10 µl acetosyringone 200mM per 100 ml and 10 mM MES +Kan. 50 mg/l) and grown untill the OD600 was between 0.5 and 1.5. Cells were centrifuged and resuspended in MMAi medium to induce competence to transfer DNA. This suspension was used for infiltrating *N. benthamiana* leaves. Infiltration was done with a syringe without a needle on the lower surface of the leaf.

Hairy root cultures of potato

Potato (line V) plants grown *in vitro* were used for transformation with *A. rhizogenes*. One cm long stem pieces were cut and placed on callus inducing medium (CIM) and incubated 4 days in a growth chamber at 24ºC in the dark. *A. rhizogenes*, carrying binary constructs of interest, were grown in MYA medium (Rif. 25 mg/l, Kan. 100mg/l). Infection solutions were made in liquid SM medium with a

final concentration of OD600 = 0.1. Stem pieces were incubated in the infection solution for 5 min, transferred to solidified SM and cocultivated for 3 days in a growth chamber at 24ºC in the dark. Next, stem pieces were washed in SM medium (containing carbenicillin 500 mg/l) and incubated on plates with SM medium (Carb. 500 mg/l and vancomycin 100 mg/l) to eliminate *A. rhizogenes*. About 10 days post *A. rhizogenes* infection, hairy roots started to appear from calli at the cutting edges. After another week, roots were cut and transferred to hairy root elongation medium with selection factors (Kan. 100 mg/l and Carb. 500 mg/l).

Nematode infection assays

Transgenic and wild type plants of the diploid potato clone *Solanum tuberosum* ssp. *tuberosum* line V were used for nematode infection tests with the populations *Globodera pallida* D383 and Rookmaker, and *G. rostochiensis* line19. For *in vitro* assays hatched second-stage juveniles were surface sterilized as described by Goverse et al., (2000). Around 100 juveniles were inoculated on the roots of a single plant. For greenhouse assays, around 1000 eggs were inoculated on roots of plants grown in a separate pot filled with sterile sandy soil (Martin W. Ganal 1995).

β-glucuronidase assay (GUS assay)

A histochemical GUS-assay was used for the proper selection of transgenic roots, and also for monitoring GUS expression in infected and non-infected plant tissues. The same procedure was used for leaves and roots. Plant material was covered with assay buffer (0.1 M phosphate buffer, pH7.0, Triton X-100, 0.1% + X-Gluc, 0.3 mg/ml) in a Petri dish. To allow a good penetration of tissue with assay buffer, samples were infiltrated with vacuum (-800 mbar for 1 min). Samples were incubated for 6 h in case of roots, and overnight for leaves at 37ºC. Additionally, leaf samples were discolored with 96 % ethanol for 72 hours at 4ºC.

Microscopic analysis

Hairy roots of transgenic potato were grown on hairy root elongation (de Greef & Jacobs) medium. Root samples were collected manually at 0, 5, 7, 14, 21 and 28 days post nematode infection. They were fixed, osmicated, dehydrated in ethanol and acetone, and embedded in epoxy resin (Fluka) as described by (Golinowski, Grundler et al., 1996). Semithin (2 μm thick) and ultrathin (70 to 80 nm thick) sections were cut with glass and diamond knives, respectively, using a Leica UCT ultramiscrotome (Leica, Bensheim, Germany). Ultrathin sections were collected on formvar coated 100-mesh copper grids and stained for 4 min with a saturated 50 % ethanol solution of uranyl acetate, followed by 6 min of staining with an aqueous solution of lead citrate. The sections were examined using FEI 268D “Morgagni” transmission electron microscope.

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Chapter 5

The Cyst Nematode SPRYSEC Protein RBP-1 Elicits Gpa2-and RanGAP2-Dependent Plant Cell Death

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SUMMARY

Plant NB-LRR proteins confer robust protection against microbes and metazoan parasites by recognizing pathogen-derived avirulence (Avr) proteins that are delivered to the host cytoplasm. Microbial Avr proteins usually function as virulence factors in compatible interactions, however little is known about the types of metazoan proteins recognized by NB-LRR proteins and their relationship with virulence. In this report, we demonstrate that the secreted protein RBP-1 from the potato cyst nematode *Globodera pallida* elicits defense responses, including cell death typical of a hypersensitive response (HR), through the NB-LRR protein Gpa2. *Gp-Rbp-1* variants from *G. pallida* populations both virulent and avirulent to *Gpa2* demonstrated a high degree of polymorphism, with positive selection detected at numerous sites. All *Gp*-RBP-1 protein variants from an avirulent population were recognized by Gpa2, whereas virulent populations possessed *Gp*-RBP-1 protein variants both recognized and non-recognized by Gpa2. Recognition of *Gp*-RBP-1 by Gpa2 correlated to a single amino acid polymorphism at position 187 in the *Gp*-RBP-1 SPRY domain. *Gp*-RBP-1 expressed from Potato virus X elicited Gpa2-mediated defenses that required Ran GTPase-activating protein 2 (RanGAP2), a protein known to interact with the Gpa2 N terminus. Tethering RanGAP2 and *Gp*-RBP-1 variants via fusion proteins resulted in an enhancement of Gpa2-mediated responses. However, activation of Gpa2 was still dependent on the recognition specificity conferred by amino acid 187 and the Gpa2 LRR domain. These results suggest a two-tiered process wherein RanGAP2 mediates an initial interaction with pathogen-delivered *Gp*-RBP-1 proteins but where the Gpa2 LRR determines which of these interactions will be productive.

Abbreviations: ARC, Apaf-1, R protein and CED4; Avr, avirulence; CC, coiled-coil; CP, coat protein; ETI, effector-triggered immunity; HR, hypersensitive response; IB, immunoblot; IP, immunoprecipitation; LRR, leucine-rich repeat; NB, nucleotide-binding; Pa, pathotype; PAML, phylogenetic analysis by maximum likelihood; PAMP, pathogen-associated molecular pattern; PTI, PAMP-triggered immunity; PVX, potato virus X; R, resistance; SEC, size exclusion chromatography; SPRY, SP1a and RYanodine receptor; TRV tobacco rattle virus; VIGS, virus-induced gene silencing.

Introduction

Gene-for-gene resistance in plants is defined by the interaction between the products of dominant plant resistance (*R*) genes and corresponding avirulence (*Avr*) genes that are often specific to a particular pathogen isolate or race [1]. Recognition of Avr proteins by R proteins triggers a defense response that limits infection, and may lead to a characteristic cell death response referred to as the hypersensitive response (HR). In the absence of recognition by a cognate host R protein, Avr proteins often play a role in pathogen virulence by subverting basal defense mechanisms, and in this context are referred to as pathogen effector proteins [2].

A variety of plant *R* genes have been identified, conferring resistance to a broad spectrum of biotrophic pathogens including bacteria, fungi, oomycete, viruses, and arthropods [3]. The most numerous type of *R* genes encode intracellular proteins with nucleotide-binding (NB) and leucine-rich repeat (LRR) domains, collectively referred to as NB-LRR proteins. Two structurally different classes of NB-LRR proteins exist that encode N-terminal domains which either share homology with the Toll/Interleukin-1 Receptor (TIR) cytoplasmic domain (TIR-NB-LRR class) or have a less conserved domain with a predicted coiled-coil (CC) structure in some members (CC-NB-LRR class).

Identification of pathogen-encoded Avr proteins from bacterial, viral, fungal and oomycete plant pathogens has yielded a remarkable list of proteins that elicit NB-LRR-mediated resistance [2,4]. Some Avr-encoding genes show hallmarks of selection pressure, manifested as sequence diversification or gene deletions that have allowed escape from host detection and signifying the evolutionary contest between plants and their pathogens [5]. Avr proteins recognized by NB-LRR proteins show little structural commonality except that they are either synthesized in (in the case of viruses), or delivered to the host cytoplasm by various microbial protein delivery systems. In the absence of a cognate R protein, most Avr proteins are thought to act as effector proteins to enhance pathogen virulence. Indeed, it has been suggested that NB-LRR proteins have evolved to "guard" cellular targets of effectors by responding to their alteration [1]. Alternatively, the decoy model suggests that NB-LRR proteins might recognize effectors not by interacting with virulence targets *per se*, but with proteins that simply resemble effector targets [6]. *Avr* genes from microbial pathogens have traditionally been identified by genetic approaches. Genetic identification of Avr genes from metazoan parasites has been challenging however, owing to the complexity of their genomes and life cycles, and a paucity of genetically tractable model organisms. This hindrance is particularly acute for plant parasitic nematodes. Alternatively, Avr candidates can be discovered by first identifying proteins likely to act as effector proteins and testing their propensity to be recognized by a given R protein [7].

Cyst nematodes of the genus *Globodera* are obligate plant parasites, spending the majority of their life cycle within roots. These nematodes develop an intimate relationship with their host *via* the induction of a complex feeding site structure, known as the syncytium, in the vascular cylinder of the potato roots. Cyst nematodes produce an assortment of parasitism proteins in order to infect plants, which in principle can be thought of as being analogous to effector proteins of microbial pathogens [8,9]. These proteins are synthesized in the oesophageal glands (two sub-ventral and one dorsal) and some of these are injected into the host cytoplasm using a specialized structure called the oral stylet. Both host range specificity and suppression of host plant resistance are thought to be controlled by nematode effector proteins [10]. Many putative nematode effector proteins have been identified by virtue of their

possession of a protein sorting signal for extracellular secretion and expression in the esophageal gland [8]. To date, however, there are no unambiguous reports of nematode effector proteins that also elicit defense responses by specific NB-LRR proteins.

Use of plant nematode resistance genes is an effective and environmentally safe method to manage these parasites. Four nematode *R* genes encoding NB-LRR proteins have been identified in *Solanaceous* species [11]. *Gpa2* is a potato gene that encodes a CC-NB-LRR protein and confers resistance against two field populations (D383 and D372) of *G. pallida* [12,13,14]. In *Gpa2*-expressing potatoes, nematodes penetrate roots, start the initiation of their feeding site and become sedentary. However, the tissue surrounding the developing feeding site subsequently becomes necrotic and collapses, suggesting the elicitation of an HR. *Gpa2* is closely related to the *Rx* and *Rx2* genes, which confer resistance to Potato Virus X (PVX), through recognition of the viral coat protein (CP). Rx function is dependent on Ran GTPase-activating protein 2 (RanGAP2), a protein shown to interact with the N-terminal CC domains of Rx, Rx2 and Gpa2 [15,16]. Domain swap experiments have shown that the N-terminal halves of the Rx and Gpa2 proteins are interchangeable for mediating HR responses in response to the PVX CP whereas the LRR domain determines recognition specificity [17].

In this report, we used a candidate gene approach to test the possibility that the *G. pallida* RBP-1 protein may possess avirulence activity towards Gpa2. *Gp*-RBP-1 possesses a secretion signal peptide, is expressed in the *G. pallida* dorsal esophageal gland, and is most closely related to a family of proteins from *G. rostochiensis*, the secreted SPRY domain (SPRYSEC) proteins, which have been shown to be present in stylet secretions [18,19,20]. RBP-1 and SPRYSEC proteins possess a SPRY domain that most closely resembles the Ran GTPase-associated protein, Ran-Binding Protein in the Microtubule-organizing center (RanBPM) [19], a multi-domain protein conserved in most eukaryotes [21,22]. The SPRY domain of *Gp*-RBP-1 is part of a B30.2 domain, an extended domain structure comprising PRY and SPRY subunits [18]. We show that *Gp*-RBP-1 variants are highly variable within and between populations and appear to be under diversifying selection, with maintenance of avirulent (recognized by Gpa2) *Gp*-RBP-1 variants in populations not controlled by Gpa2. We also present data suggesting that recognition of *Gp*-RBP-1 by Gpa2 is mediated by an initial interaction with RanGAP2 but that the Gpa2 LRR domain determines which *Gp*-RBP-1 result in activation of Gpa2. Implications for mechanisms of recognition and selective pressures on nematode effector proteins are discussed.

RESULTS

Identification of a *G. pallida* AvrGpa2 candidate

One of the hallmarks of Avr recognition by NB-LRR proteins is the induction of an HR when both proteins are present in the same cell. As such, we tested whether *Gp*-RBP-1 could induce a Gpa2-dependent HR in a transient expression assay. A *Gp-Rbp-1* cDNA derived from *G. pallida* pathotype (Pa-) 2/3 population Chavornay was cloned into the binary vector pBIN61 under control of the cauliflower mosaic virus 35S promoter as a C-terminal HA-tagged EGFP fusion (*Gp*-RBP-1:EGFP:HA), but lacking its secretion signal peptide. This protein was transiently co-expressed with *Gpa2* driven by the *Rx* genomic promoter using *Agrobacterium*-mediated expression

(agroinfiltration) in *N. benthamiana* leaves. *Gp*-RBP-1:EGFP:HA elicited an HR in the infiltration patch within three to four days (Figure 1A). An equivalent fusion protein with a SPRYSEC homolog from *Globodera rostochiensis* (*Gr*-RBP-1:EGFP:HA) [18,20], did not elicit Gpa2-mediated HR, nor did the control proteins EGFP:HA or the coat protein (CP) from potato virus X (PVX). Rx and Rx2 were also tested for recognition of *Gp*-RBP-1:EGFP:HA, but both NB-LRR proteins showed strict specificity for the PVX CP (Figure 1A). No HR was induced when the native secretion signal peptide sequence was retained in *Gp*-RBP-1 (Figure 1B), likely due to its secretion from the plant cell. Untagged *Gp*-RBP-1 also induced a Gpa2-specific HR, indicating that recognition by Gpa2 was not an artifact of the EFGP fusion protein (Figure 1B). These results indicate that the Gpa2 protein has the capacity to recognize *Gp*-RBP-1, which in turn induces a typical disease resistance response.

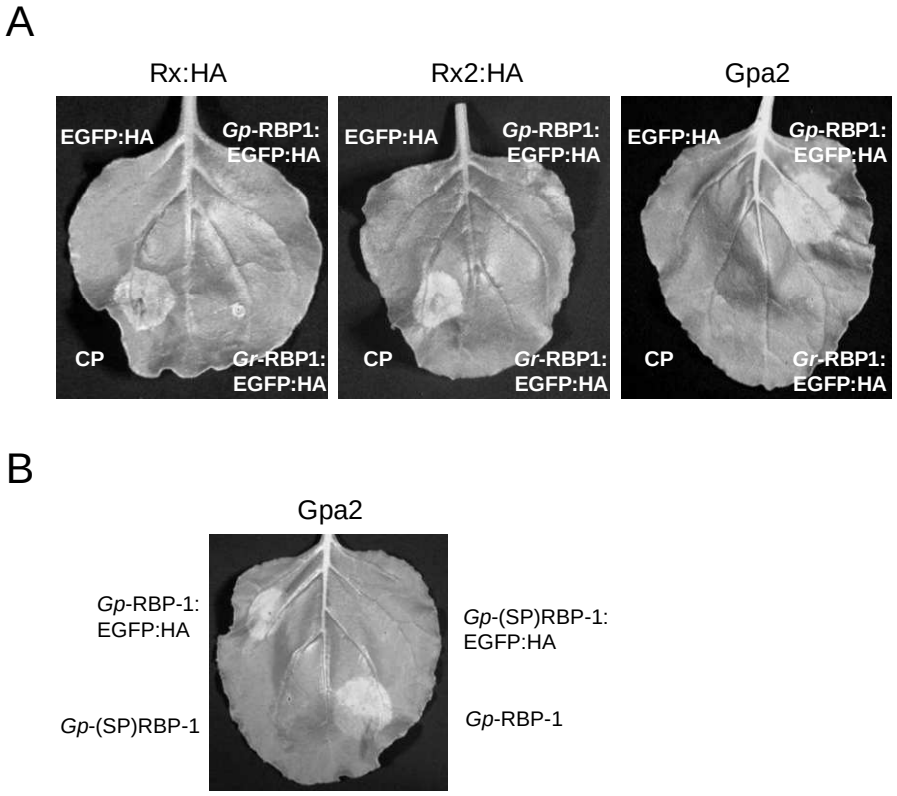

Figure 1.
Gp-RBP-1 induces a Gpa2-mediated HR in *Nicotiana benthamiana* leaves.
A - HA-tagged Rx and Rx2, or untagged Gpa2 driven by the *Rx* promoter were transiently expressed bia agro-expression in wild-type *N. benthamiana* leaves together with 35S promoter-driven PVX CP or a *G. pallida* RBP-1 protein cloned from the population Chavornay (Chav-1) fused to a C-terminal EGFP fusion and epitope tag (EGFP:HA). EGFP:HA and a *G. rostochiensis* RBP-1: EGFP:HA fusion were included as controls. HRs were observed within 2 to 3 days of ago-expression.
B - Tagged and untagged versions of *Gp*-RBP-1 were also tested that included the 23 amino acid secretion signal peptide (SP) from the predicted full-length *Gp*-RBP-1 protein [*Gp*-(SP)RBP-1 and *Gp*-(SP)RBP-1:GFP:HA]. HRs were observed within 2 to 3 days of ago-expression.

Gp-Rbp-1 is highly polymorphic and subject to diversifying selection

To investigate *Gp-Rbp-1* genetic diversity, we analyzed a number of additional sequences from several *G. pallida* populations including some from the native range of this parasite (Peru), as well as from two sequences from the very closely related species *Globodera mexicana*, which differs in host range from *G. pallida* (Figure S1). RBP-1 homologues possess an N-terminal secretion signal peptide (SP) followed by a B30.2 domain which is comprised of juxtaposed PRY and SPRY domains [18,23]. All of the *Gp*-RBP-1 variants were found to possess an additional, near-perfect repeat of the PRY domain immediately N-terminal to the B30.2 domain, whereas all variants identified to date from *G. mexicana* possess only a single PRY domain (Figures 2, 3, S1 and S2). The mean genetic distance observed between *G. pallida* and *G. mexicana* sequences was 0.07 (K2P model), but this genetic distance increased to 0.37 when comparing *G. pallida*, and *G. mexicana Rbp-1* sequences to the *G. rostochiensis* protein, SPRYSEC-19, most closely related to RBP-1 [19]. These observations strongly suggest that none of the *G. rostochiensis* sequences identified to date [19] correspond to a direct orthologue of the *Gp-Rbp-1* sequences investigated herein.

At the intraspecific level, the European *G. pallida* populations Chavornay [CH], Rookmaker [NL], D383 [NL], Guiclan [FR] and Pukekohe [NZ] demonstrated a mean genetic distance of 0.008 (K2P model), while inclusion of four additional Peruvian *G. pallida* populations (GPS4, GPS7, GPS9 and GPS10), representing different pathotypes and belonging to three of the five clades described for *G. pallida* in Peru [24], increased the mean genetic distance to 0.018 for the entire *Gp*-RBP-1 sequence dataset. This is consistent with the expectation of reduced heterogeneity in European populations that have likely been subject to a founder effect during importation in comparison to the populations existing in the native area of this nematode [25]. When examining the number of variable sites in our alignment, we found 86 out of 855 nucleotides had at least one substitution within the entire *G. pallida* data set and 67.5% of them resulted in non-synonymous mutations.

To better understand the different selective constraints acting on *Gp*-RBP-1, we carried out complementary evolutionary analyses by evaluating the non-synonymous to synonymous substitution rates per site using the SLAC, REL and FEL maximum likelihood methods implemented in HYPHY [26] as well as the CODEML program (M1 vs M2 and M7 vs M8) of the PAML package [27,28]. These models assume variable selective pressures among sites but no variation among branches in the phylogeny. The PAML M8 and M2 models of positive selection appeared to be significantly ($p < 0.001$) better adapted to the data set (Table S1A) showing that RBP-1 has indeed been subjected to positive selection (Figure 2). Ten sites were identified by both the M2 and M8 models but only three of them (23, 102 and 187) were detected with posterior probabilities > 95%. The SLAC, REL and FEL methods however, detected position 23 as a negatively selected site (data not shown). When comparing PAML to HYPHY results, four sites (59, 119, 174 and 187) came up as supported by at least two different methods (Table 1B), but the most noticeable site under positive selection was at residue 187, which is detected by all methods with strong statistical values.

In order to obtain insights into the impact of the founder event on the selective constraints acting on *Gp*-RBP-1, we tested the M8 model on a data subset corresponding only to sequences from indigenous Peruvian *G. pallida* populations and studied the distribution of the Ka/Ks ratio along the RBP-1 amino acid sequence (Figure 2). Using the Peruvian data subset, three additional sites (59, 106, and 202), previously detected using the entire data set, but with posterior probabilities < 95%

(Table 1A), were now significantly (posterior probabilities > 95%) detected as under positive selection. The higher variability observed among Peruvian *G. pallida* populations correlated to a higher number of sites under positive selection. Distribution of the Ka/Ks ratio along the protein sequence revealed a continuous distribution of sites under positive selection along the protein sequence. After alignment of *Gp*-RBP-1 with SPRYSEC-19 [21], it appeared that three of the PAML sites found under positive selection localize in the SPRYSEC predicted extended loops that shape the surface A of the SPRY domain (Figure 2).

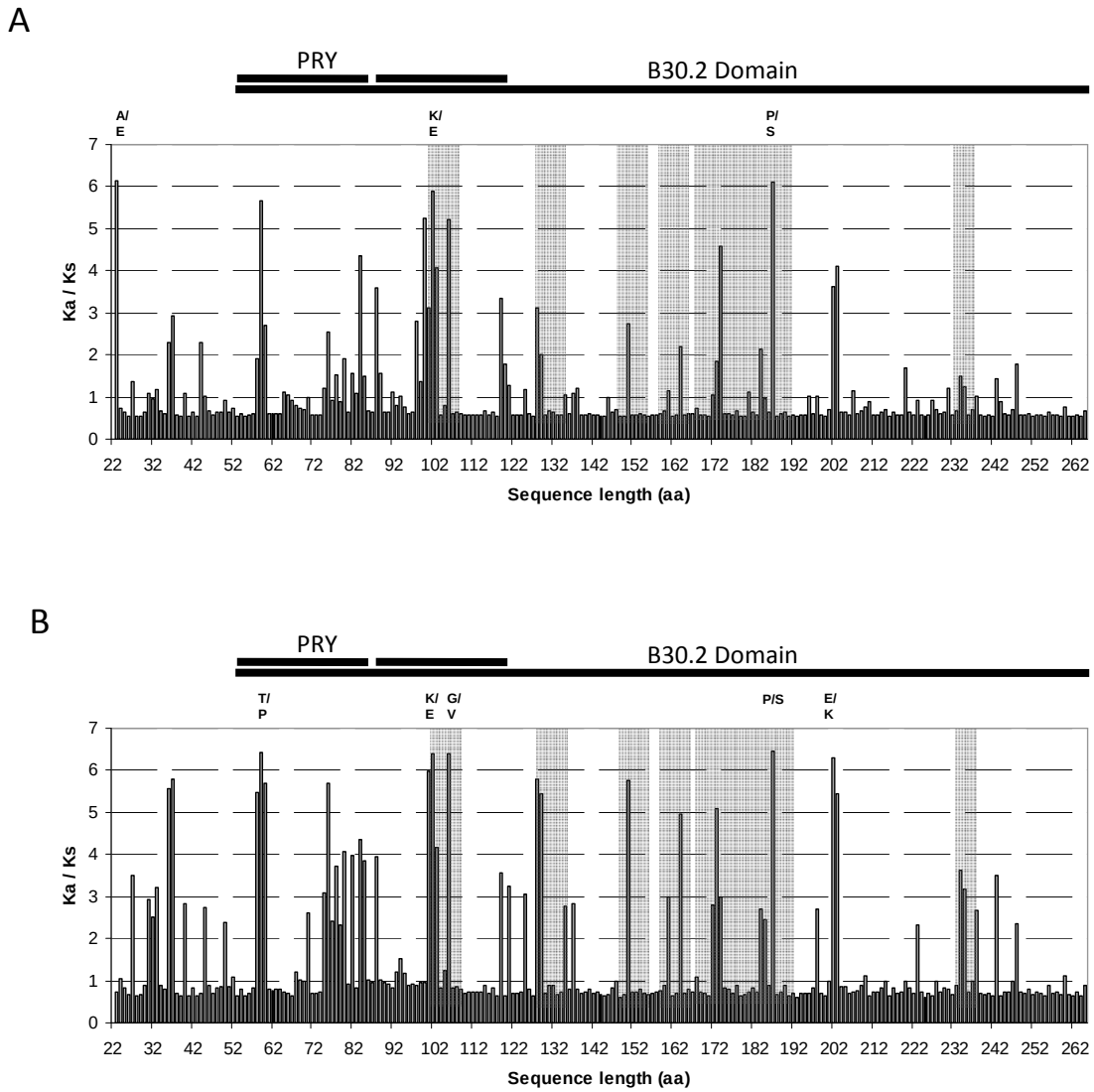

Figure 2.
Distribution of the Ka/Ks ratio along the RBP-1 amino acid sequence.
Analyses were conducted using the codeml module of PAML on the full data set of *G. pallida* and *G. mexicana* sequences (**A**) or on a subset corresponding to the sequences obtained from the four Peruvian *G. pallida* populations plus the *G. mexicana* sequences (**B**). Amino acid variants found to be subjected to positive selection with posterior probability >95% are indicated above each site. Sequence portions corresponding to the SPRYSEC extended loops in the B30.2 protein structure are highlighted in pink. The entire B30.2 domain is indicated by a bar above the graph, with the region containing the duplicated PRY domains indicated by double bars.

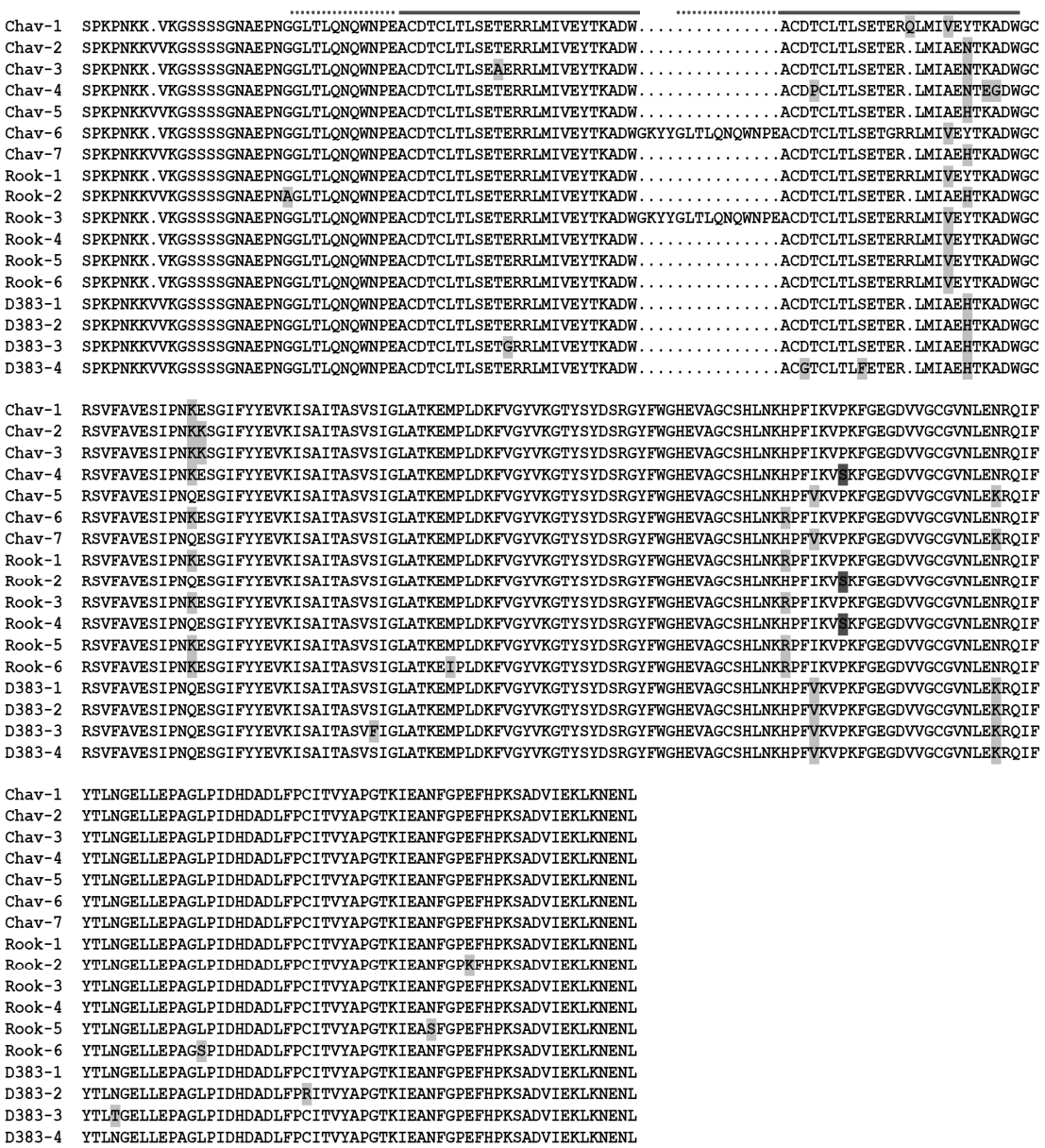

Figure 3.
Analysis of *Gp*-RBP-1 variants from virulent and avirulent populations. Alignment of deduced *Gp*-RBP-1 proteins encoded by cDNA sequences cloned from *G. pallida* populations D383 (avirulent; pathotype Pa-2), Rookmaker (virulent; Pa-3) and Chavornay (virulent; Pa-2/3). Variant residues are indicated with shading, with the critical proline/serine polymorphism indicated in red. PRY domain repeats are indicated by a red bar over the alignment, with the dashed segment of the bar corresponding to an extension of the repeat in two of the variants. The SPRY homology domain is overscored by the black bar.

Gp-RBP-1 Variants from both Avirulent and Virulent Populations Elicit Gpa2

The *Gpa2* gene restricts only a limited subset of *G. pallida* populations [14]. However, the possibility that virulent and avirulent individuals might co-exist within virulent populations has not been examined. We focused on the pathotype 2 (Pa-2) population D383, which is avirulent on *Gpa2* plants, and the virulent pathotype 3 (Pa-3) population Rookmaker [29], as well as Chavornay (Pa-2/3), to seek correlations between recognition by Gpa2 and the polymorphisms within and between these populations. Of a total of 76 sequences derived from RT-PCR from multiple individuals from either D383 or Rookmaker populations, we obtained four different sequences from D383 (D383-1, 37 times; D383-2, twice; D383-3, once; D383-4, once) and six from Rookmaker (Rook-1, 18 times; Rook-2, 8 times; Rook-3, 4 times; Rook-4, twice; Rook-5, twice; Rook-6, once). The *Gp*-RBP-1 sequences deduced from these populations showed a number of insertion/deletion polymorphisms and amino acid substitutions (Figure 2). Most notably, Chav-6 and Rook-3 showed a 15 aa indel that is highly similar in length and sequence to that encoded by *Gp-Rbp-1* intron 3 (44 bp in length) [18]. Thus, some *Gp*-RBP-1 isoforms may be expressed by alternative splicing although the possibility that these clones represent different alleles of the same gene or different gene copies cannot be discounted. Indeed, since these sequences were identified from a population of individuals, we cannot definitively conclude whether all the sequences we have analyzed derive from different alleles of the same gene or from different gene copies. However, the diversity seen herein is a characteristic often seen in pathogen Avr genes [30,31].

To test for recognition by Gpa2, the open reading frames, minus the SP, of the seventeen different *Gp*-RBP-1 variants identified from the D383, Rookmaker and Chavornay populations were cloned in frame with a C-terminal hemagglutinin (HA) epitope tag. All clones from the avirulent population D383 induced a Gpa2-specific HR on *Gpa2*-transgenic *N. tabacum* (tobacco; Figure 4A). Several *Gp*-RBP-1 variants from Chavornay and Rookmaker were also recognized by Gpa2, although some differences in HR strength were consistently observed (Figure 4A). Three variants (Chav-4, Rook-2 and Rook-4) failed to elicit a Gpa2-dependent HR despite the detection of similar protein levels of all variants by immunoblotting (Figure 4C). We also tested two RBP-1 variants (Gmex-1 and Gmex-2) from *G. mexicana*, which share high degrees of amino acid sequence similarity with *Gp*-RBP-1 proteins but encode only a single PRY domain (Figure S1). Neither of these *Gm*-RBP-1 proteins elicited a Gpa2-dependent HR (Figure 4).

A Single Residue Determines Gpa2 Recognition of *Gp*-RBP-1

Despite numerous polymorphisms in *Gp*-RBP-1 variants, only a proline/serine polymorphism at position 187, relative to the reference full-length Guic-3 sequence (Figure S1), correlated with recognition by Gpa2 (Figures 3 and 4A). This residue was also shown to be under positive selection in the evolutionary analysis of the Peruvian *G. pallida* populations and in the full dataset (Figure 2 and Table S1). To test the importance of residue 187 in recognition by Gpa2, we substituted serine and proline codons at position 187 in Rook-1, Rook-4, Chav-7, and Gmex-1. The substitution of proline 187 to serine in Rook-1 and Chav-7 abolished recognition by Gpa2, whereas substitution of serine 187 to proline in Rook-4 and Gmex-1 resulted in a gain of recognition by Gpa2, although the Gmex-1 S166P protein elicited only a very weak HR (Figure 5A). These observations are consistent with an absolute requirement for a proline residue at position 187, but suggest that other regions of the protein likely modulate the potential for recognition by Gpa2.

To explore further the role of the structurally variable RBP-1 N terminus in recognition by Gpa2, we tested constructs of Chav-7 with serial deletions of its PRY sequences, and exchanged the Gmex-1 SPRY domain for that from Chav-7 (Figure 5B). Chav-7 deletants lost their ability to elicit Gpa2, however, immunoblot detection demonstrated that these proteins accumulated to lower levels, suggesting that the deletions destabilize the protein. On the other hand, the chimeric protein comprising the single PRY domain from Gmex-1 and the Chav-7 SPRY domain was recognized by Gpa2, albeit, to a lesser degree (Fugre 5B). This result indicates that an intact N-terminus is required for recognition of *Gp*-RBP-1 by Gpa2, and that variation in this region of the protein can influence the strength of recognition by Gpa2.

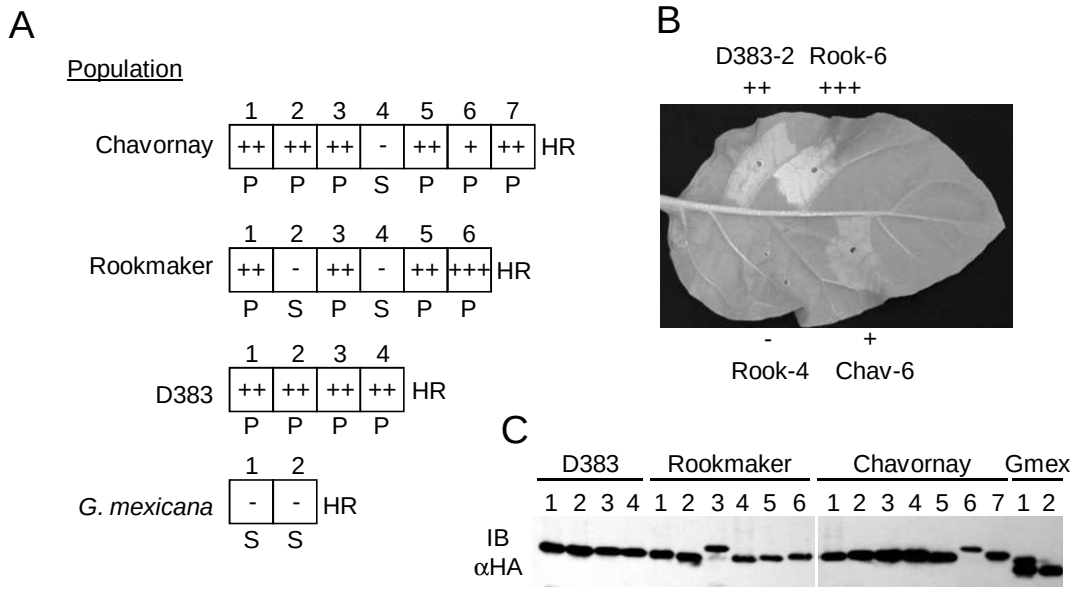

Figure 4.
Recognition of *Gp*-RBP-1 by Gpa2 corresponds to avirulence, but not virulence in *G. pallida* populations. **A** - *Gp*-RBP-1 variants (shown in Figure 3) cloned into pBIN61 as HA-tagged proteins under control by the CMV 35S promoter were transiently expressed via agro-infiltration on GPAII::Gpa2 transgenic tobacco. The responses in the infiltrated patches were scored visually with a complete lack of response scored as (-). Positive HR responses were scored as follows: complete collapse and rapid desiccation of the infiltration patch within 2 days (+++), complete collapse of the infiltration patch by 3 days post-infiltration (++), or slow and incomplete collapse with residual live cells (+).
HR phenotypes representative of the scale used herein are shown (**B**), as photographed seven days after infiltration. The presence of either a proline (P) or serine (S) residue at the position corresponding to Rook-1 residue 187 is indicated.
C - Immunoblot with horse radish peroxidase-conjugated anti-HA antibody demonstrating relative protein levels of transiently expressed RBP-1 proteins.

RanGAP2 is required for HR Induced through Gpa2

Previously, the RanGAP2 protein was shown to interact with the N-terminal CC domains of both Rx and Gpa2, and to be required for Rx-induced responses to PVX [15,16]. A lack of workable reverse genetic approaches precluded an investigation of the requirement for RanGAP2 in the potato-nematode interaction. Therefore, to test the requirement for RanGAP2 in Gpa2-mediated responses, we generated transgenic *N. benthamiana* expressing Gpa2 from the *Rx* genomic promoter as well as PVX derivatives expressing Gpa2-eliciting (D383-2 or D383-4; PVX-D2 and PVX-D4) or non-eliciting (Rook-2 or Chav-4; PVX-R2 and PVX-C4) versions of *Gp*-RBP-1. RanGAP2 expression was silenced by virus-induced gene silencing (VIGS) using a tobacco rattle virus (TRV) vector [15]. As a control, plants were inoculated with the empty TRV vector (TV:00). Rub-inoculation of TV:00-infected plants with PVX expressing either PVX-D2 or PVX-D4 resulted in the presentation of HR-type lesions in the inoculated leaves (Figure 6A). However, resistance responses induced by Gpa2 failed to prevent systemic spread of the recombinant viruses, resulting in a spreading systemic HR (SHR; Figure 6A). Although this response differs from the Rx-mediated response to most PVX strains [12] it resembles the response seen in *Rx* transgenic *N. benthamiana* infected with a strain of PVX weakly recognized by Rx [32]. Indeed, SHR-type responses are commonly seen in interactions between *R* genes that are not able to fully contain virus infection due to weak recognition [4]. In contrast, PVX-R2 and PVX-C4 did not induce HR lesions or SHR (Figure 6A). Silencing of RanGAP2 abrogated both the induction of local HR and SHR by PVX-D2 and PVX-D4, demonstrating a requirement for RanGAP2 in Gpa2 function (Figure 6A).

To complement our VIGS experiments, we also used a dominant-negative approach to block RanGAP2 function in Gpa2-mediated responses. Plant RanGAP proteins possess a plant-specific N-terminal WPP domain that includes a three amino acid signature motif (WPP) shown to be essential for concentrating RanGAP1 protein to the cytoplasmic side of the nuclear envelope as well as the cell division plane [33,34]. The Rx CC domain interacts with RanGAP2 through the WPP domain [16] as does the Gpa2 CC domain (Figure S2). We fused the WPP of RanGAP2 to EGFP:HA (WPP:EGFP:HA) and used this construct to stably transform *N. benthamiana*, with control transgenic lines generated to express EGFP:HA. Over-expression of WPP:EGFP:HA completely blocked the HR elicited by transient expression of Gpa2 plus *Gp*-RBP-1:EGFP:HA (Figure S2B). However, it had no effect on the CP-dependent HR elicited by Rx or by Pto plus AvrPto (Figure S2B). Although interference by WPP:EGFP:HA appeared to be specific to Gpa2, we do not rule out the possibility that residual endogenous RanGAP2 activity may be sufficient for Rx function, which normally mediates a more rapid and stronger HR than Gpa2.

Artificial tethering of RanGAP2 and *Gp*-RBP-1 enhances Gpa2-mediated HR

A number of proteins that interact with the N termini of NB-LRR proteins mediate Avr recognition by their cognate NB-LRR partner [35,36,37,38] and we have previously suggested that RanGAP2 may play a similar role with by Rx and Gpa2 [15]. However, we have been unable to consistently show a direct interaction between *Gp*-RBP-1 and potato RanGAP2 by yeast two-hybrid or co-immunoprecipitation (M.A.S. and P.M., unpublished data). In an attempt to demonstrate in situ interactions, we employed the bimolecular fluorescence complementation (BiFC) technique using split YFP fragments [39]. Constructs were

generated to fuse either the N-terminal or C-terminal YFP fragments, plus a FLAG epitope tag, to the C-termini of proteins of interest (nYF and cYF).

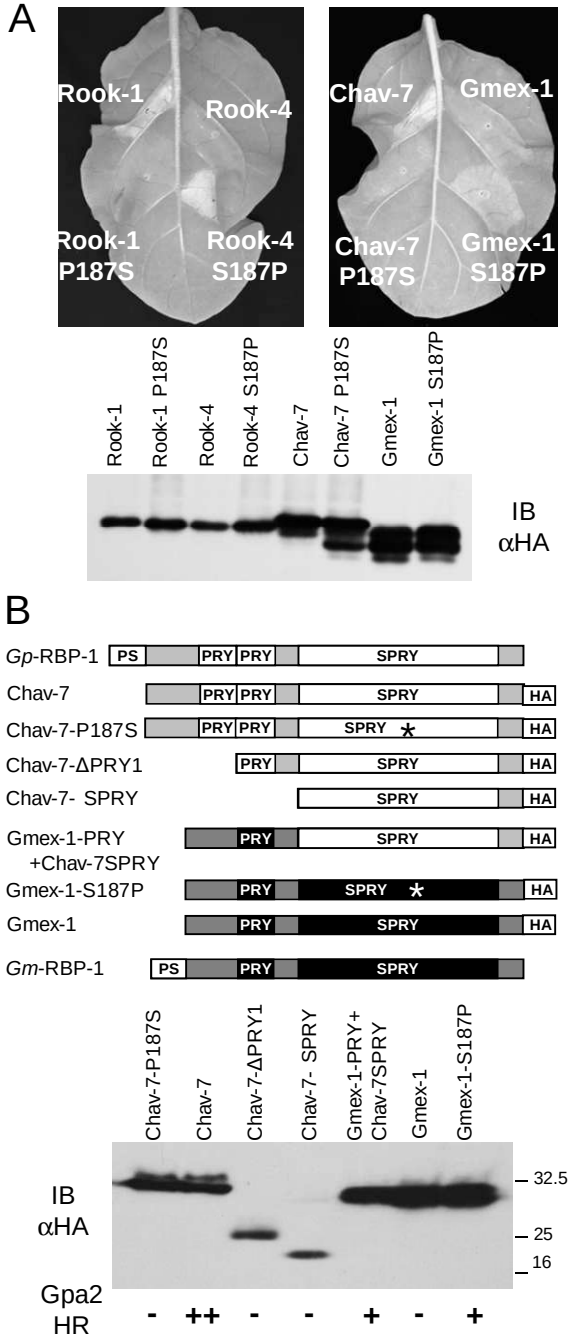

Figure 5.
A single residue in the *Gp*-RBP-1 SPRY domain is a key determinant of Gpa2 recognition.
A - Proline 187 of Rook-1 and Chav-7 was substituted for serine and serine 187 of Rook-4 and Gmex-1 was substituted for proline. The resulting *Gp*-RBP-1:HA proteins were transiently expressed in *Gpa2* tobacco leaves. Note that Rook-4 S187P induced an HR of a strength equivalent to those elicited by Rook-1 and Chav-7 (+++ as per Figure 4B), whereas Gmex-1 S187P induced a much weaker response (+ as per Figure 4B). Gp-RBP-1:HA variants were also expressed in wild-type tobacco and protein extracts were subjected to anti-HA immunoblotting (IB) to determine protein expression levels (lower panel).

B - Deletions of, and fusions between, *G. pallida* Chav-7 and *G. mexicana* Gmex-1 RBP-1:HA are represented schematically. Individual proteins were expressed in wild-type tobacco and protein extracts were subjected to anti-HA immunoblotting to determine protein expression levels (lower panel). Individual proteins were scored for their ability to induce an HR on *Gpa2*-transgenic tobacco as per the scale in Figure 4B.

BiFC fusion proteins were first tested for functionality in HR assays. Although the *Gp*-RBP-1 (D383-2) protein elicits a Gpa2-dependent HR within three days of agroinfiltration (++, Figure 4A), fusion of *Gp*-RBP-1 (D383-2) with the YFP fragments (D383-2:nYF and D383-2:cYF) resulted in a much weaker elicitation of Gpa2-mediated HR (+ as per the scale in Figure 4B). However, we observed a strong HR (+++ as per Figure 4B) upon co-expression D383-2:cYF with RanGAP2 fused to the nYFP fragment (RanGAP2:nYF) in *Gpa2*-transgenic tobacco leaves (Figure 7A). A similar, albeit less pronounced, HR enhancement was seen with the reciprocal combinations of complementing YFP fragments, D383-2:nYF and RanGAP2:cYF (Figure 7A). This weaker response, however appears to correlate with lower expression of D383-2:nYF (Figure 7B). Comparison of protein expression levels of RanGAP2:cYF, RanGAP2:nYF and RanGAP2 with only a FLAG tag (RanGAP2:F) showed that HR enhancement correlated with the presence of complementing YFP fragments, and not protein expression levels (Figure 7B). As an additional control, D383-2:nYF and D383-2:cYF were co-expressed with GUS YFP fragment fusions, GUS:nYF and GUS:cYF, neither of which showed any effect on enhancing the Gpa2-mediated HR (Figure 7A).

The reconstitution of YFP fragments is irreversible [40]. Indeed, we find that all combinations of HA or FLAG tagged nYFP and cYFP fusion proteins that we have tested interact and can be efficiently co-immunoprecipitated (Figure S4, MAS and MJJ unpublished data). Since the control protein GUS also interacted with all proteins tested in this assay (Figure S4) split YFP reconstitution appears to be highly promiscuous in plants as long as the cognate fusion proteins are stably expressed. Nevertheless, we reasoned that if the recognition by Gpa2 is mediated by a weak or transient interaction between RanGAP2 and *Gp*-RBP-1, then strengthening such an interaction would strengthen the degree of Gpa2 activation. To test the specificity of this phenomenon we introduced Gp-RBP-1 (Rook-4), which is not recognize by Gpa2 (Figure 4A) into the split YFP assay with RanGAP2. Although YFP complementation allowed these two proteins to interact physically, it did not result in a gain of recognition of Gp-RBP-1 (Rook-4) by Gpa2 (Figure S3A). Moreover, complementing pairs of *Gp*-RBP-1 and RanGAP2 did not activate the Rx protein (Figure S5). These results suggest that the artificial tethering of *Gp*-RBP-1 proteins to RanGAP2 mimics and enhances an interaction that normally occurs between these proteins, but that interaction alone is not sufficient to activate the associated NB-LRR protein. Thus, although RanGAP2 is involved in an initial phase of Avr interaction, recognition specificity is nonetheless determined by the NB-LRR protein.

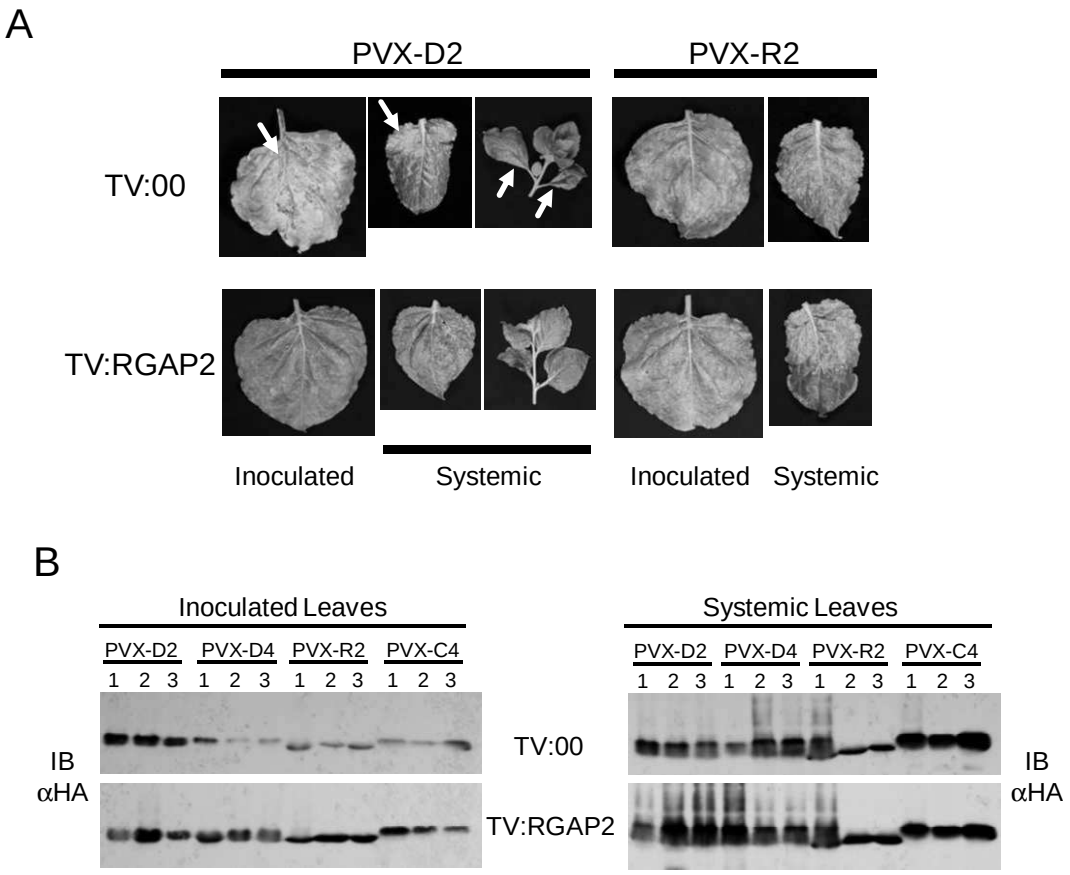

Figure 6.
Gpa2-mediated responses to PVX-RBP-1:HA requires RanGAP2. PVX vectors were generated to express two avirulent versions (D383-2 and D383-4) of *Gp*-RBP-1:HA (PVX-D2 and PVX-D4) as well as two virulent (Rook-2 and Chav-4) variants (PVX-R2 and PVX-C4).
A -Virus saps containing recombinant viruses were rub-inoculated onto *Gpa2*-transgenic *N. benthamiana* that had previously been infected with the empty TRV VIGS vector or TRV:RGAP2. Phenotypes from a representative experiment are shown for PVX-D2 and PVX-R2, photographed two weeks after PVX inoculation. Virus spread to systemic tissues was observed either by the development of systemic lesions and necrosis (PVX-D2 and PVX-D4) or PVX symptoms typical of infected wild-type plants (PVX-R2 and PVX-C4). Necrosis on local and systemic leaves is indicated by arrows.
B - Protein extracts taken from inoculated and systemic leaves of *Gpa2*-transgenic *N. benthamiana* plants, infected as in (A), were subjected to anti-HA immunoblotting (IB) to detect Gp-RBP-1:HA accumulation.

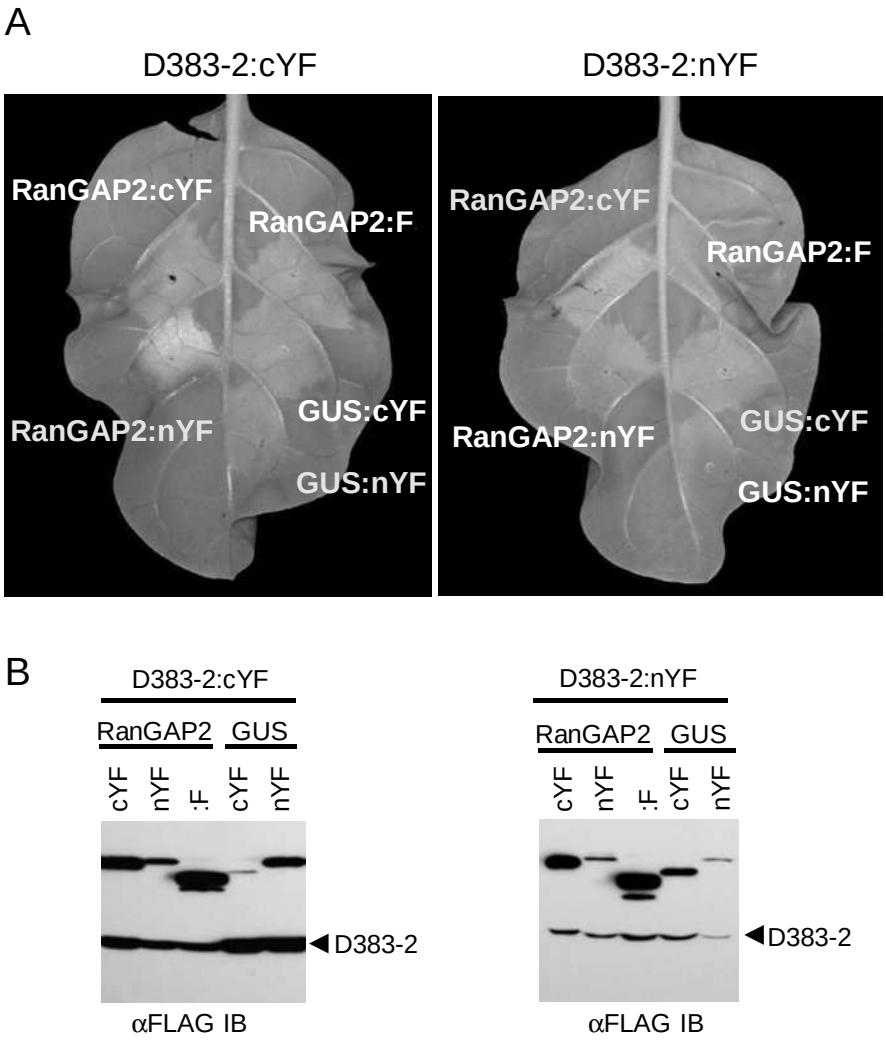

Figure 7.
Tethering of RanGAP2 and *Gp*-RBP-1 enhances Gpa2-mediated HR. The open reading frames of RanGAP2, Gp-RBP-1 clone D383-2 and GUS were fused at their C-termini to either the C-terminal or N-terminal fragments of YFP:FLAG (cYF and nYF, respectively). D383-2:cYF and D383-2:nYF were co-expressed, by agro-infiltration, in *Gpa2*-transgenic tobacco together with both complementing fusion proteins (yellow) and non-complementing YFP fusion proteins (white) as indicated (top panel). RanGAP2 with only a C-terminal FLAG tag (RanGAP2:F) was included as an additional non-complementing control. Fusions proteins were also expressed in wild-type tobacco and protein extracts were subjected to anti-FLAG immunoblotting (IB) to confirm that activation in the combinations with complementing YFP fragments did not correlate with the highest RanGAP2 levels (lower panels).

DISCUSSION

Given a lack of consistent reverse genetics tools for cyst nematodes, we have used functional assays to demonstrate avirulence activity of *Gp*-RBP-1 as defined by the ability of a protein to elicit defense responses by a specific R protein. The presence of matching R and Avr proteins is generally sufficient to induce resistance

response, the most obvious being the HR. Our data show that specific *Gp*-RBP-1 variants induce an HR only in the presence of Gpa2 but not Rx or Rx2 (Figures 1 and 4). Thus, by definition, these proteins possess Gpa2 avirulence activity and at a functional level represent a gene-for-gene relationship. Furthermore, these same *Gp*-RBP-1 proteins elicit resistance responses, manifested as systemic HR, when expressed from PVX (Figure 6). The fact that Gpa2 does not fully restrict these recombinant viruses is likely due to the relatively rapid movement of PVX from infected cells, similar to what is seen with versions of PVX that are weakly recognized by Rx [32]. This is consistent with the fact that most *Gp*-RBP-1 variants induced a Gpa2-mediated HR only after three days (Figure 4), whereas the Rx/CP-mediated HR occurs within 24 hours (P. Moffett, unpublished observations). Furthermore, even on *Gpa2* potato plants avirulent *G. pallida* induce an HR only after 7-9 days, (K. Koropacka, unpublished observations) suggesting that the Gpa2 response is relatively weak, possibly due to an inherently weak recognition of Avr proteins. Since the nematode does not move from its initial feeding site, this slow response may be sufficient for nematode resistance whereas it results in SHR in the case of a viral infection.

While *Gp*-RBP-1 alleles displayed many polymorphisms, recognition by Gpa2 could be attributed to a single proline/serine polymorphism in the SPRY domain (Figure 5). However, although a proline at position 187 appears to be absolutely necessary for Gpa2 activation, variations at other sites likely modified the strength of HR induced through Gpa2 and a nearly-intact protein is required for Avr activity (Figures 4 and 5). We only recovered avirulent variants of *Gp*-RBP-1 from the avirulent population D383, consistent with a role for this nematode protein in eliciting Gpa2-mediated resistance. However, both Gpa2-recognized and non-recognized variants of *Gp*-RBP-1 were isolated from two *G. pallida* populations (Rookmaker and Chavornay) virulent to *Gpa2*. It is possible that these versions of *Gp*-RBP-1 are not expressed although this seems unlikely as their isolation depended on the expression of their mRNAs. These data suggest rather, that field populations contain both virulent and avirulent individuals, consistent with the fact that *Gpa2* has not been effective in the field.

On the other hand, it is possible that *Gp*-RBP-1 is not the sole determinant of avirulence among different *G. pallida* populations. A recent report showed that a key gene from the root-knot nematode *Meloidogyne incognita* determining avirulence to the tomato *Mi-1* gene, designated *Cg-1*, could encode an RNA that regulates avirulence. The longest open reading frame (ORF) in *Cg-1* has the capacity to encode a polypeptide of only 32 amino acids without the appearance of signal sequence [41]. It is unlikely that a product of the *Cg-1* gene ultimately elicits the Mi-1 protein and yet silencing of *Cg-1* in the nematode compromised resistance conferred by the *Mi-1* gene. Thus, avirulence as defined genetically, may not correlate absolutely with the possession of a gene encoding avirulence activity, as defined by the elicitation of an R protein by a pathogen-derived molecule. Indeed, this concept is not without precedent. For example, in *Pseudomonas syringae* the effector protein AvrRpt2 interferes with recognition of AvrRpm1 by the NB-LRR protein Rpm1, while the effectors VirPphA and AvrPtoB are able to suppress the HR responses induced by co-delivered Avr proteins [42,43,44]. Suppression of Avr recognition by NB-LRR proteins can be highly specific as in the case of the flax TIR-NB-LRR L6 and L7 proteins which recognize the same versions of flax rust AvrL567 proteins but are differentially suppressed by the presence of the flax rust inhibitor (*I*) gene [30,45,46]. Furthermore the oomycete protein ATR13^{Emco5} confers avirulence toward the

Arabidopsis *RPP13* gene in the ecotype Nd-0 but not ecotype Ws-0, despite the ability of RPP13 to recognize bacterially-delivered ATR13^{Emco5} in both ecotypes [47]. This is reminiscent of the ability of the *Pseudomonas syringae* protein AvrPphC to suppress recognition of AvrPphF, but only in certain bean cultivars [48]. Thus it would appear that the ultimate outcome of the interaction between a given pair of Avr and R proteins can be influenced by additional factors determined by the genotypes of both the pathogen and the host. Only forms of *Gp*-RBP-1 avirulent to Gpa2 were found in population D383 suggesting that this is a prerequisite for Gpa2-mediated resistance. However, the identification of forms of *Gp*-RBP-1 avirulent to Gpa2 in the Rookmaker population might suggest that additional factors present in this population may act epistatically to *Gp*-RBP-1, either suppressing recognition of Gp-RBP-1 by Gpa2 or the ensuing defense responses.

Although this report does not fully address the extent of variability of *Gp-Rbp-1* alleles and homologues, our initial analysis shows a very high degree of amino acid variation encoded within the nematode populations examined. Evolutionary analysis suggested that a number of residues encoded by *Gp-Rbp-1* are under selective pressure. Previous analyses of genes encoding *G. rostochiensis* SPRYSEC proteins have shown that this gene family has undergone diversifying selection [19]. Whether *Gp*-RBP-1 is simply one member of a similarly expanded and diversified *G. pallida* SPRYSEC family remains to be elucidated. However, the *Gp*-RBP-1 sequences appear to be more similar to each other than to *Gm*-RBP-1 (Figure S1). As such, we suggest that the *Gp*-RBP-1 variants represent either different alleles of the same gene or the products of very recent duplications that can effectively be considered to be functionally the same. Thus, our analyses would indicate that the *Gp-Rbp-1* nematode parasitism gene has been subject to diversifying selection within nematode populations. It should be noted that sites under positive selection in *Gp*-RBP-1 were different than those identified in SPRYSEC homologs [19], although both analyses indicated selection on residues predicted to be at the surface of the protein in extended loops of the B30.2 domain (Figure 2). It has been suggested that the B30.2 domain in SPRYSEC proteins could provide a hypervariable binding surface which may be tuned to interact with a variety of protein partners [21]. For RBP-1 and SPRYSEC proteins this would presumably include plant protein targets including selection for interaction with virulence targets and/or selection for avoiding interactions with components involved in pathogen recognition. Such dual evolutionary forces may be further compounded by different selection pressures on alternate hosts and thus it may not be unexpected to find different positions under positive selection when comparing SPRYSEC and RBP-1 proteins.

Mutation and migration are two of the major evolutionary forces considered when assessing the risk of pathogen evolution in management of disease resistance and, due to their lifestyle, cyst nematodes have been associated with a low risk value for overcoming resistance [49]. However, both the high levels of gene flow shown to occur between populations [50,51] and our finding of positive selection in the *Gp-Rbp-1* gene suggest that this risk may be higher than previously thought, with consequent implications for the development of durable resistance strategies.

High levels of variability have been shown for Avr determinants from two other eukaryotic pathogens, the ATR1 and ATR13 proteins from *H. parasitica*, and the AvrL456 proteins from *M. lini*, presumably because they are under selection pressure to evade the plant defense system [52,53]. However, although ATR13 is highly variable, a single polymorphic amino acid determines recognition by RPP13, with a small number of other residues modulating the strength of this response [31].

This shows parallels to Gp-RBP-1 which also shows a great deal of variability (Figure 2) but whose recognition is ultimately determined by a single polymorphic residue (Figures 4 and 5). Thus, the *R* genes in question may not be a major factor in maintaining the diversity of these pathogen effectors. In particular, in the case of *Gp-RBP-1*, *Gpa2* does not restrict most European *G. pallida* populations, nor is it likely that *Gpa2* has exerted a significant pressure on nematode populations. Our analyses indicate that the polymorphism at position 187 in *Gp*-RBP-1 was under positive selection well before *G. pallida* was introduced into Europe (Figure 2 and Table I). Thus the variability seen in *Gp-RBP-1* may be due to selection pressures exerted in the past within the native range of the pathogen which may have included R proteins present in native hosts that recognize *Gp*-RBP-1. Alternatively, it has been proposed that *G. pallida* has adapted to new hosts on multiple occasions throughout its evolutionary history [24] and variation in *Gp*-RBP-1 may have been selected for during these adaptations. The role of RBP-1 and SPRYSEC proteins in parasitism is presently unknown. However, the *G. rostochiensis* protein SPRYSEC19 has been shown to interact physically with an NB-LRR protein without activating it suggesting that it may play a role in inhibiting host defenses or that this family of proteins may be predisposed to recognition by NB-LRR proteins.

Like Rx, Gpa2 both binds to, and requires RanGAP2 for function (Figures 6 and S3). Given the specific interaction of RanGAP2 with Rx-like proteins and a lack of obvious signaling function, we have suggested that RanGAP2 may play a role in recognition by Gpa2 and Rx [15]. Indeed, multiple examples exist where proteins that bind to the N termini of NB-LRR proteins mediate recognition of Avr proteins, including the ternary interactions of AvrPto/Pto/Prf, AvrPphB/PBS1/RPS5, AvrRpm1/RIN4/RPS1, AvrRpt2/RIN4/RPS2, and p50/NRIP1/N [36,54,55,56]. How can these observations be reconciled with domain swapping experiments demonstrating that the LRR domain determines recognition specificity [17,32,57,58,59]? The enhancement of Gpa2-mediated responses by tethering RanGAP2 to *Gp*-RBP-1 are consistent with a role for RanGAP2 as a recognition co-factor (Figure 7) that initially interacts with the Avr protein. However, tethering is not sufficient to induce activation of Gpa2 by non-recognized versions of *Gp*-RBP-1 nor is it sufficient to activate the Rx protein (Figures S4-S6). Thus, despite a prerequisite for an interaction with RanGAP2, it appears that the LRR domain determines which interactions will be productive. Such a scenario may explain apparently contradictory reports showing both direct and indirect interactions between the TIR-NB-LRR protein N and its cognate Avr determinant the p50 subunit of the tobacco mosaic virus (TMV) replicase. In the plant cell, P50 interacts with N only in the presence of the chloroplast protein NRIP1 [55], whereas there appears to be a direct interaction between N and p50 in the yeast two-hybrid system and *in vitro* [60]. A general mechanism for NB-LRR recognition of their cognate Avr determinants through a two-step process could reconcile such discrepancies. Indeed the N/p50 example would suggest that the NRIP1/TIR complex might stabilize a subsequent interaction between p50 and the N LRR domain. Furthermore, such a scenario could provide a mechanism to explain how NB-LRR proteins might evolve new recognition specificities without having to evolve to bind new cellular recognition co-factors. Further work will be required to determine whether such recognition co-factors are differentially modified by Avr proteins, resulting in activation of the NB-LRR or whether they act to somehow present Avrs to the LRR domain which in turn mediates recognition. In addition, it is of interest to determine whether RBP-1 proteins may target RanGAP2 as part of a virulence function as predicted by the guard hypothesis

[1] or whether it simply mimics the true virulence target(s) of RBP-1 as predicted by the decoy model [6].

Materials & Methods

Plant Material and Transient Expression

N. benthamiana and *N. tabacum* plants were germinated and grown in a glass house or growth chambers maintained at 23°C. All experiments were repeated at least three times. Virus-induced gene silencing (VIGS), transient expression of proteins (Agro-expression), protein extraction, immuno-precipitation and immuno-blotting were carried out as previously described [15].

Transgenic *N. benthamiana* expressing *Gpa2* from the *Rx* native promoter were generated by stable transformation using *A. tumefaciens* strain LBA4404 carrying binary vector clone pB1-Gpa2 as previously described [15]. Transgenic *N. benthamiana* were generated to stably express RanGAP2 WPP:EGFP:HA and EGFP:HA from the cauliflower mosaic virus (CaMV) 35S promoter by transforming leaf tissue using *A. tumefaciens* strain C58C1 carrying binary vector constructs pBIN61-WPP:EGFP:HA or pBIN61-EGFP:HA (described below), and selecting on kanamycin. Transgenic *N. tabacum* expressing *Gpa2* from the *GPAII* native promoter were generated by stable transformation using *A. tumefaciens* strain pMOG101 carrying binary vector *pBIN+GPAII::Gpa2*.

Plasmid Construction

For generation of expression clones, all inserts were ligated into 5´ *Xba*I and 3´ *Bam*HI sites of the pBIN61 binary vector series unless otherwise indicated. This vector series contains epitope tags, or the enhanced red-shifted variant of jelly fish green fluorescent protein (EGFP) with an HA epitope tag, positioned for carboxy-terminal tagging of inserts in frame with the *Bam*HI site [17,61,62]. To obtain the complete *Gp-Rbp*-1 ORF, cDNA prepared from *G. pallida* pathotype (Pa) 2/3 population Chavornay [18] was amplified with primers GpaRBPMForSP (5´-CTCTAGAATTTATTGCCCCCAAAATG-3´) and GpaRBPMstopRev (5´-GGATCCAGCAAACCCATCATAAATTCTCG-3´) and ligated into the pGEM-T vector. A pGEM-T clone was used to amplify fragments that 1) had the signal peptide deleted using primers GpaRBPMforXba (5´-CTCTAGACCATGGAGTCGCCAAAACCAAAC-3´) plus GpaRBPMstopRev; 2) had the stop codon changed to a *Bam*HI site for epitope tagging, using primers GpaRBPMForSP and GpaRBPMrevBam (5´-CCTGGATCCTAAATTCTCGTTTTTC-3´) or 3) had both the signal peptide deletion and the *Bam*HI site substitution for the stop codon, using primers GpaRBPMforXba and GpaRBPMrevBam. Nematodes from virulent (Rookmaker, Pa-3) and avirulent (D383 Pa-2) population of *Globodera pallida* (Pa-2/3) were hatched from eggs in the presence of potato root diffusate. Juveniles in the preparasitic stage (J2) were collected and used for RNA extraction followed by cDNA synthesis (Super Script III, Invitrogen). All additional *G. pallida* and *G. mexicana* RBP-1 clones were obtained by amplification with primers GpaRBPMrevBam plus either Chav6-7forXba (TGTCTAGAACCATGGAGTCGCCAAAACCAAAC), Gmex-1forXba (TGTCTAGAACCATGGAGTCGCCAAAAACAAAC), or Gmex-2forXba (TGTCTAGAACCATGGAGTCATCCAGTCCTGGCAATAC). A fragment without the signal peptide and with a BamHI substitution of the stop codon was amplified from cDNA prepared from *Globodera rostochiensis* pathotype Ro_1 kindly provided by X. Wang, using primers GroRBPMforXba (5´-CTCTAGACCATGGATTCGCCGCCGCCAAAAAC-3´) and GroRBPMrevBam (5´-GGATCCAAATGGGCCAAAGTTCG-3´). YFP N- and C-terminal fragments were amplified by PCR using the enhanced yellow fluorescent protein (EYFP) from the pSAT vector series as a template [63] with primers BamFor-N-YFP (5´-GGATCCGGGATGGTGAGCAAGGG-3´) plus BglRev-N-YFP (5´-CAGATCTGTCCTCGATGTTGTGG-3´) for the N-terminal fragment and BamFor-C-YFP

(5´-GGATCCATGGGCGGCAGCGTGCAG-3´) plus BglRev-C-YFP (5´-CAGATCTCTTGTACAGCTCGTCCATGC-3´) for the C-terminal fragment. Inserts cloned into pGEM-T were digested with *Bam*HI and *Bgl*II and ligated into the *Bam*HI site of pBIN61 constructs with either a FLAG:6His (FH) or HA tag [61], allowing subsequent cloning of candidate genes in frame with the epitope tagged YFP fragment using the 5´ *Bam*HI site. Site directed mutants and swap-domain constructs were generated based on extension overlap PCR. Primers were designed to change proline 187 to serine in Chav-7, and the equivalent serine to proline in Gmex-1, and to fuse aa 23-95 of Gmex-1 to aa 121-265 of Chav-7 (Figure S1). The Chav-7 deletion constructs were generated by PCR and correspond to fragments expressing residues 82-265 and 121-265 of Chav-7. A methionine and an alanine residue were added to N-terminal deletion constructs.

The GPAII:Gpa2 construct was assembled from the promoter region of the Gpa2 gene, the coding sequence, and the 3´-UTR. First, the 3´-UTR of *Gpa2* (274 bp) was amplified from pBINRGC2 [13] using the primers 5UTRkp (5´-TGGTACCTTCTGCAGCGAGTAGTTAAGGTGTTCTGAGGAC-3´) and 3UTRrev (5´-CTTAATTAACCCGGGAGATTGAGGACTCCCAAGAAAGG-3´). The amplicon was subcloned into the *KpnI* and *PacI* sites of pRAP-YFP. The *Gpa2* promoter region (GPAII; 2744 bp upstream of start codon, including the 5´-UTR) was subcloned into the *Asc*I and *Nco*I sites of the pRAP-3´UTR-YFP to generate pRAP-GPAII-3´UTR-YFP. The 5´-end of the *Gpa2* coding sequence was PCR amplified from pBINRGC2 [13] using primers 5´GpRxbn (5´-TTTTTGGATCCATGGCTTATGCTGCTGTTACTTCCC-3´) and GpRxStuRev (5´-CAAAGAAAGAAGGCCTAGGAGTAC-3´). The *Nco*I and *Pst*I fragment was ligated together with an *Avr*II-*Pst*I fragment from pBINRGC2 into the *Nco*I and *Pst*I sites of pUCAP making pUCAP-Gpa2 [64]. The *Nco*I and *Pst*I fragment from the pUCAP-Gpa2 plasmid was subsequently into the *Nco*I and *Pst*I sites of pRAP-GPAII-3'UTR-YFP, resulting in pRAP-pGPAII::Gpa2-3´UTR-YFP. As a final cloning step, the *Asc*I-*Pac*I fragment of pRAP-pGPAII::Gpa2-3´UTR-YFP was ligated into corresponding sites in the binary plasmid pBIN+ resulting in pBIN-GPAII::Gpa2.

DNA and Protein Sequences and Analysis

DNA sequences were translated to protein and aligned using the Translator and ClustalW-based Aligner programs of the JustBio suite (Pierre Rodrigues, www.justbio.com/tools/phb). New *Gp-Rpb-1* sequences functionally analyzed in this study have been deposited to GenBank/EMBL databases under the following accession numbers: AM491352 (Chav-1), AM491353 (Chav-2), AM491354 (Chav-3), AM491355 (Chav-4), AM491356 (Chav-5), FJ392678 (Chav-6), FJ392677 (Chav-7), EF423897 (Rook-1), EF423898 (Rook-2), EF423899 (Rook-3), EF423900 (Rook-4), EF4238901 (Rook-5), EF4238902 (Rook-6), EF423893 (D383-1), EF423894 (D383-2), EF423895 (D383-3), EF423896 (D383-4). New *G. mexicana Rbp-1* sequences analyzed in this study have been deposited to GenBank/EMBL databases under the following accession numbers: FJ392679 (Gmex-1), and FJ392680 (Gmex-2). Additional *G. pallida* sequences used for PAML analysis were: EU982195 (Luffness; GPE1), EU982196 (Ouessant; GPE2), EU982197 (Chavornay; GPE3), EU982198 (Duddingston; GPE5) and EU982199 (Guiclan; GPE6) from Europe; and EU982200 (Colque-cachi; GPS3), EU982201 Chamancalla; GPS5), EU982202 (Ballo-ballo; GPS7), EU982203 (Chocon; GPS8), EU982204 (Otuzco; GPS9), and EU982205 (Huamacucho; GPS10) from Peru. Additional sequences relevant for this report can be retrieved from the GenBank/EMBL databases under the following accession numbers: AJ251757 (*Gr*-RBP-1) AJ011801 (Rx), AJ249449 (Rx2), AJ249449 (Gpa2), AF172259 (PVX-CP), AF202179 (Bs2), and AM411448 (RanGAP2).

Construction of the sequence data sets

Complementary DNAs encoding *Gp-Rbp1* were amplified from 13 *G. pallida* populations (7 European and 6 Peruvian) as described, using specific primers 5'IC5.2 and 3'IC5.2 [18]. The PCR products were cloned and sent to Macrogen (www.dna.macrogen.com) for sequencing. Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin) with DNA 5-0 alignment parameters was used for multiple sequence alignment [65]. The alignment was manually corrected when necessary. The MEGA program v 3.1 was used to obtain Neighbour-Joining trees [66].

Evolutionary analysis: Identification of sites under positive selection

Selective pressures on RBP-1 sequences were evaluated using the ratio of nonsynonymous to synonymous substitution rates per site ($\omega = K_a/K_s$) using the phylogenetic analysis by maximum likelihood (PAML), single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), internal branches fixed-effects likelihood (IFEL) and random effect likelihood (REL) methods implemented in the PAML package version 3.14 [67] or in the HYPHY package [26]. A value of $\omega = 1$ reflects neutrality, $\omega < 1$ indicates purifying selection and $\omega > 1$ indicates positive selection. PAML analyses were done with the CODEML program (M1 vs M2 and M7 vs M8 models). The Bayes Empirical Bayes approach was used to calculate the posterior probabilities that each site fell into a different K_a/K_s (or ω) class [68]. PAML assigns a likelihood score to models for selection. A likelihood score for a model incorporating positive selection that is higher than that for a null model without positive selection is evidence for positive selection. The significance of the differences was estimated by comparing the null model and positive selection model ($2\Delta l$) with a chi square table (Likelihood Ratio Test, LRT).

Acknowledgments

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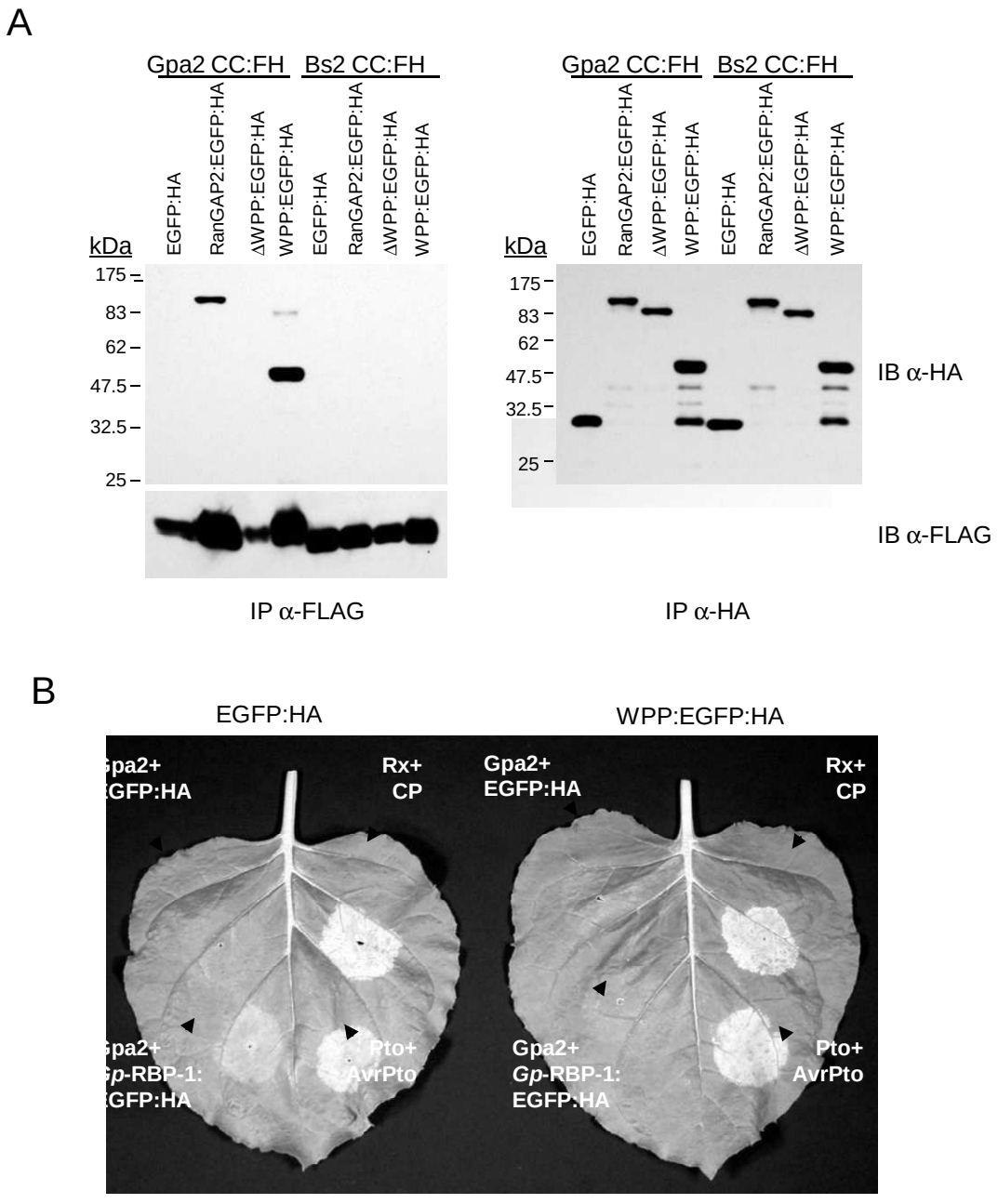

Figure S2.
Interaction between RanGAP2 and Gpa2 through their amino-terminal domains.
A - FLAG-tagged CC domains from Gpa2 and Bs2 were transiently co-expressed by agro-infiltration with RanGAP2 or fragments thereof as EGFP:HA fusion proteins in *N. benthamiana*. Reciprocal co-immunoprecipitations with anti-FLAG and anti-HA conjugated agarose beads demonstrate that the RanGAP2 amino-terminal WPP domain interacts specifically with the Gpa2 CC domain when analyzed on immunoblots detecting the epitope tags.
B - A dominant-negative version of RanGAP2, consisting of a 133 amino acid fragment from the RanGAP2 amino terminus was expressed transgenically as a GFP fusion protein in *N. benthamiana* (WPP:EGFP:HA). Control lines were also generated expressing EGFP:HA protein. Leaves were infiltrated with 35S::Pto plus 35S::AvrPto or pB1-Gpa2 plus pBin61-EGFP:HA as positive and negative HR controls, respectively. The RanGAP2 dominant-

negative effect was assayed by co-infiltration of pB1-Rx:HA with pBin61-CP, or pB1-Gpa2 with pBin61-*Gp*-RBP-1:EGFP:HA.

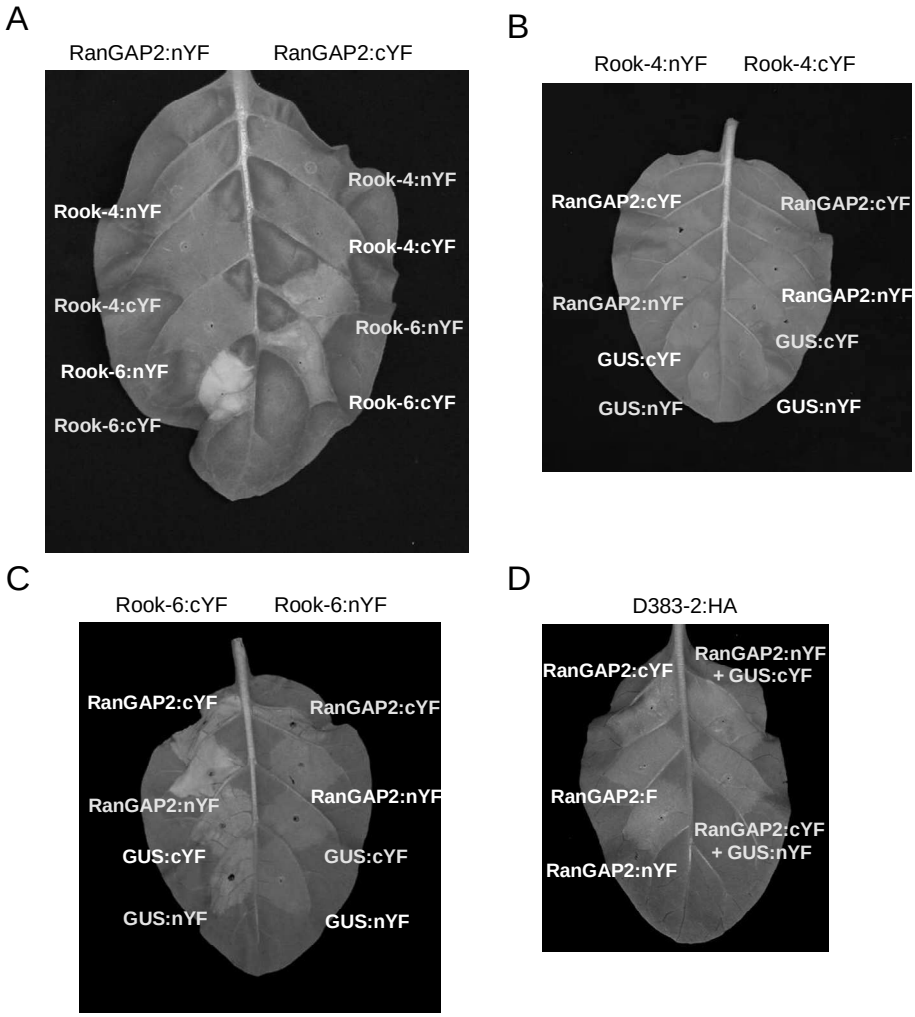

Figure S3.
Enhancement of HR through Gpa2 by complementing YFP fragments fused to RanGAP2 and *Gp*-RBP-1 is specific for avirulent variants of Gp-RBP-1. Reciprocal YFP fragment fusions of *Gp*-RBP-1 (Rook-4 and Rook-6) were co-expressed in *Gpa2*-transgenic tobacco together with the indicated nYF and cYF fusions of RanGAP2 and GUS (**A**-**C**). Complementing pairs of YFP fragment fusion proteins are noted in yellow non-complementing combinations in white. Note that Rook-6:nYF induces a weaker response than Rook-6:cYF (**A**), similar to that seen with D383-2:nYF (Figure 7A). (**D**) HR enhancement did not result simply from the co-expression of D383-2 with RanGAP2:nYF, RanGAP2:cYF or RanGAP2:F demonstrating a requirement for YFP complementation in the HR enhancement.

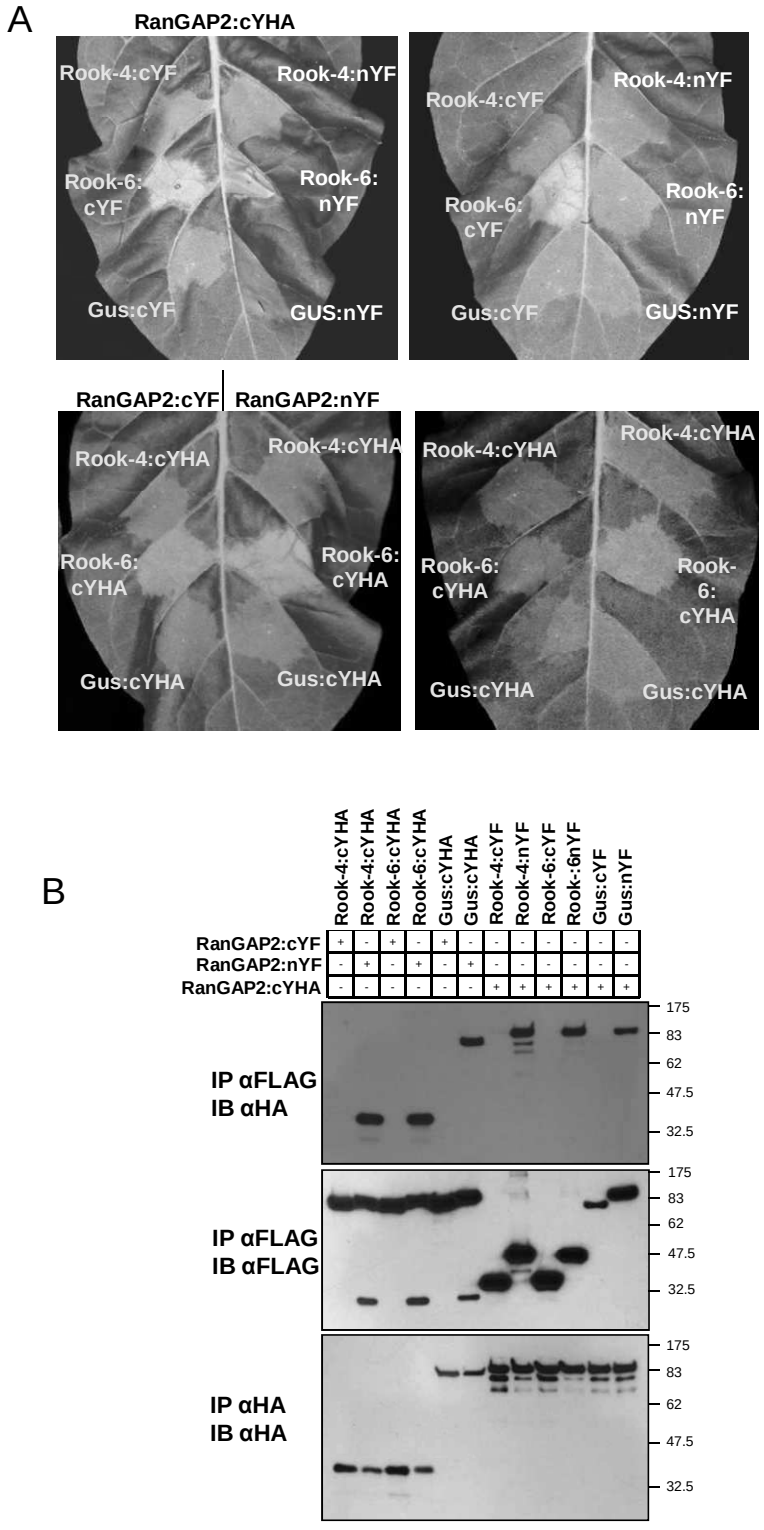

Figure S4.
Enhancement of Gpa2-mediated HR by YFP complementation correlates with physically interaction between RanGAP2 and *Gp*-RBP-1 fusion proteins. In order to demonstrate physical interaction between YFP fragment fusions, the FLAG epitope tag of nYF and cYF fusions was replaced with an HA epitope tag (nYHA and cYHA). Rook-4, Rook-6 and GUS fusions with either nYHA, cYHA, nYF or cYF were transiently expressed in Gpa2-transgenic tobacco either alone (right hand side) or together with either RanGAP2:cYHA,

RanGAP2:cYF or RanGAP2:nYF (A). HR induction results with HA fusions were similar to those obtained in experiments in which all fusions were tagged with the FLAG-epitope (compare top versus bottom panels and this figure to Figure S3). (B) Similar combinations of YFP fusion proteins were co expressed in wild-type *N. benthamiana.* Protein extracts were subjected to-immunopreciptation (IP) was performed with anti-FLAG agarose beads followed by immunoblotting (IB) with anti-FLAG and anti-HA antisera. Anti-HA immunoprecipitation followed by anti-HA immunoblotting was also performed to detect HA epitope-tagged fusions for confirmation of expression levels. Detection of co-immunoprecipitated proteins shows that only combinations with complementing YFP fragments interact.

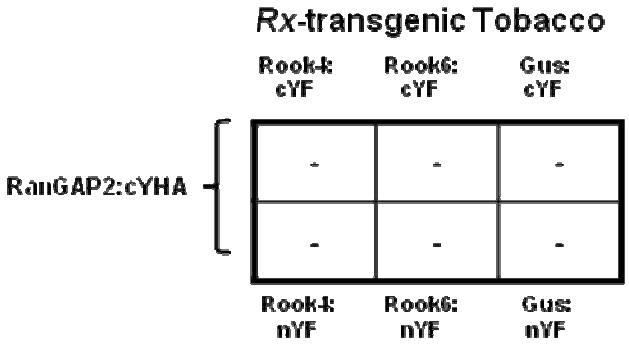

Figure S5.
Requirement for NB-LRR specificity determination for HR elicitation by YFP complemented *Gp*-RBP-1. The indicated combinations of YFP fragment fusion proteins were transiently expressed by agro-infiltration in *Rx*-transgenic tobacco leaves as in Figure S4A. A lack of HR is indicated by (-)

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Chapter 6

Gpa2-mediated activation is determined by the C-terminal end of its LRR domain and is suppressed by virulent *Globodera pallida* RBPs

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Abstract
Recently, a nematode effector (RBP-1) with a B30.2/SPRY domain was described as candidate avirulence factor recognized by the potato resistance gene Gpa2 in avirulent populations of *Globodera pallida* (Sacco et al., 2009). Domain exchanges between the two highly homologous NB-LRR genes Gpa2 and Rx1 localize RBP-1 recognition to the LRR domains of these R proteins (chapter 3). To further investigate nematode recognition, the LRR domain of Gpa2 was divided into four fragments and replaced by homologous regions from Rx1. This resulted in the identification of 18 residues required for nematode and RBP-1 recognition in a C-terminal fragment of 155 amino-acids in Gpa2. Seven of these residues are predicted to be in close spatial proximity on the protein surface of the LRR domain. All RBP-1 variants from avirulent populations elicit a hypersensitive response in plants expressing *Gpa2*. Resistance breaking populations of *G. pallida* simultaneously express RBP-1 and RBP-1 variants that do not activate Gpa2 mediated defense response. Here we show that virulent variants of RBP-1 inhibit RBP-1 activation of Gpa2 suggesting a novel mechanism for evasion and/or suppression of avirulence.

Introduction
Plants have evolved a complex multilayered immune system for self-protection. The first line of defense in plants, the PAMP-triggered immunity, is based on recognition of highly conserved and essential microbial molecules (PAMPs) at the interface of plant and pathogen. Pathogens have evolved various mechanisms involving so-called effectors to evade or to suppress PAMP-triggered immunity (PTI). Suppression of PAMP-triggered immunity may lead to the activation of a second line of defense based on the highly specific recognition of these pathogen effectors, and which is referred to as effector-triggered immunity (ETI). Pathogens have evolved yet other effectors to interfere with effector-triggered immunity so that they achieve full virulence again. However, these effectors also create new targets for novel recognition specificities in the immune receptors encoded by R genes. This phenomenon, which is described in the zigzag model (Jones and Dangl, 2006), follows the gene-for-gene concept of disease recognition specificity in resistance genes (R genes) proposed by Flor in 1971 (Flor 1971).

At present, more than 50 R genes are cloned from various plant species (van Ooijen et al., 2007). Despite acting against a wide range of unrelated pathogens, most of the immune receptors encoded by R genes share a similar overall architecture with structurally conserved domains. The major class of R protein includes either a coiled-coil or TIR domain at the N-terminus followed by the nucleotide-binding (NB) and ARC subdomains, and a leucine-rich repeat domain. The leucine-rich repeats in the LRR domain at the carboxyl terminus consist of the LxxLxxLxLxxC/Nxx consensus sequence with x representing a non-leucine residue. Each of these repeats is separated from the next one by a linker of variable length (Enkhbayar et al., 2004).
To date, six genes conferring resistance against plant parasitic nematodes, including the potato gene *Gpa2*, have been isolated from different plant species (for overview see chapter 2 of this thesis). *Gpa2* restricts in a population specific manner the development of *G. pallida*. The *Rx1* gene, which confers a resistance to potato virus X, is located in the same R gene cluster. Both genes belong to CC-NB-ARC-LRR class of R genes, and share more than 80% overall identity at protein level. Most of the differences between Gpa2 and Rx1 are found in the LRR domain.

It has been shown for several R proteins (Ellis et al., 1999; Dodds et al., 2001; Moffett et al., 2002) that the LRR domain is involved in recognition of pathogen elicitors with avirulence activity. Based on a sequence homology to the LRR of the porcine ribonuclease inhibitor the three-dimensional structure of the LRR domain in R proteins is believed to be shaped as a horseshoe. This horse shoe-like structure is built from an inner series of parallel β-sheets consisting of conserved structural amino acid residues forming the back-bone and variable solvent exposed residues that play a role in protein-protein interactions (Jones and Jones, 1997). The sites of these solvent exposed residues appeared to be subjected to diversifying selection, which supports a role for these residues in pathogen recognition (Michelmore and Meyers, 1998; Mondragón-Palomino 2002; Butterbach 2007). The biochemical mechanism underlying pathogen recognition in most cases is presumed to take place through indirect interaction with a pathogen effector.

Survival of endoparasitc nematodes within plant roots likely requires the evasion or suppression of the plant's innate immunity. Although, experimental data to substantiate this is scarce, proteins secreted in pre- and early parasitic stages probably contribute to this process. Recently, a family of proteins with a PRY-SPRY/B30.2 domain secreted (SPRYSECs) from *G. rostochiensis*. One of the SPRYSEC family members was shown to interact with the LRR domain of a CC-NB-LRR protein from a susceptible tomato. *Globodera pallida* RBP-1 encodes a related secretory protein with a SPRY domain. Transient expression of Gp-RBP-1 in *N. benthamiana* and *N. tabacum* leaves elicited a *Gpa2*-dependent hypersensitive response (Sacco et al., 2009, chapter 5). All Gp-RBP-1 variants found in the avirulent population of *G.pallida* (D383) elicit a *Gpa2*-dependent HR, while a virulent population (Rookmaker) possessed two variants of which one induces an HR while the other not. A single amino acid polymorphism between virulent and avirulent alleles determined the presence or absence of the activation of *Gpa2*-dependent HR (Sacco et al., 2009).

In chapter 3 we described a structure-function study showing that a sequence exchange of the LRR in Gpa2 for the LRR from Rx1 converts a nematode resistance gene into a virus resistance gene, and vice versa. To investigate which part of the LRR domain of Gpa2 is required for the specific R protein activation, the LRR of Gpa2 was divided into four subdomains and each of these subdomains was replaced by the homologous part from Rx1. In total sixteen chimeric combinations of LRR subdomains were fused to the CC-NBS of Rx1 and tested both in an agroinfiltration together with RBP-1 and in nematode resistance assays. We found that the region in Gpa2 LRR (808-912 aa) required for RBPs perception in co-expression experiments in *N. benthamiana* leaves is also required for nematode resistance in potato roots. Earlier work showed that Gpa2 resistance-breaking populations still have RBP-1 variants capable of inducing a hypersensitive response in an agroinfiltration assay together with Gpa2. Here we followed up on a hypothesis that nematodes evade recognition by producing modified variants of effectors which lack avirulence activity to outcompete or block homologous effectors with avirulence activity.

Results

RBP is recognized by the C-terminal end of the LRR domain of Gpa2

In chapter 5, we demonstrated that a *Gpa2*-dependent hypersensitive response can be activated by the nematode effector protein RBP-1. In chapter 3, it was pointed at the LRR domain as the part of the Gpa2 protein involved in specific nematode recognition. This raised the question whether the RBP-1-induced hypersensitive response also depends on recognition by the LRR domain of Gpa2.

To further narrow down the fragment of the LRR domain in Gpa2 required for specific RBP-1-induced HR, the LRR of both Gpa2 (G4) and Rx1 (R4) were divided into three subdomains (ggg or rrr), including three blocks of five leucine rich repeats each with a fourth fragment (G5 or R5) consisting of the acidic tail exclusively present in Rx1 (Fig. 1). In total sixteen chimeric combinations of Gpa2 and Rx1 LRR fragments were generated and subsequently fused to the CC-NB domains of Rx1 (R13) or Gpa2 (G13) and tested in an agroinfiltration assay on leaves of *N. benthamiana*. Most of the chimeric constructs fused to the CC-NB of Gpa2 were autoactive and were, therefore, excluded from further studies (data not shown).

A set of five non-autoactive LRR chimeras (Fig. 2) was agroinfiltrated into *N. benthamiana* leaves. Each construct was expressed with RBP_{ROOK6}, a functional orthologue of $RBP_{D383\text{-}1}$ with avirulence activity from the avirulent nematode population D383. RBP_{ROOK6} derives from a Gpa2-breaking population of *G. pallida*, which expresses diverse RBP variants. To test the specificity of the response, the Gpa2 non-activating RBP_{ROOK4} variant from the virulent population of *G. pallida* and GFP were used as a control. RT-PCR showed that all constructs were expressed (data not shown). None of the tested LRR chimeras gave an HR in the presence of RBP_{ROOK4}, whereas three out of five sub-LRR chimeras did result in an HR when co-expressed with RBP_{ROOK6}. We observed no HR in the presence of RBP_{ROOK6} for the LRR chimera R13ggrG5 and R13G4R5. In R13ggrG5 the Gpa2 LRR repeats 11-15 were replaced with the corresponding fragment of Rx1, whereas in R13G4R5 the stretch of amino acids after the last LRR repeats of Gpa2 was replaced by the acidic tail from Rx1 (Fig. 3).

An additional chimeric Rx1 construct (RRRRG), described by Rairdan and Moffett (Rairdan and Moffett 2006) was also co-expressed with RBP_{ROOK6} and RBP_{ROOK4}. In this construct, the inserted LRR fragment from Rx1 is 50 nucleotides shorter at its N-terminal end than R13ggrG5. This RRRRG construct still resulted in a specific response to RBP_{ROOK6}, but not to RBP_{ROOK4} (data not shown). Therefore, we conclude that the effector protein RBP from *G. pallida* activates Gpa2 via the C-terminal residues at the LRR domain between residues 808 and 912.

Domain 1-3 (CC-NB-ARC)

```
Gpa2  (1) MAYAAVTSLMRTIHQSMELTGCDLQPFYEKLKSLRAILEKSCNIMGDHEGLTILEVEIIEVAYTTEDMVD
Rx1   (1) MAYAAVTSLMRTIHQSMELTGCDLQPFYEKLKSLRAILEKSCNIMGDHEGLTILEVEIVEVAYTTEDMVD

Gpa2 (71) SESRNVFLARNVGKRSRAMWGIFFVLEQALECIDSTVKQWMATSDSMKDLKPQTSSLVSLPEHDVEQPEN
Rx1  (71) SESRNVFLAQNLEERSRAMWEIFFVLEQALECIDSTVKQWMATSDSMKDLKPQTSSLVSLPEHDVEQPEN

Gpa2(141) IMVGRENEFEMMLDQLARGGRELEVVSIVGMGGIGKTTLAAKLYSDPYIMSRFDIRAKATVSQEYCVRNV
Rx1 (141) IMVGRENEFEMMLDQLARGGRELEVVSIVGMGGIGKTTLATKLYSDPCIMSRFDIRAKATVSQEYCVRNV

Gpa2(211) LLGLLSLTSDEPDYQLADQLQKHLKGRRYLVVIDDIWTTEAWDDIKLCFPDCDNGSRILLTTRNVEVAEY
Rx1 (211) LLGLLSLTSDEPDDQLADRLQKHLKGRRYLVVIDDIWTTEAWDDIKLCFPDCYNGSRILLTTRNVEVAEY

Gpa2(281) ASSGKPPHHMRLMNFDESWNLLHKKIFEKEGSYSPEFENIGKQIALKCGGLPLAITLIAGLLSKISKTLD
Rx1 (281) ASSGKPPHHMRLMNFDESWNLLHKKIFEKEGSYSPEFENIGKQIALKCGGLPLAITVIAGLLSKMGQRLD

Gpa2(351) EWQNVAENVRSVVSTDLEAKCMRVLALSYHHLPSHLKPCFLYFAIFAEDERIYVNKLVELWAVEGFLNEE
Rx1 (351) EWQRIGENVSSVVSTDPEAQCMRVLALSYHHLPSHLKPCFLYFAIFTEDEQISVNELVELWPVEGFLNEE

Gpa2(421) EGKSIEEVAETCINELVDRSLISIHNVSFDGETQRCGMHDVTRELCLREARNMNFVNVIRGKSDQNSC
Rx1 (421) EGKSIEEVATTCINELIDRSLIFIHNFSFRGTIESCGMHDVTRELCLREARNMNFVNVIRGKSDQNSC
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Subdomain 4A (LRR)

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Gpa2(489) AQSMQCSFKSRSRISIHNEEELVWCRNSEAHSIITLCIFKCVTLELSFKLVRVLDLGLTTCPIFPSGVLS
Rx1 (489) AQSMQRSFKSRSRIRIHKVEELAWCRNSEAHSIIMLGGFECVTLELSFKLVRVLDLGLNTWPIFPSGVLS

Gpa2      LIHLRYLSLRFNPRLQQYRGSKEAVPSSIIDIPL (592)
Rx1       LIHLRYLSLRFNPCLQQYQGSKEAVPSSIIDIPL (592)
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Subdomain 4B (LRR)

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Gpa2(593) SISSLCYLQTFKLYHPFPNCYPFILPSEILTMPQLRKLCMGWNYLRSHEPTENRLVLKSLQCLNELNPRYC
Rx1 (593) SISSLCYLQTFKLNLPFPSYYPFILPSEILTMPQLRTLCMGWNYLRSHEPTENRLVLKNLQCLNQLNPRYC

Gpa2      TGSFLRLFPNLKKLEVFGVKEDFRNHKDLYDFRYLYQLEKLAFSTYYSSSACFLKNTAPLGSTPQDPLRFQ
Rx1       TGSFFRLFPNLKKLQVFGVPEDFRNSQDLYDFRYLYQLEELTFRLYYPYAACFLKNTAPSGST-QDPLRFQ

Gpa2      METLHLETHSRATAPPTDVPTFLLPP (755)
Rx1       TEILHKEIDFGGTAP----PTLLLPP (750)
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Subdomain 4C (LRR)

```
Gpa2(756) PDCFPQNLKSLTFSGDFFLAWKDLSIVGKLPKLEVLQLSHNAFKGEEWEVVEEGFPHLKFLFLDSIYIRYW
Rx1 (751) PDAFPQNLKSLTFRGEFSVAWKDLSIVGKLPKLEVLILSWNAFIGKEWEVVEEGFPHLKFLFLDDVYIRYW

Gpa2      RASSDHFPYLERLFLSDCFYLDSIPRDFADITTLALIDIFRCQQSVGN (873)
Rx1       RASSDHFPYLERVILRDCRNLDSIPRDFADITTLALIDIDYCQQSVVN (868)
```

Domain 5

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Gpa2(874) SAKQIQQDIQDNYGSSIEVHTRYLYRNGAFLVV------------------------------
Rx1 (869) SAKQIQQDIQDNYGSSIEVHTRHLFIPKSVTTVEDDDDSVTTDEDDDDDDFEKEVASCRNNVE
```

Figure 1.
Alignment of Gpa2 and Rx1 proteins with indicated junctions used to create a chimeric sub-LRR swap constructs. The Gpa2 and Rx1 unique aminoacids are displayed in black

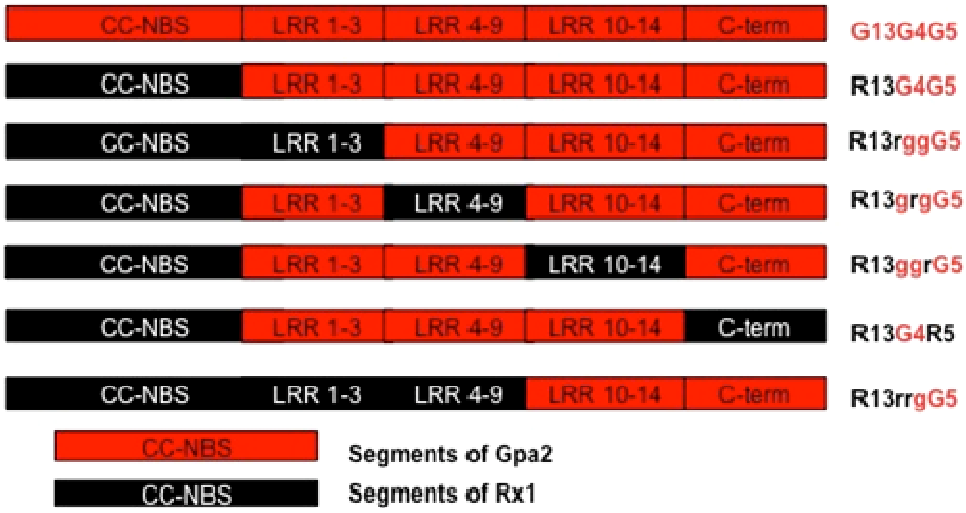

Figure 2.
Schematic picture of sub-LRR swap constructs used in agroinfiltration in leaves of *N. benthamiana* and potato transformation (except for RRRRG)

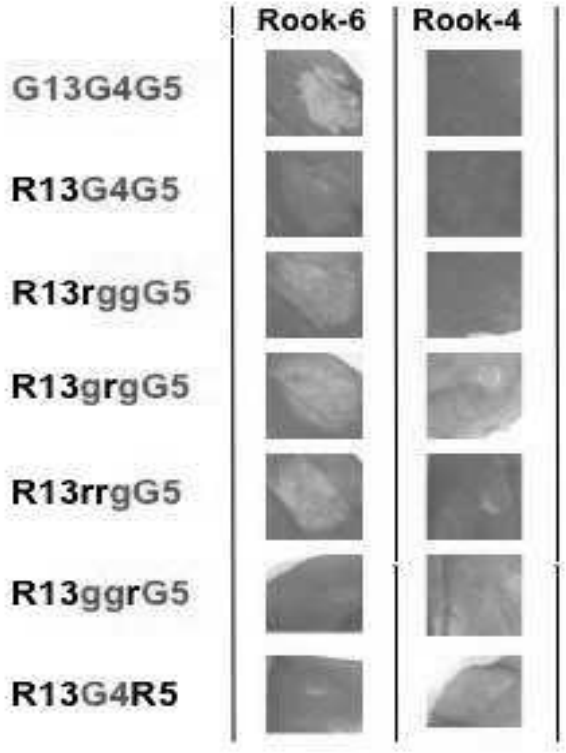

Figure 3.
Phenotypes observed upon coinfiltration of chimeric Gpa2/Rx1 constructs with *G.pallida* avirulent (RBP_{Rook-6}) and virulent (RBP_{Rook-4}) effectors. Pictures were taken 7 days post infiltration.

Gpa2 mediated nematode resistance is determined by the same LRR region

To investigate whether RBP recognition by Gpa2 and the specificity of Gpa2-mediated nematode resistance are depending on the same region in the LRR domain, we tested four LRR chimeras described above for nematode resistance. We generated transgenic potato plants carrying the constructs, R13G4R5, R13ggrG5, R13grgG5 and R13rggG5 expressed from the CaMV 35S promoter. RT-PCR followed by sequencing of the PCR products confirmed that all the constructs were properly expressed (data not shown). Two independent transgenic lines were selected for each construct and 8 replicates of each line were challenged with the avirulent population of *G. pallida* (D383). Transgenic plants carrying the complete genomic sequence of the Gpa2 gene (GPAII::Gpa2) and the pBIN+-empty vector were used as controls. The nematode infection assay was performed *in vitro* and the number of developed females was counted 6 weeks post inoculation (Fig. 3 and 4). Plants transformed with the empty vector were fully susceptible to nematodes resulting in an average of 22 (±6.45) fully developed adult females per plant. Plants expressing the Gpa2 gene were resistant to *G. pallida* D383 as no adult females did develop. Plants transformed with R13ggrG5 and R13G4R5 harbored 17.75 (±-3.3) and 17 (±-2.6) fully developed adult females per plant, respectively (Fig. 4). In contrast, the LRR chimeric constructs R13rggG5 (0.88±1.05) and R13grgG5 (1.28±1.25) were resistant to nematode infections. These results demonstrate that the recognition of the RBP-1 effector protein in an agroinfiltration assay on leaves of *N. benthamiana* corresponds with specific nematode resistance in potato to the avirulent population of *G. pallida* and determined by the same region in the C-terminal end of the LRR domain of Gpa2.

Gpa2 specific residues involved in recognition map on the surface of the LRR domain

The LRR domain of Rx and Gpa2 is the most variable part of these proteins and most differences accumulate especially in the C-terminal half of this domain (van der Vossen et al., 2000; Bendahmane et al., 1999). A pair wise alignment of the region 808-912 in Gpa2 and Rx1 revealed the presence of 10 amino acid substitutions in the leucine-rich repeat part and 9 amino acid substitutions in the most distal C-terminal end of the Gpa2 protein, corresponding to the region encoding for the acidic tail in Rx (Fig. 5).

To investigate how these residues involved in RBP and nematode recognition are positioned in the protein structure, we made a computer aided 3D model of the LRR domain of Gpa2. Figure 6 shows that the LRR of Gpa2 is forming as a typical contiguous horseshoe. The critical step in modeling the LRR domain of Gpa2 was to locate the correct positions of the LRR motifs along the sequence. Whereas Rx1 has a true *lrr* motif in the first repeat (507-512aa), Gpa2 has an imperfect first *lrr* - with the first L in LxxLxL replaced by a glutamic acid - suggesting that either the entire domain is shifted or at least the first *lrr* distorted, as often happens in marginal repeats of other documented LRRs. We mapped the physico-chemical variability of amino-acids (using BLOSUM62 similarity matrix) and the site undergoing positive selection (DNA analysis Butterbach 2007) onto the Gpa2-LRR 3D model. The LRR domain appears to be divided in two parts by a plane parallel to the horseshoe which passes through the middle of the *lrr* motif, separating the Lxx parts and xxN parts of the lrr motifs. Both the variability and positive selection pattern show a strong separation between Lxx and xxN regions with a strong bias towards the xxN part of the lrr motifs in the LRR domain. This suggests that the xxN surface is most probably responsible for the specific protein-protein interactions.

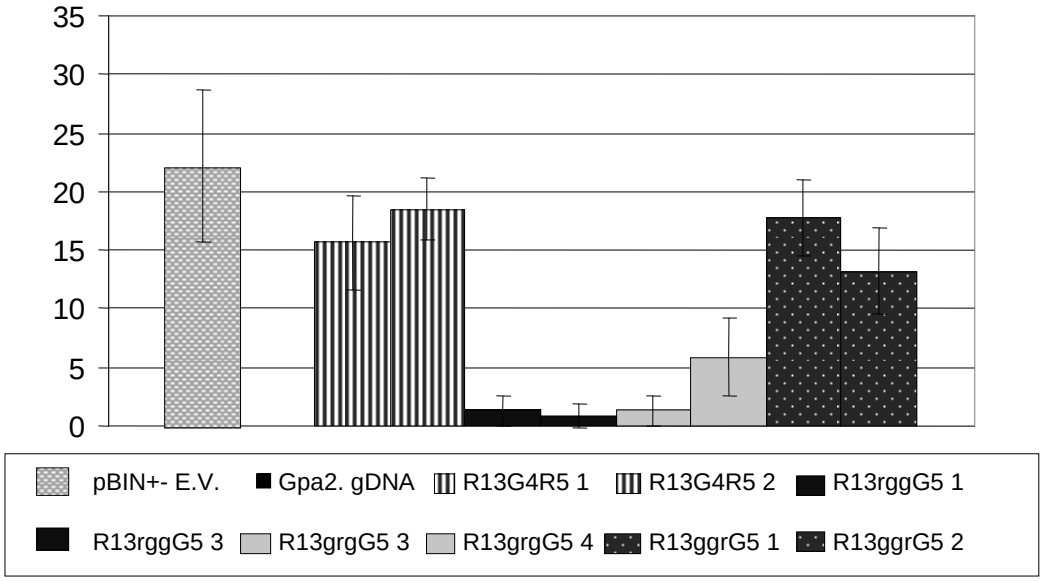

Figure 4.
Nematode resistance assay on transgenic potato plants infected with the avirulent *G. pallida* population D383. Transgenic plants harboring Gpa2 gDNA clone (Chapter 3) were used as resistant control, whereas empty vector plants E.V. were used as a susceptible control. Two independent transgenic lines were used per intra-LRR construct and 8-10 replications per line. Plants were scored as resistant if the average number of developed adult females was lower than eight.

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                                Subdomain 4C

                LxxLxLxx (LRR 11)          LxxLxxLxL  (LRR 12)
Gpa2 (756) PDCFPQNLKSLTFSGDFFLAWKDLSIVGKLPKLEVLQLSHNAFKGEEWEVVEEG
Rx1  (751) PDAFPQNLKSLTFRGEFSVAWKDLSIVGKLPKLEVLILSWNAFIGKEWEVVEEG

               LxxLxLxxNxL (LRR 13)
Gpa2 (810)FPHLKFLFLDSIYIRYW  (826)
Rx1  (805)FPHLKFLFLDDVYIRYW  (821)

               LxxLxLxxNxxL (LRR 14)   LxLxxLxxN (LRR 15)
Gpa2 RASSDHFPYLERLFLSDCFYLDSIPRDFADITTLALIDIFRCQQSVGN (873)
Rx1  RASSDHFPYLERVILRDCRNLDSIPRDFADITTLALIDIDYCQQSVVN (868)

                                Domain 5

Gpa2  SAKQIQQDIQDNYGSSIEVHTRYLYRNGAFLVV----------------------
Rx1   SAKQIQQDIQDNYGSSIEVHTRHLFIPKSVTTVEDDDDSVTTDEDDDDDDFEKEVASCRNNVE
```

Figure 5.
Alignment of the Gpa2 C-terminal region, consisting of the most distal leucine rich repeats (subdomain 4c) and a C-terminal extension (domain 5) of unknown structure. The amino acid residues unique for Gpa2 are highlighted. The Gpa2 LRR fragment which was shown to be irreplaceable for Gpa2 functioning, is underlined and specific Gpa2 amino-acid residues mapped on the protein surface in Fig. 6 are colored in green and bold.

Mapping of the Gpa2-specific residues on the structure model of the LRR of Gpa2 showed that seven reside within the leucine-rich repeats and shape into a single cluster at the concave surface of the horseshoe-like structure of the LRR domain. It is known that this surface serves as a platform for protein-protein interactions and hence, this cluster of Gpa2 specific residues at the C-terminal end could be directly or indirectly be involved in the specific recognition of the cognate effector protein RBP-1. The most C-terminal region of Gpa2 protein after the last repeat of the LRR was not included in the model because this stretch of amino acids does not have any known structure and no template for modeling was found, but it still might be involved in RBP-1 recognition as shown in the functional assays.

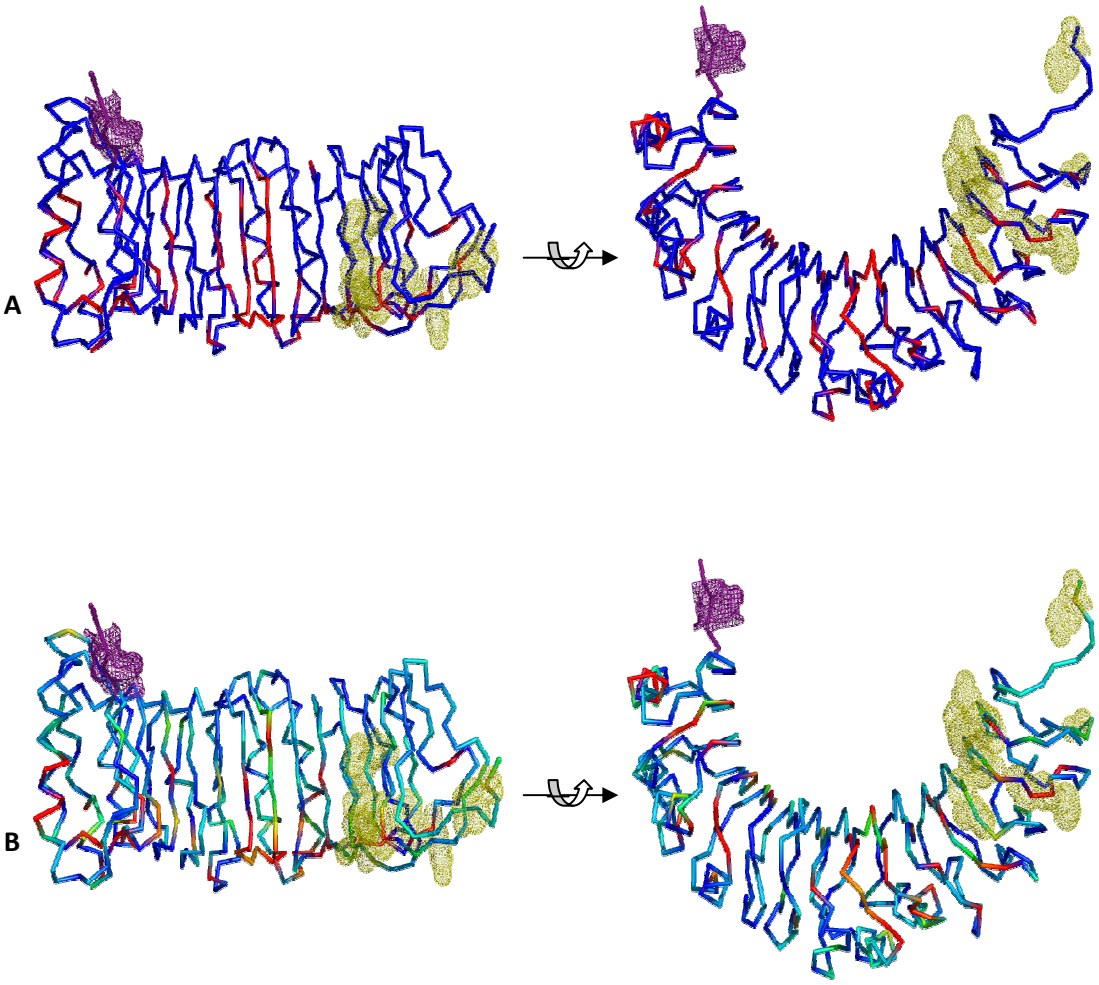

Figure 6.
Sequence mapping onto the Gpa2-LRR 3D model. The 11 mutations separating Gpa2 from Rx in the region 808-903 are represented with yellow dots. The V507E mutation affecting the stability of the first LRR motif in Gpa2 is shown in purple.
A. Shows in red the positively selected sites (Butterbach 2007) mapped onto the 3D model.
B. Shows the aminoacid variability mapping of the Rx family (Butterbach 2007). This is shown in color scale varying from blue (conserved) to red (most variable). As can be seen positive selection results in unequal variations of the physical properties of the surface.

Loss of function mutation in RBPs localizes to the protein surface

To better understand the structural basis of the significant functional differences between RBP_{ROOK4} and $RBP_{ROOK6,}$ two computer aided 3D models were built to pin down the location of the S/P mutation. The RBP-1s isolated from *G. pallida* were identified having a B30.2/SPRY domain, which are distorted beta sandwiches with a core of antiparallel beta-strands connected by highly variable loops similarly to SPRYSECs of *G. rostochiensis* (Rehman et al., 2009). A protein sequence alignment of RBP-1s with SPRYSECs showed an uneven distribution of the sequence similarities. Regions with a nearly perfect match are interspersed with highly diverse regions. Apparently, the overall framework of the SPRY domain is conserved in both RBP-1s and SPRYSECs, and we therefore could use the three dimensional protein structure model of the SPRYSEC (Rehman et al., 2009) to localize the residues associated with *Gpa2*-dependent HR-induction in RBP_{ROOK6}. Interestingly, all sequences of *G. pallida* RBP-1s (Chapter 4) have a repetition of a 25 amino acid stretch, ACDTCLTLSETERRLMIVEYTKADW, twice, at positions 37 and 62. These were called the N-terminal extensions, NTE1 and NTE2 respectively (Fig. 7). And while NTE2 overlaps with the beginning of the GUSTAV template, NTE1 does not.

The secondary structure prediction indicates that NTEs have a strong propensity for extended beta-strand formation, which is confirmed by the local configuration of NTE2 template into the GUSTAV structure. In addition, the contact propensity profiles of NTE1 and NTE2 are very high, suggesting putative extended contacts with the rest of the beta strands of the core of this fold. Besides, sequence profiling shows that the stretch linking NTE1 and NTE2 has a high accessibility propensity suggesting a possible fold-over of this repeat, forming a contiguous extended beta sheet with the core.

Sequences of both virulent and avirulent RBP-1 variants from Rookmaker (RBP_{ROOK1} until RBP_{ROOK6}) are highly similar with only 5 mutations in 247 amino acids (i.e. Q/K^{100}, M/I^{127}, H/R^{162}, S/P^{166} and L/S^{201}). However, only the proline to serine substitution at position 166 (in RBP_{ROOK2} and RBP_{ROOK4}) is absolutely correlated with a loss of *Gpa2*-dependent HR in RBP-1 variants. This S to P mutation is the only one that in $RBP\text{-}1_{ROOK4}$ that is predicted to be in the so-called extended beta strand configuration. The extended beta strand configuration is lost in $RBP\text{-}1_{ROOK6}$ due to the presence of proline instead of serine at position 166. Hence, a loop was generated at this position and further refined using the different phi/psi values in $RBP\text{-}1_{ROOK4}$ and $RBP\text{-}1_{ROOK6}$ to fulfill the constraint suggested by our secondary structure predictions. Consequently, a serine at position 166 allows the elongation of the short extended stretch in this region, while a proline at this position forces a bend, which will induce a significant local change in the so-called surface A of the structure of the RBP-1 protein (Rehman et al., 2009). As seen from Fig.7, the S166P mutation decreases significantly the local contact forming surface in RBP_{ROOK6}, as compared to the virulent RBP_{ROOK4} .

The complete RBP-1 polypeptide is required for activation of Gpa2

Ten RBP-1 variants have been identified in virulent and avirulent populations of *G. pallida* consisting of a central PRY-SPRY domain of 130 residues with an ancillary N-terminal extension of 142 residues (Pro-domain software). To investigate whether either the PRY-SPRY domain in RBP-1 or the N-terminal extension alone is able to activate Gpa2-dependent HR we generated two constructs to express them separately in plants. However, co-expression of neither the N-terminal extension nor the PRY-SPRY domain alone with Gpa2 resulted in a hypersensitive response in *N. benthamiana* leaves (data not shown). In addition, co-expression of Gpa2, the N-terminal extension, and the PRY-SPRY domain in a single infiltration assay did not lead to a HR suggesting that the N-extension and the PRY-SPRY domain of RBP cannot trans-complement each other.

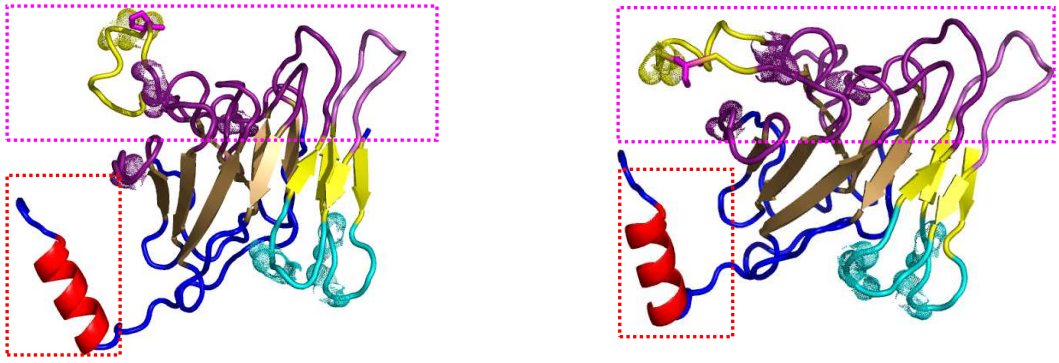

Figure 7.
3D models of RBP_{Rook-4} (A) and RBP_{Rook-6} (B). The magenta box encloses the surface A loops while the red box encloses the BC box C-terminal helix. The B surface consists in the blue loops and is extended by the two cyan loops from NTE1 and NTE2. The last light magenta loop in the A surface is the loop added by the N-terminal extension. The S/P loop is in yellow with the S and the P represented as sticks in magenta. Positively selected aminoacids are showed in dots.

RBP variants from virulent nematodes suppress Gpa2-dependent HR

The avirulent RBP-1_{ROOK6} and virulent RBP-1_{ROOK4} variants are both expressed in the *Gpa2* resistance-breaking Rookmaker population of *G. pallida*. To investigate why this resistance-breaking population is not recognized by *Gpa2* we tested two hypotheses. First, as the Rookmaker is a field population, it may contain a mixture of virulent and avirulent individuals. Therefore, we assumed that avirulent individuals can be selected out when developing on a resistant plant thus reducing the heterogeneity of RBP-1 variants within a population. To verify this, we collected fully developed adult females of the resistance-breaking strain from roots of the *Gpa2* resistant potato. The composition of RBP-1 variants in these females was then compared with the RBP-1 variants present in the pre-parasitic nematodes used for inoculation of the roots to see if indeed selection had occurred. If the Rookmaker population had been a mixture of virulent and avirulent genotypes, we would expect to eliminate genotypes having the RBP-1_{ROOK6} variants and other HR inducing RBP variants (1, 2, 3, and 5) with a proline residue at position 166. Around 100 young adult females were pooled for genomic DNA extraction. In addition, the progeny of the females, which were harvested from resistant roots, were used for inoculation of fresh resistant roots to obtain the next generation of virulent adult females. We used nested PCR to increase the amplification specificity because of the high abundance of SPRY containing genes in the nematode genome. The PCR products were cloned and a random sample of 96 clones was sequenced from two successive nematode generations. We found the same Gpa2 activating RBP variants in the Rookmaker population before and after reproduction on Gpa2 resistant plants suggesting that no selection against the HR-inducing RBP-1_{ROOK6} had taken place (data not shown).

Secondly, we investigated the hypothesis that the inactive RBP-1 variants from the resistance-breaking Rookmaker population are capable of suppressing the HR induced by RBP-1_{ROOK6}. Again, when Gpa2 was co-expressed with RBP-1_{Rook6} in *N. benthamiana* leaves a strong HR was clearly visible in the infiltrated patch at 3 dpi. Remarkably, co-expression of Gpa2 and RBP-1_{Rook6} did not elicit a strong HR anymore when either RBP-1_{Rook2} or RBP-$1_{Rook4,}$ or a combination of both, were included in the infiltration mix (Fig. 8 A-D). We excluded that the suppression was the result of a dilution of each component in the infiltration

mix by testing all combinations with the same bacterial concentration while compensating with a GFP construct to achieve a same transgene load.

To investigate whether RBP_{Rook2} and RBP_{Rook4} had an inhibitory effect down- stream of the Gpa2 immune receptor, we also tested the effect of inactive RBP-1 variants on the HR induced by Rx1 and its elicitor coat protein CP106 (Fig. 8 B) and an autoactive chimera $Gpa_{CC\text{-}NBS}Rx_{LRR}$ (Fig. 8 D). Including $RBP\text{-}1_{Rook2}$ or/and $RBP\text{-}1_{Rook4}$ in the infiltration mixtures did not affect the HR mediated by Rx1 and the autoactive mutant of Gpa2. Co-infiltration of Inf1 from *Phytophthora infestans* with either RBP_{Rook2} or RBP_{Rook4} did not suppress the HR induced by this elicitin in *N. benthamiana* leaves either (Fig. 8 C). Therefore, the observed suppressive effect of $RBP\text{-}1_{Rook2}$ and $RBP\text{-}1_{Rook4}$ on Gpa2-dependent HR is mediated most likely through the Gpa2 protein and not by interfering with conserved downstream signaling pathways.

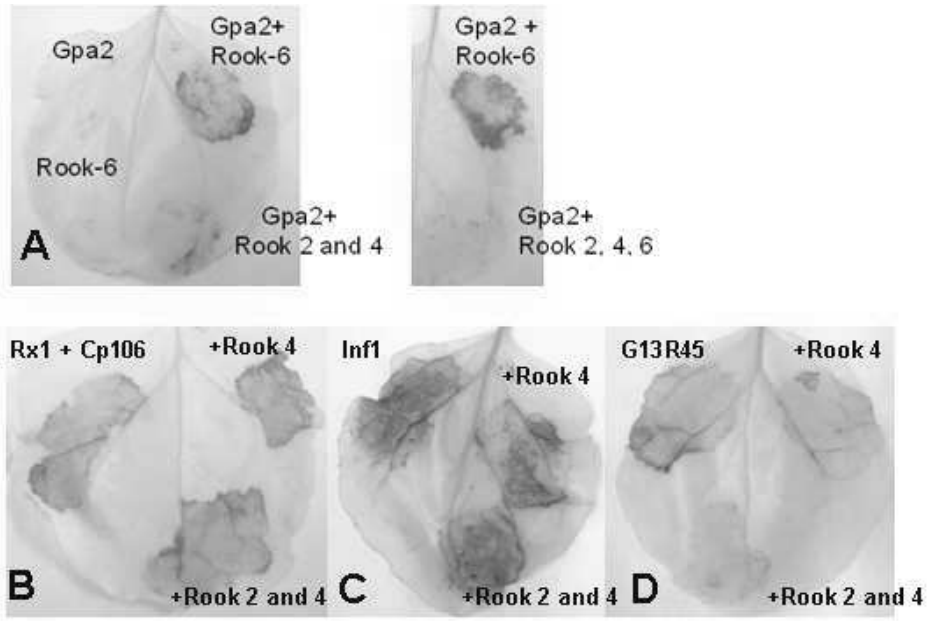

Figure 8.
Suppression of Gpa2 mediated HR (A) triggered upon recognition of $RBP_{Rook\text{-}6}$ by $RBP_{Rook\text{-}2}$ and $RBP_{Rook\text{-}4}$.
This effect was not observed for HR triggered by the R gene-effector combination Rx1-Cp106 (B), the elicitin Inf1 (C) or an autoactive chimeric construct G13R45 (D).

Discussion

Rx1 and Gpa2 are two neighboring and highly similar resistance genes from the same R gene cluster in potato, which recognize two completely different pathogens. Rx1 recognizes the coat protein from the avirulent strains of *potato virus X* (PVX) and recognition is lost by a single amino acid substitution in the coat protein of the resistance-breaking strain. Recently, we showed that RBP-1 effectors from *G. pallida* activate a Gpa2-dependent HR in *N. benthamiana* leaves (Chapter 5). Here, we report that Gpa2 recognition of its cognate effector RBP-1 is determined by a stretch of approximately one hundred residues located at the C-terminus of the LRR domain. The same C-terminal region of Gpa2 is required for nematode resistance to the avirulent population D383 of *G. pallida* in potato and hence, Gpa2-mediated cell death induced by RBP correlates with Gpa2-mediated nematode resistance. Strikingly, nematodes from the resistance-breaking population Rookmaker express both active RBP-1

and inactive RBP-1 variants. We found that RBP-1 variants from the resistance-breaking population Rookmaker with a serine at position 166 instead of a proline are able to suppress Gpa2 activation by RBP-1.

The LRR domain determines recognition specificity in Gpa2

Our agroinfiltration experiments in *N. benthamiana* and the nematode resistance tests in potato with the Rx1 and Gpa2 LRR domain chimeras showed that the Gpa2 recognition specificity is determined by the C-terminal end of the LRR domain. Despite a high homology between Rx1 and Gpa2 protein our experimental data suggest that a similar but not identical region of LRR is required for pathogen recognition. For Gpa2 the LRR region between amino acids 808 and 912 is irreplaceable, whereas the Rx1 protein did not recognize the virus coat protein when the LRR fragment (amino acids 593-937) was replaced with the Gpa2 corresponding part. Additional difference in Rx1 and Gpa2 recognition platform is the acidic tail, which can be deleted without disrupting the Rx1 functionality (P. Butterbach and J. Roosien, pers comm). As opposed to Rx1, the Gpa2 protein is very sensitive to any modification at its C-terminus, because fusion of GFP or other affinity tags as well as deletion of the 3'UTR (see chapter 4) results in the loss of function of the gene.

Our finding that Gpa2 recognition specificity resides in the C-terminal half of the LRR domain is in line with other studies with NB-LRR immune receptors, suggesting that the LRR domain can be functionally divided in two subdomains. The C-terminus seem essential for pathogen recognition, while residues in the N-terminus of the LRR are probably involved in intramolecular interactions with the adjacent ARC domain to maintain the R protein in an inactive stage (Rairdan and Moffett, 2006; chapter 3 of this thesis). Specific effector recognition via the C terminus of the LRR may trigger a conformational change, which allows a change in nucleotide-binding status of the protein and the binding of other molecular components to achieve down-stream disease resistance signaling (Moffett et al., 2002; Hwang and Williamson, 2003; Takken et al., 2006). Our previous data on Gpa2 supports this model, for Gpa2 is kept in self-inhibitory state in the absence of nematodes as was shown with several domain exchanges between Rx1 and Gpa2 leading to autoactivity (Rairdan and Moffett, 2006).

It has been shown for several R proteins (Ellis et al., 1999; Dodds et al., 2001; Moffett et al., 2002) that the LRR domain is involved in direct or indirect interaction of pathogen elicitor. The ability to recognize RBP is encoded in the residues between sites 808-912 of Gpa2, including LRR 13 to 15. In this region ten residues within the leucine-rich repeats and additional 9 amino acids at the 3' end of the protein are different from the same region of the Rx1. When these residues are projected on the 3D model of the Gpa2 LRR most of them, while appearing randomly dispersed in the primary sequence, map to the protein surface creating a potential recognition site for RBP. It has been shown that this region of the LRR is subject to positive selection (van der Vossen et al., 2000; Bendahmane et al., 1999) which provides additional support for a role of the C-terminus of the LRR in determining pathogen recognition specificity. The patterns of positive selection in the set of RBP-1 variants (chapter 5) suggest that the nematodes apparently benefit from a loss of recognition by Gpa2. The recently characterized SPRYSEC19 from *G. rostochiensis* also exhibits footprints of positive selection in surface A of the protein. Interestingly, the residues under positive selection are located in two regions of RBP-1, viz. surface A and the N terminal extension, which may suggest that RBP-1 may interact with two different proteins.

Is RBP recognition based on a direct interaction with the LRR of Gpa2?

Rehman and coworkers (2009) have shown a direct interaction between SPRYSEC19 – a homolog of RBP-1 in *G. rostochiensis* - and the C-terminal leucine-rich repeats of the LRR domain of the CC-NB-LRR protein SW5F. We have used yeast-two-hybrid to test whether a direct interaction between the LRR of Gpa2 and RBP_{ROOK4}, RBP_{ROOK6}, and RBP_{D383} takes place as well. To this purpose a Gpa2 LRR fragment (amino acids 800-912) was used as a bait to test for interaction with the RBP variants, including several controls. None of the combinations, except for the positive control, showed an interaction in yeast (data not shown). While the direct interaction between this region of Gpa2 LRR and RBPs was not evident in this yeast-two-hybrid experiment, the possibility of a direct interaction in planta cannot be excluded. We have tested only a fragment (800-912 aa) of the LRR for an interaction with RBP-1 variants. Perhaps a full-length LRR is necessary for physical binding or this fragment may require the presence of CC-NB-ARC domains for proper folding and sensing the nematode effector. The Gpa2 protein in plant cells most likely exists in a multiprotein complex with other proteins such as RanGAP (Sacco et al., 2007). The absence of these interactors in yeast could influence the binding capacity of the LRR to RBP-1 variants. A similar phenomenon has been observed before with Pto, for which the active conformation of Pto proteins was essential for AvrPto binding (Xing et al., 2007). We therefore cannot exclude that binding of RBP-1 to Gpa2 occurs but only when Gpa2 protein is in the proper conformation.

S166P mutation changes the shape of surface A in RBP protein

Because the autoactive mutants Gpa2 is not suppressed by the inactive forms of RBP-1, the mechanism of suppression or inhibition likely operates on a functional Gpa2 protein, but not on Gpa2 activated signaling. When a similar experiment is done with Rx1 and the coat protein of the breaker strain of PVX, which differs only in one residue from the avirulent strain, this suppressive effect is also not observed. Further research is required to resolve the mechanism of possible competitive interactions of the active and the inactive RBP variants on the Gpa2 protein. Essentially, there are two models that could explain this phenomenon. First, the inactive variants could physically out-compete the active RBP for binding at the target of RBP. The binding target could be directly in Gpa2 protein or in the virulent target monitored by Gpa2. Alternatively, the inactive RBP may intercept RBP by forming an inactive heterodimer complex rendering it essentially undetectable for the Gpa2 immune receptor.

Interestingly, the differences between *G. rostochiensis* SPRYSECs and *G. pallida* RBP-1 SPRY sequences are mainly located on surface A in the structural models of the proteins. Five out of seven loops of surface A are significantly different among these closely related SPRY proteins. This may suggest that specificity of these proteins is linked to the properties of surface A. The S/P^{166} mutation is mapped in one of the loops forming surface A. This conformational change is apparently crucial for RBP recognition and Gpa2 activation (Chapter 5) and in agreement with a competition model, in which serine RBP-1 variants are able to outcompete the Gpa2 activating P variants by an increase in their interaction surface with a host protein.

Having the model allowed us also to understand why the truncated RBP-1 constructs (SPRY domain alone or the N-terminal extension) failed to initiate the Gpa2 dependent HR. N-terminal extension creates an additional loop in RBP-1 protein. By adding a loop the N-terminal extension repeats NTE1 and NTE2 increase the surface A of the PRY-SPRY domain. This surface is known to interact with other proteins (Nicholson 1998). Therefore, removing this extension from the protein, although it might not disrupt the core, splits the interaction surface and compromises RBP activity.

Inactive RBP variants cause loss of avirulence in nematodes

The question, however, is whether the nematodes from the breaker populations have evolved inactive RBP-1 variants to loose avirulence, or whether this phenomenon is an artifact created by our experimental design. Remarkably, we found that the resistance breaking population expressed both active and inactive forms of RBP-1s. One could argue that since this Rookmaker as a field population, not a pure line originated from a single female, might comprise of largely virulent individuals with a small proportion of avirulent individuals accountable for the active RBP-1s in the samples. We did not find supporting evidence for such a mix of virulent and avirulent genotypes in the Rookmaker population, because females collected from roots of Gpa2 resistant potato expressed the same RBP-1 variants as individuals used for the inoculation. This might be explained by the fact that the late and relatively mild syncytium degradation in Gpa2 roots (see chapter 4) put no selection pressure on males and avirulent genotypes are maintain in the virulent population. RBP-1s were identified based on amplification from RNA extracted from pooled individuals and sequencing of 96 clones, where different RBP classes were differently represented. Inactive RBPs were found in 10 clones from 48 sequenced clones of Rookmaker juveniles. Active RBP variant Rook-6, which is the strongest activator of Gpa2, was identified as a singleton among 48 sequenced independent clones, which can indicate that the strong avirulence are maintain in the virulent population, but at a low levels.

Phytopathogenic bacteria deliver a cocktail of effectors via their type III secretion system into the host cells. These proteins are known to manipulate the plant immunity using various mechanisms like subverting host ubiqutination system, modulating host proteins, transcription machinery, or hormone signaling (reviewed in Cuhna et al., 2007). Survival of endoparasitc nematodes within plant roots requires the plant immune evasion and proteins secreted in pre- and early parasitic stages may contribute to this process. Few examples of immuno suppressive effectors have been reported for animal parasitic nematodes (Giacomin et al., 2008). Additionally, a chorismate mutase secreted by cyst nematodes was shown to play a role in altering host defense response elicited by nematode infection and has been associated with R gene based immunity (Lambert et al., 2005). The potential function of secreted proteins similar to SKP-1 and RING-H2, as well as ubiquitin extension proteins (Davis et al., 2004), (Baum et al., 2007) suggests that nematodes may actively and selectively regulate host cell protein degradation to their parasitic advantage. Finally, a similar suppressive effect as described in this study for the nematode effector RBP-1 is demonstrated for *Fusarium oxysporum* races expressing Avr1 next to Avr2 and Avr3, which are able to colonized plants possessing two functional R genes, I2 and I3 (Houterman et al., 2008). Surprisingly, in case of RBP-1 the suppression seems to act specifically on its own cognate immune receptor Gpa2.

Materials and Methods

Domain-swap constructs

An unique conserved ApaLI site at the beginning of the LRR region (Domain4 and 5) and a PstI site at the end of the LRRs of the Gpa2 and Rx1 encoding regions allowed exchange of the ApaLI-PstI LRR fragments resulting in R1-3G4G5 and G1-3R4R5 swaps in pRAP, pRXI and pGPAII. The sequence of the LRR domains of Rx1 and Gpa2 carries conserved restriction sites before the first LRR repeat (ApaL I), after repeats 1-3 (Cla I), 4-9 (Acc III) and 10-14 (EcoR I). The C-terminal end after the LRR repeats (domain 5 (G5 or R5) is delimited by PstI. Using these enzymes in combination with the unique Asc I or Pac I in the pRAP vector, gene segments from the Gpa2 LRR can be introduced into the Rx1 background and vice versa. In the nomenclature of the constructs lowercase g or r are used to represent the LRR segments. So G1-3rgrR5 contains the CC-NBS of Gpa2, then ApaLI – Cla I (LRR1-3) from Rx1, ClaI-Acc III (LRR4-9) from Gpa2, Acc III-EcoRI (LRR10-14) from Rx1 and the C-terminal segment (EcoR I-Pst I from Rx1. Other intra-LRR swap constructs were created in a similar way.The swap constructs were introduced into the binary pBIN+ vector using the unique AscI-Pac I sites and were finally transformed to *Agrobacterium tumefaciens* for expression studies in plants.

Agrobacterium-mediated transient transformation

Agrobacterium tumefaciens strain pMOG101 (Van der Hoorn 2000) was transformed with binary (pBIN+) constructs and grown in YEB medium. Cells were pelleted and resuspended in Minimal Medium for *Agrobacterium* (MS, 10mM MES 2% sucrose, pH 5.6) and infiltrated at OD_{600}=0.5 into leaves of four-week old *Nicotiana benthamiana*.

Agrobacterium-mediated stable transformation of potato

Agrobacterium tumefaciens strain pMOG10 was transformed with binary (pBIN+) constructs and grown in YEB medium. Bacteria cultures were used to transform the diploid potato line V as described by (van Engelen, Schouten et al., 1994).

DAB staining

Agroinfiltrated leaves of *N. benthamiana* were vacuum infiltrated for 20 minutes in 1mg/ml 3,3'-diaminobenzidine in PBS and discolored in pre-warmed 80% Ethanol of 60^0C.

Yeast two hybrid screening

The MATCHMAKER two-hybrid system 3 (Clontech) was used to construct bait and prey constructs. A segment of the Gpa2 LRR (1990-2455 nt) was cloned as BspeI-BamH1 fragment into pGADT7 vector (bait) and *G.pallida* RBPs (Rook-4, Rook-6 and D383-1) were cloned as PCR products amplified with primers ForXbaI and RevBam into pGBKT7 vector (prey). Yeast (AH109) cells were transformed with combinations of generated bait and prey vectors, empty vectors as control according to the protocol from Clontech. Transformants were selected on plates deficient in Leucine and Tryptophan, respectively. Selected yeast colonies were spotted on plates lacking histidine and/or adenine to verify protein interactions. As alternative method to confirm interactions, yeast cells co-transformed with both pGADT7 and pGBKT7 vectors and assayed with LacZ blue-white screening (Chien 1991).

Nematode resistance assay

In vitro cultures of transgenic lines and wild type potato clones were grown in Petri dishes on B5 medium and 3 week old roots were inoculated with pre-parasitic second stage juveniles (J2) of potato cyst nematodes *Globodera pallida* pathotype Pa3 (Rookmaker) and Pa2 (D383). J2 were hatched from dry cysts in filter-sterile potato root diffusate (de Boer et al., 1992). Collected J2 were surface sterilized using 0.5% (w/v) streptomycin-sulphate and penicillin (Duchefa) for 20 min. and 0.1% (w/v) ampicilin-gentamicin (Sigma) for 20 min, and 0.1 % chlorhexidine-digluconate (Sigma) for 3 minutes. After final rinsing with sterile tap water approximately 300 individuals were transferred to the root tips and plates kept for 4 weeks at 18^0C in dark.

RBP-1 variants in females selected on Gpa2 plants

Wild type potato genotype containing *Gpa2* gene grown in vitro was inoculated with sterilized juveniles of *G. pallida* population Rookmaker. Six weeks after inoculation females which appear from the roots were collected and pooled for genomic DNA extraction. This extracted DNA was used as a template for nested amplification with Gpa2RBPMforXba (5' – CTCTAGACCATGGAGTCGCCAAAACCAAAC-3') plus GpaRBPMstopRev (5' – GGATCAGCAAACCCATCATAAATTCTCG-3') and in the second round RBP101for (5'-GGAATCCCGAAGCATGTGAC) plus GpaRBPMstopRev. PCR product of the second round of amplification was cloned into TOPO2.1 vector (Invitrogen) and sequenced.

Computer aided modeling of the Gpa2 LRR domain

For pattern and profile searches and domain recognition we used the InterPro programs that uses Pfam, Prints, Prodom, SMART, TIGR and Prosite databases (Mulder 2007).

For the secondary structure prediction, the following programs best ranked by CASP4 experiment were used: GOR IV (Ellis 1994), Jpred (Cuff 1998), HNN (Guermeur), PROF (Ouali 2000), Porter (Pollastri and McLysaght, 2005), SOPMA (Geourjon 1995), NNPredict (Kneller 1990), PsiPred (Jones 1999). For inter-domain linker prediction, DLP was used (Miyazaki 2002).

For contact forming and accessibility propensity profiles ACCpro (Pollastri 2002) and CMAPpro program were used (Pollastri 2001).

For sequence to structure alignment and refined threading special in-house software SLIDE was used. LRR modelling protocol used a suite of scripts and programs developed in-house which includes SLIDE.

For the refined modelling Insight II software package from Accelrys was used. The Homology module was used for coordinate transfer and loop generation. Local simulated annealing and energy minimization during modelling steps were performed via the Discover module with cvff force field.

Based on the consensus sequence, the variability at a given position in the sequence was defined as the average of the Blosum62 substitution matrix values between every sequence and the consensus.

$$\sum_i \frac{M(S_{ij}, C_j)}{i}$$, where

S_i - sequence i, C - consensus sequence, j - position

These values were scaled to the limits of b-factors in pdb files, which reflect the local structural disorder. In this way similarly to the b-factor color code, a 3D mapping of sequence variability was obtained in which with increased variability color shifts from blue (highly similar stretches) to red (highly variable stretches)

Sequence characterization: To delimit the Gpa2-LRR domain boundaries domain linker prediction has been performed. Because DLP method failed to predict linkers between Gpa2 domains, Rx family sequences were scanned against Interpro database collection. Although 513 was set as the first aa position of the LRR in Gpa2 (SUPERFAMILY entry SSF52058), for convenience of modelling we chose 505 as the first aa position.

Locate LRR motifs: The critical step in modeling the LRR domain of Gpa2 was to locate the correct positions of the LRR motifs along the sequence. To this end all the putative motifs of the target sequence were identified.

Template identification and modelling: Although fold recognition returns hits comprising of LRR domains such as": 2z7xA - $3.6e^{-25}$; 1ziwA - $5.3e^{-25}$; the alignment with these sequences show signifficant insertions at the level of many repeats, with severe gap penalty. To overcome this problem we rather used used an alternative approach consisting in modelling locally group of repeats starting from the best local template, followed by assembling these fragments in an overall model. Following this protocol Schizosaccharomyces pombe RNA1P (1yrg), Bos taurus decorin (1xku) and human Toll-like R3 (2a0z) have been found as the best templates and used as follows: -a) *lrr*2-5 (aa507-626) ↔

1yrg(62-188); -b) *lrr*6-8 (627-700) ↔ 1xku(102-172); -c) for *lrr*9-10 (701-765) ↔ 1yrg(246-304); d) for *lrr*11-15 (766-903) ↔ 2a0z(409-540). The four local template fragments were superimposed along their *lrr* motifs to form a continuous LRR framework and the standard remote homology modelling procedures were applied. The model LRR-Gpa2 model was then refined using repeated rounds of simulated annealing and energy minimization.

Model analysis: The overall properties of the model such as the curvature and the twist were first collectively acquired from the templates by coordinate transfer and then refined by minimizing the energy of the whole structure.

Computer aided modeling of RBP

Fold recognition was carried out with Phyre (Bennett-Lovsey 2008). Patterns, profiles and domain recognition were performed with InterPro, based on Pfam, Prints, Prodom, SMART, TIGR and Prosite databases (Mulder et al., 2007). Secondary structure prediction was profiled with Jpred (Cuff et al., 1998), HNN (Guermeur,), PROF (Ouali and King, 2000) Porter (Pollastri and McLysaght, 2005), SOPMA (Geourjon and Deleage, 1995), NNPredict (Kneller et al., 1990), PsiPred (Jones 1999). Contacts and accessibility propensities were profiled with ACCpro (Pollastri et al., 2002) and CMAPpro (Pollastri et al., 2001). DLP (Miyazaki et al., 2002) was used for inter-domain linker prediction. Sequence to structure alignment and refined threading were performed with SLIDE a special software developped in the lab. The refined global model generation was carried out with Insight II from Accelrys. The Homology module was used for coordinate transfer and loop generation. Local simulated annealing and energy minimization during modelling steps were performed with Discover cvff force field.

Domain identification: Rookmaker and D383 sequences from *G. pallida* were identified as being B30.2/SPRY domains, pfam00622 and smart00449, which are distorted beta sandwiches with a core of antiparallel beta-strands connected by highly variable loops. Sequences from both virulent and avirulent Rookmaker populations - Rook4 and Rook6 - are highly similar with only 5 mutations in 250 aminoacids: Q/K^{100}, M/I^{127}, H/R^{162}, S/P^{166} and L/S^{201}.

Template identification: Fold recognition returned as the best hit Drosophila melanogaster GUSTAVUS protein (PDB code - 2fnj) with an e-value of $2.9E^{-13}$. Also 2afj, 2vol and 2fbe were 100% estimated precision hits. The template and target overlap over a region of 187 aminoacids that do not comprise an N-treminal stretch of 59 aa in Rook-4 and 61 aa in Rook -6.

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Chapter 7

Concluding Remarks

Introduction

The most crucial aspect of plant, and any other living organisms, survival is the ability to distinguish between "self" and "non-self". Especially plants, due to their sedentary lifestyle and simultaneous exposition to a wide range of pathogens had to evolve a dynamic and versatile defense system to survive in a hostile environment. Plants lack an adaptive immune system, but employ several cell autonomous mechanisms to react to their intruders, which resembles the mammalian and insect innate immune system. Invading pathogens can be recognized by either pattern recognition receptors (PRR) or resistance proteins (R proteins) resulting in PAMP-triggered immunity (PTI) or effector-triggered immunity (ETI), respectively (see Chapter 2 for an overview).

In turn, pathogens have evolved various means to overcome these plant defense responses. Especially biotrophic pathogens like cyst and root knot nematodes, which are fully depending on living host tissue for their development and reproduction, have found sophisticated ways to evade and manipulate the host's immune system. Pathogens secrete proteins and small molecules that alter the host-cell structure and that are currently known as effectors (Hogenhout et al., 2009). These alterations either facilitate infection of the host (virulence factors) or trigger defense responses (avirulence factors and elicitors) or both (Huitema et al., 2004; Kamoun 2006).

Cyst and root-knot nematodes establish an intimate relationship with their hosts for months to complete their life cycle. In susceptible plants, they are able to modify a differentiated host cell into a multinucleate feeding structure from which they obtain their nutrients. To achieve such drastic host cell modification plant parasitic nematodes have to manipulate fundamental elements of plant cell development. Thereto, secretions produced in the esophageal glands of plant parasitic nematodes are injected into the host cell via their protrusible stylet throughout their life cycle. Secretions from the two subventral glands of cyst and root knot nematodes are often involved in the penetration of the host and migration towards the appropriate feeding site, while the dorsal gland secretions seem to be involved in feeding cell formation and maintenance (Davis et al., 2008).

For endoparasitic nematodes, more than 60 proteins are potentially secreted and implicated in different processes, including modulation of the host defense response (Davis et al., 2004, Jauber et al., 2002; Huang et al., 2003). For example, a secreted chorismate mutase from *G. pallida* seems to play a role in altering host defense responses upon nematode infection and has been associated with *R* gene based immunity (Lambert et al., 2005). Additionally, venom allergen-like proteins secreted by animal-parasitic nematodes are known to invoke host immune response. They are conserved and were found in phytoparasitic nematodes and animal parasitic nematodes (Zhan et al., 2003); however their role remains unclear (Baum et al., 2007).

The number and diversity of putative nematode effectors and limited knowledge about nematode resistance mechanisms make it very difficult to predict which proteins secreted by cyst and root knot nematodes can play a role in plant immunity. Here, we will give an overview of parasitism genes encoding cyst and root knot nematode effector proteins and discuss their potential role in modulating the plant's defense system.

Nematode avirulence genes

The gene-for–gene hypothesis states that host resistance specificity is determined by complementary pair of a pathogen effector encoded by avirulence genes (*Avr* gene) and a receptor encoded by plant resistance genes (*R* gene). Only for the interaction between the potato cyst nematode *G. rostochiensis* and potato Mendelian proof of a gene-for-gene interaction has been demonstrated (Janssen et al., 1991). Selection of pure parasitic and non-parasitic lines of *G. rostochiensis* and subsequent reciprocal crosses using these lines have

shown that parasitism is recessively inherited at a single locus and that the inheritance is not sex-linked (Janssen 1990; Janssen et al., 1991). A dominant locus *H1* present in resistant potato cultivars was demonstrated as only being effective against certain pathotypes of *G. rostochiensis*, while nematodes carrying recessive virulence alleles could reproduce normally on these plants. Because of a segregation pattern of 3:1 non-parasitic to parasitic, which is typical for single gene inheritance and a proven dominant nature of the *H1* resistance, it has been proposed that this interaction classifies as a gene-for-gene type of mechanism (Janssen et al., 1991).

For a limited number of other incompatible plant-nematode interactions, the genetic basis of nematode virulence was investigated. Studies using three highly homozygous inbred lines were performed for the soybean cyst nematode *H. glycines* (Dong 1997). The inbred line crosses clearly demonstrated that parasitic ability is inherited in a Mendelian fashion. Both dominant and recessive genes were found and proven by linkage analysis to be unlinked loci (Dong 1997). In the case of root-knot nematodes, analysis of virulence segregation in progeny of a controlled cross of *M. hapla* indicated that virulence in the nematode is inherited as a single recessive trait, and that the nematode-bean interaction might be classified as a gene-for-gene interaction (Chen and Roberts, 2003). Aiming to facilitate map-based cloning of genes that mediate plant-nematode interactions, a genetic map has been constructed for the potato cyst nematode *G. rostochiensis* (van der Voort et al., 1999). Due to the outcrossing nature of *G. rostochiensis* and technical limitations in using individual offspring genotypes for map construction, this map was made with a bulked offspring population. To our knowledge, this map has not yet been used to map parasitism or avirulence genes.

In an attempt to identify avirulence gene products involved in *Mi*-mediated resistance in tomato, cDNA-AFLP fingerprinting was used for pair wise comparison of the expression profiles of near-isogenic lines from the root-knot nematode *M. incognita*, avirulent and virulent on *Mi-1* resistant plants (Semblat 2001). This resulted in the identification of several differentially-expressed genes including *map-1*, which was shown to encode for a protein containing a predictive signal peptide for secretion and two classes of repetitive motives. Immunolocalization experiments confirmed that the MAP-1 protein is secreted by the amphids, which are the principal chemosensory organs of the nematode. The role of *map-1* in avirulence, however, has never been demonstrated. In addition, a transcript present in avirulent but absent in virulent lines of *M. javanica* has also been identified. Curiously, this gene does not resemble *map-1*, suggesting that there may be more than one gene that can mediate nematode recognition in tomato plants with the *Mi-1* gene (Williamson and Gleason, 2003).

Genes encoding avirulence factors or effectors are believed to be direct targets of selection forces that drive evolution between host and pathogen. Effector alleles that increase the reproductive success of the pathogen will be immediately favored by natural purifying selection. Naturally selection acts not only on diversifying or conserving nucleotides within genes, but also on copy number polymorphism. For example, *P. infestans Avr3b-Avr10-Avr11* locus exhibits remarkable copy number variation resulting in amplification of up to 25 truncated copies of the candidate Avr gene *pi3.4* (Jiang et al., 2006). Indeed, the SPRYSECs and RPB-1s from cyst nematodes are encoded by multigene families, which products are being under diversifying selection (Rehman et al., 2009; Chapter 6/Sacco et al., 2009). The SPRYSEC family consists of more than 20 genes being expressed in different life stages of the nematode (Rehman et al., 2009). Similarly, several highly similar RBP-1s were amplified from cDNA obtained from *G. pallida* pre-parasitic stages. Remarkably, it was noticed that only nematodes virulent on Gpa2 plants expressed truncated copies with premature stop codons next to full length RBPs (Koropacka, unpublished data). The role of these truncated

copies is unclear, but perhaps they act as natural dominant negative mutants inside the host cells.

Many pathogen effectors were identified and named based on their avirulence activity. However, it is assumed that they must contribute to pathogen fitness, for example, by contributing to virulence on a susceptible host. Therefore, it was anticipated that RBP-1 could play a role in nematode parasitism. However, overexpression of a *Gpa2* non-eliciting and *Gpa2* eliciting RBP-1 in susceptible potato plants challenged with virulent and avirulent nematodes from *G. pallida* had no impact on nematodes virulence (Chapter 6).

Recognition of nematode effector proteins by R proteins

The molecular mechanism by which plant the NB-LRR type of R proteins recognize pathogen attack remains unclear despite intensive work done by numerous research groups. However, there is accumulating evidence that the LRR domain is involved in pathogen recognition. In the simplest model a direct receptor-ligand interaction determines the recognition of the pathogen by the plant's immunesystem. This model was supported by the finding of several pairs of Avr products that bind directly to an R protein. For example, the multigenic loci (K, L, M, N, P) for flax rust resistance recognize about 30 effectors from the flax rust fungus and the direct physical interaction between soma of these effectors and R proteins was demonstrated in yeast-two-hybrid assay (reviewed in Ellis et al., 2007). Additional supportive evidence came from research done on AvrPita and Pita (Jia et al., 2000) and PopP2 and RPS-1 (Deslandes et al., 2003), which were also shown to physically interact. In the alternative molecular model for effector recognition the R proteins are associated with a host protein or so-called virulence target, which is guarded by the R protein. Guarded proteins can be a cellular target for effectors (guard model) or mimic such a target (decoy model). In the presence of a pathogen effector the guardee is modified and this modification results in the activation of the R protein (Van der Biezen and Jones, 1998; Dangl and Jones, 2001; Van der Hoorn and Kamoun, 2008).

Interestingly, the NBS-LRR protein SW5F was found to interact with SPRYCES-19, a RanBPM homologue from *G. rostochiensis* (Rehman et al., 2009). This is the first example of direct binding of a nematode effector to a plant NB-LRR protein. The structural similarities between both RBP-1 and SPRYSEC19 at the one hand and the LRR domains of Sw5F and Gpa2 at the other side of the interaction prompted us to test whether the eliciting or non-eliciting RBP-1 interacts directly with C-terminal fragment of Gpa2 protein in yeast (Chapter 6). However, no such binding was found, but we cannot exclude that direct binding requires a full-length Gpa2 protein or additional plant proteins, which together may form the functional complex. For our understanding of the mechanisms underlying Gpa2 activation it would be of great value to uncover the cellular target for nematode RBP-1 in plants.

R proteins are activated upon recognition of nematodes effectors secreted into host cell. In theory nematodes could remain undiscovered by mutations in their effectors that abolish recognition. However the primary role of effectors in nematode parasitism defines the functional constraints for such mutations, because of their possible negative impact on nematode fitness in susceptible hosts plants. We observed that the avirulent population of *G. pallida* (D383) expresses four different RBP-1 variants of which all trigger a Gpa2-dependent defense response. Nematodes from the virulent *G. pallida* population Rookmaker express additional RBP-1 variants that have a P166S mutation and as a result are no longer recognized by Gpa2. These data suggest that loss of avirulence activity in RBP-1 is correlated with a single nucleotide polymorphism, which may have limited impact on the fitness of the nematode.

The fast majority of RBP-1s identified to date have a signal peptide at the N terminal end, which suggests that the proteins are secreted through the stylet into the host plant cell. *In situ*

hybridization experiments showed that RBP-1s are present exclusively in the dorsal gland, suggesting that these proteins play a role in nematode parasitism (Blanchard et al., 2005). A GFP fusion of $RBP_{D383\text{-}1}$ was transiently expressed in *N. benthamiana* leaves, which resulted in the detection of the fluorescent protein in the cytoplasm and nucleus of the cells (data not shown). This suggests that RBP-1s, once inside a host cell, are equally distributed within the host cell and not targeted to one specific compartment. Gpa2 is also present in both the cytoplasm and nucleus (Dees et al., unpublished data) like other NB-LRR proteins including N (Burch-Smitch et al., 2007) and Rx1 (Slootweg et al., in prep). These results suggest that RBP-1 and Gpa2 indeed co-localize in the host cell. Manipulation of the subcellular distribution of RBP-1 and Gpa2, for instance with nuclear targeting signals, will help us to determine in which cell compartment RBP-1 recognition takes place. In addition, it will be interesting to test whether the subcellular localization of the RBP-1s with a serine is different to that of RBP-1 with a proline at position 166, to see if the lack of Gpa2 activation correlates with a difference in subcellular localization in the host cell.

Options for modulation of *R* gene mediated host responses by nematodes

R gene recognition and signaling are attractive targets for pathogens to overcome the plant's defense system. Indeed, suppression of plant innate immunity has emerged as the primary function of effectors. For many known effectors the mechanism through which they interfere with plant immunity remains unknown. So far, two cellular processes seemed to be key targets for pathogens: transcription and RNA homeostasis, and targeted protein degradation.

Pathogenic bacteria secrete a wide range of effectors into the host cell via type III secretion system that target among others also the plant nucleus and therefore the gene expression machinery. Phytopathogenic species of *Xanthomonas* and *Ralstonia* harbor multiple effectors that encode transcription activator-like proteins (TAL). TAL effector *AvrBs3* of *Xanthomonas campetris pv. Vesicatoria* (Xcv) is translocated to plant cell nuclei where they initiate transcription of several genes named *upa* (upregulated by AvrBs3) (reviewed in Saijo and Schultze-Lefert, 2008). A core sequence in gene promoters was determined and named the upa-box, which is a direct target for *AvrBs3* (Kay et al., 2007). As cyst and root-knot nematode infections cause severe changes in cell cycle regulation and metabolism, it is very likely that they secrete proteins that can directly affect the plant transcription machinery. This was supported by the identification of a parasitism gene encoding a peptide (16D10) with CLE (CLAVAT3/ESR-like) signature from the subvental esophageal gland cells of root-knot nematodes (Huang et al., 2003). A yeast-two-hybrid screen and immunoprecipitation of 16D10 demonstrated a specific interaction with the SAW domain of plant SCARECROW-like (SCL) transcription factors (Huang et al., 2006), which provides the first evidence that a secreted nematode parasitism gene product can regulate host activity via binding to an intracellular plant transcription factor.

Targeted protein degradation may provide a powerful and unique means for regulation of the host cell phenotype by pathogens (review in da Cuhna et al., 2007). A well defined example of a pathogen effector with a potential role in suppressing host defense responses is demonstrated by the activity of a domain of *Pseudomonas syringes* AvrPtoB that functions as a mimic of host plant E3 ubiquitin ligase (Janjusevic et al., 2006). The potential function of secreted proteins similar to SKP-1 and RING-H2, as well as ubiquitin extension proteins (Baum et al., 2006; Davis et al., 2004), suggests that nematodes may also actively and selectively regulate host cell protein degradation to their parasitic advantage. The presence of a BC box at the C terminus of SPRYSEC19 (Rehman et al., 2009) and RBP-1 (Chapter 6) suggests that this type of nematode effector proteins could be involved in specific degradation of their targets as well.

Recent findings indicate that several oomycete RXLR effectors also mitigate host immunity. For example, *P. infestans* Avr3a suppresses the hypersensitive cell death induced by another *P. infestans* protein, INF1 elicitin (Bos et al., 2006). Recently, it was shown that *Fusarium oxysporum* secretes an effector that can both trigger and suppress R gene-based immunity. This effector, Avr1, triggers disease resistance when the host plant, tomato, carries an *I* or *I-1* gene and simultaneously suppress *I-2* and *I-3* (Houterman et al., 2008). Interestingly, we were able to show that virulent nematodes from *G. pallida* express both Gpa2 activating and inactivating RBP-1 variants, which are able to suppress the Gpa2 dependent HR when they are present together in *N. benthamiana* leaves. Currently, we are investigating whether the ability of RBP-1_{ROOK2} and RBP-1_{ROOK4} to suppress Gpa2 mediated HR in *N. benthamiana* leaves corresponds with breaking Gpa2 resistance by virulent nematodes from the *G. pallida* population Rookmaker upon infection of transgenic potato plants harboring the Gpa2 gene and the genes encoding these non-activating RBP-1s.

This inhibition probably targets the *Gpa2* gene at the activation stage rather than its downstream signaling pathway as the effect was specific only for the *Gpa2* wild type gene and not for the autoactive mutant (D460V) of *Gpa2* or the highly homologous gene *Rx1* (Chapter 5). One possible explanation could be that these effectors affect the transcriptional regulation of the *Gpa2* gene as we have observed an inhibitory effect of virulent nematodes on GUS expression driven by the endogenous *Gpa2* promoter (Chapter 4). In Chapter 3 the quantitative nature of the Gpa2 and Rx proteins was shown and hence, decreasing the *Gpa2* gene expression level might be sufficient to avoid the defense response. This was also demonstrated for the expression of the rice gene *Xa3*, encoding a LRR receptor kinase type of protein, which is associated with a range of resistant activities in different genetic backgrounds and different developmental stages. A higher expression level of *Xa3* results in a wider resistance spectrum, a strong resistance level and a whole growth stage resistance (Cao et al., 2007).

As an alternative explanation for RBP mediated Gpa2 suppression, we propose the competition model. In this model, the RBP-1 molecule has a dual function. One is to bind the cellular target, which might be the Gpa2 protein itself or the guardee. This binding occurs for both activating RBPs and suppressive RBPs, but only activating RBPs are able to modify the host target or translocate it to different cell compartment, what results in activation of the Gpa2 triggered defense. When we scanned the RBP-1 sequences with posttranslational modification software (Abgent, Sumoplot), we have found that the stretch of amino acids including the proline (but not the serine) is recognized as two overlapping SUMOylation motifs (VKVP- score 0.82 and PKFG- score 0.43). Small ubiquitin-like Modifier (SUMO) proteins are a family of small proteins that bind covalently to other proteins to modify their function. This posttranslational modification is involved among others in cytoplasmic-nuclear trafficking, transcriptional regulation and protein stability. Hence, it will be interesting to investigate whether RBPs are specifically SUMOylated in plant cells and how this affects the role of RBP in nematode parasitism.

Perspectives

The importance of suppressing plant defense and cell death for the survival of cyst and root knot nematodes is undeniable. Most information about host plant targets and mechanisms employed to subvert the defense response comes from studies on pathogenic bacteria, which secrete diverse proteins via the type III secretion system. Numerous examples of secretions able to interfere with plant responses were identified. Repertoires of effectors determine the bacteria's ability to infect a host and therefore, host specificity. Our knowledge about the role of plant parasitic nematode effectors in modulating plant immunity is still limited, but in this

thesis we have presented a first example of a nematode effector gene family, of which specific members have shown the potential to activate and inhibit specific *R* gene mediated responses. The recent progress in nematode genome sequencing, including the genome of *G. pallida*, will deliver a huge amount of data concerning nematodes effectors. The complete sequence of the *M. incognita, H. glycines, and M. hapla* genome (Elling et al., 2009; Abad et al., 2008) will undoubtedly contribute to the identification of genes not represented among the available ESTs libraries. This will allow us to unravel the various modes of action employed by cyst and root knot nematodes to manipulate their hosts.

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Summary

***Gpa2* recognition specificity**

Among all the multicellular animals, nematodes are the most numerous. In soil, a high variety of free living nematodes feeding on bacteria can be found as well as species that parasitize insects, animals or plants. The potato cyst nematode (PCN) *Globodera pallida* is an important pest of cultivated potato. Upon infection of the roots, the nematode induces a feeding cell complex or so-called syncytium, on which the immobilized nematode fully depends for its development and reproduction. Due to the sophisticated feeding manner and ability to survive for a long time in the absence of a host plant, the best way to control these soil-born pathogens is the exploitation host resistance. Natural resistance to nematodes is based on single dominant resistance genes (R) or quantitative trait loci (QTL). Several nematode resistance genes have been identified and mapped. This includes the potato gene *Gpa2* (Van der Vossen et al., 2000) that confers resistance against the population D383 of *G. pallida*. The *Gpa2* gene is highly homologous to *Rx1*, which confers resistance against potato virus X (Bendahmane et al., 1999). Both genes encode a protein with a nucleotide-binding leucine-rich repeat (NB-LRR) domains and a short coiled-coil domain at the N-terminus, which are in 88% identical at the amino acid level. The vast majority of the differences between Gpa2 and Rx1 is found in the predicted solvent exposed regions of the LRR domain. In chapter 2, we have shown that the LRR domain is essential for the recognition specificities of Gpa2 and Rx1, whereas the CC-NBS domains can be exchanged without affecting the specificity. In chapter 5, we have used a series of chimeric constructs in which segments of the Gpa2 LRR were replaced by the corresponding segments from Rx1. These constructs allowed us to narrow down the region required for nematode recognition to a stretch of residues between 808 and 912 amino acid residues in Gpa2, including 10 amino acids that differ between Gpa2 and Rx1. Furthermore, a computer-aided 3D model of the LRR domain is presented in which 7 of the Gpa2 specific amino acid residues map in a cluster onto the concave surface of the horseshoe-like structure of the LRR domain.

Gpa2-mediated nematode resistance

The research described in chapter 3 aimed to understand the mechanisms underlying Gpa2-mediated resistance to the potato cyst nematode *G. pallida*. The extreme resistance response conferred by the close homologue Rx1 results in the blocking of the potato virus X (PVX) at the infection sites and hence, the prevention of systemic spreading throughout the plant. Surprisingly, an entirely different defense mechanism was observed for resistant potato plants infected with juveniles of the avirulent *Globodera pallida* population D383. In susceptible plants, both the virulent population Rookmaker and the avirulent population D383 formed normal developing syncytia and nematodes were able to complete their life cycle as described in previous studies. Infection of resistant plants with the avirulent population showed no differences between susceptible and resistant potato plants in the early stages of *G.pallida* parasitism (root entering, migration, syncytium initiation). Syncytium induction took place in parenchyma cells, but rarely in other tissues. In samples collected 7 days later, however, the first necrotic cells in the surrounding of the syncytium were noticed including symptoms of degradation in the ultra structure of the syncytium itself in case of resistant plants infected with avirulent nematodes. Samples collected 10 days post infection had already a layer of necrotic cells, which separates the syncytium from the vascular bundle. At 14 days post infection, it was observed that the parenchyma cells not incorporated directly in the syncytia started to divide fast. Groups of hyperplastic cells surrounding the degrading syncytium resulted in pushing it away to the outer part of the root. This unique phenomenon, which was

not observed before, can be part of the Gpa2-mediated defense response or a secondary reaction to the presence of necrotic, dead cells and a way to exclude them from the healthy conductive tissue of the root.

Transcriptional regulation of the Gpa2 promoter

To look in more details into the transcriptional regulation and expression of *Gpa2*, the native promoter was fused to the reporter gene GUS and this construct was introduced into susceptible potato. In chapter 3, the activity of the *Gpa2* promoter was observed and shown to be restricted to the vascular system and the root tips in uninfected plants. Roots were challenged with *G.pallida* and the localization of the GUS expression was observed at the infection sites at different parasitic stages. During infection with virulent nematodes - but not the avirulent ones - this activity seems to be down regulated in vicinity of the syncytium. Such a local inhibition of Gpa2 promoter activity is in line with observations made on resistant roots when necrotic cells were only present around the feeding cell complex, distantly from the feeding nematode.

The effector protein RBP-1 elicits a Gpa2 dependent HR

Recently, a RBP-1protein with strong similarity to the SPRY domain of the Ran-binding protein RanBPM in juveniles of *G. pallida* was identified as a putative Gpa2 elicitor. Transient expression of RBP-1 in *N. benthamiana* leaves elicits a Gpa2-dependent cell death typical for the *R*-gene associated hypersensitive response (HR). Total RNA isolated from two populations of *G.pallida*, D383 (avr to Gpa2) and Rookmaker (vir to Gpa2) was converted into cDNA and screened for the presence of RBP-1s. This screening allowed the identification of in total 10 classes of closely related homologs of RBP-1. All identified classes were tested for their ability to elicit the Gpa2-dependent HR in an agroinfiltration assay. The capacity to induce an Gpa2-dependent HR was shown to correlate with a single amino acid substitution in RBP-1. No response was observed for two classes, which were obtained from the virulent population (RBP-1_{ROOK2}, RBP-1_{ROOK4}). For the other homologous RBP-1 classes – both deriving from the virulent and avirulent population - the response was ranging from a mild to a strong and fast HR. Both in-active RBP-1 variants have a serine substitution at position 166 (S166P) within the SPRY domain. When this residue was projected on a computer aided 3D model of RBP, we noticed that this amino acid is in a loop extending from the protein core. Replacing the proline into a serine is predicted to change the shape of the loop and hence, to affect the potential surface for protein-protein interactions.

Non-eliciting RBP-1 variants suppress RBP-induced Gpa2 activation

It was shown that the non-eliciting variants (RBP-1_{ROOK2} and RBP-1_{ROOK4}) can suppress the activation of a Gpa2-mediated HR by the eliciting RBP-1 variants. This effect was specific for the Gpa2-mediated HR, and not observed with a Rx1-induced HR. As autoactive mutants of Gpa2 and Rx1-mediated cell death are not blocked by the inactive variants of RBP-1, the mechanism of suppression or inhibition likely operates on a functional Gpa2 protein, instead of downstream Gpa2-activated signaling pathways. Further research is required to resolve the mechanism underlying the possible competitive interactions of the active and the inactive RBP-1 variants on the Gpa2-mediated HR. Essentially, two possible models that could explain this phenomenon. First, the inactive variants could physically out compete the active RBP-1s. The binding target of active and inactive variants of RBP-1 variants could be directly in the Gpa2 protein or in the virulence target monitored by Gpa2. Alternatively, the inactive variants of RBP-1 may intercept active RBP-1 variants by forming an inactive heterodimer complex rendering it essentially undetectable for the Gpa2 protein.

Samenvatting

De specifieke herkenningskarakteristieken van Gpa2

Van alle meercellige dieren zijn de nematoden het talrijkst. In de bodem komen diverse vrijlevende nematoden voor die zich voeden met bijvoorbeeld bacteriën, maar ook soorten die als parasiet leven in dieren, insecten of planten. Het aardappelcystenaaltje (ACA) *Globodera pallida* is een plantenparasitaire nematode die grote schade toebrengt aan de aardappelteelt. Na infectie van de wortels induceren deze nematoden een voedingscel, die ook wel syncytium wordt genoemd. De nematode verliest zijn vermogen om te migreren en is volledig afhankelijk van deze voedingscel voor zijn verdere ontwikkeling en reproductie. Door zijn vernuftige manier van voeden en zijn vermogen om langdurig te overleven in de grond in afwezigheid van de waardplant, is het gebruik van resistente planten een goede manier om deze parasiet te bestrijden.

Natuurlijke resistentie tegen cystenaaltjes is gebaseerd op zogenaamde enkelvoudige, dominante resistentiegenen (R genen) of kwantitatieve resistentie loci in het genoom van de plant (QTL's). Diverse nematode resistentiegenen zijn inmiddels gekarteerd en geïdentificeerd. Hiertoe behoort het aardappelgen Gpa2 dat resistentie verschaft tegen de populatie D383 van *G. pallida*. Het Gpa2 gen vertoont sterke homologie met Rx1 (88%), een gen dat resistentie tegen het aardappelvirus X geeft (PVX). Beide genen coderen voor een eiwit met een nucleotiden-bindend domein (NB), een domein dat bestaat uit een repeterende serie leucine-rijke sequenties (LRR) en een zogenaamd coiled-coil domein (CC). Het merendeel van de verschillen tussen Rx en Gpa2 zijn te vinden in het LRR domein. In hoofdstuk 2 is aangetoond dat de herkenningsspecificiteit van Rx en Gpa2 door het LRR domein bepaald wordt, terwijl de CC-NBS uitgewisseld kan worden zonder dat de specificiteit aangetast wordt. In hoofdstuk 5 zijn een aantal constructen getest waarin delen van de LRR van Gpa2 en Rx waren uitgewisseld. Hiermee was het mogelijk om de regio die bepalend is voor de herkenning van *G. pallida,* terug te brengen tot een gebied tussen de aminozuren op positie 808 en 912, inclusief de 10 residuen die specifiek zijn voor Gpa2. Door deze residuen te projecteren op een 3D model van het LRR domein van Gpa2, bleken 7 aminozuren in een cluster te liggen op het gebogen oppervlak van de hoefijzervormige structuur van dit domein.

Gpa2 geïnduceerde nematode resistentie

Het onderzoek dat in hoofdstuk 3 beschreven is, had als doel om meer inzicht te verkrijgen in de resistentiereactie door Gpa2 tegen het aardappelcystenaaltje. Het nauw verwante gen Rx1 geeft een extreme resistentierespons tegen PVX op de infectieplek, waardoor verdere verspreiding van het virus voorkomen wordt. Gpa2 blijkt echter een geheel andere reactie te geven. Hoewel er geen verschil te zien was tussen de inductie en vroege ontwikkeling van het syncytium door nematoden van de virulente (Rookmaker) en avirulente populatie (D383), waren 7 dagen na infectie met de avirulente nematoden de eerste afgestorven cellen zichtbaar rondom de voedingscel. Analyse van de ultrastructuur van deze cellen toonde symptomen aan die wezen op de degradatie van het bijhorende syncytium. Na 10 dagen was er een ring van dode cellen gevormd rondom het syncytium waardoor het contact met het vaatweefsel verbroken werd. Na 14 dagen bleken de parenchyma cellen in de buurt van het syncytium zich snel te delen, waardoor deze naar de buitenkant van de wortel werd geduwd. Deze unieke reactie, die voor zover ons bekend nog niet eerder is beschreven, kan deel uitmaken van de Gpa2 afweerreactie of een secundaire reactie zijn die gericht is op het afschermen van gezond weefsel.

Activiteit van de Gpa2 promoter
Om meer inzicht te krijgen in de plaats en tijd waarop het Gpa2 gen tot expressie komt in de plant, is de promoter van Gpa2 gefuseerd met het reportergen GUS voor het transformeren van aardappel. In hoofdstuk 3 beschrijven we dat de activiteit van de promoter zich beperkt tot de wortelpuntjes en het vaatsysteem van de plant. Na infectie met *G. pallida* kon de verandering in dit patroon geobserveerd worden. Dit resulteerde in de observatie dat de promoteractiviteit van Gpa2 op en rond de infectieplek verminderde in wortels die geïnfecteerd waren met virulente nematoden, terwijl in wortels met de avirulente nematoden er nog steeds promoter activiteit werd waargenomen.

De nematode effector RBP-1 activeert Gpa2
Onlangs is er een eiwit (RBP-1) gevonden in juvenielen van *G. pallida* dat grote overeenkomsten vertoont met het SPRY domein van het Ran bindend eiwit RanBPM, en dat in aanmerking kwam als mogelijke elicitor van Gpa2. Transiënte expressie van RBP-1 in bladeren van *Nicotiana benthamiana* induceert een specifieke celdood reactie in aanwezigheid van Gpa2. Totaal RNA was geïsoleerd uit de avirulente en virulente populatie D383 en Rookmaker, dat vervolgens is omgezet in cDNA om te zoeken naar de aanwezigheid van RBP-1. De screening resulteerde in de identificatie van 10 klasses van gerelateerde RBP-1 homologen. Van elke klasse is een RBP variant getest op het vermogen om Gpa2 te activeren in een agro-infiltratie assay. Twee van de 10 klassen bleken geen Gpa2 activiteit te induceren en deze varianten waren beiden afkomstig van de virulente populatie. De andere varianten waren in staat om in meer of mindere mate een specifieke reactie te induceren. Het vermogen om Gpa2 te activeren bleek te correleren met een enkele aminozuur substitutie. Beide niet-inducerende varianten beschikten over een serine in plaats van een proline op positie 166 in het SPRY domein. Analyse van de 3D structuur van RBP liet zien dat deze substitutie in een van de lussen ligt en het vervangen van een S naar een P resulteert in een verandering van de vorm van deze lus en dat daarmee het interactie oppervlak waarschijnlijk verandert wordt.

Onderdrukking van de RBP-geïnduceerde Gpa2 reactie
De niet-activerende RBP varianten bleken in staat om de celdood reactie, die door de andere RBP varianten geactiveerd was, te onderdrukken. Deze reactie bleek specifiek, want de Rx reactie kon niet onderdrukt worden. Ook de celdoodreactie van autoactieve mutanten kon niet worden onderdrukt, dus vermoedelijk werkt de onderdrukking direct in op Gpa2 in plaats van de signaaltransductieroutes die door Gpa2 worden geactiveerd. Vervolgonderzoek moet uitwijzen of er sprake is van een competitiemodel, waarbij de activerende en niet-activerende varianten elkaar verdringen. Dit zou kunnen bij zowel een interactie met Gpa2 zelf of een interactie met een virulentietarget dat door Gpa2 bewaakt wordt. Een andere optie is de vorming van heterodimeren, waardoor er een inactief complex ontstaat dat niet herkend kan worden.

Acknowledgments

Finally, I have reached the last part of this thesis (frankly speaking I still cannot believe that I've done it). Despite the fact that only my name is written on the cover, it was a real team effort even if some of the team members are not aware of their contributions.

First of all, I want to acknowledge my promoter and co-promoters, Jaap Bakker, Aska Goverse and Geert Smant. Thank you for giving me the opportunity to be a part of your group. I appreciate that you were able to see a potential researcher in that 24 year old girl who came to Wageningen directly from University with no practical experience.

My external supervisor, Frank Takken, you have helped me a lot with the last bits of writing by keeping up my spirits. Thank you for this support! And of course we have shared many nice scientific and social events including the day when you became a tireless shopaholic in Keystone ☺.

When looking back at my lab masters I cannot name anybody else but Jan Roosien. Jan, it was always a great pleasure working next to you and learning some of your tricks. As you know, having you as a supervisor was noticed as a plus during my interview with my current employer Enza Zaden. And I also benefited from what you have taught me during my daily challenges in the lab.

So many people have passed through the Nematology group at Wageningen University during the last 5 years that I am not able to name all of you, my apologies for this. When I came for my intership there was a strong Polish minority at Nema - Ula, Aneta, Agnieszka, Ania T., Ania P., Ania K and several MSc students. It was great to have you there at the beginning of my adventure otherwise known as living and working in Wageningen. Of course Nema is an international working platform where many nations are sharing the same working space and I was very lucky to have funny, friendly and helpful colleagues. Rikus, Sven, Joost, Hein, Lotte, Ruud, Ana, Jan vdV., Patrick, Erik, Erin, Hans K., Willemin, Jose, Wiebe, Olga, Andre, Koen, Casper, Paul, Agata, Kasia, Nikkie, Mirjam, Tineke, Erwin, Sajid (and probably others that I have forgot, please forgive me), thank you all! There were countless moments we had fun together that I will remember. Some of you became good friends and I hope that we will keep it this way in the future ☺. Wiebe – I'm very happy that you are in Arnhem – good luck with all of your plans!

Special thanks goes to Lissette, the best secretary ever. You guided me through the legalization of my stay in the Netherlands, organizing housing, health insurance, congress registrations and finally my thesis submission. It was always very pleasant talking with you.

Moving to Wageningen was an experience in its own. When I came for my first intership I was very naïve thinking that changing my country of residence would be a piece of cake ☺. In reality I have faced many obstacles such as finding a place to live, organizing the paperwork (read to convince clerks that Poland is actually a part of EU), using a bike for everyday transport (even when rains or snows), living in a small town, adapting to Dutch culture and food – you can place the above in your own order of importance ☺.

I was very grateful to come across the Polish (mainly but with exceptions) community in Wageningen! You guys were the best source of information and the best companions – Aneta, Ula, Marysia S., Agnieszka, Marysia T-K., Piotrek, Pisiek and Pikutek. Bardzo sie ciesze, ze Was poznalam! Duzo sie wydarzylo przez te lata, niezapomniane przebierane imprezy, wspolne wigilie, urodziny, sluby, nowe domy, zmiany prac, narodziny dzieci - I mam nadzieje, ze pomimo rozproszenia nas po Holandii I nie tylko (patrz Marysia S.) zostaniemy w kontakcie.

When writing this part I have realized how many people have I met. Wageningen is a special place for making new friends; so many people come from all over the word, staying for a period of time and moving forward. Thanks to this multiculturalism and mobility, I had a chance to know people from

different countries. Nikkie and Dave, we became good friends and we will try to keep in touch despite your busy life, full of family life, work and house renovation. Good luck with all the projects you have running and those you still have in mind!

Ana, Lotte, Annemarie and Ruud – great that we had chance to come across each other! You are my best Wageningen club, always ready for socializing. We have done a lot together and I'm waiting for more to come ☺. You are the great examples how colleagues become friends.

The same counts for Ewa and Aneta – you are my special girls….Ewa- bardzo sie ciesze, ze nasza znajomosc, ktora zaczela sie od wylaczonego PCRa w Warszawie przetrwala studia w Wageningen i doktorat w Amsterdamie, wliczajac w to twoj slub w Indiach☺ Jestes jedna z nielicznych osob zdolna do zaskarbienia sobie sympatii absolutnie wszystkich. Powodzenia z twoim doktoratem I z organizacja zycia malzenskiego (mam nadzieje, ze uda Wam sie w koncu zamieszkac ze soba na dluzej niz kilka miesiecy). Buzka!

Aneta- ktoz jest lepszym partnerem do rozmow niz ty? Dziekuje Ci za nasze wszystkie kawki, winka, lekcje tanca brzucha, maile i dyskusje telefoniczne. Dla Ciebie tez duza buzia!
Skoro jestem w polskim paragrafie, to nie moge ominac moich przyjaciolek z ery przed Holandia. Agata – super patrzec jak wspolnie przechodzimy przez zycie zaczynajac od nastolatek z Dobisza przesiadujacych w Bagatelce po dziewczyny 30+ mieszkajace niby daleko od siebie a jednak blisko na tyle zeby byc w dobrym kontakcie. Tak trzymac ☺
Moja druga ulubiona kolezanka – Beatka- nie wiem ile wieczorow I nocy przegadalysmy, ile wina wspolnie wypilysmy i ile jeszcze przed nami…..Uwielbiam twoje sarkastyczne poczucie humoru!

Na koniec zostawilam sobie najwazniejsze osoby. Mamo i tato bez waszego wsparcia I wiary we mnie nie doszlabym do tego miejsca! Dziekuje za wszystko.
Karolina, moja mala siostrzyczka, ktora w mgnieniu oka wyrosla na powazna studentke budownictwa. Zycze ci duzo sukcesow i trzymam kciuki!

Tomek- to ze jestem tu i teraz jest w znacznej mierze twoja zasluga. Na zawsze bede pamietac ten telefon, kiedy zadzwoniles z pytaniem czy nie chcialabym pojechac na stypendium do Holandii. To prawie jak scena z filmu- jak jeden telefon i decyzja podjeta w minute determinuje dalsze losy bohaterow ☺. Od tamtej chwili podazamy razem przez zycie zaczynajac od scinania oliwek i kopania dziury na wloskiej prowincji, poprzez przeprowadzki (zmienilismy mieszkanie 6 razy plus moja ostatnia przeprowadzka w Enk), wyjazdy, pumpy, inwestycje, remonty, imprezy i niekonczace sie rozmowy o wszystkim. Nie chce nic zmieniac w naszym zyciu, kocham cie bardzo i bede ci kibicowala w drodze do zostania potentatem w produkcji ziemnio-kulek ☺.

When the PhD period was approaching the unavoidable finish it was a time for me to find a new job. Fortunately, there was a vacancy in the Phytopathology group at Enza Zaden. When leaving Arnhem for my first interview the remote control of our garage refused to work and I was already running late before even leaving the house. Finally I managed to arrive on time, but it was a bit stressful. For my second interview, I chose to take public transport but it didn't help much - the train had a delay. Now I believe that these were signs to how my work in Enkhuizen would look like – struggling with transportation ☺. Seriously speaking, I am very happy that I have joined the Enza team. Karin and Phyto-group (Guurtje, Rein, Hill-Jan, Sonja and Ronald) thank you for a nice welcome! I like the research I'm doing and there are million things I have to learn or improve so you keep me busy ☺. To my new labmates (Ilja, Marieke, Bart, Ruud, Geert-Jan and Jan Willem) - thank you for guiding me through the secrets of the Enza labs.

Wrapping up, I went a long way during the last years and I am the person who I am today because of all of you! From each single person I have met there is something what I have learned and it remains with me. Now I am ready to close the period of my PhD and I'm looking forward what life will bring next ☺.

List of publications:

Koropacka K.B., Sobczak M., Janakowski S., Van Schaik C., Overmars H., Prins P., Roosien J., Smant G., Bakker J., Goverse A., "Gpa2 resistance disconnects the nematode feeding site from nutrition source, but virulent nematodes seem to overcome this by local suppression of the Gpa2 gene" – manuscript in preparation

Slootweg E., **Koropacka K.B.,** Roosien J., Dees R., Van Schaik C., Pomp R., Bouwman L., Schots A., Smant G., Bakker J., Goverse A., "Domain exchange between Rx1 and Gpa2 in potato reveals flexibility of CC-NB-LRR genes to switch between virus and nematode resistance"
-manuscript in preparation

Sacco M.; **Koropacka K.B**.; Grenier E.; Jaubert M.; Blanchard A.; Goverse A.; Smant G.; Moffett P (2009) "The Cyst Nematode SPRYSEC Protein RBP-1 Elicits Gpa2- and RanGAP2-Dependent Plant Cell Death" PLoS Pathogens 5 (8). - p. 1 - 14.

Tomczak A.; **Koropacka K.B**.; Smant G.; Goverse A.; Bakker E (2009) "Resistant Plant Responses" In: Plant Cell Monographs / Robinson, D.G, . Springer-Verlag Berlin Heidelberg, (Cell Biology of Plant Nematode Parasitism 15).

Koropacka K.B.; Sobczak M.; Roosien J.; Sacco M.; Moffett P.; Smant G.; Bakker J.; Goverse A. (2008) "Specific activation of the Gpa2 gene by members of the RBP effector gene family from the potato cyst nematode Globodera pallida" In: Keystone Symposia on Molecular and Cellular Biology, Abstract Book.

Koropacka K.B.; Sobczak M.; Roosien J.; Smant G.; Bakker J.; Goverse A. (2008) "Specific activation of the Gpa2 gene by members of the RBP effector gene family from the potato cyst nematode Gobodera pallida" In: PhD Summer School, Wageningen, The Netherlands.

Koropacka K.B.; Slootweg E.; Roosien J.; Sobczak M.; Dees R.; Sacco M.; Moffett P.; Smant G.; Bakker J.; Goverse A. (2008) "Structure-function analyses of Gpa2-mediated resistance against the potato cyst nematode Globodera pallida" In: Proceedings of the second COST 872 Annual Meeting, Postojna, Slovenia.

Butterbach P.; Slootweg E.; **Koropacka K.B**.; Spiridon L.; Dees R.; Roosien J.; Bakker E.; Arens, M.; Petrescu A.; Smant G.; Bakker J.; Goverse A. (2007) "Functional constraints and evolutionary dynamics of the Rx1/Gpa2 cluster in potato" In: Proceedings of the First Annual Meeting of Cost 872, 9 - 5 May, 2007, La Colle-sur-Loup, France.

Koropacka K.B. (2007) "Conversion of nematode resistance into virus resistance by intramolecular recombination between two potato genes, Rx1 and Gpa2" Lunteren: ALW Meeting Experimental Plant Sciences.

Koropacka K.B.; Slootweg E.; Roosien J.; Dees R.; Sacco M; Moffett P.; Smant G.; Bakker J.; Goverse A. (2007) "Structural and functional analysis of GPA2-mediated resistance against the potato cyst nematode Globodera pallida" In: XIII International Congress on Molecular Plant-Microbe Interactions. July 21-27, 2007, Sorrento, Italy.

Tomczak A.; **Koropacka K.B**.; Roosien J.; Smant G.; Bakker E.H.; Bakker J.; Goverse A. (2006) Structural and functional genomics of cyst nematode resistance in potato In: European Society of Nematologists XXVIII International Symposium, Blagoevgrad, Bulgaria, 5-9 June 2006.

Curriculum vitae

Kamila Koropacka was born on March 29th, 1979 in Warsaw, Poland. Between 1999 and 2004 she has studied at Warsaw Agricultural University in the Interdisciplinary Studium of Biotechnology with specialization in Plant Science.

In 2003, for a period of nine months she has done a Master project at the Laboratory of Nematology in Wageningen. Her research was focused on the transcriptional regulation of the potato resistance gene *Gpa2* in response to nematode infection.

Back to Poland, she has completed her university education with the master thesis entitled – "Evaluation of the sunflower potential in phytoremediation of soil contaminated with lead".

In February 2005, she was appointed at Laboratory of Nematology as a Phd student (assistant in opleiding, AIO). Her research was focused on studying molecular aspects of interactions between potato cyst nematodes (*Globodera pallida*) and their host potato.

Since April 2009, she is working as a Molecular Phytopathology Researcher at Enza Zaden in Enkhuizen.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Kamila Koropacka**
Date: **5 February 2010**
Group: **Laboratory of Nematology, Wageningen University**

1) Start-up phase		*date*
►	**First presentation of your project**	
	Structure-function study on the potato resistance gene, Gpa2.	Feb 12, 2005
►	**Writing or rewriting a project proposal**	
►	**Writing a review or book chapter**	
	Resistant Plant Responses in: Cell Biology of Plant Nematode Parasitism Springer-Verlag, (Plant Cell Monographs 15) 2008	May-Jun, 2007
	Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure		*date*
►	**EPS PhD student days**	
	EPS PhD student day, Nijmegen	Jun 02, 2005
	EPS PhD student day, Wageningen	Sep 19, 2006
	EPS PhD student day, Wageningen	Sep 13, 2007
►	**EPS theme symposia**	
	Theme 2 Interactions between Plants and Biotic Agents, Leiden University	Jun 23, 2005
	Theme 1 Developmental Biology of Plants, Wageningen University	Apr 26, 2005
	Theme 2 Interactions between Plants and Biotic Agents, Utrecht University	Jan 22, 2009
►	**NWO Lunteren days and other National Platforms**	
	NWO-ALW Experimental Plant Sciences meeting, Lunteren	Apr 04-05, 2005
	NWO-ALW Experimental Plant Sciences meeting, Lunteren	Apr 03-04, 2006
	NWO-ALW Experimental Plant Sciences meeting, Lunteren	Apr 02-03, 2007
	NWO-ALW Experimental Plant Sciences meeting, Lunteren	Apr 07-08, 2008
	Bioexploit, project meeting, Ede	Dec 16, 2007
►	**Seminars (series), workshops and symposia**	
	Real Time PCR and Gene expression analysis, seminar series from Biorad	Jun 07, 2005
	Symposium on intracellular signalling, Amsterdam	Feb 02, 2006
►	**International symposia and congresses**	
	8th Conference of the European Foundation for Plant Pathology, Copenhagen	Aug 13-17, 2006
	XIII International Congress on Molecular Plant-Microbe Interactions,	Jul 21-27, 2007
	Keystone symposia, Plant Innate Immunity	Feb 10-15, 2008
►	**Presentations**	
	NWO-ALW Experimental Plant Sciences meeting, poster	Feb 04-05, 2005
	NWO-ALW Experimental Plant Sciences meeting, poster	Apr 03-04, 2006
	8th Conference of the European Foundation for Plant Pathology, Denmark, poster	Aug 13-17, 2006
	NWO-ALW Experimental Plant Sciences meeting, oral presentation	Apr 03, 2007
	XIII International Congress on Molecular Plant-Microbe Interactions, oral presentation	Jul 21-27, 2007
	Keystone Symposia, Plant Innate Immunity, poster	Feb 10-15, 2008
►	**IAB interview**	Sep 14, 2007
	Subtotal Scientific Exposure	*14.1 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Confocal microscopy, UvA	May 15-19, 2006
	O-mics data analysis course, Nijmegen	Nov 07-10, 2005
	EPS Summerschool Signaling in Plant Development and defence: towards Systems Biology	Jun 19-21, 2006
	EPS Summerschool: On the evolution of plant pathogen Interactions: From principles to practice	Jun 18-20, 2008
►	**Journal club**	
	PhD student literature discussion group Nematology	2005-2008
►	**Individual research training**	
	Visit in the Boyce Thompson Institute, Lab of Peter Moffett, practical lab training	May 23-26,2006
	Subtotal In-Depth Studies	*8.7 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Scientific writing	Oct 01-Nov 26, 2007
	Career perspective	Mar-May 2008
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*3.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**33.9**

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

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

The reseach presented in this thesis was performed at the Laboratory of Nematology of the Wageningen University with financial support from the European Union 6th framework project BIOEXPLOIT (FOOD-CT-2005-513959).

Cover image depicts a second stage juvenile of *Globodera pallida* inside the root of the potato plant harbouring the Gpa2 gene.