Nematode effectors suppress plant innate immunity

Dr. Geert Smant

Laboratory of Nematology, Wageningen University, P.O. Box 8123, 6700 ES, Wageningen, The Netherlands

+31(0)317485254

geert.smant@wur.nl

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

Wiebe J. Postma, Erik J. Slootweg, Sajid Rehman, Anna Finkers-Tomczak, Tom O.G. Tytgat, Kasper van Gelderen, Jose L. Lozano-Torres, Jan Roosien, Rikus Pomp, Casper van Schaik, Jaap Bakker, Aska Goverse, and Geert Smant*

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Authors’ present address: Laboratory of Plant Breeding, Wageningen University, 6708 PB Wageningen, The Netherlands.

Authors’ present address: Ecogenomics, Institute for Water and Wetland Research, Radboud University Nijmegen, 6525 AJ Nijmegen, The Netherlands.

Authors’ present address: Department of Molecular and Developmental Genetics, Institute of Biology, Leiden University, 2333 EB Leiden, The Netherlands.

*Corresponding author; e-mail geert.smant@wur.nl.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Geert Smant (geert.smant@wur.nl).
Abstract

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

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

The majority of plant resistance proteins are members of the NB-LRR receptor family, which consist of a central nucleotide-binding (NB) domain and a leucine-rich repeat (LRR) domain at the carboxyl terminus (Eitas and Dangl, 2010). At their amino-termini, the NB-LRR plant immune receptors either carry a coiled-coiled (CC) domain, or a Toll/Interleukin-1 receptor like (TIR) domain. The NB domain, which is also referred to as the NB-ARC (nucleotide-binding adaptor shared by APAF-1, certain Resistance proteins, and CED-4) domain, most likely changes from a closed ADP bound state to an open ATP bound state when the resistance protein detects a pathogen (Lukasik and Takken, 2009). The LRR domain is thought to act as the sensor in NB-LRR receptors, which in the absence of the cognate effector keeps the resistance protein in an autoinhibited “off” state. In this model, the recognition of a pathogen effector induces a conformational change in the LRR domain that lifts the inhibition of the NB domain in the core of the resistance protein. Artificially induced mutations in NB-LRR immune receptors suggest that the two functions of the LRR domain, pathogen recognition and negative regulation of the NB domain, reside in different parts of the domain. Several sequence exchanges and deletions at the N-terminus of the LRR domain switch NB-LRR immune receptors into a permanent effector-independent autoactive state (Rairdan and Moffett, 2006). By contrast, mutations in repeats at the C-terminus of the LRR domain do not lift the autoinhibition, but instead change the recognition specificity of NB-LRR immune receptors (Farnham and Baulcombe, 2006).
The molecular mechanisms underlying effector recognition by plant immune receptors are not well understood. NB-LRR immune receptors may activate signaling pathways that lead to effector-triggered immunity when they physically associate with their cognate effectors (Krasileva et al., 2010). However, the fact that such direct interactions seem to be exceptional inspired the formulation of the ‘guard’ model in which immune receptors activate host defenses by detecting effector-induced perturbations in other plant proteins (Van Der Biezen and Jones, 1998). Plant immune receptors may thus efficiently expand the spectrum of disease resistances of a plant by guarding common virulence targets of multiple effectors (Chung et al., 2011). In the recently proposed intermediate ‘bait-and-switch’ model a pathogen effector may still directly interact with NB-LRR immune receptors but only after binding to an accessory protein that functions as co-factor for the receptor (Collier and Moffett, 2009).

There are only few examples of plant immune receptors that directly interact with their cognate pathogen effector (Jia et al., 2000; Deslandes et al., 2003; Ellis et al., 2008; Krasileva et al., 2010; Tasset et al., 2010; Chen et al., 2012). For only three of these resistance proteins a physical association with the effector was demonstrated in planta. The TIR-NB-LRR resistance protein RPP1 of Arabidopsis thaliana associates via its LRR domain with the effector ATR1 of Peronospora parasitica (Krasileva et al., 2010). This interaction results in a defense-related programmed cell death in leaves of Nicotiana tabacum. Also in Arabidopsis, the association of the TIR-NB-LRR resistance protein RRS1-R with the PopP2 effector of Ralstonia solanacearum results in immunity (Tasset et al., 2010). Similarly, the physical association of the CC domain of the resistance protein RB from potato with the IPI-O1 effector of P. infestans triggers a programmed cell death in N. benthamiana (Chen et al., 2012). Recently, we found that the effector SPRYSEC-19 of G. rostochiensis interacts in yeast with the seven C-terminal repeats of the LRR domain of the CC-NB-LRR protein SW5F of tomato (Rehman et al., 2009). The SW5 resistance gene cluster in tomato confers resistance to a broad range of tospoviruses (Boiteux and de Giordano, 1993). Five other SW5 resistance gene homologs have been identified in tomato. The homolog SW5B confers resistance to tomato spotted wilt virus (TSWV), whereas the functions of SW5A and SW5C-F are currently unknown (Spassova et al., 2001).

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

In this paper we report the functional characterization of the effector SPRYSEC-19 of *G. rostochiensis*, and its interaction with SW5F, in plants. We first tested the hypothesis that SPRYSEC-19 activates SW5F-dependent programmed cell death and nematode resistance. However, co-expression of SPRYSEC-19 and SW5F by agroinfiltration in leaves of *N. benthamiana* and in tomato did not trigger a defense-related programmed cell death. Moreover, nematode infection assays on tomato plants harboring SW5F showed no resistance to *G. rostochiensis*. Next, we tested the alternative hypothesis that SPRYSEC-19 modulates host defense responses in plants. Our data demonstrated that SPRYSEC-19 selectively suppresses CC-NB-LRR-mediated programmed cell death and disease resistance.

**RESULTS**

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

Previously, we showed that the effector SPRYSEC-19 of *G. rostochiensis* interacts with a C-terminal fragment of the LRR domain of SW5F (SW5F-LRR7-13) in a yeast-two-hybrid screen on tomato root cDNA (Rehman et al., 2009). An *in vitro* pull-down assay confirmed that SPRYSEC-19 and SW5F-LRR can interact without cofactors (Rehman et al., 2009). This specific association of SPRYSEC-19 and SW5F was confirmed *in planta* by bimolecular fluorescence complementation (BiFC) and co-immunoprecipitations (CoIP) (Supplemental Fig. S1). The only other known physical association of a pathogen effector and the LRR domain of a resistance protein *in planta* triggers a defense-related programmed cell death in *N. tabacum* leaves (Krasileva et al., 2010). We expected that co-expression of SPRYSEC-19 and SW5F would also trigger a cell death response in agroinfiltrated leaves of *N. benthamiana*. However, no local cell death was observed within 10 days after transient overexpression of SW5F with either 4MYC–tagged SPRYSEC-19 or untagged SPRYSEC-19 (Supplemental Fig. S2). The fragment of SW5F (SW5F-LRR7-13) that interacted with SPRYSEC-19 in the yeast-two-hybrid screen derived from the near-isogenic line CGR161 of
S. lycopersicum cultivar MoneyMaker. We reasoned that other close homologs of SW5F either in CGR161 or in the parent cultivar MoneyMaker might be able to mediate a SPRYSEC-19-triggered cell death in N. benthamiana. A PCR using SW5F specific primers resulted in the identification of three SW5F homologues (Supplemental Fig. S3). Transient co-expression of none of the SW5F homologues with either SPRYSEC-19 (Fig. 1A) or 4MYC-SPRYSEC-19 resulted in a local programmed cell death in agroinfiltrated areas of N. benthamiana leaves. The three SW5F variants are polymorphic at nine amino acid positions in the LRR region (Supplemental Fig. S3). Despite these differences, 4MYC-SPRYSEC-19 captured on anti-MYC beads pulled-down the transiently expressed LRR domain of all three SW5F variants (Fig. 1B). This demonstrated that the absence of programmed cell death is not caused by lack of a physical interaction between SPRYSEC-19 and the LRR domains of the SW5F homologs.

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

SW5F from tomato might not be able to mediate programmed-cell death in N. benthamiana, because it requires accessory proteins that are absent in N. benthamiana. However, transient expression of SPRYSEC-19 by agroinfiltration in leaves of the S. lycopersicum cultivar MoneyMaker harboring the SW5F gene did not result in a local cell death either (Fig. 2). Not all functional disease resistance proteins trigger a local cell death at the infection site of avirulent pathogens (Bendahmane et al., 1999; Bulgarelli et al., 2010), and SW5F might therefore still confer resistance to G. rostochiensis in tomato. To test whether SW5F mediates resistance to the population of G. rostochiensis from which SPRYSEC-19 was isolated (Ro1 line 19), we inoculated 7 days old seedlings of the tomato cultivar from which SW5F was cloned (i.e. MoneyMaker) with infective second juveniles. Three weeks post-inoculation on average 29 (S.E.M ±1.1) juveniles per tomato plant developed into the adult female stage, which is consistent with a normal susceptibility to G. rostochiensis in tomato (Sobczak et al., 2005).

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

Next, we reasoned that SPRYSEC-19 interacts with SW5F to suppress effector-triggered activation of SW5F-mediated immune signaling. The SW5F gene has not been linked to a particular disease resistance trait, and by consequence the elicitor of the pathogen that might activate SW5F-mediated signaling is also not known. The TSWV resistance mediated by SW5B is currently the only phenotype linked to the SW5 cluster in tomato. However, the elicitor of the virus that activates SW5B has not been identified either. To be able to test if SPRYSEC-19 suppresses SW5-mediated programmed cell death, we introduced a D-to-V
mutation at position 879 in SW5F and at position 857 in SW5B to make the proteins autoactive (Bendahmane et al., 2002; De La Fuente Van Bentem et al., 2005; Tameling et al., 2006; Van Ooijen et al., 2008). Only the expression of SW5B-D857V resulted in an effector-independent cell death response following agroinfiltration of N. benthamiana leaves (Fig. 3A). Co-expression of 4MYC-SPRYSEC-19 suppressed the effector-independent cell death response mediated by the SW5B-D857V mutant protein in agroinfiltrated leaves of N. benthamiana (Fig. 3B). This outcome suggested that SPRYSEC-19 suppresses SW5B-mediated activation of effector-triggered immunity.

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

Next, we investigated whether SPRYSEC-19 also suppresses the programmed cell death mediated by other CC-NB-LRR resistance proteins. The SPRYSEC effector GpRBP-1 of the white potato cyst nematode G. pallida triggers a Gpa2-mediated cell death in N. benthamiana (Sacco et al., 2009). To investigate a possible SPRYSEC-19 controlled suppression of Gpa2-mediated programmed cell death, we co-expressed 4MYC-SPRYSEC-19 together with GpRBP-1 and Gpa2 by agroinfiltration in leaves of N. benthamiana. GpRBP-1 transiently expressed with Gpa2 and 4MYC-GFP triggered a strong cell death response in the infiltrated leaf areas within 4-7 days post infiltration. By contrast, no local cell death was observed following the co-expression of GpRBP-1, Gpa2, and 4MYC-SPRYSEC-19 in N. benthamiana. We therefore concluded that SPRYSEC-19 suppressed elicitor dependent programmed cell death mediated by Gpa2. Gpa2 is highly similar to the virus resistance protein Rx1 that recognizes the coat protein of the avirulent PVX strain UK106 (Cp106) (Bendahmane et al., 1995). Cp106 shares no sequence similarity with GpRBP-1 or with other SPRYSEC effectors. We used the Rx1-mediated cell death response in N. benthamiana to investigate whether SPRYSEC-19 suppresses the action of a homologous CC-NB-LRR protein that is not triggered by a SPRYSEC. As expected, co-expression of Rx1, Cp106, and 4MYC-GFP resulted in a local cell death response in agroinfiltrated leaf areas of N. benthamiana (Fig. 4). By contrast, replacing 4MYC-GFP with 4MYC-SPRYSEC-19 completely abrogated the Rx1/Cp106-triggered cell death response in N. benthamiana leaves. SPRYSEC-19 of G. rostochiensis thus also suppresses programmed cell death mediated by the CC-NB-LRR resistance proteins Gpa2 and Rx1.

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

We also co-expressed SPRYSEC-19 with R3a (Huang et al., 2005) and Rpi-blb2 (Van Der Vossen et al., 2005; Oh et al., 2009) from potato and their cognate elicitors from Phytophthora infestans in N. benthamiana leaves to test whether SPRYSEC-19 also modulates the cell death responses mediated by more distantly related CC-NB-LRR resistance proteins (see Supplemental Fig. S4 for an identity matrix). The co-expression of R3a and Rpi-blb-2 and their cognate elicitors resulted in a local cell death response in N. benthamiana, which was not suppressed in the presence of SPRYSEC-19 (Fig. 4). Similarly, the effector-triggered cell death response mediated by a resistance protein of the TIR-NB-LRR class (i.e. BS4) and the extracellular LRR class (i.e. Cf-4 and Cf-9) was also not affected by co-expression of SPRYSEC-19 either (Fig. 4). The P. infestans secreted elicitin INF1 has features of pathogen-associated molecular patterns and autonomously elicits a strong cell death response in leaves of N. benthamiana (Heese et al., 2007). The expression of SPRYSEC-19 did not suppress INF1-induced cell death in agroinfiltrated leaves of N. benthamiana (Fig. 4). The CC-NB-LRR protein NRC1 likely operates in signaling pathways downstream of different types of resistance proteins (e.g. Rx1, Mi-1.2, Cf4, and Cf-9) (Gabriels et al., 2007). To investigate whether SPRYSEC-19 modulates immune signaling downstream of resistance proteins, we co-expressed SPRYSEC-19 with an autoactive mutant of NRC1(D481V) by agroinfiltration in leaves of N. benthamiana. Expression of NRC1(D481V) caused a strong cell death response within 24 hours after agroinfiltration in N. benthamiana leaves, which was not suppressed by SPRYSEC-19 (Fig. 4). Altogether, our data demonstrated that SPRYSEC-19 suppresses the programmed cell death mediated by a group of closely related CC-NB-LRR resistance proteins.

SPRYSEC-19 suppresses disease resistance mediated by Rx1

The local cell death mediated by resistance proteins may be a consequence rather than a prerequisite of disease resistance in plants (Coll et al., 2011). To determine if SPRYSEC-19 also suppresses disease resistance mediated by a CC-NB-LRR protein, we assessed the replication of the avirulent PVX strain UK106 in the presence of both the resistance protein Rx1 and SPRYSEC-19, and in the presence of Rx1 alone. To this purpose, PVX was introduced into N. benthamiana leaves by agroinfiltrating the complete viral amplicon

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including GFP (PVX::GFP). Virus replication was first deduced from the accumulation of GFP in mesophyll cells in infiltrated leaf areas (Fig. 5A). As expected, the co-expression of Rx1, PVX::GFP, and GUS resulted in poor accumulation of GFP in agroinfiltrated areas. However, replacing GUS with 4MYC-SPRYSEC-19 in the agroinfiltration mix led to a strong GFP signal. We also co-expressed PVX::GFP and 4MYC-SPRYSEC-19 alone in N. benthamiana mesophyll cells to demonstrate that 4MYC-SPRYSEC-19 targeted the action of Rx1 and not the replication of PVX directly (Fig. 5A). To confirm that the accumulation of GFP reflects PVX replication in mesophyll cells, we also quantified the accumulation of PVX coat protein by using a specific antibody in an ELISA on total protein extracts isolated from agroinfiltrated leaf areas (Fig. 5B). We concluded that the suppression of Rx1-mediated immune signaling by SPRYSEC-19 also results in loss of disease resistance.

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

To investigate whether stable overexpression of SPRYSEC-19 enhances the susceptibility of plants to plant pathogens, we first inoculated transgenic potato plants (line V) overexpressing untagged or 4MYC-tagged SPRYSEC-19 with *G. rostochiensis*. Four weeks post inoculation the number of adult females per plant was not significantly higher in at least twelve independent transgenic potato lines overexpressing SPRYSEC-19 as compared to transgenic plants harboring the corresponding empty binary expression vector (Supplemental Fig. S5). The draft genome sequence of the sister species *G. pallida* suggests that potato cyst nematodes carry over 200 different SPRYSEC genes (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/g_pallida). We therefore reasoned that the overexpression of one specific SPRYSEC gene family member in a host plant might have little impact on the virulence of *G. rostochiensis*. However, the potato line V is resistant to *Verticillium dahliae*, and the *V. dahliae* genome does not harbor homologues of nematode SPRYSEC effectors. We therefore challenged the transgenic potato lines overexpressing either SPRYSEC-19 or 4MYC-SPRYSEC-19 with *V. dahliae* strain 5361, to test whether SPRYSEC-19 alters the resistance of potato plants to this fungus. Four weeks post inoculation with *V. dahliae* the SPRYSEC-19 overexpressing plants showed a strong reduction in shoot growth as compared mock-inoculated plants, and as compared to the empty vector plants inoculated with *V. dahliae* (Fig. 6A). To further quantify the level of resistance to *V. dahliae* in the transgenic potato lines, we measured the accumulation of fungal biomass in plants harboring either SPRYSEC-19, 4MYC-SPRYSEC-19, or the empty expression vector by specifically amplifying the internal transcribed spacer (ITS) region of *V. dahliae* with PCR (Fradin et al., 2011). The ITS region of *V. dahliae* was amplified from plants overexpressing either SPRYSEC-19 or 4MYC-SPRYSEC-19 three weeks post inoculation with fungal spores (Fig.
6B). As expected, no amplification product of the ITS region in *V. dahliae* was observed in the empty vector plants three weeks post inoculation with fungal spores. These data suggest that SPRYSEC-19 suppresses a yet unidentified fungal resistance in potato, rendering these plants susceptible to an otherwise avirulent strain of *V. dahliae*.

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

To investigate whether the suppression of Gpa2, Rx1, and autoactive SW5B requires a physical interaction with SPRYSEC-19, we co-expressed 4MYC-SPRYSEC-19 and the LRR domains of these proteins fused to a 4HA tag in leaves of *N. benthamiana* for co-immunoprecipitation. Capturing 4MYC-SPRYSEC-19 in total protein extracts of agroinfiltrated leaf areas with anti-MYC beads did not result in the co-immunoprecipitation of the LRR domains of Sw5B, Rx1, and Gpa2 (Fig. 7). We therefore concluded that SPRYSEC-19-mediated suppression of CC-NB-LRR-mediated programmed cell death and resistance does not require a physical interaction of SPRYSEC-19 with the LRR domains of these resistance proteins.

**DISCUSSION**

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

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

We have demonstrated with four different experimental designs that the physical association between SPRYSEC19 and the LRR domain of SW5F is robust. That this association does not activate effector-triggered programmed cell death and resistance may indicate that SW5F is an inactive gene duplicate of a paralogous functional CC-NB-LRR resistance protein to *G. rostochiensis*. In this scenario the lack of functional constraints on the SW5F gene may have rendered its activation domains (i.e. CC-NB) dysfunctional, while binding to the sensor (i.e. LRR) domain is still intact (Takken and Goverse, 2012). We tried to make SW5F, along with SW5B, constitutively active by introducing mutations at positions that switch several other CC-NB-LRR resistance proteins into a permanent “on”-state. However, these mutations only induced autoactivity in SW5B, which is thus far the only member of the SW5 cluster linked to a known resistance (Spassova et al., 2001). The lack of autoactivity in SW5F mutants therefore favors the hypothesis that SW5F is a dysfunctional paralogue of a functional nematode resistance gene.

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

As the suppression of autoactive mutant CC-NB-LRR proteins demonstrated, SPRYSEC19 most likely does not disturb the recognition of specific cognate pathogen effectors that activates these resistance proteins. It is nonetheless conceivable that SPRYSEC-19 is able
to outcompete other SPRYSEC effectors of *G. rostochiensis* that trigger the activation of a functional homolog of SW5F. Such a mechanism seems to determine the virulence of *P. infestans* strains on potato plants harboring the RB resistance protein (Chen et al., 2012). Alternatively, as discussed earlier SPRYSEC-19 may also suppress CC-NB-LRR resistance proteins by targeting the immune receptors to the proteasome for degradation (Rehman et al., 2009). However, western blots of total protein extracts of agroinfiltrated leaf areas revealed no enhanced breakdown of CC-NB-LRR proteins or parts thereof in the presence of SPRYSEC-19. We therefore conclude that our current data does not support a model in which SPRYSEC-19 interacts with CC-NB-LRR resistance proteins to alter their turnover rate.

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

Next to the ability to induce and maintain feeding cells, the survival and reproduction of sedentary plant-parasitic nematodes is most likely determined by their ability to suppress host defenses. The molecular mechanisms underlying the suppression of host defense responses by plant-parasitic nematodes are not known. All known plant immune receptors conferring resistance to *G. rostochiensis* belong to the CC-NB-LRR class of resistance proteins (Molinari, 2011). Here we showed that *G. rostochiensis* has evolved several SPRYSEC effectors that selectively suppress CC-NB-LRR mediated programmed cell death and disease resistance. The SPRYSECs in the potato cyst nematodes *G. rostochiensis* and *G. pallida* constitute the largest effector family found in a plant parasitic nematode to date. If the SPRYSEC effector family functions as suppressors of effector-triggered immunity, the expansion of this effector family may reflect adaptations to functional diversifications in plant immune receptors. As the SPRYSEC effector GpRBP1 of *G. pallida* suggests, on their turn plants may have evolved novel NB-LRR plant immune receptors (e.g. Gpa2) that recognize and neutralize SPRYSEC effectors again. It will be highly interesting to investigate if GpRBP1 also suppresses CC-NB-LRR resistance proteins, and if the activation of Gpa2-
mediated resistance also involves a physical association between the LRR domain of Gpa2 and GpRBP1.

MATERIALS AND METHODS

Plant material

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

Cloning and plasmid construction

SPRYSEC-19 was subcloned from pGBK7-A18-2 (Rehman et al., 2009) as a BspMI-BamHI fragment an inserted jointly with the complementary oligo pair A18For + A18Rev (Supplemental Table S1) into pRAP digested with NheI-BglII. The coding regions of the mature peptides of other SPRYSECs without their native signal peptides for secretion were PCR-amplified from G. rostochiensis cDNA. The full-length SW5F genes of tomato cv. GCR161 were PCR-amplified as described before (Rehman et al., 2009). The regions of R-genes coding for the LRR domain were subcloned from existing plasmids: SW5A and –B (Spassova et al., 2001), SW5F (Rehman et al., 2009), Gpa2 (Rairdan and Moffett, 2006) and Rx1 (Slootweg et al., 2010). PCR-amplification products were cloned into vector pRAP using specific restriction sites and confirmed by DNA sequencing. The fragments cloned into the pRAP vector were cloned in frame with the CaMV 35S promoter and N- or C-terminal 4HA or 4MYC affinity tags, or no additional tags. Primers and restriction sites used for the cloning of novel genes are listed in Supplemental Table S2. Expression cassettes of pRAP, including promoter, affinity tags and the gene of interest, were subcloned into binary vector pBINPLUS (Van Engelen et al., 1995) using Ascl and PacI restriction sites. All SW5F genes were cloned with the 3’ UTR (polyadenylation signal and terminator) of the SW5F gene from isolated from MoneyMaker (Rehman et al., 2009). Autoactive SW5 mutants were made by inserting the annealed oligo pair D879V-1 and D879V-2 (Supplemental Table S3) between the BspHI and XbaI restriction sites of the SW5 genes in pRAP. All the above described constructs were mobilized to A. tumefaciens strain MOG101 (Hood et al., 1993), which was selectively grown on 50 mg/L kanamycin and 20 mg/L rifampicin. For the expression of SPRYSEC-19 in
tomato, the coding region for the mature peptide of SPRYSEC-19 without its signal peptide was PCR-amplified from *G. rostochiensis* cDNA using primers listed in Supplemental Table S1 and cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA). After confirmation of the sequence by DNA sequencing, SPRYSEC-19 was subcloned to the expression vector SOL2085 (kindly provided by Patrick Smit) using LR clonase (Invitrogen, Carlsbad, CA, USA), resulting in vector SOL2085:SS19. For agroinfiltrations in tomato leaves the constructs were mobilized to *A. tumefaciens* strain 1D1249 (Wroblewski et al., 2005) which was selectively grown on 100 mg/L kanamycin, 100 mg/L spectinomycin, and 1 mg/L tetracyclin.

**Agroinfiltrations**

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

**Suppression of programmed cell death**

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

**SPRYSEC-19 in tomato**

SOL2085:GFP (kindly provided by Patrick Smit), SOL2085:SS19 (see above), pBIN61:Rx(D460V) (Bendahmane et al., 2002) in *A. tumefaciens* strain 1D1249 were
agroinfiltrated in leaves of *L. esculentum* cv. MoneyMaker. Agroinfiltrated leaves were monitored up to 10 days for visual assessment of cell death.

**Bimolecular fluorescence complementation**

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

**Co-immunoprecipitation**

Total protein extracts of transient transformed *N. benthamiana* leaves were made by grinding leaf material in protein extraction buffer (50 mM Tris-HCl pH7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 2% polyclar-AT PVPP, 0.4 mg/ml Pefabloc SC plus (Roche, Basel, Switzerland), 5 mM DTT) on ice. For co-immunoprecipitation the total protein extract was first passed over a sephadex G-25 column (GE Healthcare, Little Chalfont, UK). The protein extract was treated with rabbit-IgG agarose (40 μl slurry per mL protein extract). After preclearing the protein extract was mixed with 25 μl anti-Myc agarose beads (Sigma, MO, USA) or anti-HA agarose beads (Roche) and incubated for 2 hours at 4°C. After washing six times with washing buffer (protein extraction buffer with 0.15% Igepal CA-630) the beads were resuspended in Laemmli buffer (Sambrook, 1989) and the bound protein was separated by SDS-PAGE and blotted on PVDF membrane. For immuno detection we used antibodies goat anti-Myc (Abcam, Cambridge, UK) and HRP-conjugated donkey anti-goat (Jackson, West Grove, PA, USA) or HRP conjugated rat anti-HA (Roche). Peroxidase activity was visualized using Thermoscientific Supersignal West Femto or Dura substrate and imaging the luminescence with G:BOX gel documentation system (Syngene, Cambridge, UK).

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

**Nematode resistance assay**

Dried cysts of *G. rostochiensis* pathotype Ro1 Mierenbos were soaked on a 100-μm sieve in potato root diffusate to collect hatched pre-J2s (De Boer et al., 1992). Freshly hatched preparasitic second stage juveniles in suspension were mixed with an equal volume of 70% (w/v) sucrose in a centrifuge tube and covered with a layer of sterile tap water. Following centrifugation for 5 min at 1,000 × *g*, juveniles were collected from the sucrose–water interface using a Pasteur pipette and washed three times with sterile tap water. The nematodes were surface sterilized by incubation for 20 min in 0.5 % (w/v) streptomycin/penicillin solution, for 20 min in 0.1% (w/v) ampicillin/gentamycin solution, for 5 min in sterile tap water, and for 3 min in 0.1% (v/v) chlorhexidine solution. The nematodes were subsequently washed three times in sterile tap water, resuspended in sterile 0.7% solution of Gelrite, and pipetted along the roots of 3-week-old *in vitro* grown plants. Routinely, we used between 150 and 200 pre-J2s per plate containing one plant. Adult females per plate were counted six to eight weeks after inoculation. For each transformant tested at least 15 independent lines were used.

**PVX resistance assay**

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

**Verticillium dahliae resistance assay**

*Verticillium dahliae* isolate 5361 (kindly provided by Richard Cooper) was grown on 4% potato dextrose media (Duchefa, Haarlem, The Netherlands) at 28°C for 2 weeks. Fungal
spores were transferred to sterile de-ionized water to a concentration of 1 x 10^6 spores/ml. The roots of three-week old in vitro grown transgenic potato plants were soaked in spore suspension for five minutes and transferred to pots with soil in a greenhouse. For each transformant at least 10 independent lines were used with 4 biological controls. At 20 days post inoculation pictures were taken and to determine the fungal biomass in infected plants, stem pieces were cut from the potato plants just above ground level and flash frozen in liquid nitrogen. Total DNA was extracted from plant tissues using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). A 200-bp fragment of the ITS gene of V. dahliae was PCR-amplified using primers ITS1-F (Gardes and Bruns, 1993) and ST-VE1 (Lievens et al., 2006) on DNA samples using FirePol polymerase (Solis BioDyne, Tartu, Estonia). As an internal control potato actin was amplified from the same templates using primers StActinF and StActinR (Nicot et al., 2005).

**Accession numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: JX026913-JX026918 (SPRYSEC-4 – SPRYSEC-15); JX026924 (SPRYSEC-16); JX026920 (SPRYSEC-19); JX026925 (SW5F GCR161-1.1); JX026926 (SW5F GCR161-1.2); JX026927 (SW5F MoneyMaker).

**Supplemental data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** BiFC and CoIPs showing specificity of SPRYSEC-19-SW5F interaction.

**Supplemental Figure S2.** Photograph of an agroinfiltrated N. benthamiana leaf co-expressing SPRYSECs and SW5F.

**Supplemental Figure S3.** Protein alignment of SW5F isoforms identified in tomato.

**Supplemental Figure S4.** Amino acid identity matrix of R-proteins compared in this study.

**Supplemental Figure S5.** Susceptibility of transgenic potato lines overexpressing SPRYSEC-19 to G. rostochiensis.

**Supplemental Table S1.** Oligonucleotides used to subclone SPRYSEC-19

**Supplemental Table S2.** Primers and restriction sites used to clone SPRYSECs, SW5F, and R-gene LRR regions.
Supplemental Table S3. Oligonucleotides used to construct autoactive SW5 mutants.

Supplemental Table S4. Primers used to clone SPRYSECs and LRRs into BiFC vectors.

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**Figure 1.** The physical association of SPRYSEC-19 and three SW5F variants in planta does not trigger a programmed cell death. A, Transient expression of SW5F variants (GCR161-1.1 and -1.2, and Moneymaker) by agroinfiltration in *N. benthamiana* leaves together with empty expression vector (EV, left side of leaves) or SPRYSEC-19 (SS19; right side of leaves). Pictures were taken at 10 days post infiltration. B, Co-immunoprecipitation of HA-tagged LRR domains of the SW5F variants by 4MYC-SPRYSEC-19 or 4-MYC-GFP. SPRYSEC-19 and GFP were captured anti-MYC agarose beads (IP MYC/IB MYC) in total protein extracts of agroinfiltrated leaves of *N. benthamiana* transiently co-expressing the proteins. LRR domains pulled-down by either 4-MYC-SPRYSEC-19 or MYC-GFP (IP MYC/ IB HA) were detected on western blots with anti-HA serum.

**Figure 2.** Transient expression of SPRYSEC-19 in tomato cv. MoneyMaker harboring the SW5F gene does not result in local cell death. SPRYSEC-19, GFP, and autoactive mutant of the resistance gene Rx1 (i.e. Rx1[D460V]) were transiently expressed by agroinfiltration in leaves of tomato plants. Photograph was taken seven days post infiltration.

**Figure 3.** SPRYSEC-19 suppresses SW5B activated programmed cell death. Leaves of *N. benthamiana* were agroinfiltrated with wildtype and autoactive mutant SW5 genes under 35S CaMV promoter and monitored for the initiation of cell death over 10 days. The suspensions of the bacteria carrying the constructs were infiltrated in a 1:1 ratio with a combined OD600 as indicated. Photographs were taken five days post infiltration. A, Wildtype (wt) SW5B and – F genes overexpressed next to SW5 mutants with a D-V mutation. B, Autoactive mutant SW5B(D857V) co-infiltrated with equal amounts of 4MYC-SPRYSEC-19 (left side of leaf) or 4MYC-GFP (right side of leaf).

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

**Figure 5.** SPRYSEC-19 suppresses resistance to Potato virus X mediated by the CC-NB-LRR protein Rx1. A, Transient expression of GFP-labeled PVX (PVX:GFP) in leaves of *N. benthamiana* after agroinfiltration together with the β-glucuronidase gene (GUS) or SPRYSEC-19 (SS19), with (top panel) or without (lower panel) the resistance gene Rx1 under control of a leaky scan 35S CaMV promoter. GFP expression was visualized under a UV lamp four days post infiltration. Agroinfiltrations with the empty binary expression vector (EV) were included as control. B, Quantification of PVX replication by ELISA directed against
the PVX coat protein. Bars represent ELISA signal intensity; error bars represent standard error of mean.

**Figure 6.** SPRYSEC-19 overexpression renders a resistant potato genotype susceptible to *Verticillium dahliae*. Stable transgenic potato (*S. tuberosum* line V) overexpressing SPRYSEC-19 (19.3) or 4MYC-tagged SPRYSEC-19 (M19.1 and M19.7) under control of the 35S CaMV promoter infected with *V. dahliae* strain 5361. A, Shoot growth of *V. dahliae* (V) and mock (M) -inoculated transgenic potato lines four weeks post inoculation. EV is a transgenic potato line harboring the corresponding empty binary expression vector. B, Quantification of *V. dahliae* biomass by PCR-amplification of the internal transcribed spacer region of *V. dahliae* in total DNA extracts of *V. dahliae* and mock-inoculated transgenic potato lines (top panel). SPRYSEC-19 and actin genes were PCR amplified as internal controls (middle and bottom panels). 1kb+: DNA size marker.

**Figure 7.** SPRYSEC-19 does not bind the LRR domains of suppressed R-proteins. Co-immunoprecipitation of different 4HA-tagged LRR domains of SW5F, -A, -B, Rx1, and Gpa2 with 4MYC-SPRYSEC-19 (SS19) transiently co-expressed in *N. benthamiana*. The LRR domains pulled-down (IP MYC/IB HA) by 4MYC-SPRYSEC-19 on anti-MYC agarose beads (IP MYC/IB MYC) were detected on western blots with anti-HA serum.