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Functional analysis of potato cyst nematode

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Functional analysis of potato cyst nematode effectors

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Summary - Potato Cyst Nematodes (PCN) live in an intimate association with their host plants. The outcome of the interaction is solely dependent on the interplay between molecules at the interface of the host plant and the nematodes. Nematodes secrete protein molecules, termed effectors, which modify the host physiology to gunner complete host plant colonization. Most significant effectors are the proteins injected into plant cells to suppress the host defence response. Other effectors target gene expression to modify host morphology and physiology for the nematodes to acquire unlimited supply of nutrients for the whole parasitic phase. Little is known on how PCN suppress the host defence. However recently, a gene family, Gr1106 has been identified and proven to suppress the plant innate immunity and therefore it was named Nematode Suppressor of Immunity 1 (NSI-1). In order to elucidate the biological role of NSI-1 gene family in nematode virulence, transgenic potato plants with susceptible background harbouring overexpression constructs of NSI-1 variants were challenged with a virulent Globodera rostochiensis line 22. The susceptibility was determined after fuschin staining at 2 weeks post inoculation and a similar trend was noted after counting the cysts six weeks post inoculation. The assays showed a hyper-susceptibility of the transgenic potato plants (carrying NSI-1 variants E4 and E7) to nematodes when compared to the control that was transformed with an empty vector. However, plant carrying variant C4 showed no significant difference with the control plant (GUS). Additionally, two in planta RNA interference approaches: hairpin RNA (hpRNA) and artificial micro RNA (amiRNA) were used to silence NSI-1. The constructs were engineered and transformed into a potato genotype with a susceptible background. Nematode infection assay on all transgenic potato plants harbouring NSI-1 hpRNA resulted in reduced virulence of Globodera rostochiensis line 22. On average, the virulence was reduced by almost 43.5%. Altogether, by combining these two studies we concluded that, NS1-1 gene family in PCN is crucial for nematode parasitism. In this study, we further used ATTA to rapidly screen NSI-1 effectors from an avirulent population on resistant potato genotype and the aim was to identify potential avirulent gene candidates. SH83-92-488, a diploid potato genotype containing H1 and Gpa2 was used and of all the eight constructs of NSI-1 variants screened, only E7 and N10 seemed to be recognized by the R- gene since a consistent hypersensitive response was observed 7 days post infiltration.

Keywords: RNA interference, hpRNA, amiRNA, transgenic plants, ATTA

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Among all described plant parasitic nematodes, potato cyst nematodes (PCN): *Globodera rostochiensis* and *Globodera pallida* are the most economically important and outstanding parasites of solanaceous plants (Turner & Rowe, 2006; Mugniéry & Phillips, 2007). Annually, *G. rostochiensis* and *G. pallida* cause a huge loss of the potato crop and both species are listed as quarantine pests (http://www.eppo.org). These devastating plant parasitic nematodes are obligatory, biotrophic and soilborne endoparasites. Strikingly, at the end of their life cycle, the mature females die and harden their cuticle thereby forming cysts, a structure that protect a pool of eggs from biotic and abiotic constraints. This attribute therefore, allows PCN to survive in a dormant state for a considerable number of years (Williamson & Hussey, 1996). When proper host cues (root exudates) are sensed, the infective second-stage juveniles (J2s) hatch from the eggs, and with the help of two chemoreceptors -amphids- they successfully locate their host (Jones *et al*., 2000). Subsequent to host location, the J2s penetrates the root just behind the apex, preferentially in the differentiation and elongation zone (close to the root tip) by using their robust stylet. Following this, the J2s migrate intracellularly towards the vascular cylinder in a destructive manner, rupturing the plant cells (Golinowski *et al*., 1996). This predisposes the invaded roots to secondary infections by other plant pathogens (Nicol *et al*., 2011; Perry & Moens, 2011). Ingeniously, the J2 select a favoured site, induce and form multinucleate feeding site - syncytium- in the vicinity of the vascular bundle. From this metabolic sink, the nutrients for development and reproduction are acquired at the hosts’ expense (Hussey & Grundler, 1998; Gheysen & Mitchum, 2009).

The complex plant-nematode interactions involve nematode offence, host defence and nematode subverting the plant attack to facilitate a compatible interaction. This interaction like other plant-pathogen interactions, involve the interplay of molecules secreted by host plant and the parasite. However, most plants are resistant to a sundry array of pathogens, due
to the possession of the sophisticated, complex and multiple layers of defence systems that wards off the pathogens (Chisholm, et al., 2006; Jones & Dangl, 2006; Dodds & Rathjen, 2010). These defence systems include pre-existing and induced defences. The pre-existing defence system involves the use of preformed, constitutive structural and chemical barriers. When the plant pathogens bypass the pre-existing defence system, they are confronted with induced defence systems, viz. basal defence and effector-triggered immunity (Jones & Dangl, 2006). Upon the recognition of the conserved microbial or pathogen associated molecular patterns (MAMPs or PAMPs) by transmembrane pattern recognition receptors (PRRS), a non-specific defence response referred to as PAMP triggered immunity (PTI) is triggered (Jones & Dangl, 2006; Zipfel, 2009). When the pathogens further circumvent the basal defence system, the host plant deploys a final and mostly effective line of defence called effector-triggered immunity (ETI). Hereby, a resistance gene ($R$ gene) encodes for polymorphic nucleotide binding site leucine rich repeat proteins (NBS-LRR), which recognize pathogen-secreted effectors. The recognition of pathogenic effectors by these protein receptors activates the ETI (Jones & Dangl, 2006; Bent & Mackey, 2007; Thomma et al., 2011). The ETI is coupled with programmed cell death, referred to as hypersensitive response (HR). Since plant-pathogen interactions are arms race, plant pathogens circumvent the ETI by the formation of new effector proteins through positive selection pressure and mutations (Jones & Dangl, 2006; Metraux et al., 2009). Direct suppression of effector-triggered immunity by plant pathogens is also achieved by production of novel effectors (Kelley et al., 2010; Kim et al., 2010; Oh et al., 2010). As an alternative to the above methods of overriding ETI, some plant pathogens lose their virulence proteins after being unsuccessful and compensate the lost effectors to regain pathogenic fitness (Jones & Dangl, 2006). Nevertheless, the mechanism underlying the suppression of the plant innate immunity by the highly advance biotrophic nematodes like PCN is still elusive.
Since the inception of the idea of nematode parasitism genes, enormous achievements have been reported, and a catalogue of genes coding for putative parasitism proteins from plant-parasitic nematodes is still growing. To date, a spectrum of nematode effectors have been identified and functional studies state that some nematode effectors are involved in plant cell wall modification, syncytium development and immunity modulation (reviewed by Haegeman et al., 2011). Surface coat, amphids, excretory-secretory pore and three oesophageal glands are reputed to be potential source of the interacting nematode molecules (Vanholme et al., 2004). Among these effectors source, oesophageal glands cells are the major suppliers and effectors from these glands are introduced into plant cells through the protrusible stylet (Davis et al., 2004; 2008). Plant cell wall degrading enzymes are by far the most studied and striking is their hypothesized or possible origin - horizontal gene transfer from bacteria (Smant et al., 1998; Davis et al., 2000; Jones et al., 2005; Danchin et al., 2010).

Potato Cysts Nematodes, being obligatory sedentary endoparasites are continuously exposed to host plant defence systems. Nevertheless, despite the complex and multiple layers of plant defence systems that wards off pathogens, the PCN surprisingly manage to form the syncytium, which is a result of cell wall dissolution and subsequent fusion of protoplasts (Grundler et al., 1998). Further, the PCN moult, develops and survive inside the host plants for several weeks. This ability implies that PCN either avoid or manipulate the panoply of host plant defences. This is likely achieved through the use of a spectrum of effectors either deposited on the cuticle or injected through the oral stylet. So far, little information is known on how cyst nematodes modulate plant innate immunity. Nevertheless, 10A06 from Hetoredera schachtii (Hewezi et al., 2010), SPRY domain-containing proteins (SPRYSEC) (Rehman et al., 2009b) and venom-allergen like proteins (VAPs) (Lozano-Torres et al., 2012) from G. rostochiensis, are thought to suppress plant defence system. The effector 10A06, which interact with Spermidine Synthase 2 (SPDS2), is believed to disrupt salicylic acid (SA)
signal pathway in *Arabidopsis thaliana* by interfering with the biosynthesis of polyamines (Hewezi *et al.*, 2010). The SPRYSEC effectors from *G. rostochiensis* have been proven to interact with a CC-NB-ARC-LRR protein encoded by a resistance gene homolog SW5F (Rehman *et al.*, 2009b). However, it stays elusive how this interaction with non-functional resistance protein homolog may suppress the plant innate immunity. It was shown, however, that SPRYSEC suppress the HR triggered by specific R/AVR combinations when it is co-expressed with them (Postma *et al.*, 2012).

Despite the financial losses brought down by the PCN, the control measures and management strategies available seem to be insufficient. For many years, people were using nematicides as an only control strategy. Their use however, is now restricted and almost banned due to adverse effects they impose to the environment (Lilley *et al.*, 2011). Management through crop rotation has a serious limitation since hatching of J2s only occur in response to host root exudates, and they remain dormant for many years protected by the cyst (Williamson & Hussey, 1996). Therefore, banning or restrictions of nematicides use urgently calls for other management strategies that are more sustainable and durable. Among these options is the use of resistant cultivars. Interestingly, in some crops and related wild species, natural plant resistance against nematodes has been observed and even though conventional plant breeding has some limitations, it appears to provide durable protection (Williamson & Hussey, 1996; Hammond-Kosack & Jones, 1997; Williamson & Kumar, 2006). The better form of protection may however, be identified in the future upon a clear understanding of plant-nematode interactions through characterization of PCN effectors. The understanding of intricate ins and outs of pathogenesis and attributes that make PCN vulnerable to plant innate immunity is therefore a priority; this will allow the design and development of excellent management strategies. A range of molecular techniques are available and could be used to elucidate or unravel the role of nematode parasitism genes in plant-nematode interaction.
Currently, RNA interference (RNAi) is under scrutiny for it is showing a convincing efficacy and is a promising tool for functional genomics (Urwin et al., 2002; Bakhetia et al., 2005; Chen et al., 2005b, Yadav et al., 2006, Huang et al., 2006b, Kimber et al., 2007; Sindhu et al., 2009). Briefly, RNAi is homology gene specific silencing mediated by short RNAs (sRNAs). The sRNAs are classified into two categories, viz. short interfering RNAs (siRNAs) and micro RNAs (miRNAs). Their mode of action is similar and they only differ in their biogenesis: siRNAs are processed from long, perfectly double-stranded RNA whereas miRNAs are produced from fold-back precursors (MIR genes) transcribed from imperfect inverted repeats in the genome. When the double stranded RNA (dsRNA) is recognized, the dsRNA and its homologue are degraded following a chain of biological processes. The dsRNA is cleaved by Dicer, an RNaseIII enzyme into short interfering RNAs (siRNAs) of 21-25 base pairs (Myers et al., 2003; Lilley et al., 2007). Subsequent to the cleavage, the siRNAs are guided to RNA-induced silencing complex (RISC) where one of each fragment, known as the guide strand, is incorporated into the RISC and base pairs with the complementary messenger RNA (mRNA) (Martinez et al., 2002, Grishok, 2005). Resultantly, the mRNA is cleaved by argonaute, a catalytic component of RISC and subsequently results in gene silencing. In case of miRNA, the precursors preferentially produce one siRNA duplex, the miRNA–miRNA* that will be incorporated into the silencing complex upon processing (Schwarz et al., 2003). An alternative tool for functional studies of putative nematode parasitism gene is a constitutive overexpression of the parasitism genes in host plant (Goverse & Karczmarek 1999; Hewezi et al., 2010, Lee et al., 2011; Souza et al., 2011). The overexpression of a foreign gene in a plant can give rise to a different morphology when compared to wild type or will lead to plant susceptibility to the specific parasite.

Recently, Finkers-Tomczak et al., (2011) identified a new nematode gene family Gr1106 from G. rostochiensis. The Gr1106 effectors were proved to suppress the ETI and, owing to
this, the family is now referred to as, Nematode Suppressors of Immunity (NSI-1). These effectors are exclusively expressed in the dorsal oesophageal gland. This gene family showed a 92% nucleotide identity to that harbouring in G. pallida and has not been reported in other plant parasitic nematodes of economic importance. Several variants were noted in NSI-1 gene family and it was proven that the family is under diversifying and positive selection (Finkers-Tomczak et al., 2011). The importance of NSI-1 expression for nematode survival was proven using RNAi, where the NSI-1 gene silencing by soaking pre-parasitic J2 in NSI-1 dsRNA resulted in reduced nematode virulence (Finkers-Tomczak et al., 2011). However, this way of RNAi delivery is transient and might lead to regain of nematode virulence. Therefore, another RNA-mediated genetic interference, based on dsRNA/hpRNA and amiRNA produced in planta was used to investigate the effect of silencing NSI-1 gene in PCN, since the syncytium provide an ideal route for dsRNA/amiRNA delivery. This will pave a way to the PCN control by the transgenic silencing of the genes whose expressions are essential during feeding site formation and maintenance. So far, targeting early parasitism genes using transgenic plants expressing dsRNA seemed to be less feasible since the genes are expressed before or soon after hatching, so the protein is present before nematodes enter the plant and start initiating the syncytium. Although taking up of dsRNA targeting early expressed genes might occur during invasion, a better approach would be to target genes whose expression is essential after the resumption of feeding (Bakhetia et al., 2005). The NSI-1 genes are expressed in all parasitic stages; however, they are strongly up-regulated during feeding (sedentary stage) and therefore, are good candidates for in planta silencing.

Since NSI-1 variants had been proven to suppress plant immunity possibly by interfering with signalling pathways of ETI when transiently co-expressed with avirulence factors; RBP-1/Gpa-2 (Sacco et al., 2009), R3alAvr3a (Armstrong et al., 2005) and AvrBlb2/Rpi-blb2 (Oh et al., 2009), it can be hypothesized that, some NSI-1 family members could be recognized by
co-evolving plant immune system. This might be so because as pathogens gain new effectors, plants may also generate new R proteins to detect the pathogenic effectors (Anderson et al., 2010). Conversely, some of NSI-1 effectors may have been recognized by the R-proteins before, so to gain pathogenic fitness, the potato cyst nematodes might have abolished these avirulence effectors. Owing to this, it might have resulted in the formation of new effectors to suppress the plant innate immunity. The possibility of NSI-1 recognition by the co-evolving plant innate immunity was verified by screening NSI-1 effectors on resistant potato genotype that harbours genes conferring resistance to nematodes using Agrobacterium Transient Transformation Assay (ATTA). The technique is a quick, robust and versatile tool for studying R-gene dependent response to their cognate avirulent (Avr) effectors from a pathogen (van der Hoorn et al., 2000; Rietman et al., 2011). Since ATTA is widely used, it could be used as a replacement of classical nematode infection assays for checking crop resistance. This reliable technique is based on the induction of HR upon the detection of an elicitor by the R gene and thus, it gives a phenotype (necrotic lesion) that is easy to score. Owing to this property, ATTA was used to screen effectors of NSI-1 family originating from avirulent populations of G. rostochiensis.

In the present study, our focus was on functional analysis of a NSI-1 gene family of G. rostochiensis. The importance of NSI-1 gene family was investigated using heterologous gene overexpression and RNAi in susceptible potato genotype background. This was done to check the effect of overexpressing NSI-1 variants on G. rostochiensis infectivity and verify the effect of NSI-1 variants suppression on plant immunity by the use of RNAi constructs for gene knockdown in susceptible crop plants. Additionally, ATTA was used to screen NSI-1 variants coming from the avirulent population on a resistant potato genotype (SH) to identify Avr gene candidates. The information generated from our current study will not only increase our understanding of plant-nematode interaction but could be exploited for transgenic control
of the outstanding potato parasites, the PCN and ATTA could be used for selection of resistant cultivars in a faster and reliable way.

Materials and Methods

Nematode infection assay on plants overexpressing NSI-1 variants

Inter-nodal and top shoot cuttings of transgenic potato lines overexpressing NSI-1 variants (E4, E7 and C4) and empty vector (GUS) were grown in 6 well culture plates (one plant/well) containing B5 medium (3.28g/L Gamborg B5, 20g/L sucrose, 15 g/L bacto agar, pH 6.2) for three weeks at 21°C. Each treatment was replicated 24 times. Three weeks after in vitro culturing, each plant was challenged with about 200 J2s of G. rostochiensis line 22 (Janssen, 1990). The invasive stage J2s were obtained after soaking dried potato nematode cysts for 4 days in tap water followed by incubation in potato root diffusate (PRD) for 3 days. Freshly hatched J2s were cleaned following a sucrose gradient centrifugation and washed with sterile tap water. Prior to infection, invasive nematodes were surface sterilized by incubation in 0.5 % (w/v) streptomycin/penicillin solution for 20 min, 0.1 % (w/v) ampicillin/gentamycin solution for 20 min, 5 min in sterile tap water and 0.1 % (v/v) chlorhexidine solution for 3 min. Following this, nematodes were washed thrice in sterile tape water and subsequently suspended in 0.7 % Gelrite solution. The sterilized nematodes were observed under the Leica dissecting microscope to check if the J2s were still alive before inoculation.

Two weeks post inoculation, infected plant roots were stained in acid fuchsin in order to determine the number of nematodes that had invaded the roots. Briefly, the roots were washed and soaked in 10 ml of 2.5% Sodium hypochlorite (NaOCl) in 100 ml beakers for 5 min with an occasional stirring. The bleached roots were subsequently rinsed and soaked in
tap water to remove residual NaOCl. Acid-fuschin was diluted in a ratio of 30 ml distilled water to 1 ml from the acid stock staining solution (0.35 g acid-fuchsin, 25 ml acetic acid, 75 ml water). From this dilution ratio, a volume of 10 ml was added to each treatment, boiled for 30 sec in a microwave oven and let to cool down to room temperature. The staining solution was drained and roots were rinsed in running tap water. Roots were again brought to boil in 20 ml of 70 % acidified glycerol for distaining and placed in Petri dishes for analysis. Observation and counting of the invaded stained nematodes were done under a Leica dissecting microscope. The adult females were counted 6 weeks post inoculation. Average numbers of nematodes invading the roots and adult females were calculated and statistically significant differences among the lines overexpressing NSI-1 variants and empty vector were determined using Student’s $t$-Test (one tailed distribution, homoscedastic).

**SILENCING OF NSI-1 GENE BY IN PLANTA GENERATED RNAi**

Validation and functional importance of NSI-1 parasitism gene family was done using *in planta* generated RNAi. Two modes of RNAi: siRNA from hairpinRNA and artificial microRNA were used to silence *NSI-1* gene.

**HAIRPIN NSI-1 SILENCING CONSTRUCTS DESIGN**

Based on the sequence of *NSI-1* gene, two conserved parts across the gene family variants (see Appendix I) were selected in C-terminus and N-terminus of *NSI-1* gene giving rise to two different constructs designated A and B respectively. The polymerase chain reaction (PCR) of selected gene fragments (A and B) was carried using two primer sets. To form the hairpin after transcription, each sequence was cloned in sense and antisense orientation. The sense fragment for each construct was amplified using a primer with gene specific restriction sites XbaI (forward primer) and SmaI (reverse primer) and inserted as
XbaI/SmaI fragment in pSUPERMD-RNAi (pSMD) vector (Fig. 1), a modified version of the pSuperRNAi vector. The antisense fragment for each construct was amplified using gene specific primers having restriction sites SacI (forward primer) and KpnI (reverse primer) and was inserted in an inverted manner as a KpnI/SacI fragment into pSMD vector. Therefore, the resultant transcripts form hairpin dsRNA and the fragment of GUS gene serves as an intron.

Table 1. Primers used for amplification of the conserved NSI-1 regions for hairpin RNA engineering.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer</th>
<th>Restriction</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Forward (ANfw)</td>
<td>TCTAGA</td>
<td>CGTCCACACCAGCAAAAGGA</td>
</tr>
<tr>
<td></td>
<td>Reversed (ANrv)</td>
<td>CCCGGG</td>
<td>GGCGACTTTGTATCTTTTA</td>
</tr>
<tr>
<td>Reversed</td>
<td>Forward (ARfw)</td>
<td>GAGCTC</td>
<td>TCCACACCAGCAAAAGAAA</td>
</tr>
<tr>
<td>complement</td>
<td>Reversed (ARrv)</td>
<td>GGTACC</td>
<td>GTCGGCGACTTTGTATCTTT</td>
</tr>
<tr>
<td>Normal</td>
<td>Forward (BNfw)</td>
<td>TCTAGA</td>
<td>CGATGCCAAAAACCATCAAAGC</td>
</tr>
<tr>
<td></td>
<td>Reversed (BNrv)</td>
<td>CCCGGG</td>
<td>GTTCAGCTAACACTGGAG</td>
</tr>
<tr>
<td>Reversed</td>
<td>Forward (BRfw)</td>
<td>GAGCTC</td>
<td>ATGCCAAAAACCATCAAGC</td>
</tr>
<tr>
<td>complement</td>
<td>Reversed (BRrv)</td>
<td>GGTACC</td>
<td>TACGTCCCGTTCAGCTACAA</td>
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</table>

**CLONING NSI-1 HAIRPIN CONSTRUCTS**

Per each construct, the PCR was done to amplify the desired fragments from *G. rostochiensis* NSI-1 (variant N11) gene subcloned in TOPO vector. All used primers per each sequence are shown in Table 1. The PCR products were resolved on agarose gel and gel purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, U.S.A.) following manufacturer’s instructions and ligated into TOPO vector using TOPO TA cloning® Kit (Invitrogen, Breda, The Netherlands). The recombinant TOPO vectors carrying the amplified fragments were transformed into electro-competent TOP10 *Escherichia coli* and successful transformations were verified by colony PCR, restriction digest of the plasmid and sequencing. Recombinant plasmids, TOPO vector containing the constructs were digested and the excised constructs were cloned into pSMD vector. The recombinant vector, pSMD carrying putative constructs and an empty vector were transformed into *A.
*tumefaciens* (AGL1-virG) in separate mix through electroporation at 2, 5 kV, 400 Ω, 25 µF. The electroporated mixtures were quickly re-suspended in 400 µl YEB and pipetted back into respective Eppendorf tubes and incubated for 2 hrs at 28°C, 250 rpm. Following this, the bacteria were plated out and incubated at 28°C for 2 days. To verify the presence of the cloned inserts and empty vector, colony PCRs were performed using primers which had been used to amplify each of the cloned fragments (see Table 1) in combination with P1101, GUS4 and GUSP2 that have complements located at the promoter and the GUS insert (Fig. 2).

**Fig. 1.** The structure of pSUPERMD RNAi vector. This vector is a modified version of the pSuperRNAi vector. It harbours a kanamycin resistance gene for selection, a super promoter and multiple cloning sites with unique restriction sites that are separated by GUS fragment.

**Fig. 2.** Diagramatic representation of hairpin construct (not drawn to scale) showing all primer combinations used and regions they amplify during verification of successful cloning. These primer combinations were also used to screen putative transformants after stable potato transformation.
ARTIFICIAL miRNAs CONSTRUCTS DESIGN

The Web MicroRNA Designer (wmd3.weigelworld.org) was used to design 21mer amiRNAs using NSI-1 gene sequence as the target. Two candidate targets were selected (amiRNA1 - TATTTTTAGGTTTCGTGGCG and amiRNA2 - TAATGCTCATCGTTAGCGG) from the possible amiRNA candidate sequences basing on their binding energy and target gene specificity. The WMD3 interface, ‘Oligo’ software tool automated four oligonucleotide primers I-IV per amiRNA construct (I: miRNA sense, II: miRNA antisense, III: miRNA* sense, IV: miRNA* antisense; Table 3). The site-directed mutagenesis on precursors of endogenous miRNAs was performed using overlapping High Fidelity PCR. The three sets of High Fidelity PCR per amiRNA construct were initially carried using different sets of primers following the PCR scheme (Table 2) and the plasmid pRS300 harbouring Arabidopsis thaliana MIR319a (Appendix 2) precursor was used as a template to yield fragments of 272, 170 and 272 bp lengths respectively. The mixture for each PCR contained 25 µl of 2X Phusion® High-Fidelity PCR Master Mix (Phusion DNA Polymerase, dNTPs, optimized reaction buffer and MgCl2), 2.5µl of RS300_A-IV, III-II and I-RS300_B (10mM), 2µl pRS300 and 18 µl sterile MQ water to a final volume of 50µl. Thermo cycling conditions were as follows: a hot start at 98°C for 30 sec, 30 cycles; 98°C for 10 sec, 55°C for 20 sec, 72°C for 10 sec and 10 min at 72°C. Resolution of the three individual PCR products was done on 2% TAE (1×) buffered agarose gel (Invitrogen™, UK) at 70V for 1 hr 30 min. The PCRs products were gel purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, U.S.A.) and the DNA was eluted in 6 µl sterilized MQ water. The DNA fragments were then used as templates to generate amiRNA precursor in which endogenous miRNA and miRNA* of A. thaliana miR319a were replaced with amiRNA and amiRNA* derived from NSI-1 gene respectively. The PCR mixture contained 25 µl of 2X Phusion Master Mix, 2.5µl of RS300_A and RS300_B (10mM), 0, 5 µl PCR (a), (b) and (c), 18, 5 µl
sterile MQ water to a final volume of 50µl. Thermo cycling conditions were similar as described above. The High Fidelity enzyme was used in construction of the amiRNA constructs because of its greater accuracy than Taq DNA polymerase. The PCR products were resolved on 1% TAE (1×) buffered agarose gel with 4 µl ethidium bromide (Sigma, St Louis MO, USA) along with 1Kb Plus DNA ladder at 70 V for 1 hr 30 min and visualized under UV light.

**Table 2. PCR scheme used to engineer amiRNAs**

<table>
<thead>
<tr>
<th>forward oligo</th>
<th>reverse oligo</th>
<th>template</th>
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<tbody>
<tr>
<td>(a) A</td>
<td>IV</td>
<td>pRS300</td>
</tr>
<tr>
<td>(b) III</td>
<td>II</td>
<td>pRS300</td>
</tr>
<tr>
<td>(c) I</td>
<td>B</td>
<td>pRS300</td>
</tr>
<tr>
<td>(d) A</td>
<td>B</td>
<td>(a)+(b)+(c)</td>
</tr>
</tbody>
</table>

**Fig. 3.** Engineering of NSI-1 amiRNAs. The mutagenesis on precursors of endogenous miRNAs was executed by overlapping PCRs. Oligonucleotides I to IV were used to introduce and replace miRNA and miRNA* regions (red) of A. thaliana miR319a precursor with artificial sequences (blue). Primers RS300_A and RS300_B were based on template plasmid (pRS300) sequence. The resultant and functional miRNA precursors were generated by an overlapping PCR that combined PCR products A-IV, II-III, and I-B in a single reaction with primers RS300_A and RS300_B.

**CLONING ARTIFICIAL miRNAs CONSTRUCTS**

The fusion products of 500 bp were gel purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, U.S.A.) following manufacturer’s instructions and ligated into TOPO vector using TOPO TA cloning® Kit (Invitrogen, Breda, The Netherlands). A-tailing of the 3.0 µl PCR fragments was done in a mix containing 0.5 µl MgCl₂, 0.5 µl 10x BD buffer, 1.0 µl of 10 mM dNTPs (Invitrogen) and Firepol (Solis BioDyne, Estonia) to final volume of 5 µl and incubated at 72°C for 20 min in the PTC-200 thermal cycler. The product from A-tailing (1 µl) was mixed with 0.5 µl pCR® 2.1-TOPO® vector (0.1 ng/ µl), 1.0 µl salt mix (1.2M NaCl + 0.06M MgCl₂) and 3.5 µl MQ to a final
volume of 6 μl and incubated for 5 min at room temperature. The TOPO vector containing
the amiRNA construct were transformed into electrocompetent TOP10 *E. coli*, subsequently
suspended in 250 μl S.O.C medium and incubated for 1 hr at 37°C, 250 rpm. 50 μl and 100
μl of bacteria suspension were spread on LB agar plates supplemented with 50μg/ml
kanamycin for selection and incubated overnight at 37°C. The success of cloning and
transformation was verified with colony PCR using the primers, which had been used to
amplify each of the cloned fragments under the same PCR conditions as described above.
The plasmid DNA was isolated with Wizard® Plus SV Minipreps System (Promega, USA).
To avoid false positive results from the PCR, the plasmids containing the inserts were
sequenced and further digested using SmaI and KpnI. Further, a set of PCRs were performed
using the two recombinant TOPO vectors as a templates to introduce new restrictions sites to
make the amiRNA compatible with pSMD vector. The primer pair used were, pRS_SmaI
(changing KpnI site into SmaI site) and pRS_KpnI (changing NotI site into KpnI site). The
thermo cycling conditions, gel purification, TOPO cloning, transformation of
electrocompetent TOP10 *E. coli* and colony PCRs were done as previously described.
Sequencing of amiRNA constructs inserted in TOPO vector was done by BaseClear, Leiden,
The Netherlands. For analysis, 10 plasmid samples per putative construct originated from
different colonies were used and the volume per sample was 20 μl that contained 750 μg
plasmid DNA and 25 pmol primers. The constructs were sequenced from both sides of the
vector using pRS_SmaI forward primer (TATAGGCGAATTGGGTCCCGGGC) and
pRS_KpnI reverse primer (GGTACCGCTCTAGAATCTTAGGA). Per each amiRNA
construct, the sequences were aligned using BioEdit software along with the pRS300
sequence confined to the A to B region that harbours the construct. The recombinant
plasmids containing new restriction sites were then subjected to a restriction digest.
Sequential digest was done due to enzyme specific temperature and buffer requirements for
optimal activity. The first restriction digest mix contained 10 µl of plasmid DNA, 2 µl of 10X NEBuffer 4, 1 µl SmaI and 2.8 µl MQ and incubated at 25°C for 2 hrs. Subsequently, 1 µl of KpnI, 2 µl of 10X NEBuffer 1 and 0.2 µl (100 µg/ml) bovine serum albumin (BSA) were added to the mix to a final volume of 20 µl and further incubated at 37°C for 2 hrs. The digested plasmids DNA were separated on 1% TAE (1×) buffered agarose gel (Invitrogen™, UK) and the resulting amiRNAs and pSMD (GUS removed) fragments were cut and gel purified. The precursor fragments (chimeric sequences) were cloned into the corresponding restriction sites (Sma1 and Kpn1) of pSMD vector (Fig. 1). Ligation mix contained 2 µl (45ng/µl) linearized pSMD, 1 µl (11ng/µl) amiRNA construct, 0.5 µl 10 x PRS buffer, 0.5 µl 10 m ATP and 0.5 µl T4 DNA Ligase, with the proportion 3:1 (insert to vector both with sticky ends) and incubated at 16°C overnight. The recombinant products were further transformed into electrocompetent TOP10 E. coli (Invitrogen) in pre-cooled 0.5 ml cuvette using Gene Pulser (Biorad) with electrical field of 2.5kV, 25µF, 200Ω. Subsequent to electroporation, the bacteria were suspended in 350 µl SOC medium and incubated for 1 hr at 37°C, 250 rpm. The bacteria were then plated out on solid LB medium agar supplemented with kanamycin for selection. Colony PCRs were performed (colonies were chosen randomly) using a forward primer (p1101) from the super promoter and a reverse primer from the amiRNA construct (Ilami-Rs-a, gene specific). Only one positive colony per each amiRNA construct was cultured overnight in LB medium supplemented with kanamycin followed by plasmid isolation as previously described. Additionally, confirmation of the desired constructs was done by restriction digest using SmaI and KpnI under similar conditions as previously described. The pSMD plant expression vectors containing the different constructs were further transformed into A. tumefaciens (AGL1-virG) by electroporation at 2, 5 kV, 25 µF, 400 Ω. Colony PCRs were performed again as previously explained and stable plant transformation followed.
**Fig. 4.** A snap shot of amiRNA construct (not drawn to scale) cloned into pSMD vector showing primer combination used to check its presence in the RNAi vector. The vector was modified (removal of GUS insert) to position the amiRNA constructs next to the super promoter. This will allow an enhanced gene expression of the amiRNA constructs.

**Table 3.** Predicted amiRNA sequences and oligonucleotides used to engineer amiRNA targeting NSI-1 gene family.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| I miR1-s        | forward   | gaTAATTTTTAGTGTTCGAGGG |}
| II miR1-a       | reverse   | gaCCGCCACGAAACCTAATATTAaagacagtcgaatga |
| III miR1*a      | forward   | gaCCACCACGAAACCAAAAATTtcacatgctctatag |
| IV miR1*a       | reverse   | gaAAATTTTTGTTTCGAGGGGgctcatacatatattcct |
| amiRNA1         |           | CTGCAAGGCGATTAAGTTTGGGTAAGCGG |}
| Oligonucleotide | Direction | Sequence |
| I miR2-s        | forward   | gaTAATGGGTCATCGTGAAGCGG |}
| II miR2-a       | reverse   | gaCCGGCTTAACGATGACCGATTAtcaagacagtcgaatga |
| III miR2*a      | forward   | gaCCACTTAACGATGTCGATTtcacagtgtcatattcct |
| IV miR2*a       | reverse   | gaAAATGGGGAACATCGTAAAGTGGGgctcatacatatattcct |
| amiRNA1&2       |           | CTGCAAGGCGATTAAGTTTGGGTAAGCGG |}
| RS300_A         | forward   | CTGCAAGGCGATTAAGTTTGGGTAAGCGG |}
| RS300_B         | reverse   | GCCGAATACAAATTCACACAGGAACAG |
| pRS_SmaI        | forward   | TATAGGGGCGAATTGGTTCCCGGCGG |
| pRS_KpnI        | reverse   | GGTACCAGCTCTAGAACTAGTGGGA |

**STABLE POTATO TRANSFORMATION**

Successful cloning was subsequently followed by stable potato transformation. Shoots from 5 weeks old *in vitro* diploid potato genotype line V were excised and explants of approximately 0.5 cm to 1 cm long were prepared. The sterile Whatman filter papers were soaked in PACM (MS30 medium, 2 g/l casein hydrolysate, pH 6.5, 1mg/l 2, 4-D and 0.5 mg/l kinetin) and the explants were transferred to 2 PACM saturated sterile Whatman filter papers overlaid on R3B medium (MS30 medium, pH 5.8, 8 g/l plant-agar, 2 mg/l NAA and 1 mg/l BAP) in 12 cm square Petri dishes. The square Petri dishes were sealed with Leucopore tape, wrapped in aluminium foil and incubated at 24ºC. After 2 days the explants were incubated
for 10 min in 50 ml of overnight cultures of *A. tumefaciens* harbouring pSMD vectors with respective constructs, viz. hairpin RNA constructs, amiRNAs constructs and an empty vector (pSMD). Following this, explants were dried on a sterile Whatman paper. The first PACM filter paper from each Petri dish per each construct was removed and 50 explants per construct were placed on the second PACM-soaked filter paper. Petri dishes were again sealed with Leucopore tape and incubated for 2 days in light at 24°C. At day 4, explants were transferred to ZCVK medium (MS20, 8 gram/l plant agar, 1 mg/l zeatin, 100 mg/l kanamycin and 200 mg/l cefotaxim, 200mg/l vancomycin) that induce callus and incubated at 24°C for 2 weeks. Consecutively, the clear-green calli from the explantates were dissected and transferred to fresh ZCVK plates, sealed with Leucopore tape and once more incubated at 24°C for 2 weeks. The shots were transferred to MS20 medium supplemented with 100 mg/L kanamycin and incubated for further two weeks after which, the shoots were cut and transferred at least twice on selective MS20 medium in sterile plastic tubes.

**GENOMIC DNA EXTRACTION AND PCR ANALYSIS**

The genomic DNA of selected lines of Line V putative transformants with hairpin constructs and empty vector was extracted from frozen leaves, grinded in liquid nitrogen and purified with DNeasy Plant Mini Kit (Qiagen) following the manufacturer’s instructions. Confirmation of the presence of the gene cassettes of the vector carrying the cloned constructs was done using a PCR. The primer set used was, p1011 - 5’-GCCTGTGGTCTCAAGATGGA-3’ (forward) and GUS 4 - 5’-GACAAAAACCCACCCAAGCGTG-3’ (reverse). The primer pair, Actin-F TGCTGCGCTGTGTGTTCTCT and Actin-R ACCAGCCTTCACCATTCCAGTTCC was used to verify quality of the genomic DNA of potato plants. Thermo cycling conditions for the PCR were as follows: initial denaturation at 94°C, 30 cycles denaturation of 30 s at 94°C,
annealing at 53°C for 30 s, polymerization at 72°C for 1 min 30 s and final extension at 72°C for 10 min. The PCR mixture contained 0.5 µl (10 mM) of each primer, 1µl (0.2 mM each) dNTP’s, 2.5 µl (2 mM) of MgCl2, 0.1 µl (1.25 u / 50 µl) Firepol, 14.2 sterile MQ water and 1µl DNA to a final volume of 20µl. The PCR products were then resolved on 1% TAE (1×) agarose gel (Invitrogen™, UK).

**GENE EXPRESSION ANALYSIS**

Total RNA was extracted from shoots of 21 days old putative transformants using RNeasy Plant Mini Kit (QIAGENBenelux B.V., Venlo, The Netherlands) following the manufacturer’s instructions. Lysis buffer of choice was Buffer RLT (including 1 % β-Mercaptoethanol) and 96% ethanol was used. Briefly, approximately 100 mg of fresh plant shoots were harvested and quickly frozen in liquid nitrogen before RNA extraction. The leaf materials were ground with glass pestles in 2 ml eppendorf tubes in liquid nitrogen. DNase treatment of RNA was done using Turbo DNase enzyme (Ambion, Austin, TX, U.S.A). The treated RNA was reverse transcribed using Superscript™ III First-Strand Synthesis (Invitrogen) following manufacturer’s instructions. The synthesized complementary DNAs (cDNA) were diluted to a concentration equivalent to 10 ng/µl and used as templates in PCR using three sets of primers: XbaRNA2F-5’-GCCTGTGGTCTCAAGATGGA-3’ (forward) and GUS 4: 5’-GACAAAAACCACCCAAGCGTG-3’ (reverse), GUSPP2 (forward) and SacRNA2F-GAGCTCTCCACACCAGCAAAAGGAAA (reverse). To check the presence of the gene cassette, 5’ Kan F-ATGATTTGAAACAAGATGGATTGCAC and 3’ Kan R-TCAGAAGAACTCGTCAAGAAG GCG primers were used. Themocycling conditions for the PCR were as described in the previous PCR. The PCR products were resolved on 1% TAE (1×) buffered agarose gel (Invitrogen™, UK) with 4µl ethidium bromide (Sigma, St Louis MO, USA) at 70 V for 1 hr 30 min and visualized under UV light.
IN PLANTA RNAi OF NSI-1 ASSAY

Putative Line V transformants that revealed to harbour the hairpin construct and empty vector (pSMD) were multiplied in MS20 medium. From these stocks, top shoots and inter-nodal cuttings of 10 independent transgenic lines expressing hpRNA and the control (transformed with an empty vector) were in vitro cultured in 6 well plates containing Garmborg B5 medium. Three weeks later, the plants were challenged with about 200 surface sterilized J2s of *G. rostochiensis* line 22. Each treatment was replicated 6 times. Surface sterilization and inoculation of J2 were performed as described in NSI-1 variants overexpression assay. Three weeks after nematode inoculations young females were counted. Verification of silencing was also done using Quantitative real-time PCR (qRT-PCR) to detect and quantify the transcript of targeted gene (*NSI-1*). To confirm this, total RNA from roots and nematodes was extracted two weeks post inoculation. Briefly, two root systems per treatment were harvested, snap-frozen in liquid nitrogen and homogenized into powder using TissueLyser (QIAGEN) following manufactures' instructions. Following this, total RNA was extracted using Maxwell® 16 LEV simplyRNA Tissue Kit following manufacturer’s instructions and quantified using Nanodrop 1000 Spectrophotometer, (Isogen, Life Science). The total mRNA was reverse transcribed to cDNA using Superscript III and oligo-dT primers (Invitrogen), diluted 5 times and used as templates for *NSI-1* expression analysis. Quantitative real-time PCR (qRT-PCR) was performed using MyIQ™ Real-Time PCR Detection System (Bio-Rad Laboratories) and SensiMix™ SYBR & Fluorescein (Bioline). The qPCR mixture per sample contained 22 µl of SensiTemplateMix (10 µl SensiMix and 1 µl cDNA) and 2µl of primer mix to a final volume of 25µl. Two primer mixes used were; NSI1 u_qpcr_f and NSI1 u_qpcr_r (for the target gene) and GrKin F and GrKin R (for the reference gene (cAMP)) sequences are shown in Table 4. Each treatment had 2 technical repeats. The thermo cycling conditions were as follows: 95 °C for 15 min, followed by 35
cycles of 30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. Subsequent to the amplification, the
reaction mixes were subjected to temperature ramping to create the dissociation curve and
melting curve was monitored from 50°C to 95°C in 0.5°C increments using the 10 s hold at
every step. To check for the amplification specificity, the products were resolved on 2% TAE
(1×) buffered agarose gel (Invitrogen™, UK) and additionally, analysis of the melting curve
was done. During the amplification, all data were captured and recorded by MyiQ Optical
System Software, version 1 (Bio- Rad) as a function of threshold cycle (Ct) and were
exported to Microsoft Excel for analysis, that is, to calculate gene-specific fold changes. The
cAMP-dependent protein kinase (cAMP) was used as an internal control (endogenous
reference gene) and NSI-1 from EV (absence of dsRNA) as a calibrator. The fold change in
NSI-1 expression was calculated using the $2^{-\Delta \Delta Ct}$ method (Livak & Schmittgen, 2001). The
NSI-1 gene expression was normalized to an endogenous reference gene and relative to the
EV (empty vector line infected with nematodes). The comparative $\Delta \Delta Ct$ calculation
involves finding the difference between each sample’s $\Delta Ct$ and the reference’s $\Delta Ct_{NSI-1}$
target) - $\Delta Ct_{cAMP}$ (calibrator) = $\Delta \Delta Ct$ were $Ct_{GOI}$ - $Ct_{norm}$ = $\Delta Ct$ sample and $Ct_{GOI}$ - $Ct_{norm}$ = $\Delta Ct$
calibrator. The values obtained were therefore transformed into absolute values using $2^{\Delta \Delta Ct}$.

**Table 4.** List of primers used in quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSI1u_qpcr_f</td>
<td>ATCGGTGCCCGGACCATCCA</td>
</tr>
<tr>
<td>NSI1u_qpcr_r</td>
<td>CCTCCTCCTCGCATAATCGT</td>
</tr>
<tr>
<td>GrKin_1F</td>
<td>ATCAGCCATTCAAATCTACG</td>
</tr>
<tr>
<td>GrKin_1R</td>
<td>TTCTTCAGCAAGTCTTCAAC</td>
</tr>
</tbody>
</table>
SCREENING OF NEMATODE EFFECTORS USING ATTA

Agroinfiltration assays were performed on potato genotype, SH83-92-488 (SH) clonally maintained in vitro. Twelve top shoots of SH plants were excised and in vitro cultured in MS20 medium at 21°C for three weeks, and afterwards were transferred to the greenhouse and grown in pots. Three weeks later, the potato plants were ready for NSI-1 effectors screening assays using ATTA.

All the NSI-1 variants used in the assays were subcloned into different pK2GW7, pK7FWG2 and pK7WGF2 vectors (Karimi et al., 2002) and transformed into A. tumefaciens strain AGL1, with virG helper plasmid (Lazo et al., 1991; Wu et al., 2008). Prior to ATTA, a start culture for each construct (shown in Fig. 5) was grown in 10 ml YEP medium supplemented with 10 μl spectinomycin (50mg/ml) and 10 μl carbenicillin (50mg/ml) for selection of A. tumefaciens cells containing, E3, E4, E6, E7, E9, N10, N11, C4, EV; 10 μl kanamycin (50mg/ml) and 10 μl carbenicillin (50mg/ml) to select transformed A. tumefaciens cells with Gpa2, RBP1, GUS. The culturing was done over night in a shaker (200 rpm) at 28°C. From the starter culture, 100 μl of each construct was further cultured in 10 ml YEBi medium (5 g/l beef extract, 5 g/l bactopeptone, 5 g/l sucrose, 1 g/l yeast extract, 0.002 M MgSO₄, selective antibiotics (as previously done in the starter culture), 1000μl MES and 20 μM acetosyringone (Sigma-Aldrich). Before infiltration, the cultures were centrifuged at 4000 rpm for 15 min and the pellets were resuspended in 10 ml MMAi medium (5 g/l MS, 1,95 g/l MES, 20 g/l sucrose, pH 5.6 , 200 μM acetosyringone). Subsequent to this, the optical density (OD) of each bacterial suspension with the desired constructs was measured at 600 nm and brought to OD₆₀₀ of 0.3 following serial dilutions with MMAi medium. For co-infiltration, a positive control, Gpa2 and RBP-1 were mixed in a 1:1 ratio to a final OD₆₀₀ of 0.6 (0.3 per construct). After getting the desired OD, two fully-grown young leaves per each plant were infiltrated with bacterial suspensions of all treatments using a 1-ml syringe
without a needle through the abaxial leaf surface. Infiltrated leaves were harvested 7 days post infiltration and HR was checked as scored using the scale shown in Table 6.

**Table 5.** NSI-1 variants from an avirulent *G. rostochiensis* population screened on SH potato genotype harbouring *H1* and *Gpa2* genes.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Variant/treatment</th>
<th>ATTA Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK7FWG_C4</td>
<td>C4</td>
<td>8</td>
</tr>
<tr>
<td>pK2GW7_E3</td>
<td>E3</td>
<td>7</td>
</tr>
<tr>
<td>pK2GW7_E4</td>
<td>E4</td>
<td>5</td>
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<tr>
<td>pK2GW7_E6</td>
<td>E6</td>
<td>11</td>
</tr>
<tr>
<td>pK2GW7_E7</td>
<td>E7</td>
<td>2</td>
</tr>
<tr>
<td>pK2GW7_E9</td>
<td>E9</td>
<td>6</td>
</tr>
<tr>
<td>pK7WGF2_N10</td>
<td>N10</td>
<td>9</td>
</tr>
<tr>
<td>pK7WGF2_N11</td>
<td>N11</td>
<td>3</td>
</tr>
<tr>
<td>pK2GW7_EV</td>
<td>EV (control)</td>
<td>10</td>
</tr>
<tr>
<td>pK7FWG_EV</td>
<td>EV (control)</td>
<td>1</td>
</tr>
<tr>
<td>pBin+:RBP1-D383-1-GFP</td>
<td>control</td>
<td>4</td>
</tr>
<tr>
<td>pBin+:GpaII:Gpa2</td>
<td>control</td>
<td>4</td>
</tr>
<tr>
<td>pBin+:35S:GUS</td>
<td>control</td>
<td>12</td>
</tr>
</tbody>
</table>

As a control to check for successful infiltration of the used constructs, GUS (*β*-glucuronidase) staining was done. This allows an observation of protein expression in leaves infiltrated with GUS constructs. In case that expression occurs, the infiltrated spot after incubation in GUS substrate (X-gluc) turns to a blue colour. Seven days after agroinfiltration, the leaves were excised from the plant, placed into a 30 ml syringe and a volume of 10 ml GUS staining solution was added and vacuum pressure was applied. The staining solution per each treatment was comprised of 10 ml GUS buffer (64 ml 0.1 M Na2HPO4, 26 ml 0.1 M NaH2PO4, 90 µl Triton-x100) containing 100 µl x-gluc solution (30 mg x-gluc in 1 ml DMSO). Vacuum pressure was applied with a syringe until the leaves absorb GUS staining solution. This was followed by incubation in GUS solution overnight, at 37°C. After the overnight incubation, the leaves were de-coloured with 100% ethanol resulting in white coloured leaves. This allowed a clear observation of the blue colour and the necrotic spots.
Table 6. Visual ATTA scoring scale

<table>
<thead>
<tr>
<th>No HR</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak HR</td>
<td>1</td>
</tr>
<tr>
<td>Moderate HR</td>
<td>2</td>
</tr>
<tr>
<td>Strong HR</td>
<td>3</td>
</tr>
<tr>
<td>Very Strong HR</td>
<td>4</td>
</tr>
</tbody>
</table>

**DATA ANALYSIS**

All statistical analysis of the data obtained from *NSI-1* variants over-expressing assay, *in planta* RNAi nematode assay and ATTA were subjected to Student’s *t*-Test (one tailed distribution, homoscedastic). Statistical differences were considered significant when the p-values were < 0.05. In case of the qPCR, $2^{\Delta \Delta Ct}$ method was used (Livak & Schmittgen, 2001).
Results

*Nsi-1 OVEREXPRESSION IN POTATO ENHANCES SUSCEPTIBILITY TO G. rostochiensis INFECTION*

Constitutive overexpression of a pathogen gene in host plant and assaying host susceptibility is a promising research tool for parasitism gene functional validation. In order to check whether the *NSI-1* alters the potato plants susceptibility, several *NSI-1* (*Gr1106*) variants were cloned into a binary vector and transformed into potato using *A. tumefaciens* strain AGL1 virG. After the transformants selection and expression analysis one line per construct was challenged with 200 *G. rostochiensis* J2 infective juveniles. Two and six weeks post inoculation, the mean number of juveniles and mature females *G. rostochiensis* per plant were determined and used to asses genotype susceptibility. Two out of three genotypes overexpressing *NSI-1* variants, E4 and E7 had high average numbers of juveniles and cysts that were significantly higher as compared with GUS (empty vector) (Fig. 5; p-value<0.05 in Student’s *t*-Test). C4 showed susceptibility to nematode infection however the susceptibility was not significantly higher when compared to GUS (p-value>0.05) in Student’s *t*-test at both two and six weeks post inoculation. Therefore we conclude that, *Gr1106* variants differ in their ability to enhance susceptibility of potato plants to *G. rostochiensis*, with some variants causing plant hyper-susceptibility.
Fig. 5. Overexpression of NSI-1 variants in potato. Transgenic potato plants overexpressing NSI-1 variants revealed an enhanced susceptibility to G. rostochiensis infection. The assay included three plant genotypes that were transformed to overexpress different respective NSI-1 variants (E4, E7 and C4) and an empty vector (GUS) as a control. Three weeks old transformed potato plants were each inoculated with about 200 infective juveniles. Each treatment was replicated 12 times. Data are presented as the mean ± SE. A. Average number of fuschin stained parasitic juveniles that invaded plant roots determined 2 weeks post inoculation. B. Average number of nematodes that developed into cysts 6 weeks post inoculation. Error bars indicate the standard error of means. Different letters indicate significant differences at P < 0.05.

SELECTION OF PUTATIVE LINE V HAIRPIN TRANSFORMANTS

In order to verify the presence of hairpin construct and an empty vector in the putative transformant plants, the shoots were excised and grown on selective medium supplemented with kanamycin and only transgenic plants containing the alien DNA bearing the kanamycin resistance were selected. Additionally, in order to avoid false positive results from kanamycin selection, genomic DNA and gene expression were analyzed using PCR to check for presence of hairpin. From genomic DNA analysis of thirteen lines, only three lines (B1/4, B1/8 and B1/9; Fig. 6) didn’t reveal to harbour the hairpin construct (absence of 1000 kb fragment) and were discarded from NSI-1 silencing assay. This was observed after agarose gel resolution of the PCR products as depicted in Fig 6. Concomitantly, the genomic DNA of control plants (transformed with an empty vector) were analyzed using similar primers as used in hairpin transformant plants DNA. After agarose gel electrophoresis, the DNA from all the plants transformed with an empty vector (pSMD) showed an expected band size (750 kb) (Fig.7).
Comparing the PCR analysis, we conclude that, the hairpin construct was successfully integrated into the potato genome (different fragment sizes after PCR; Fig. 6 and 7). The DNA of plants harbouring the hairpin construct were further tested by PCR analysis to check the presence of the gene cassette of the vector using kanamycin primers and all the plants previously shown to contain hairpin construct gave positive results (see Appendix 3) denoting that the hpRNA construct was successfully integrated into the plant genome. In gene expression analysis, PCR using gene specific primers and GUS specific primers (XbaRNA2F and GUS 4, GUSPP2 and SacRNA2F) gave no band and probably this suggest that hpRNA is not stable.

![PCR analysis of DNA isolated from putative transgenic potato lines originating from different callus transformed with the same hairpin construct (B) using a primer pair of P1011 (forward primer from the promoter) and GUS 4 (reverse primer from the GUS insert). The fragments were resolved on agarose gel. M stands for 1kb Plus DNA ladder, B1/1-B1/13 represents the PCR amplification from thirteen transgenic plants that grew in kanamycin selective medium.](image)
Fig. 7. PCR analysis of the genomic DNA isolated from plants (EV1-EV10) that were transformed with an empty vector to be used as control in NSI-1 silencing assays. The fragments were resolved on agarose gel. M stands for 1kb Plus DNA ladder. The primers used were P1011 (forward primer from the promoter) and GUS 4 (reverse primer from the GUS insert) an all the twelve lines originated from different callus gave a positive band, smaller than the one observed in hairpin putative transformants.

NSI-1 GENE FAMILY IS CRUCIAL FOR NEMATODE VIRULENCE

To investigate the importance NSI-1 gene family in G. rostochiensis biology, transgenic potato plants expressing hpRNA targeting the conserved regions in the gene family transcripts were engineered. The hpRNA constructs were cloned into pSMD vector and their expression was controlled by a constitutive SUPER promoter and in parallel transgenic plants with an empty vector were generated and used as control. All the plants used in the silencing assay showed no phenotype difference to the untransformed line V genotype. The J2 were inoculated onto potato plants expressing NSI-1 hpRNA and the control plants. Two weeks post inoculation the effect of NSI-1 hpRNA on the inoculated nematodes was monitored. To check whether NSI-1 was silenced, qPCR was carried to detect and quantify the transcript of targeted gene (NSI-1). This allowed us to check if the transcripts are less abundant in feeding sites on plants expressing NSI-1 dsRNA. The fold changes were calculated using $2^{-\Delta\Delta Ct}$ method and variable silencing effect of NSI-1 was revealed in all transgenic lines expressing hpRNA (Fig. 8). Despite the fact that NSI-1 was silenced in all tested lines, silencing was much stronger in the four lines (greater than 1 fold), B1/2, B1/3,
B1/6 and B1/13 (Fig. 10). To check the effect of taking up the NSI-1 hpRNA by nematodes on their development, females were counted three weeks post inoculation. The grand mean of females recovered in lines: B1/1, B1/2, B1/3, B1/5, B1/7, B1/10, B1/11, B1/12, B1/13 were 24±8, 24±9, 26±6, 21±7, 32±6, 16±5, 27±8, 28±5, 21±6, 27±7 respectively whilst 40±8 and 45±7 cysts were found on control treatments, EV3 and EV9 respectively. The percent reduction of cysts in transgenic lines expressing NSI-1 dsRNA ranged from 25% to 62%. This shows that all tested transgenic lines expressing NSI-1 hpRNA were less susceptible to nematode infection than lines containing the empty vector and this was statistically significant (P-value<0.05 in Student’s t-test; Fig. 10). It therefore appeared logic that the reduction in nematode virulence was due to NSI-1 transcripts silencing and the results demonstrate that NSI-1 gene family has an important role in G. rostochiensis parasitism. The results firmly supported our project hypothesis. To check for primer specificity, the qRT-PCR products were resolved on agarose gel and expected fragment sizes were observed (Fig.9).

![Fig. 8. NSI-1 gene expression analysis using quantitative PCR. The silencing of NSI-1 is presented as a ratio (2^ΔΔCt) between the expression levels of NSI-1 in plants that express dsRNA and control lines (empty vector plants). Fold change greater than 1 was considered significant when compared to the empty vector. * represent a significant difference when compared to the EV (control).](image-url)
Fig. 9. Quantitative RT-PCR performed on cDNA from transgenic lines infected with *Globodera rostochiensis* line 22. A, Amplified product of *NSI-1* gene using NSI1u_qpcr_f NSI1u_qpcr_r (gene specific primers) and the fragment size is 134 bp. B, Amplification of the reference gene *cAMP* using GrKin_1F and GrKin_1R primers (91 bp). *M* stands for 1kb Plus DNA ladder.

Fig. 10. RNAi of NSI-1 affects *G. rostochiensis* (Line 22) virulence. The assay included 12 plant genotypes, 10 independent transgenic lines transformed to express dsRNA from the same construct (B1/1, B1/2, B1/3, B1/5, B1/7, B1/10, B1/11, B1/12, and B1/13) and 2 empty vector (EV3 and EV9) as controls transformed with pSMD. Three weeks old transformed potato lines were each challenged with about 200 infective juveniles of *G. rostochiensis* (Line 22). Each treatment was replicated 6 times. Three weeks post inoculation, the number of nematodes that developed into cysts were counted and the mean number was determined. Data are presented as the mean ± SE. The error bars indicate the standard error of means. The difference between the lines expressing dsRNA and EV control treatments was statistically different (Student’s t-test; P-value<0.05). Different letters indicate significant differences at P < 0.05.
ARTIFICIAL miRNAs CONSTRUCTS DESIGN, CLONING AND STABLE TRANSFORMATION

To further investigate the functional importance of NSI-1 gene family of *G. rostochiensis*, amiRNA constructs were engineered and integrated into potato line V genotype to induce post-transcriptional *NSI-1* silencing as an alternative to hpRNA. The sequences suggested from Web MicroRNA Designer (wmd3.weigelworld.org) of the amiRNA are shown below.

1. TATTTTTAGGTTTCGTGGCGG-amiRNA1

2. TAATGCGTCATCGTAAAGCGG-amiRNA2

Two amiRNA sequences targeting *NSI-1* were engineered following a set of PCRs. To construct the amiRNA, three sets of PCRs per amiRNA were done and the products were resolved on 2% TAE (1×) buffered agarose gel and the expected fragments sizes per each amiRNA were observed. The first, second and third PCRs were designated A, B, and C respectively resulting in fragment sizes of 272 bp, 170 bp and 272 bp (Fig. 11A, 11B). These fragments were isolated, gel purified and used as templates for respective overlapping PCR (amiRNA1 and amiRNA2).

![Fig. 11. Agarose gel electrophoresis of the PCR amplification of the fragments used in amiRNAs engineering. M. 1kb Plus DNA ladder, A. amiRNA1 A1 (Primer A & IV), B1 (Primer II&III), C1 (Primer I&B), B. amiRNA2 A2 (Primer A & IV), B2 (Primer II & III), C2 (Primer I & B). The plasmid pRS300 harbouring Arabidopsis thaliana MIR319a was used as a template in all the PCRs.](image-url)
Fig. 12. Agarose gel electrophoresis of overlapping PCRs products. The overlapping PCR was carried using gel purified fragments depicted in Fig. 8 as DNA templates. M (1kb Plus DNA ladder), D₁ (amiRNA1), D₂ (amiRNA2) and C (Control-no DNA template).

After the overlapping PCRs, the amplified fragments were resolved on 1% TAE (1×) buffered agarose gel and expected band size of 500 bp for both constructs was observed (Fig. 12). The fragments were extracted from the gel, purified and cloned as separate fragments into E. coli high copy vector pTOPO-2.1. Subsequent to transformation, colony PCR, plasmid sequencing (Appendix 4) and restriction digestion confirmed the presence of the amiRNA constructs. The RNAi expression vector and pTOPO-2.1 harbouring amiRNA constructs were subjected to a restriction digest to cut their SmaI and KpnI sites and the products were resolved on 1% TAE (1×) buffered agarose gel. The resultant fragment sizes correlated well with the expectation (Fig. 13). The GUS insert from the pSMD is however faint (500 bp).
Fig. 13. Restriction digests of pSMD and the two amiRNA constructs cloned in pTOPO-2.1 (high-copy vector) using Smal and KpnI restriction enzymes. pSMD (RNAi expression vector) restriction resulted in the excision of GUS insert (500 bp) and the fragment with bp greater than 1200, was used for amiRNA construct insertion, M 1kb Plus DNA ladder, pD1 (digested pTOPO to give amiRNA1) and pD2 (digested pTOPO to give amiRNA2), both fragments of 500 bp were then ligated to the linearized pSMD.

After ligation with pSMD and transformation to E.coli, colonies were checked by PCR with a primer combination of super-promoter specific and gene specific primer (amiRNA). For the amiRNA1, the primer used were P1101 and II miR1-a and P1101 and II miR2-a for amiRNA2. Successful cloning of the two amiRNA into pSMD was revealed by the amplification of the expected fragment of 950 kb after colony PCR (Fig 14A, 14B). We concluded that, amiRNA1 construct was present in 6 colonies (1, 3, 4, 5, 6 and 7) and amiRNA2 was only present in colony 4. The plasmids from colony 3 (amiRNA1) and colony 4 (amiRNA2) were isolated and once again used as templates in PCR and revealed the presence of the amiRNA constructs (results not shown). The plasmids harbouring the desired constructs were subsequently transformed into A. tumefaciens (AGL1-virG) and colonies were checked by PCR as previously done on E.coli transformants. All colonies were positive (950 bp fragments were observed) as depicted in Fig. 15. Constructs were introduced into Line V potato genotype through Agrobacterium-mediated transformation and the plants are already growing in the selection medium. The selected transformants will be tested in future
in the same way as was done to hpRNA lines to compare the effect of silencing using hpRNA and amiRNA.

**Fig. 14.** Agarose gel electrophoresis of the colony PCR amplifying amiRNA1 in pSMD. M (1kb Plus DNA ladder), A. amiRNA1: Numbers 1-7 represents colonies transformed with pSMD harbouring amiRNA1, 8(Control-MQ). B. amiRNA2: Numbers 1-7 represents colonies transformed with pSMD ligated with the second amiRNA construct. Only colony 4 showed positive results.

**Fig. 15.** Resolution of colony-PCR products of *A. tumefaciens* (AGL1-virG) transformed with pSMD harbouring the amiRNA constructs. M 1kb Plus DNA ladder. A. 1 and 2 represent colonies transformed with amiRNA1. B. 1-4 represent colonies transformed with amiRNA2, 5 is a control (no DNA).
SCREENING OF NEMATODE EFFECTORS USING ATTA

In order to deliver the proof on our hypothesis that some NSI-1 family members could be recognized by co-evolving plant immune system, the NSI-1 effector variants were screened on a selected potato genotype, SH that harbours H1 and Gpa2 resistance genes using ATTA. The NSI-1 variants were cloned into binary vectors pK7FWG (N10, N11, C4), pK2GW7 (E3, E4, E6, E7, E9) and transformed into A. tumeficiens virG. In this assay, the positive controls used were, RBP-1/Gpa-2 (Sacco et al., 2009) for HR and pBin+:35S:GUS for protein expression. The assay included two negative controls, viz. A. tumeficiens with binary empty vectors pK2GW7 and pK7FWG that were used in cloning of NSI- variants. Each construct was infiltrated through abaxial leaf surfaces of fully developed and young leaves (5th and 6th leaf). In all cases, gene expression of each construct was under the influence of CaMV 35S promoter. In case of a proper in planta expression of NSI-1 variants that are avirulent to H1 gene, an HR was expected. Seven days post infiltration, visual scoring was done using four different severity categories, viz. no HR (0), weak HR (1), moderate HR (2), strong HR (3) and very strong HR (4) (Table 6). After transforming the scores into percentage and a statistical analysis, we observed that among the variants cloned into pK2GW7, only E7 induced an HR that was significantly higher than the negative control-pK2GW7 (Student’s t-test; p-value< 0.05) . This was observed in both ATTAs (Fig. 16 A, B). On the other hand, C4 and N10 induced HR that was significantly higher when compared to the negative control (pK7FWG) in the first ATTA and in the second ATTA only N10 induced an HR, which was significant (Student’s t-test; p-value< 0.05). Although there was a trend of HR in E4, E6 and E9 this was not statistically significant in the two ATTAs performed. We also noted that even A. tumeficiens transformed with empty vector result in HR (pK2GW7) and this HR was consistent in both assays. As anticipated, the co-infiltration of Gpa2:RBP1 gave an HR and surprisingly, a protein expression control (GUS/YFP) in the
second ATTA resulted in an unexpected HR and furthermore, GUS staining in all infiltrated leaves did not yield expected blue colour (Fig. 17). Therefore, we concluded that E7 and N10 are good candidates for Avr effectors against *H1* gene, but in the future experiment need to be repeated in the way that unspecific HR, given by empty vectors and non-sense protein, can be eliminated.

![Graph A](image1)

**A**

![Graph B](image2)

**B**

**Fig. 16.** The graphs A and B depict the percentage of spots that developed a clear hypersensitive response (HR) after infiltration with respective constructs 7 days post infiltration. Agroinfiltration assays were repeated twice with the same constructs except GUS that was only used in the second ATTA. In each experiment, each construct was infiltrated on 24 spots (n=24). Data are presented as the mean ± SE. The error bars indicate the standard error of means. The * above the error bars indicate statistically significant deference (Student’s t-test; p-value< 0.05) from the empty vector (E3,E4,E6, E7 and E9) compared with pK2GW7, C4, N10 and N11 compared with pK7FWG).
Fig. 17. Transient expression of NSI-1 gene variants from an avirulent *G. rostochiensis* on SH, a potato genotype harbouring H1 and Gpa2 genes. A, Transient expression of E3, E4, E6, E7, E9, EV (pK2GW7), GUS and Gpa2:Rbp1 in SH leaf. A weak HR showed on spots infiltrated with E6, E4, E7 and E9 whilst no HR showed on spots infiltrated with E3. Aspecific HR appeared on spots infiltrated with EV (pK2GW7). B, Transient expression of C4, E7, N10, N11, EV (pK7FWG), GUS and Gpa2:Rbp1 in SH leaf. Severe necrosis was observed on spots infiltrated with Gpa2:Rbp1 (expected), E7, N10 and GUS. As expected, the spots infiltrated with EV (pK7FWG) showed no HR. In all cases, GUS staining did not show, however a strong and aspecific HR was observed on all spots infiltrated with GUS construct. The OD$_{600}$ 0.3 for all treatments except for Gpa2:Rbp1 that were mixed in 1:1 ratio to OD$_{600}$ 0.6.
Discussion

A common attribute of almost all plant pathogens is the secretion of virulence proteins/effectors into the host cells and these effectors, singly or when in concert, render host plant susceptibility. Cyst nematodes, just like other plant pathogens deploy an arsenal of effectors into the host plant apoplast and cytoplasm during their parasitic phase through the stylet. Among these nematode effectors, there are proteins that modulate plant innate immunity (Smant & Jones, 2011). These effectors are able to suppress or trigger (Avr) plant resistance response. A good example is that of Gp-Rbp1 from *G. pallida*. Sacco *et al.*, (2009) noted that Gp-Rbp1 is recognized by the resistance protein Gpa2 of potato, leading to HR response, thus triggering resistance. However, some of the Gp-Rbp1 variants from a virulent population suppressed the Gpa-2 dependent HR. The information about the virulence function of PCN effectors is still limited; however, scientists are putting much effort to mine effectors that have immunity modulating properties and others that aid plant parasitism. A good example in this case is the recent discovery of NS1-1 gene family expressed in dorsal gland of *G. rostochiensis* (Finkers-Tomczak *et al*., 2011). Currently, four nematode resistance genes have been identified in solanaceous species (Williamson & Kumar, 2006). Among these resistance genes is *H1* and since it confers resistance to some *G. rostochiensis* pathotypes, we could hypothesise that NSI-1 family can harbour a putative *H1* defence suppressor as well as *H1* elicitor(s).

In the current study, we report the functional analysis to determine the importance of NSI-1 gene family, recently identified in *G. rostochiensis* (Finkers-Tomczak *et al*., 2011) for nematode virulence. In an overexpression functional study of NSI-1 gene family, transgenic plants engineered to overexpress NSI-1 variants (E4, E7 and C4) were challenged with *G. rostochiensis* line 22 (virulent line). The results displayed an enhanced potato plant susceptibility to nematode infection (E4 and E7). However, C4 did not show hyper-
susceptibility, it showed no significant difference with the control plant (GUS). This might suggest that NSI-1 variants have different roles to play in nematode infection process. The results obtained from this study after determining the number of J2s that invaded the roots two weeks post inoculation mirrored the findings of Finkers-Tomczak et al. (2011) when they counted the number of cysts six weeks post inoculation. Together, this consistent observation from the two independent studies suggests that overexpression of NSI-1 variants enhances plant susceptibility to nematode infection. Furthermore, in previous studies, overexpression of some NS1-1 variants in resistant potato plants further made transgenic potato plants more susceptible to the fungus Verticillium dahliae, distantly related to nematodes. This was correlated with the down-regulation of pathogenesis-related protein-1 (PRI), a marker of the activation of SA-mediated defence pathway (Finkers-Tomczak et al., 2011). Collectively, these two independent assays suggest that, NSI-1 is possibly involved in suppression of the plant effector triggered immunity. In other studies on cyst nematodes, heterologous overexpression of putative nematodes parasitism genes resulted in suppression of plant defence system and a good example is a constitutive overexpression of Hs-10A06 from H. schachtii, an ortholog of Heterodera glycines in A. thaliana which resulted in increased susceptibility to nematode attack and to other distant pathogens. The conclusion drawn here is that, 10A06s’ target is the repression of salicylic acid (SA) defence signalling (Hewezi et al., 2010). Hewezi et al., (2008) observed that, overexpression of cellulose binding protein - Hs CBP (ortholog of Hg CBP) in A. thaliana has resulted in an enhanced plant susceptibility to H. schachtii. Also, Souza et al. (2011) reported that, an ectopic overexpression of an effector protein from the dorsal gland in tobacco plants resulted in higher infection, more galls and higher reproduction rate of M. incognita. Patel et al., (2010) noted that, an overexpression of Hs4F01 (an annexin) in A. thaliana enhances plant susceptibility to H. schachtii. Contrary to the above cases, Lee et al. (2011) reported that, an ectopic
overexpression of *Hs19C07* from *H. schachtii* in *A. thaliana* resulted in a low infection rate and further compromise female development. Altogether, these results suggest that an overexpression of a gene of interest in the host plant followed by the specific nematodes infection gives a clue about the function of that particular gene. All these reports suggest that, there is a cocktail of effectors deployed by plant parasitic nematodes that act as plant defence suppressors.

Furthermore, RNAi, an invaluable tool was used to verify the importance of NSI-1 gene family in *G. rostochiensis* parasitism. The transgenic plants with susceptible background were engineered to express NSI-1 hpRNA and nematode infection assays on transgenic plants displayed a reduced nematode virulence in all lines tested carrying B1 hpRNA construct. All the ten lines displayed significant differences from the empty vector control lines, showing a reduction in female numbers. In the previous research done by Finkers-Tomczak *et al.* (2011) on NSI-1, silencing of infective juveniles by soaking in dsRNA corresponding to *NSI-1* resulted in 33% reduction of cysts on plants challenged with the treated nematodes. However, RNAi using this method is transient and this might have resulted in re-gaining of virulence. In the current study, *in planta* generated hpRNA resulted in reduced virulence ranging from 25% to 62% amongst the transgenic lines (Fig. 10). These results show some consistency with previously done *in vitro* assays after soaking the J2s in dsRNA corresponding to NSI-1. Moreover, even higher silencing effect was observed in some lines when the hpRNA was continuously delivered by plant. This study showed that PCN are capable of ingesting bioactive form of hpRNA from plant cells, regardless of the early concerns of constrains imposed by the feeding tubes (Bakhetia *et al.*, 2005). Therefore, we conclude that, the syncytium is an ideal route for dsRNA delivery since the dsRNA will be delivered to the nematodes throughout the parasitic phase. The expression of NSI-1 variants in dorsal esophageal gland was observed to coincide with sedentary stages (syncytium initiation,
development and maintenance), and this evidence likely support that NSI-1 variants functions in protecting the nematode from plant defence system. NSI-1 silencing by *in planta* RNAi resulted in less females that develop on potato roots (reduced nematode virulence) suggesting that NSI-1 gene family plays an important role in parasitism. The possible reason for the observed differences in silencing efficiency among the transgenic lines might be due to that, the lines originated from different transformed stem cells (calli). Therefore, the integration position of the alien DNA in the potato genome might be different henceforth may result in different *NSI-1* hpRNA expression. However, we cannot state with absolute confidence that the difference in silencing effect amongst the ten B1 lines is due to positioning of the alien DNA in the plant genome since the mechanisms underlying gene silencing is still elusive. Moreover, we were not able to check for the expression level of hpRNA and compare it between lines; probably it is because of instability of transgenic hpRNA. Before, successful functional studies of nematode parasitism genes using *in planta* RNAi have been reported in root-knot and other cyst nematodes. Transgenic tobacco plants expressing dsRNA complimentary to a splicing factor protein and integrase gene resulted in compromised galls formation and reproduction of *Meloidogyne incognita* (Yadav *et al*., 2006). Also, Arabidopsis plants expressing *16D10* dsRNA targeting *16D10* - a conserved and essential root-knot parasitism gene resulted in an effective resistance against the four major RKN species (Huang *et al*., 2006b). Fairbairn *et al.* (2007) reported that a putative transcription factor MjTis11 of *Meloidogyne javanica* was down-regulated after feeding on transgenic tobacco lines expressing *MjTis11* dsRNA. Also, the knock-down effect of four parasitism genes in *H. schachtii* led to the reduction in parasitism success and number of mature females in Arabidopsis (Sindhu *et al*., 2009). Reduced by 90% number of galls was observed in transgenic soybean after targeting four *M. incognita* genes (Ibrahim *et al*., 2011). Steeves *et al.*, (2006) also found that hpRNA targeting major sperm protein gene of *H. glycines* resulted
in 68% reduction of egg production. The degree of NSI-1 silencing observed in our study was less when compared to other *in planta* silencing of putative parasitism genes studies. A number of factors such as less efficiency of the promoter in driving high levels of hpRNA and the expression pattern of NSI-1 genes might have thwart complete silencing of NSI-1. However, we cannot state with an absolute confidence since the factors influencing successful gene silencing are poorly understood. Combining all the *in planta* silencing of plant parasitic nematodes genes using RNAi, we therefore concluded that the technique is useful in functional genomics and it reveals a prospect in transgenic control of plant parasitic nematodes.

As an alternative to gene silencing using hpRNA, another aim of the study was to design and clone artificial microRNA for *in planta* silencing of *NSI-1*. The cloned amiRNAs in pSMD, an RNAi vector were stably transformed to potato line V genotype with a susceptible background. This alternative method of gene silencing has not been demonstrated or tested yet for nematode genes. Therefore the transgenic lines generated during this project will be used in the future to compare both systems (hpRNA and amiRNA) to find out the best method to silence nematode genes. In a distantly related study, amiRNAs were proven to be more effective in gene silencing than hpRNA (Butardo *et al.*, 2011). This might be due to the size and specificity of a single stable small RNA that is incorporated into the silencing complex. The technology exploits endogenous miRNA precursors that preferentially produce one siRNA duplex, the miRNA–miRNA* and this duplex directs gene knock out in either plants or animals. Due to high similarity and specificity of amiRNAs and endogenous miRNAs, amiRNAs can be easily optimized to silence one or several target transcripts without effect on the expression of other genes.

Beside functional analysis of NS1-1 gene family through constitutive overexpression and RNAi, functional screening of effectors coming from avirulent population was done. Hereby,
ATTA, a popular tool used to investigate gene function as a good alternative to stable transformation was used. One of the advantages of ATTA, is that it is much faster tool when compared to classical bioassays that run for months and the visual scoring can be done easily in case of hypersensitive response. However, the efficiency of this method is often variable and dependent on the species (Rietman et al., 2011). In this study, eight NS1-1 variants from an avirulent population of G. rostochiensis were screened on PCN host plant (SH–potato genotype). Induction of hypersensitive response (HR) by effector Avr candidates was observed 7 days post infiltration. We observed that only two variants, viz. E7 and N10 resulted in a consistent HR, which was statistically significant when compared to their respective controls. This suggests that, probably the effectors were perceived by R protein, thus a gene-to-gene response occurred. Our finding that only two variants out of eight are recognized as potential Avr proteins might be due to the fact that, NS1-1 family is under positive selection and it might have influence the variants recognition. This suggestion is well supported by the findings of Sacco et al., 2009. They found that, although all Gp-Rbp1 variants from an avirulent population were recognized by Gpa2, in virulent populations, some Gp-Rbp1 variants were recognized and some not. They stated that the variation was attributed to positive selection on numerous sites and recognition by Gpa2 was attributed to single amino acid polymorphism. Moreover, the variants that are not being recognized by Gpa2 could act epistaticaly on Avr variants or the outcome of their recognition by Gpa2. Surprisingly in our study, one of the empty vectors (pK2GW7) consistently induces a weak HR on all infiltrated spots and, a similar observation was made by Rietman et al (2011) while screening late blight effectors on different potato genotypes. They noted that, although SH genotype is in general suitable for ATTA some weak necrosis or cell death occurs even when negative controls are infiltrated. This aspecific cell death might be due to recognition of PAMPs or elicitors of A. tumefaciens and leaf architecture might have also thwart convenient
infiltration, and thus, results in mechanical damage (Rietman et al., 2011). As anticipated, the co-infiltration of Gpa2:RBP1 gave an HR and surprisingly, a non-sense protein expression control (GUS:YFP) in the second ATTA also resulted in an HR that was even much greater than Gpa2:RBP1.

Since SH harbours Gpa2, it is expected that an HR would be observed when SH is infiltrated only with Rbp1. However recent studies have shown that an HR will be present only when Gpa2 is co-infiltrated with Rbp1 (data unpublished). From this outcome, it can be hypothesized that, the Gpa2 expression level, which can be lower in leaves, is the determining factor for HR induction. This could be also the case with H1 expression after infiltration of NS1-1 variants and therefore, HR observed could be only due to either mechanical damage or unspecific response to A. tumefaciens. However, the consistent HR induction by E7 and N10 in the two ATTAs suggests that, there is a perception of these effectors by the R protein. The absence of GUS staining was surprising, however, it might have been caused by a strong unspecific HR. Logically, high HR resulted in extensive cell death and therefore, the protein (enzyme) was no longer present during GUS staining. In the course of ATTAs, minor mechanical damages were observed on SH genotype and positive control (Gpa2 and Rbp1) gave expected response and these observations together suggest that, SH is a good genotype for ATTA as reported in previous ATTA optimization experiments on potato genotypes. Conclusively, screening of NS1-1 effectors through ATTA is feasible. However, there is need to further optimization of this assay and including a potato genotype that would act as a negative control to cater for response specificity.
Conclusion and Future Perspectives

From our studies, overexpression of NSI-1 variants and *in planta* RNAi revealed that, the NSI-1 effectors are important for *G. rostochiensis* virulence. This is also supported by the previous assays when *NSI-1* dsRNA was delivered to the infective juveniles by soaking. Combining these facts with the evidence that some of the NSI-1 variants suppress R-Avr HR of well renowned HR elicitors suggest that, NS1-1 gene family harbour plant immune modulators. Ideally, the cellular target or interactor of NSI-1 needs to be deciphered before confirming this hypothesis. This can be done for example through protein-protein interactions screening (Yeast two hybrid (Y2H) or affinity purification immunoprecipitation combined with LC-MS/MS (AP-IP LC-MS/MS)) systems. Also, a biological replicate assay should be conducted on the *in planta* NSI-1 silencing assays, such that we can have an absolute confidence in the effect of NSI-1 silencing on nematode virulence. NSI-1 effectors function could be explored by monitoring their subcellular localizations once they are introduced into a plant cell. This localization may shed light about the function of parasitism protein. From ATTA experiments we found that, even an empty vector construct and non-sense genes (GUS) can elicit HR and this was not the first report of unspecific recognition. This observation therefore suggests that there is always need for using multiple negative controls in this kind of experiment. Also a negative control genotype (without nematode R genes) can be used to verify the response specificity in case that an HR is triggered on plants that harbours a nematode resistance gene. As anticipated, the co-infiltration of Gpa2:RBP1 gave an HR and using the similar HR control can be maintained in future assay.
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REFERENCES


Appendix

Appendix 1

NSI-1 variants proteins sequences and their structural features. For hairpin RNA construction, two conserved parts on the C-terminal and N-terminal were selected, thus resulting in two constructs designated hpRNA A and hpRNA B (as highlighted in the figure). The amiRNA were also designed by The Web MicroRNA Designer (wmd3.weigelworld.org) targeting two conserved region across the gene family.

Appendix 2

Map of pRS300 A. thaliana miR319a precursor used as a template in the first three sets of PCR during the engineering of the 2 amiRNA to target NSI-1 gene family silencing.
PCR analysis using vector cassette specific primers for kanamycin gene. The PCR was carried using the DNA isolated from putative transgenic potato lines originating from different callus transformed with the same hairpin construct (B) using a primer pair of Kan R and Kan F. The fragments were resolved on agarose gel. M stands for 1kb Plus DNA ladder, B1/1-B1/13 represents the PCR amplification from ten transgenic plants that grew in kanamycin selective medium.
Appendix 4

Sequencing results of plasmid DNA from different colonies that harbour the two amiRNAs cloned in TOPO vector aligned with the *A. thaliana* miR319a precursor region (A to B).