Techniques for characterization and eradication of potato cyst nematode: a review

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Abstract Correct identification of species and pathotypes is must for eradication of potato cyst nematodes (PCN). The identification of PCN species after completing the life cycle is very difficult because it is based on morphological and morphometrical characteristics. Genetically different populations of PCN are morphologically same and differentiated based on the host differential study. Later on these traditional techniques have been replaced by biochemical techniques viz, one and two dimensional gel electrophoresis, capillary gel electrophoresis, isozymes, dot blot hybridization and isoelectric focusing etc. to distinguish both the species. One and two dimensional gel electrophoresis has used to examine inter- and intra-specific differences in proteins of *Globodera rostochiensis* and *G. pallida*. Now application of PCR and DNA based characterization techniques like RAPD, AFLP and RFLP are the important tools for differentiating inter- and intra specific variation in PCN and has given opportunities to accurate identification of PCN. For managing the PCN, till now we are following integrated pest management (IPM) strategies, however these strategies are not effective to eradicate the PCN. Therefore to eradicate the PCN we need noval management practices like RNAi (RNA interference) or Gene silencing.

Keywords PCN · Biochemical techniques · DNA based characterization · Gene silencing

Introduction

The potato cyst nematodes (PCN) namely *Globodera pallida* (Stone) pale potato cyst nematode and *Globodera rostochiensis* (Wollenweber) golden potato cyst nematode are highly specialized and economically important pest of potato (Jensen et al. 1979). Worldwide PCN alone causes estimated losses up to 30% in potato crop yield (Oerke et al. 1994). A number of pathotypes within each species have been distinguished based on their capacity to multiply on different host (Kort et al. 1977). In India, PCN was first reported from Nilgiri hills in Tamil Nadu (Jones 1961). Now it's an important biotic constraint in sustainable production of potato with quarantine significance in the Nilgiris region of Tamil Nadu. It remains a serious endemic pest of potato in this region due to intensive cultivation of potato and favourable climatic conditions. The differential host reactions of PCN populations from Nilgiris and Kodaikanal hills revealed that the pathotypes Ro1 of *G. rostochiensis* and Pa 2 of *G. pallida* are the most prevalent forms accounting for 75% of the total populations. The other prevalent pathotypes are Ro2 (7%) and Pa1 (15%) and least prevalent pathotypes are Ro5 and Pa3 accounted only for 3% (Prasad 1996). The reproductive potential of PCN is high, in addition it survives for extended periods under adverse conditions that make them difficult to eradicate once established. The second-stage juvenile (J₂) hatches and exits from the cyst (Marks and Brodie 1998), stimulated by host plant root diffusates. In the presence of host plant, PCN can hatch from 60 to 80%, but in the absence of host plant, 30% PCN hatching occurs spontaneously (Oostenbrink 1950) and in cold soils, it is less only up to 18% (Turner and Evans 1998).

Commonly, symptoms caused by PCN infection are not specific and are similar to those caused by other biotic or

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abiotic stresses. PCN infected potato plants are short, tubers are much smaller and tubers yield is much lower than that obtained from healthy plants. Control of these nematodes is very difficult and expensive because they live and overwinter in soil. Traditionally, species of PCN are distinguished based on morphological characters like immature female cyst colour and morphometric characters of juveniles like tail length, stylet length, stylet knob shape and difference in vulval cone or perineal regions of cyst (Colin and Thomas 1998). The variants within each species are identified by the detail studies with differential host reactions of the population, but it is time consuming. Besides this, some new biochemical and molecular approaches have become popular among the nematologists which give accurate results without taking much time. For controlling PCN population till now we are practicing integrated pest management (IPM) approaches in combination of cultural (Crop rotation, trap cropping, bio-fumigants, resistant varieties), physical (soil solarization), biocontrol and chemical control. But current control measures are inadequate for a variety of reasons. Resistant varieties suppress or inhibit the reproduction but resistance is specific to species and pathotypes, due to development of new virulence in the PCN species the existing resistant cultivars becomes susceptible. Growing of susceptible potato variety as a trap crop is a better option for eradication of PCN. Trap crop stimulates hatching of nematodes and attracts J_2 therefore uprooting of potato crops (after 37–42 days) before completing PCN life cycle so that female would not be fertilized and no new eggs will be formed but use of potato as a trap crop is not a cost effective option. Additionally, if the trap crop is not destroyed in time newly formed PCN cysts will mature which in turn increase the PCN population rather than decreasing (Alan and Susan 1998). In temperate area during summer season under glass house condition soil solarization using transparent polythene film will kill PCN up to 20–30 cm depth. But under field condition the required temperature can be achieved only in top 10 cm layer of soil. The other disadvantage of this technique is that it can not be used for larger areas and labour consuming. Chemical control is widely used which involves the application of volatile (fumigants) and non-volatile nematicides which reduces nematode populations up to a limited period. But utility of fumigants and nematicides will increase the costs associated with plant protection and hazardous to the soil biomass (Alan and Susan 1998). To overcome the limitations of current control measures transgenic approaches can be utilized effectively for developing durable polygenic resistance in agronomically acceptable cultivars against nematodes. Transgenic plants, is a defence strategy that delivers proteinase inhibitors which can impart effective resistance against several

nematode species. Proteinase inhibitors which expressed in transgenic potato influences the sexual ratio of juveniles (J_3 stage) of *G. pallida*, later which become male but doesn't reduce the fecundity of the females so formed (Atkinson et al. 2013). RNA interference technology (RNAi) is an exciting and emerging strategy for managing plant parasitic nematodes. It is an eco-friendly defense strategy to identify target genes and also provide a further basis for transgenic resistance (Lilley et al. 2008). By this review, we have made an effort to summarize the available scientific progress regarding identification and eradication of PCN.

Protein electrophoresis and isoelectric focusing (IEF)-based characterization

Protein electrophoresis has been used rigorously during the early 1970s, in the separation of *Globodera* spp. Several electrophoretic techniques developed to detect protein variation in both the PCN species: one-dimensional gel electrophoresis for the separation of proteins; isoelectric focusing (IEF) for protein fractionation at different pH; two-dimensional gel electrophoresis (2-DGE) for electrophoretic resolution of proteins and sodium dodecyl sulphate-capillary gel electrophoresis (SDS-CGE) for the separation of micro-amounts of proteins and peptides (Isabel et al. 2004).

A diagnostic probe with the capability of separating *G. pallida* from *G. rostochiensis* was also developed (Marshall and Crawford 1987). A similar approach with two diagnostic probes was developed which were specific to *G. pallida* and differentiated two PCN species (Burrows and Perry 1988). One-dimensional SDS gel electrophoresis of proteins used to distinguish species and pathotypes of PCN (Den Nijs and Lock 1990). Later two-dimensional gel electrophoresis (2-DGE) has been used to examine inter- and intra-specific differences in proteins of *G. rostochiensis* (De Boer et al. 1992; Bossis and Mugniéry 1993) and *G. pallida* (Fullaondo et al. 2001). Dot-blot hybridisation with species-specific probes was used to distinguish pathotype Ro1 *G. rostochiensis* and Pa2/3 *G. pallida* in mixed population of nematodes (Marshall 1993). Individual diagnostic probes (pRo-67, pPa-60, and pB10-1) were isolated (Stratford et al. 1992) that could distinguish both the species of PCN and pathotype of *G. pallida* P₁A in simple dot-blot hybridization (Phillips et al. 1992; Gonzalez et al. 1995). High performance capillary electrophoresis was tried to discriminate populations and differences in protein profiles of pathotypes of *G. rostochiensis* (Ro1, Ro2 and Ro5) and *G. pallida* (Pa1 and Pa2/3) (Hinch et al. 1998; Cunha et al. 2002). Another technique for nematode characterization and identification is IEF, which is a modification of standard electrophoretic technique in

which the proteins are separated on the basis of their charge and resolved into sharp bands. This technique has proved to be sensitive enough to identify samples of PCN. During IEF, proteins are separated in a pH gradient gel and focused at the position in the gradient where they become electrically neutral. IEF used on thin layers of polyacrylamide gel to separate proteins from both the species of PCN. They used protein patterns to identify the two species, even form single cysts (Fleming and Marks 1983). IEF analysis used for detection of PCN from Russia, Ukraine and other countries. *Globodera rostochiensis* and *G. pallida* banding profile produced consistent banding patterns on mini-IEF gels over the pH range 5.0–8.0. Species identification based on the presence or absence of diagnostics bands for *G. pallida* (pI 5.7) or *G. rostochiensis* (pI 5.9) (Subbotin et al. 1999).

Immunoassays-based characterization

Serological technique offers the opportunities for large scale detection of PCN species. Polyclonal antibodies (PABs) which present in antiserum are able to recognize multiple antigenic determinants in nematodes (Scott and Riggs 1971; Wharton et al. 1983). But due to general cross-reactivity of polyclonal antisera, limits their value in species-diagnosis and directed the development of species-differentiating monoclonal antibodies and which can be used for routine purposes. Monoclonal antibodies quantified cysts from processed soil samples and from soil samples containing up to 14–20% of organic matter (Curtis et al. 1998). For the use of monoclonal antibodies, species specific proteins should be available in appropriate amount for immunization. Monoclonal antibodies used to quantify the two species of PCN from clean extracts of cysts (Schots et al. 1992). Later, two monoclonal antibodies used in cross-reactivity tests with a wide range of soil nematode species, the antibodies reacted strongly only with species of the genus *Globodera* and recognized proteins of the same molecular weight (34 kD) in both species of PCN (Robinson et al. 1993). These proteins had isoelectric points at pH values of 5.7 in *G. pallida* and 5.9 in *G. rostochiensis*. The development of life-stage-specific monoclonal antibodies to *G. pallida* was reported by Backett et al. (1993).

DNA-based characterization

Molecular approach is important for performing complementary diagnostics. For the taxonomic purposes, DNA technique was first reported in 1985 but this method was time-consuming, required a generous amount of DNA and not sensitive. The development of polymerase chain reaction (PCR) made it possible to discriminate only when

minute amounts of material were available. PCR is more appropriate for nematode DNA study. DNA analysis based on the use of DNA probes or PCR offers both sensitivity and genetic specificity (Fleming et al. 1993; Chacon et al. 1994). Using PCR technique, primers are developed that bind to the target sites on each DNA strand and replicate a specific region of DNA for separating the two PCN species (Mulholland et al. 1996; Zouhar et al. 2000).

RAPD-PCR (random amplified polymorphic DNA-PCR) based characterization

Nine decamer primers tested to amplify species specific DNA sequences by using RAPD. Primer OPG-05 produced 2 discrete DNA fragments which were consistently present in five *G. rostochiensis* populations and absent in five *G. pallida* populations (Roosien et al. 1993). RAPD technique does not require any specific probes, making it possible to work with species for which DNA libraries are not available (Rafalski et al. 1994). Analysis of RAPD based on the PCR amplification of DNA segments using arbitrary oligonucleotide primer that bind to homologous base sequences in the genome has been used to detect genetic variability in PCN (Folkertsma et al. 1994; Blok and Phillips 1995; Bendezu et al. 1997; Phillips et al. 1998) (Table 1).

A significant step in the development of markers for virulence was used to analyze a population of *G. pallida* for increased virulence and compared with an avirulent population using RAPD techniques (Patrik et al. 1995). A hybridization study showed that, RAPD product would bind only to the DNA of virulent populations, suggesting that it is tightly linked to a gene involved in virulence. RAPD analysis used to differentiate *G. pallida* populations, including some P₁A isolates, which were distinct from the typical UK gene pool (Burrows et al. 1996). Similar results were also reported in highly virulent *G. pallida* isolate from Scotland which was genetically distinct from other P₄A/P₅A isolates (Blok et al. 1997). Two primer combinations were developed and used (Table 2) for PCR which amplify only one specific band in each species *G. rostochiensis* and *G. pallida* (Fullaondo et al. 1999). The generation of unique amplification products facilitates the application of automatic detection methods.

Umarao et al. (2002) used random operon primer OPG 5 to characterize the gene pool similarity of the field populations of PCN from Nilgiri Hills. Sedlak et al. (2004) used RAPD analysis for studying genetic variability of nine different European and Czech populations of PCN. Typical RAPD profiles of individual populations were obtained by primers OPG 08 and OPG 13 (Table 3). Hlaoua et al. (2008) used RAPD to identify PCN species with in Tunisian populations and compared with European ones. They

Table 1 The oligonucleotide decamer primers used to assess the inter and intra specific variation of *G. rostochiensis* and *G. pallida* and the number of DNA fragments produced per primer

Primer	Sequence	Number of DNA fragments	
		<i>G. rostochiensis</i>	<i>G. pallida</i>
OPG-02	GCCACTGAGG	9	9
OPG-03	GAGCCCTCCA	6	10
OPG-04	AGCGTGTCTG	3	9
OPG-05	CTGAGACGGA	4	10
OPG-06	GTGCCTAACC	8	5
OPG-08	TCACGTCCAC	4	15
OPG-10	AGGGCCGTCT	15	16
OPG-11	TGCCCGTCGT	9	7
OPG-12	CAGCTCACGA	10	10
OPG-13	CTCTCCGCCA	8	10
OPG-15	ACTGGGACTC	6	7
OPG-16	AGCGTCCTCC	14	12
OPG-17	ACGACCGACA	8	9
OPG-19	GTCAGGGCAA	10	13

Table 2 Sequences of primers pairs specific for potato cyst nematode species

<i>Globodera rostochiensis</i>	<i>Globodera pallida</i>
5'-GCAAGCCCAGCGTC AGCAAC-3'	5'-TGTCCATTCCTCTCCACCAG-3'
5'-GAACATCAACCTCC TATCGG-3'	5'-CCGCTTCCCCATTGCTTTTCG-3'

Table 3 Used oligonucleotides

Primer	Sequence
OPG 03	5'-GAGCCCTCCA-3'
OPG 05	5'-CTGAGACGGA-3'
OPG 08	5'-TCACGTCCAC-3'
OPG 10	5'-AGGGCCGTCT-3'
OPG 13	5'-CTCTCCGCCA-3'
OPA 07	5'-GTGGGCAAAG-3'

found the presence of the two species *G. rostochiensis* and *G. pallida* belonging respectively to Pa2/3 and Ro1/4 pathotypes.

AFLP/RFLP analysis based characterization

The Amplified Fragment Length Polymorphism (AFLP) technique provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity (Zouhar et al. 2000). Folkertsma et al. (1996) used the powerful technique of AFLP to examine the genetic

relationship among *G. rostochiensis* and *G. pallida* populations. Manduric (2004) used seven AFLP primer combinations to amplify genomic DNAs extracted from PCN species *G. rostochiensis* and *G. pallida*. Banding patterns observed show both important similarities and differences among the populations analyzed and also detects clear population level variation. The same level of distinction is found between two Pa1 populations of *G. pallida*, confirming genetic specificity of this pathotype (Bendezu et al. 1997) which found 92.2 and 82% similarity among *G. rostochiensis* isolates representing three different pathotypes from Europe. RFLP analysis used on extracted and purified PCN DNA and detected restricted fragments from repetitive DNA. Difference between *G. pallida* and *G. rostochiensis* in the band number and size indicated the potential value of this analytical approach to PCN diagnostics (Burrows and Boffey 1986). Combining RFLP analysis with probe hybridization has also proved a useful approach for quantifying the genetic heterogeneity between PCN species and among *G. pallida* populations (Schnick et al. 1990) and also able to detect variant cyst (Reid and Pick up 2005). RFLP of the ITS I ribosomal DNA region from *Globodera* species produced diagnostic profiles that allow identification of both species and quantitative PCR determined the population sizes of cyst nematode species (Fleming and Powers 1998).

ITS-region and multiplex/real-time PCR based characterization

PCR amplification of the Internal Transcribed Spacer Regions (ITS 1 and ITS 2) combined with restriction enzyme analysis (PCR–RFLP) is a powerful tool for nematode diagnostics. The ITS1 and ITS2 located between the repeating array of nuclear 18S and 28S rRNA genes and separated by the 5.8S rRNA gene are variable genetic markers which have been used successfully for the analysis of population structure and systematic in a wide range of organisms. The amplification of specific genomic regions is highly effective way of detecting inter- and intra-specific variations between genera, species and within species. The two most common repeated regions for taxonomic and diagnostic purposes are ribosomal DNA (rDNA) and mitochondrial DNA (mt DNA) (White et al. 1990). Primers located in 28S and 18S genes amplified PCN DNA, generating a 1200 bp region encompassing the ITS1 and ITS2 regions and 5.8S gene (Fleming et al. 1993). Primers specific for trans-spliced ladder gene and the 5S ribosomal gene to develop a PCR assay based on amplified product size differences in the two species of PCN (Stratford and Shields 1994). A remarkable discovery in *Globodera* is the unique structural organization of the mitochondrial genome of *G. pallida*. A primer pair which amplify regions

Table 4 Primer sequences and PCR conditions for amplification of *Globodera* DNA

Primer name	Region targeted	Primer sequence 5'–3'	PCR conditions	References
SL	Trans-spliced leader RNA	CTC AAA CTT GGG TAA TTA AAC C	30 cycles of 30 s at 94 °C	Shields et al. (1996)
5S	5S RNA	CGC GGA TCC TTA CGA CCA TAC CAC G	1 min at 40 °C, 2 min at 72 °C	
rDNA1	28S rRNA	TTA CAC TCG CCG TTA CTA AGG	40 cycles of 1 min at 94 °C 1 min at 45 °C, 2 min at 72 °C	Fleming et al. (1993)
rDNA2	18S Rrna	TTG ATT ACG TCC CTG CCC TTT	30 cycles of 1 min at 94 °C 50 s at 60 °C, 1 min at 72 °C	Thiery and Mugniery (1996)
rDNA2	18S rRNA	TTG ATT ACG TCC CTG CCC TTT	35 cycles of 45 s at 95 °C	Szalansky et al. (1997)
rDNA 1.58S	5.8S rRNA	ACG AGC CGA GTG ATC CAC CG		
rDNA 2	18S rRNA	TTG ATT ACG TCC CTG GCC TTT	1 min at 50 °C	Powers et al. (1997)
rDNA 2.144	28 S rRNA	GAT CCG TAG GTC AAC CTG CAG ATG GAT	1 min at 72 °C	
Universal	5.8S rRNA	GCA GAA GGC TAG CGA TCT TC	Use Stoffel fragment of <i>Taq</i> DNA polymerase. Initial denaturation of 5 min at 95 °C, then 35 cycles of 30 s at 94 °C, 10 s at 95 °C, 30 s at 72 °C	Mulholland et al. (1996)
<i>G. pallida</i>	ITS1	GGT GAC TCG ACG ATT GCT GT		
<i>G. rostochiensis</i>	IT2	TGT TGT ACG TGC CGT ACC TT		
ITS5	18S rRNA	GGA AGT AAA AGT CGT AAC AAG G	Initial denaturation of 2 min at 94 °C, then 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C	Bulman and Marshall (1997)
PITSr3	ITS1	AGC GCA GAC ATG CCG CAA		
PITSp4	ITS1	ACA ACA GCA ATC GTC GAG		

between the 5S rRNA and spliced ladder RNA genes generating species specific banding patterns for both *G. rostochiensis* and *G. pallida* (Shields et al. 1996). The potential of ITS size and sequence variation were evaluated for identification of *Globodera*, *Heterodera* and *Cactodera*. They used two sets of primers to amplify ITS1 and ITS1-ITS2 products and demonstrate that PCR product size could be used to distinguish many nematode genera (Szalansky et al. 1997; Powers et al. 1997). ITS restriction patterns could be useful to follow and to control in vitro and in vivo hybridization knowing that DNA amplification of one individual is possible (Ferris et al. 1993). Subsequent restriction digestion of the PCR product size could then resolve genera exhibiting products of similar size as well as separating out many specimens at the species level. Being based on DNA sequence, this technique can be used on both adult and juvenile life stages.

The amplification and analysis of the ITS region has many advantages like rapidity to obtain profuse and clarity of the results and allow easy species identification. Isolated individual diagnostic probes (pRo-67, pPa-60, pB10-1) could distinguish *G. rostochiensis*, *G. pallida* and *G. pallida* pathotype P1A in simple strategy for the identification of putative pathogenicity factors from *G. rostochiensis*.

ITS-RFLP and two satellite DNA sequences used as taxonomic tool for confirming inter and intra specific similarities of Peruvian PCN population and used 2-DGE and RAPD data sets for comparing the imported European populations (Grenier et al. 2001). The ITS fragment size amplified 1.2 kb in *Globodera* species, parasites of Solanaceous plants. Two 21-mers oligonucleotide primers were used to amplify the ITS. Restriction digestion of the amplified ITS regions of the ribosomal DNA revealed species specific patterns. Further sequencing of the ITS was done to search for intra-specific polymorphism (Thiery and Mugniery 1996). A multiplex PCR assay used with species-specific primers located in ITS 1 for both species of PCN (Bulman and Marshall 1997; Madani et al. 2010). The ITS1 rDNA region is the target region which is used to detect morphologically identified *Globodera* spp. (Fleming et al. 2000). The D3 expansion region of the 28S gene and the ITS1-5.8S-ITS2 region of rDNA sequences from *G. rostochiensis*, *G. pallida*, *G. tabacum tabacum*, *G. tabacum virginiae* and *G. tabacum solanacearum* were aligned and compared. There are no nucleotide differences in the D3 region sequences between *G. rostochiensis* and *G. pallida*. Sequence analysis and RFLPs of ITS-PCR products showed that several haplotypes are present in the genomes

of *G. rostochiensis* and *G. pallida* populations. RFLP profiles and sequences of the rDNA used for diagnostics and phylogeny of *Globodera* (Subbotin et al. 2000) (Table 4).

Species-specific sets of primers were designed for ITS-1 amplification on the basis of known sequences of ITS-1 for both PCN species by using freeware primers. By using set of Fro1- Rro1, the product 411 bp was detected only *G. rostochiensis* and by using of set Fpa2-Rpa1, the product 239 bp was detected only *G. pallida* (Vejl et al. 2002). Sequence of partial 18S, ITS1, 5.8S, ITS2 and partial 28S sequence from *G. rostochiensis* and *G. pallida* were obtained and compared to the sequence obtained from the PCR of Ukrainian population. The sequence from the Ukrainian population had 99.03–99.57% similarity with other *G. pallida* P5A where the similarity was 97.8% and the similarity for *G. rostochiensis* was 96.82% (Blok et al. 1998; Subbotin et al. 2001). Using a novel approach based on melting peak analysis of PCR products, a semi-quantitative assay developed to measure the relative proportions of *G. pallida* and *G. rostochiensis* in a sample of PCN. The method depends on a competitive multiplex PCR where the products of each species can be separated by their distinct melting temperatures (T_m). The melting curves of the products are measured by continual fluorescence monitoring in the presence of the intercalating dye SYBR Green 1 which gradually increasing the temperature. Varying proportion of cysts of each PCN species gave rise to melting curves with different peak heights, which reflected the relative amounts of each DNA in the sample (Jayne et al. 2002). Multiplex-PCR amplification of specific regions of the PCN genomes (ITS1 and ITS2) is a sensitive method which can be used for taxonomic purposes of cyst samples which vary in age. This technique could be used for nematode populations which were directly sampled from potato field and resulted in both young and old cysts in the soil (Pylypenko et al. 2005).

RFLP of the rDNA ITS region, sequence-specific multiplex PCR and DNA sequence were used for comparisons and confirmed the identity of the Idaho population as *G. pallida*. The ITS rDNA sequence of the Idaho isolate was identical to those from York, England and the Netherlands isolates (Skantar et al. 2007). Two populations of *G. pallida* from Ukraine with the other European and South American populations compared by using cytochrome b gene partial sequence. They found that Ukrainian populations were identical to each other and to a number of European populations and they all clustered in first clade from the south of Peru (Pylypenko et al. 2008). Species-specific primers provided a new assay for distinguishing this species from *G. pallida* and *G. rostochiensis*. Isolates of *G. rostochiensis* amplified a single 914 bp product and were distinguishable from *G. pallida* isolates which

Table 5 Sequence of TaqMan primers and probes for amplification of rDNA internal transcribed spacer I region of *Globodera* spp

Primer or probe name	5'–3' Sequence
PITSpfa	ACGGACACATGCCCCGCTA
PITSp4b	ACAACAGCAATCGTCGAG
GFAMpa	FAM/ACATGAGTGTGGGGTGTAAAC/BHQ-1
PGrtfa	TCTGTGCGTCGTTGAGC
Prostorc	CGCAGACATGCCGCAA
GTETpa	TET/CGCAGATATGCTAACATGGAGTGTAG/BHQ-2
Ptab-rt2fa	TCGTTGAGCGGTTGTTGC
PITSt3mrd	AGCGCAGATATGCCGCG

amplified 914 bp and 853 bp products. Some PA1 isolates amplified both 914 and 853 bp products. This type of test can also be used for direct detection of target species without cyst morphological detection (Reid et al. 2010). The primers PCN280f and NEPCN398r were designed for the quantitative detection of *G. rostochiensis* using real-time PCR. 1, 5, 50, 125 and 250 individuals of the second-stage juveniles (J_2) of *G. rostochiensis* were mixed with various stages of vermiform *Caenorhabditis elegans* to make a total of 500 individuals and DNA was extracted from the nematode mixture. There was a significant correlation ($r^2 = 0.9355$, $P < 0.001$) between the threshold cycle values and number of *G. rostochiensis* added. There was correlation in the numbers of *G. rostochiensis* J_2 from the real-time PCR method. Real-time PCR sensitively detected a single *G. rostochiensis* J_2 out of 1000 individuals of free-living nematodes (Koki et al. 2008). TaqMan primer-probe sets developed for the detection and identification of *G. rostochiensis* and *G. pallida* using two-tube real-time PCR. The ITS rDNA-based system was specific for the identified *Globodera* spp (Nakhla et al. 2010) (Table 5).

Twelve new microsatellite markers were developed and used to know the genetic diversity and origin of fifteen populations of PCN which originating from Quebec, Canada introduced in North America. Two Quebec populations were very similar to each other and to the population found in newfound land, but they were significantly different from three other North American populations including those from New York and British Columbia (Bossis and Mugniéry 1993). Universal primers were used for identification of mixed occurrence of two *Globodera* spp. in Central Europe by multiplex PCR (Douda et al. 2014). The internal transcribed spacer region (ITS 1–5.8S–ITS2) of the rRNA gene and D2–D3 expansion segments of the 28S rRNA gene were amplified and sequenced for confirmation of PCN species which collected from Europe (Madani et al. 2010) (Table 6).

Table 6 Sequence of primers used for amplification selected region

Primer name	Sequence (5′–3′)	Amplified region	Source
rDNA1	TTGATTACGTCCCTGCCCTTT	ITS1-5.8s-ITS2 rRNA	Vrain et al. (1992)
rDNA2	TTTCACTCGCCGTTACTAAGG	ITS1-5.8s-ITS2 rRNA	
INRAcytL	GGGTGTGGCCTTGTTATTTTC	cytb of mtDNA	Picard et al. (2007)
INRAcytR	ACCAGCTAAAACCCCATCCT	cytb of mtDNA	
D2A	ACAAGTACCGTGAGGGAAAGTTG	D2-D3 of 28S rRNA	Nunn (1992)
D3B	TCGGAAGGAACCAGCTACTA	D2-D3 of 28S rRNA	

Table 7 Summary of parasitism genes of potato cyst nematodes

Accession no.	Gene name	Gene product	References
Protection against plant defence response			
AJ243736	Gr-tpx-1	Peroxiredoxin	Robertson et al. (2000)
Y09293	Gp-far-1	Fatty acid and retinol binding protein	Prior et al. (2001)
AJ493677	Gr-gpx-1	Glutathione peroxidase	Jones et al. (2004)
Cell wall modifying proteins			
AF004523	Gr-eng1	β -1,4-endoglucanase	Smant et al. (1998)
AF004716	Gr-eng2		
AF127915	Gr -pel-1	Pectate lyase	Popeijus et al. (2000)
AJ311902	Gr-expb2	Expansin	Qin et al. (2004)
Putative parasitism genes			
AJ271910	Gr-sxp-1	SXP/RAL-2	Jones et al. (2000)
AJ270995	Gr-ams-1		
AJ300178	Gp-nex	Annexin	Dalzell et al. (2009)
AJ251758	Gr-A41	RanBPM homologue	Qin (2001)
AJ251757	Gr-A18		
AJ457834	Gp-cm-1	Chorismate mutase	Jones et al. (2003)

Identification of pathogenicity factor/parasitism gene

The genes that encode nematode secretions directly involved in the parasitism of the host are collectively termed “parasitism genes” (Davis and Tylka 2000). The generation of cDNA libraries and expressed sequenced tags (ESTs) from parasitic stages of nematodes is a powerful approach to discover genes involved in nematode parasitism (Table 7). Expressed sequence tags coupled with bioinformatic tools were used to deduce potential gene identities from *G. rostochiensis* and revealed parasitism genes such as pectate lyase (Popeijus et al. 2000).

Putative pathogenicity factors, A18 (Gr-A18) and A41 (Gr-A41) were identified from the dorsal or sub-ventral esophageal glands of *G. rostochiensis*. The partial sequence of these factors showed no significant homology to any known gene. Their presence in the dorsal glands of infective juveniles suggests that these proteins could be involved in feeding cell initiation or in protection against plant defense responses (Qin et al. 2000) Peroxiredoxin gene (Gr-tpx-1) in

sub-ventral gland cells of J₂s of *G. rostochiensis* catalyses the breakdown of hydrogen peroxide therefore, it plays a role in protection of the parasite from plant defence responses (Robertson et al. 2000). Gp-far-1 protein secreted from *G. pallida*, a member of the nematode-specific fatty-acid- and retinol-binding (FAR) family of proteins, localizes to the surface of this species. Gp-far-1 binds two lipids (linolenic and linoleic acids) that are precursors of plant defense compounds and the jasmonic acid signalling pathway (Prior et al. 2001). Annexins are calcium dependent phospholipid binding proteins, these proteins have a strong affinity towards acidic phospholipid membranes and actin filaments in a calcium-dependent manner. It is present in excretory/secretory products from *G. pallida* J₂. Gp-nex encodes annexins and was immunolocalised in the amphids, genital primordium and in the constraining muscles of *G. pallida* J₂ (Fioretti et al. 2001).

Collagen gene, gp-col-1 and gp-col-2 were reported in adult virgin and gravid females of PCN causing various changes in cuticular structure which accompany nematode growth and reproduction (Gray et al. 2001). The third gene

isolated, gp-col-t, consists of a partial collagen sequence which represent an unexpressed pseudo gene. Glutathione peroxidases (GpXs) encode two proteins in PCN, one protein has a functional signal peptide that targets the protein for secretion from animal cells while the other is predicted to be intracellular. The intracellular protein is likely to have a role in metabolism of active oxygen species derived from internal body metabolism while the secreted protein may protect the parasite from host defenses (Jones et al. 2004). A secreted protein (β -1,4-endoglucanase) were purified and characterized first time from the subventral glands of PCN (Smant et al. 1998). The four cellulase genes from *G. rostochiensis* (*Gr-eng1*, *Gr-eng2*, *Gr-eng3* and *Gr-eng4*) are specifically expressed in the sub-ventral esophageal glands of the nematodes during their invasion of host plants (Rehman et al. 2008). The discovery of an expansin-like protein in the secretions of *G. rostochiensis* was the first report of expansins outside of the plant kingdom and a benchmark example of potential molecular mimicry of host molecules by a phytoparasitic nematode. Expansins are a group of plant cell wall loosening proteins which (exp B1) act as the parasitism factors or effectors in *Globodera rostochiensis*. Molecular variability of another expansins (exp B2) genes evaluated in diverse-populations of the *G. rostochiensis* (Qin et al. 2004). *Gr-CLE* genes were specifically expressed within the dorsal esophageal gland cell of PCN parasitic stages. *Gr-CLE* genes encode CLE proteins to facilitate parasitism and initiate feeding structure in the host root. Five genes which contained multiple CLE motifs were identified from the PCN. *Gr-CLE-1* was unregulated in the early parasitic stages with a level much lower than that of *Gr-CLE-4* genes and declined in later parasitic stages, whereas *Gr-CLE-4* genes were dramatically up regulated in early parasitic stages and remained high in later parasitic stages (Shun-Wen et al. 2009, Lu et al. 2009). Gp-RBP-1 possessing a secretion signal peptide is expressed in dorsal esophageal gland. Secreted protein RBP-1 from *G. pallida* elicits defense responses through the NB-LRR protein Gpa2 from cell death typical of a hypersensitive response (HR) in host plant (Sacco et al. 2009). Secreted SPRYSEC effector proteins which contain the B30.2/SPRY domain are specifically expressed in the dorsal esophageal gland of *G. rostochiensis* in the parasitic cycle (Qin et al. 2000) and having key role in biological processes, including regulation of cytokine signaling, RNA metabolism, calcium release and developmental processes (Rehman et al. 2009).

RNA-mediated interference (RNAi)

An exciting and emerging alternative eradication strategy, is the use of RNA-mediated interference (RNAi) to identify target genes, silence gene expression of target (Fire et al. 1998) and assess its potential effects on parasitism (Baum

et al. 2006; Bakhetia et al. 2005; Davis et al. 2004; Lilley et al. 2007). RNAi is a natural, novel and eco-friendly defense strategy that delivers a cost effective control of PCN. The birth of RNAi technology from classical *Caenorhabditis elegans* studies has shown the ways and means to explore the possibilities of this mechanism for protecting plants from nematode damage (Godelieve and Vanholme 2007). RNAi pathway elicits sequence-specific silencing of targeted mRNA transcripts through the introduction of homologous double-stranded (ds) RNA to a feeding nematode to incapacitate or kill it. In this way, RNAi represents an attractive prospect as a reverse genetic tool. The utility of RNAi for functional analysis of plant-parasitic nematode genes was first reported in 2002.

Genes encoding a cysteine proteinase, *gpcp-1* (Urwin et al. 2002) and major sperm protein *msp-1*, *msp-2*, *msp-3* (Novitski et al. 1993) of PCNs were targeted by soaking infective J₂s in a solution of dsRNA with the addition of the neurochemical octopamine to stimulate ingestion of dsRNA. The major sperm protein (MSP) had no effect on either PCN development or sexual fate after 14 days of treatment. In addition to this, reduction in transcript abundance for targeted mRNAs in the infective juvenile. Targeting cysteine proteinases did not reduce the number of parasites but caused a shift from the normal female/male ratio of 3:1–1:1 by 14 days post infection (dpi). They reported that by increasing the incubation periods (4 h to 7 days) of pre parasitic nematodes to dsRNA resulted effective silencing for some genes in cyst nematodes (Urwin et al. 2002). Knockout of the β -1,4-endoglucanases reduced the ability of the PCN to invade roots. They also used RNAi for *gr-ams-1* gene which is a secreted protein of the main sense organ (the amphids) and essential for searching the host location (Qing et al. 2005). The *gr-ams-1* dsRNA treated nematodes were almost completely unable to locate host plants compared with controls. In addition to 16D10 (Davis et al. 2008) in *G. rostochiensis*, reduction of the transcript-encoding amphidial secretory protein AMS-1 by dsRNA soaking of J₂ greatly reduced the ability of worms to locate host plants (Chen et al. 2005). Exapansins (Exp gene) are a group of plant cell wall loosening proteins found among the parasitism factors of plant parasitic nematodes. Gr-Exp genes were demonstrated to have cell-wall extension activity *in planta* (Qin et al. 2004; Kudla et al. 2005). In vitro secreted chorismate mutase (CM) from PCN can balance a CM-defective bacterial mutant and modify the cellular shikimic acid pathway to affect a number of outcomes. Over expression of nematode CM in plant roots leads to altered plant tissue development and potential modification of cellular partitioning of IAA (Jones et al. 2003).

RNAi of CM gene and pectate lyase (*Gr-pel-1*) individually resulted in the increased number of males (Bakhetia et al.

Table 8 List of Gp-flp-12 siRNAs for gene silencing in *G. pallida*

Gene (Gene bank accession no.)	Species	Si RNA strand sequence (S: 5′–3′; A:3′–5′)	Construct size (bp)
Gp-flp-12 siRNA 1 (Aj300488)	<i>G. pallida</i>	S: UGAACGGAGUUGAUGGGGAUU A: UUACUUGCCUCAACUACCCCU	21
Gp-flp-12 siRNA 1a (Aj300488)	<i>G. pallida</i>	S: aGAACGGAGUUGAUGGGGAUU A: UUaCUUGCCUCAACUACCCCU	21
Gp-flp-12 siRNA 1b (Aj300488)	<i>G. pallida</i>	S: UGAACGGAGUUGAUGcGGAUU A: UUACUUGCCUCAACUACcCCU	21
Gp-flp-12 siRNA 2 (Aj300488)	<i>G. pallida</i>	S: CGCUUCUGAUGCUGUCGGGUU A: UCGCGAAGACUACGACAGCCC	21
Gp-flp-12 siRNA 3 (Aj300488)	<i>G. pallida</i>	S: GGAAUCCCGACUUUUGGGCUU A: UACCUUAGGGCUGAAAACCCG	21
Gp-flp-12 siRNA 4 (Aj300488)	<i>G. pallida</i>	S: AGAUGCUCCCAUCGUCCCCUU A:UCUCUACGAGGGUAGCAGGGG	21
Gp-flp-12 siRNA 5 (Aj300488)	<i>G. pallida</i>	S: ACGACCGACCAAUGAACGGUU A: UUUGCUGGCUGGUUACUUGCC	21

2007). The FMRFamide-like peptides (FLPs) form the largest and most diverse family of neuropeptides present in across nematode species and FLPs modulate sensory and motor functions of nematode. Neuromuscular function is a well-established target for parasite control, parasitic nematode FLP signaling has significant potential in novel control strategies. In attempts to evaluate flp function in *G. pallida*, it was discovered that, silencing of the five siRNAs, (Gp-flp-1, -6, -12, -14, or -18) in *G. pallida*, obtained abnormal behavioral phenotype changes (Dalzell et al. 2010). Further delineation of these effects revealed that double-stranded RNA exposure time (≥ 18 h) and concentration (≥ 0.1 $\mu\text{g/ml}$) were critical to the observed effects which were reversible. The flp genes in *G. pallida* are essential to coordinate locomotory activities, do not display redundancy and are susceptible to RNAi, paving the way for the investigation of RNAi-mediated flp gene silencing as a novel plant parasite control strategy (Kimber et al. 2007). This inhibitory phenomenon is time and concentration-dependent, a tenfold increase in dsRNA to 1 mg/ml shown comparable irregularity of phenotype and inhibition of mortality in *G. pallida*, following a 24 h soak in 0.1 mg/ml dsRNA (Dalzell et al. 2009) (Table 8) (Fig. 1).

Gp-flp-32 encodes the peptide AMRNALVRF amide and it is expressed in the brain and ventral nerve cord of *G. pallida* (Fig. 2). After treatment with dsRNA, migration rate of worms and the ability to infect potato root systems was increased.

Due to increased rates of migration and infection would more rapidly deplete the finite energy reserves in the non-feeding J₂s which resulted premature death. A novel putative Gp-flp-32 receptor (Gp-flp-32R) is expressed in *G. pallida* and Gp-flp-32R-silenced worms also display an increase in migration rate (Atkinson et al. 2013) (Table 9).

PCN live in an intimate association with their host plants, the outcome of interaction is solely dependent on

the interplay between molecules at the interface of the host plant and nematodes. Nematodes secrete protein molecules, termed effectors, which modify the host physiology to complete host plant colonization. Most significant effectors are the proteins injected into plant cells to suppress the host defense 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) (Makunde 2012).

Expression of the aldicarb-like peptides as secretory products in transgenic potato resulted in root exudates with acetylcholinesterase-blocking activity, which in greenhouse trials reduced *G. pallida* infection with cyst number declining 36–48% relative to vector controls (Liu et al. 2005). Peptide mimics of levamisole also reduced *Globobera* infection in a potato hairy root system. In an alternative approach, (Fioretti et al. 2002) characterized monoclonal antibodies directed against *G. pallida* amphidial secretions and found that J₂ soaked in these antibodies showed diminished mobility and delayed invasion of potato roots.

Conclusions

Protein analysis is an effective tool for the characterization and identification of plant-parasitic nematodes. It is important for direct examination of genetic material or DNA analysis by using RAPD, AFLP, RFLP markers. ITS region of rRNA and rDNA is potentially the most powerful

Fig. 1 Double-stranded (ds) RNA produced in the plant can lead to RNA silencing in plant-parasitic nematodes. A transgenic plant is generated that expresses dsRNA (a) targeting a specific nematode gene. The dsRNA is processed by the plant DICER enzyme (b) into small interfering (si) RNAs (c). The dsRNA and/or the siRNAs are ingested by the nematode (d) when feeding from the plant cell. If longer dsRNAs are ingested, the processing from dsRNA to siRNA can be performed by the nematode DICER. siRNA (e) is recognized by the RISC complex of the nematode and unwound in sense and antisense strands. Part of the RISC complex loaded with the antisense strand interacts with the corresponding mRNA in the nematode (f). The mRNA is cleaved (g) and degraded (h). Alternatively, the targeted mRNA can be made double-stranded after binding of the siRNA, and this dsRNA is then processed to form additional siRNAs, amplifying the initial silencing signal. In plants as well as in nematodes, RNA silencing also generates a systemic signal that can be transported across cell boundaries. Reproduced from Godelieve and Vanholme (2007)

method for PCN diagnosis and to measure genetic variability. Many works have published on DNA methods for detection and quantification of PCN at species level, but not at pathotype level. The current methods are not yet more sensitive for determining pathotypes in species. However, there is a focused need for further improvement of these technologies for DNA identification at pathotype level in PCN. Correct identification of species and pathotypes by these techniques will be opened the new ways for resistance breeding by pyramiding resistance gene against PCN and will structure an important and available component of IPM. Apart of this, the current approaches like expression of effectors genes in plants, multiple nematode effectors function in parasitism and identification of target proteins secreted from PCN may be of wonderful tool for

Fig. 2 Gp-flp-32 express in the brain and ventral nerve cords of pre-parasitic juveniles (J₂s). Reproduced from Atkinson et al. (2013)

Table 9 Gp-flp-32 Short interfering (si) RNA sequences

siRNA sequence	siRNA name
Gp flp32	AAGCGATGCGAAATGCGCTCG
Gp32R flp	TAAGGTCGCAAGTGCAATTTT
Mi flp32	AAAAGAAATTCAACGATGGAA
Positive control Gp ace	AATAGTACGGTAGCCAGTAGG
Non native negative control	AACCCTCCCCCTTGACTCATT

managing and eradication of PCN. By using RNAi or gene silencing approach can silence specific gene which responsible for producing pathogenicity proteins in phytoparasitic nematodes. RNAi has proved as an outstanding tool for the functional analyses of cyst nematode parasitism genes. Although it has not been showed on field level but it is possible that dsRNA can be delivered to the feeding nematode to interfere with normal gene function in the nematode. Hence, it will further built up support for innovative research towards the development of PCN resistant transgenic plants.

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