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Decades of researches in biochemical and molecular nematology at IARI

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A B S T R A C T

Like any other branch of Agricultural Sciences, the Division of Nematology at IARI, initiated researches in the exciting field of biochemistry and molecular biology of plant parasitic nematodes during the early seventies of last century. The main research areas were biochemical mechanism of resistance against phytonematodes and diagnostics of economically important plant parasitic nematodes. Specific isozymes have been identified for differentiating the important root knot nematodes species of India belonging to the genus *Meloiodogyne*. Further, molecular markers like RAPDs, AFLP, SSRs and PCR–RFLP of rDNA have been employed to study the molecular genetic diversity in major root knot and cyst nematode species of India to assist the breeding program for developing resistant varieties and other purposes like regulatory and nematode diagnostics. Attempts have also been to made to understand the molecular signaling events during compatible plant nematode interactions by identifying the genes during the transition from preparasitic to parasitic stage in ear cockle nematode and molya disease of wheat. Currently attempts are being made to utilize RNAi technology to identify the genes targets for the management of root knot and cyst nematodes. In this paper some of the success stories of the technology developed by the scientists at IARI are discussed.

Introduction

Problems related to the availability of effective single gene resistance to nematodes, their commercial viability and durability under threat from different species, pathotypes or races forced the different workers around the globe to apply biotechnology and molecular biology in the field of plant nematology. Further, concern over the environmental impact of some highly toxic non-specific nematicides and their expenses prompted a greater emphasis on alternative control methods, especially the development of nematode-resistant crop cultivars by way of genetic engineering. In addition, the formation of feeding cell structure a unique feature on which sedentary endoparasitic nematodes sustain attracted the molecular biologists to study the gene regulation of the events. Rapid progress in the development of molecular techniques has made it possible to work with minute quantities of DNA extracted from microorganisms like phytonematodes for molecular characterization. In this article an overview on the progress on biochemical and molecular research at IARI on plant parasitic nematodes and some of the recent developments in molecular nematology elsewhere have been presented.

Biochemical and molecular basis of plant nematode interaction

Biochemical and Molecular Nematology started at IARI in early seventies with modest research facilities. Initially studies relating to mechanism of host resistance to the root-knot nematode *Meloidogyne incognita* were made to elucidate biochemical basis of resistance in tomato and cowpea. Activities of several enzymes like Peroxidase (PO), Indole acetic acid (IAA), Phenylalanine ammonia lyase (PAL), Thyrosine ammonia lyase (TAL), Poly phenol oxidase (PPO), Superoxide dismutase (SOD) and Acid phosphate as the expression of host reaction to nematode inoculation were worked out.

Time course studies of some of these enzymes demonstrated that the biochemical mechanism got activated soon after nematode inoculation. The initial responses were very important in deciphering the fate of the nematode plant relationship. The resistant cultivars showed a hypersensitive effect wherein the biochemical activity of these enzymes was very high. Peak in the activity of these enzymes reached in between 72 to 96 hours of nematode inoculation.

On the basis of results reported from our laboratory and elsewhere a model was proposed by Premachandran and Dasgupta (1983) to explain the plant nematode interaction. This model has three important aspects: (i) Emphasis on the significance of early biochemical events which occur soon after the infection of host with the nematode; (ii) the role of DNA dependent RNA polymerase and RNA synthesized de

novo in coding altered pattern of transcription of synthesis of polypeptides required to set in either compatible or incompatible host reaction to the parasite and (iii) the significance and decisive role of the host besides that of the parasite in deciding the course of disease development. Further, the model predicts that the resistant expression in root knot nematode infected plan is mediated by gene expression at transcriptional level.

Hydrolytic enzymes

The first reports in literature on isolation, purification and characterization of trypsin like protease from the root-knot nematode, *M. incognita* was submitted from this Division (Dasgupta and Ganguly,1975). In addition various hydrolytic enzymes including amylase, esterases, trehalose, cellulose and proteases were detected from the tissue homogenates of the infective stage of the root-knot nematode, *M. incognita*. The results of these investigations revealed that the second-stage juveniles of *M. incognita* have the potentiality of hydrolyzing major host components like carbohydrates and proteins. These findings signify the possible role of digestive enzymes from this nematode in altering host metabolism following parasitic invasion which ultimately leads to diseased condition of the hosts.

Phyto-nematodes Diagnostics

Plant parasitic nematodes are microscopic in size making it a formidable task even for well-trained nematode taxonomists to make precise and reliable identification. Moreover many of the genera exhibit little morphological diversity. Correct identification is very important for every aspect of nematological research and to provide effective management strategy. The application of protein and especially DNA based diagnostics has increased interest of the scientific community in nematode taxonomy and systematics and has revolutionized nematode phylogenetic analysis. During the last two decades molecular techniques both protein and DNA based have been widely used to confirm the variability of existing nematode species and to assist in the identification and description of new species. Division of Nematology have started using esterase enzyme banding pattern for identification of species of *Meloidogyne*, the most common root-knot nematode problem using soluble protein extract of a young single female or from the galled root pieces harboring a female (Ganguly and Dasgupta, 1989). The enzyme banding patterns are also used for *Heterodera* spp., *Rotylenchulus* spp. etc. Intraspecific variation in terms of races/pathotypes/biotypes is found in *Meloidogyne, Heterodera, Globodera* etc. Use of DNA-based diagnostics has several advantages over protein based methods especially to exclude the effect of environmental factors, developmental variation and expression of a particular gene. The current practice of identification of races using host differential is time-consuming and environment-dependent. Molecular diagnostics can overcome limitation of identification based on morphological characters which are dependent on particular life stages (usually adult and sometime both sexes) for critical characters and the availability of trained taxonomists with particular skills needed to identify. Plant nematodes are approximately 1 mm in size and composed of 1000 to 2000 cells the size is suitable for a polymerase chain reaction (PCR) with a minimum effort required for preparation of DNA. Using PCR it is now possible to produce millions of copies of targeted DNA sequence and the product can be subsequently separated by electrophoresis. Nematode DNA can be further characterized by RFLP, AFLP, dot blotting or sequencing. Also DNA-based techniques do not rely on expression of a gene product as required for isozyme analysis.

Most DNA finger-printing studies have been done on nematode species that are economically important such as species of *Meloidogyne*, *Pratylenchus*, *Radopholus*,

Table 1
Enzymes used in plant nematode identification

Enzymes	Nematodes
Esterase and Malate dehydrogenase	*Meloidogyne*
	Heterodera
	Bursaphelenchus
	Rotylenchulus
	Aphelenchoides
	Radopholus
Alkaline phosphatase	*Steinernema* *Heterorhabditis*
Superoxide dismutase	*Heterodera*
Phosphate glucose	Banana race of *Radopholus*

Heterodera, *Globodera* that offer regulatory concerns. Further these studies also extended the understanding of the evolutionary and phytogenetic relationships among the species of a taxon. Diagnostic methods with individual juvenile, female, cyst or egg are routinely used which are sensitive enough to detect specific species in a given sample.

The DNA sequence variation of the four races of *M. incognita* helps us in differentiating the four races. Phylogenic tree obtained by alignment of the sequences of internal transcribed spacers of ribosomal DNA gene complex (ITS of rDNA) for the four races indicated that race 1 and race 4 are most related and race 3 is least related to other races. Further race 2 is more closely related to race 1 and race 4 than race 3. Different restriction sites for the four races were also obtained using DNA map of DNA star software, which can be used to have RFLP pattern of different races for discrimination of the races. Enzymes like *Bst*UI, *Bst*XI, *Mlu*I, *BsaH*I, *Hga*I, *Sfa*NI, *BsmF*I, *BSiE*I and *DdeE*I which give different restriction sites in nucleotide sequence of different races can be used to differentiate the four races. But these enzymes were needed to be tried on unknown populations to conclude that these generate race-specific PCR-RFLP pattern facilitating them to be used in nematode diagnostics. The information regarding different restriction sites in nucleotide sequence of different races may be utilized for quarantine purposes i.e. the PCR-RFLP pattern of four Indian races can be used as reference to compare the PCR-RFLP of ITS of any population of *M. incognita* that might come as a contaminant with any planting material that is being imported.

To determine the intraspecific variability seven populations of *Heterodera avenae* from Delhi, Sirsa, Jhansi, Tikamgarh (MP), Jaipur, Udaipur and Ludhiana were subjected to random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis. The dendrograms obtained based on UPGMA clustering of a similarity matrix generated by Jaccard's indicated that populations of Sirsa, Tikamgarh, Udaipur and Ludhiana are more closely related forming one cluster to which Jaipur population is related. This indicates that there is a tremendous amount of genetic variability among the different geographical populations with a possibility of more variability within a population hitherto unknown.

Studies were undertaken on the utility of a molecular diagnostic method for potato cyst nematode (*Globodera pallida* and *G. rostochiensis*) of the Nilgiri Hills. A random operon primer OPG 5 that can differentiate between the two species of *Globodera* has been used to characterize the gene pool similarity of the field populations of potato cyst nematode from Nilgiri Hills. Work was carried on molecular characterization of *Steinernema thermophilum* Ganguly and Singh (Nematoda: Steinernematidae). This species has been compared with four indigenous isolates (one of *S. glaseri* and three unidentified) of *Steinernema* for determining the genetic relatedness by random amplified polymorphic DNA (RAPD) markers. Eleven decamer oligonucleotide primers were used to generate random amplified polymorphic DNA fragments from five populations. The primers generated 177 fragments, ranging from 3000 base pairs to 200 base pairs in size. *S. thermophilum* has been found to be very different from all other isolates and two isolates S1 and S2 originating from Gujarat are the most closely related with only 40% similarity. The present findings give clear indication that *S. thermophilum* from IARI, New Delhi is genetically different from other isolates/spp. analyzed. Table 2 has furnished some of the success of utilizing the molecular diagnostic in Nematology.

Table 2
Examples of using molecular diagnostics in Plant Nematology

Nematodes	Authors
Meloidogyne mayaguensis	Block *et al*. 2002
Meloidogyne izalcoensis	Carneiro *et al*. 2005
Meloidogyne paramoensis	Carneiro *et al*. 2005
Bursaphelenchus xylophilus	
Heterodera filipjevi	Uma Rao *et al*. 2004
Ditylenchus africanus	Wendr *et al*. 1995
Meloidogyne incognita, M. javanica, M. arenaria	Zijlstra *et al*. 2000
M. hapla, M.chitwoodi, M. fallax *Radopholus citri, R. brigei* and *R. similis*	Kaplan *et al*. 2000
Globodera pallida and *G.. rostochiensis*	Subbotin *et al*. 2000
Pratylenchus	Waeyenberge *et al*. 2000

Molecular characterization of crops against *Meloidogyne* and *Heterodera* spp.

The management of plant parasitic nematode has relied upon three primary methods, *viz*., rotation with alternative crops, use of nematicides and resistant cultivars. Crop rotation is neither effective against polyphagous nematodes nor economical from the growers' point of view. Use of nematicides is becoming restricted due to health and environment concerns. The use of host resistance is, therefore, the most environmentally and economically acceptable method. Unfortunately genetic resistance to plant parasitic nematodes does not occur in all cultivated host species. Engineered resistance to nematodes has been the subject of research and development for several years.

Engineering resistance has been classified into three categories:

Transfer of existing resistance

Transferring *R* genes to susceptible hosts would seem to offer an effective and long-term solution to disease. The methodology developed eventually led to the cloning of the *Hs1^{pro1}* locus (Cai *et al*. 1997). The *Mi* gene, which confers resistance to *M. Incognita* and several other species of *Meloidogyne*, as part of a larger DNA fragment (Milligan *et al*. 1997) of which the *Mi1.2* element was shown to be involved in resistance not only to nematodes but also to aphids, *Gpa2* (van der Vossen *et al*. 2000), m*Hero* (Ernst *et al*. 2002) and *Gro1-4* (Paal *et al*. 2004), all of which conferred resistance to cyst nematodes (Table 3). With an increase in the understanding of how the resistance genes exert their function, it may be possible in future to broaden the specificity of a resistance or increase its durability by direct modification.

Table 3
Cloned nematode resistance genes

Gene	Resistance
Hs1^{pro1}	Resistance to best cyst nematode from *Beta procumbens* (Cal *et al*.1997)
Hi gene of tomato	Resistance to *Meloidogyne incognita* and *M. javanica* isolated from tomatoes (Milligan *et al*. 1998)
Gpa2	Resistance to *Globodera pallida* identified in potato (van der Vossen *et al*. 2000)
Hero	Resistance to *Globodera* isolated from tomato (Ernst *et al*. 2002)
Gro1-4	*Resistance gene* against *Globodera rostochiensis* (Paal *et al.* 2004)

Targetting Nematode as antinematode resistance gene

An alternative method to engineer broad spectrum resistance which is not only active against sedentary nematodes but also to many damaging species of migratory nematodes employs effective antinematode genes.The key opportunities expressing anti-nematode genes occur during (a) invasion, (b) feeding site initiation, (c) early feeding of the parasite, (d) feeding and digestion of pre-adults, (e) feeding and digestion of the adult female, and (f) its reproductive development offer opportunities for engineered resistance. Many of these opportunities are common to endoparasitic nematodes irrespective of their precise mode of feeding. Specific expression of genes that affect plant cells may hinder invasion (i) prevents feeding site initiation, (ii) or full development of the feeding site into a plant transfer system (iii). Other nematodes that modify plant cells offer similar opportunities for engineered resistance. Anti- nematode gene is defined as one that produces a peptide/protein that is toxic, damaging or inhibitory to nematodes but is not deleterious or significantly less so to the host plant or to the animals/humans that will eventually feed on the plant or its products. Several classes of potential anti-nematode genes encoding lectins, enzymes and enzyme inhibitors are being evaluated for their ability to confer broad-spectrum nematode resistance.

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A number of proteinase inhibitors expressed in plants have been reported affecting the growth and development of plant parasitic nematodes (Table 4). In fact, this approach has resulted in the production of plants with altered response to cyst nematodes. It has been observed that tomato root cultures expressing the rice oryza cystatin proteinase inhibitor gene did not support normal growth and development of the potato cyst nematode, *Globodera pallida*. Vain *et al*. (1998) have

Table 4
Protease inhibitors as anti-nematode genes

Protease inhibitors	Crop	Effect of nematode
CpTi	Transgenic potato	Reduced the fecundity of *Meloidogyne* spp., sex reversal of *G.. pallida* to produce less damaging males
CpTi	Transgenic tobacco	Alternation of sex in *G.. tabaccum*
Cystine proteinase inhibitor (Oryzacystatin-I)	Transgenic tomato	Slow growth of *G.. tabaccum*
Cystine proteinase inhibitor (Oryzacystatin-I)	Transgenic tomato	Slow growth of *G.. pallida*, size of female reducede and egg production prevented
Serine protease	Transgenic *Arabidopsis*	Slow growth of female of *Heterodera schachtii*
Cysteine proteinase inhibitor (*S. tuberosum tuberosum*)	Transgenic potato	*G.. pallida*

engineered rice with a gene-encoding oryzacystatin. Transformed plant expressed with a proteinase inhibitor up to 0.2% of the total soluble protein. This resulted in a significant 55% reduction in egg production by *M. incognita*. Alteration of the sex ratio by CpTI (Cowpea trypsin proteinase inhibitor) was also noted for tabacum on transgenic tobacco and is presumably triggered by nutritional stress experienced by juveniles soon after initiation of feeding (Burrows and De Waele, 1997). Cystine Pls are likely to pose a valuable class of anti-nematode genes, especially since cystine proteinase inhibitor expressed in transgenic potato, provided resistance against *Globodera pallida* under field conditions. The advantage of these strategies is that they are pre-formed defense like α-terthienyl and do not require any nematode infection for induced resistance.

Antibodies

Antibodies produced in plants (plantibodies) are being investigated as a means of conferring resistance to pests and pathogens. Antibodies against nematode esophageal gland secretions may be utilized to disrupt either feeding site formation or feeding behavior (Hussey, 1989) via expression of functional antibodies in plants (Hiatt *et al*. 1989). Plantibodies that selectively neutralize such enzymes could disrupt this aspect of host parasite relationship. The monoclonal antibody specific to stylet secretions of *M. incognita* were expressed in tobacco (Baum *et al*. 1996). Unfortunately, despite the antibodies binding to the nematode pharyngeal glands and stylet secretions, no reduction in the ability of the nematode to parasitize the tobacco roots was seen. Although it is not entirely clear why the plantibodies failed to disrupt giant cell formation by inhibiting stylet secretion action, one feasible explanation is that the plantibodies accumulated apoplastically, whilst nematode stylet secretion was delivered to the cytoplasm. Plantibodies have also been generated that recognize secretory-excretory proteins on the cuticle surface and the amphids of *Globodera pallida* and *G. rostochiensis*. The plantibodies were shown to affect nematode movement and resulted in delayed root penetration. However, these effects were temporary as turnover of the secreted proteins resulted in loss of the bound antibody (Fioretti *et al*. 2002).

Targeting the nematode feeding site

In the case of endoparasitic nematodes, such as species of root-knot and cyst nematodes, an alternative strategy to targeting the nematode directly is to disrupt the feeding sites that these nematodes initiate within the plant. The aim of this approach is to destroy the nutrient supply by expressing nematicidal gene product resulting in feeding cell death or site metabolic attenuation. Once the larvae have set up a feeding site it becomes sessile and its continued existence and development are entirely dependent on the feeding cells. Therefore, killing the feeding cell would result in the death of the nematode and thus confer resistance in the host plant. This could be achieved theoretically by expressing a cell death gene specifically within the feeding site upon its induction. Primarily, this approach would require a promoter that would drive the cell death system specifically in the nematode feeding cell; the value of such a promoter is self-evident.

Disruption of feeding structures can be achieved by (1) Tissue specific expression of a toxic protein, and (2) Down regulation of a component necessary for development or maintenance of feeding cells by anti-sense inhibition or cosuppression. Another possibility is to suppress the normal

abundance of a mRNA species that is essential for its maintenances (3) To activate the plant's internal capacity for programmed cell death or apoptosis, which occurs during normal development and also associated with environmental stress or pathogen attack. So far, transgenic approach for nematode resistance by triggering apoptosis selectively in nematode–induced feeding structure has not been reported. But as the understanding of the signals and components in this process increases, such an approach may be feasible in the near future.

Exploitation of RNA Interference nematode management

RNA interference (RNAi) is one of the biggest scientific breakthroughs of recent times. RNAi offers to be one of the easiest ways to unravel the functions of a gene in a disease or interaction by switching it off easily. As a result it can be used to characterize the biological function of the genes contained in their genomes.

The RNAi technology offers a promising future to manage the damage caused by plant parasitic nematodes to crop plants. Plants can be designed to produce dsRNAs which can then silence specific essential genes contributing to development and growth or parasitism of the nematode. On feeding these engineered plants, the nematode would ingest these dsRNA, or its siRNAs, from the plant cytoplasm, and once inside the nematode the RNAi process would inactivate the gene targeted by the dsRNA and stop further development or parasitism of the nematodes. This strategy has already been successfully demonstrated and coming few years will see lots of research development in this area. As and how genomic sequences of plant parasitic nematodes are reported, specific RNAi constructs could be created to unravel the functional genomics of specific genes. This information will lead to very focused and specific nematode management strategy. A number of examples are available in literature to indicate its significance in Nematode management (Table 5).

Table 5
RNA interfetrence and plant parassitic nematodes

Nematode (treatment)	Activity & effects
Heterodera glycines, Globodera pallida (Soaking in ds RNA containing preparation)	Targeting Cystein-proteinase. Alters the sex ratio female to male from 3:1 to 1:1 (Urwin *et al*. 2002).
Meloidogyne incognita (Soaking in ds RNA solution)	Knock down of two genes in subventral gland (Rosso *et al*. 2005)
Meloidogyne incognita TransgenicTobacco	Poor infection and development (Yadav *et al*. 2006)
Meloidogyne javanica Transgenic tobacco expressed in eggs and eggs producing Female	Affected the fecundity & development (Fairbairn, 2007)

Conclusion

The unprecedented movement of plant derived commodities due to globalization, impact of climate change on geographical distribution, behavior of known population with regard to consequent changes in land use and agricultural practices pose a new threat to agricultural production either from new or recently distributed species of plant parasitic nematodes. Molecular techniques may be the only practical approach to recognize these modern identification methods must provide accuracy, speed, reliability and affordability. Further research in this are will lead to develop suitable strategies for nematode management.

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