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ВО СПОМЕН НА
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На нашиот незаборавен,
Почитуван научен работник, колега, соработник,
Драг другар и пријател - Васил Коцевски.

ЈНУ ИНСТИТУТ ЗА ЈУЖНИ ЗЕМЈОДЕЛСКИ КУЛТУРИ - СТРУМИЦА

To our unforgettable,
Respectful, scientific worker, colleague, collaborator,
Dear companion and friend -Vasil Kocevski.

INSTITUTE OF SOUTHEREN CROPS - STRUMICA

СОДРЖИНА
CONTENTS

Одделение за заштита на растенијата од болести штетници и плевели
Department of protection of the plants from diseases, pests and weeds

ОДДЕЛЕНИЕ ЗА ЗАШТИТА НА РАСТЕНИЈАТА ОД БОЛЕСТИ, ШТЕТНИЦИ И ПЛЕВЕЛИ

DEPARTMENT OF PROTECTION OF THE PLANTS FROM DESEASES, PESTS AND WEEDS

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SINGLE PASS cDNA SEQUENCING – A POWERFUL TOOL TO ANALYSE GENE EXPRESSION IN PREPARASITIC JUVENILES OF THE SOUTHERN ROOT KNOT NEMATODE *MELOIDOGYNE INCOGNITA*

Dautova Makedonka [1], Marie-Noelle Rosso[2], AbadP.,[2] Gommers F.,[1] Bakker J.[1] and Smant G.[1]

2001, Nematology 3(2): 129 – 139.

Key words - chitinase, parasitism gene, plant parasitic nematode, random sequencing, secretion.

Abstract - Expressed sequence tags (EST) have been widely used to assist in gene discovery in various organisms (*e.g.*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Mus musculus*, and *Homo sapiens*). In this paper we describe an EST project, which aims to investigate gene expression in *Meloidogyne incognita* at the onset of parasitism. Approximately 1 000 5'-end sequence tags were produced from a cDNA library made of freshly hatched preparasitic second stage juveniles (J2). The EST were identified in the primary transformants of the cDNA library, and clustered into nine functional groups including (candidate) parasitism genes. A large fraction of the EST (45%) did not have a significant homologue in public databases and could not be clustered in a functional group. Sixty five percent of the EST that could be clustered into a functional group had homologues in other nematode species. EST were for virtually all parasitism related genes that have been cloned from *M. incognita* to date. In addition, several novel genes were tagged, including a xylanase and a chitinase gene. The efficiency of EST projects, which produce sequence data for thousands of genes in months time without any difficult pre-selections of mRNA pools, makes random sequencing of good quality cDNA libraries a superior method to identify candidates for parasitism related genes in plant-parasitic nematodes. The sequences in this paper are retrievable from Genbank with the accession numbers BE191640 to BE191741, BE217592 to BE217720, BE225324 to BE225598, BE238852 to BE239221, and BE240829 to BE240865.

ЕДИНЕЧНО kDNK СЕКВЕНЦИОНИРАЊЕ - МОЌЕН МЕТОД ЗА АНАЛИЗИРАЊЕ НА ГЕНИТЕ ИЗРАЗЕНИ ВО ПРЕПАРАЗИТСКИ ЛАРВИ ОД *ЈУЖНАТА* ГАЛОВА НЕМАТОДА *MELOIDOGYNE INCOGNITA*

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2001, Nematologija 3(2): 129 - 139.

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Краток извадок

"Expressed sequence tags' (EST) се широко утребувани за откривање на гени во различни организми (на пример *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Mus musculus*, и *Homo sapiens*). Во овој труд опишан е EST проект, со цел да се испитуваат гени експресирани во *Meloidogyne incognita* во почетокот на паразитирање. Приближно 1000 5'-end секвенци беа добиени од кДНК банка конструирана од свежи препаразитни втор ларвен стадиум ($Л_2$).

ESTs беа идентификувани во примарни трансформанти од кДНК банката, и беа категоризирани во 9 функционални групи, вклучувајќи ги кандидати за паразитизам гените. Голем дел од ESTs (45%) не покажаа сличност со гените од светската банка. Шеесет и пет проценти ESTs кои можеа да се класифицираат во функционална група имаа хомолог со гени од други нематодни видови. ESTs за сите гени вклучени во паразитизам кои се клонирани од *M. incognita* до денес, беа детектирани. Неколку нови гени беа пронајдени, како на пример генот за ксалиназа и хитиназа. Ефикасноста на EST проектот, кој произведе секвенциони податоци за илјада гени за период од еден месец без било каква пре-селенција на мРНК, го прави рандомното секвенционирање на кДНК банките супериорен метод за идентификација на кандидати гени вклучени во паразитирањето на растително-паразитните нематоди. Секвенците од овој труд може да се пронајдат во светската ген банка (GenBank) под следните акцесии**:** BE191640 до BE191741, BE217592 до BE217720, BE225324 до BE225598, BE238852 до BE239221, и BE240829 до BE240865.

1. INTRODUCTION

ROOT KNOT NEMATODES (*MELOIDOGYNE* SPP.) ARE POLYPHAGOUS ENDOPARASITES, RESPONSIBLE FOR BILLIONS OF DOLLARS IN ANNUAL CROP LOSSES. THE MAJORITY OF THE PLANT SPECIES THAT ACCOUNT FOR THE WORLD'S FOOD SUPPLY ARE SUSCEPTIBLE TO ROOT KNOT NEMATODE INFECTION. THE HALLMARK OF THE COMPLEX NEMATODE-PLANT INTERACTION IS THE FEEDING CELL STRUCTURE THAT IS INDUCED BY THE NEMATODE IN THE HOST PLANT. THE FEEDING CELL STRUCTURE – THE GIANT CELL – FACILITATES A PERMANENT FLOW OF PLANT NUTRIENTS FROM THE VASCULAR TISSUE TO THE FEEDING NEMATODE. INADEQUATE FEEDING CELLS RESULT IN POOR DEVELOPMENT AND REDUCED FECUNDITY OF THE NEMATODES. KNOWLEDGE OF NEMATODE GENES THAT ARE INVOLVED IN HOST PENETRATION, MIGRATION AND FEEDING MAY HELP TO DESIGN RESISTANCE STRATEGIES FOR PEST CONTROL (WILLIAMSON & HUSSEY, 1996).

The haploid genome size of *Meloidogyne* is estimated to be 51 Mb (Pableo & Triantaphyllou, 1989) and it seems reasonable to expect that the gene number is in the same order of magnitude as in *Caenorhabditis elegans* (~19,000). To date various approaches have been applied to investigate gene expression in plant parasitic nematodes in order to identify candidate parasitism genes. In summary, these approaches include screening of cDNA libraries either with monoclonal antibodies specific for nematode secretions (Hussey *et al*., 1990; Davis *et al.*, 1992, 1994) or with homologous plaque hybridization (Koltai *et al*., 1997), PCR based cloning using degenerate primers (Smant *et al*., 1998), RNA fingerprinting (Ding *et al*., 1998), and differential screening of cDNA libraries (Rosso *et al*., 1999; Lambert *et al*., 1999). Although these methods have proven to be successful for a limited number of genes, all require prior knowledge of candidate genes or technically advanced pre-selections in mRNA pools.

Random sequencing of cDNA libraries of various developmental stages has been applied to animal parasitic nematodes such as the filarial nematodes *Brugia malayi* and *Onchocerca volvulus* in order to identify expressed sequence tags (ESTs)

of nematode genes (http://helios.bto.ed.ac.uk/mbx/fgn/net/librarylist.html and http://math.smith.edu/~sawlab/fgn/net/librarylist.html). In the *Brugia* genome project, a combination of expressed sequence tag sequencing from multiple cDNA libraries representing the complete filarial nematode lifecycle, and comparative analysis of the sequence dataset has proven to be very effective in gene discovery. With the advent of high throughput sequencing facilities, the affordable prices of a single sequence run make similar EST projects feasible for plant parasitic nematodes too. Hence, approximately 1000 ESTs were recently produced from the potato cyst nematodes, *Globodera pallida* and *G. rostochiensis,* and proved to be efficient tools for identifying novel parasitism genes (Popeijus *et al*., 2000a, b).

This paper describes an EST project to investigate gene expression in *Meloidogyne incognita* at the onset of parasitism. We have chosen to start with pre-parasitic second stage juveniles to cover the initial phases of the parasitic cycle, plant penetration and intercellular migration. Single-pass sequences were obtained from the 5' end of the cDNA library inserts, because the encoded N-terminal sequences are usually more informative in terms of homology. The EST were identified in the primary transformants of the cDNA library, and grouped into 9 functional groups, including (candidate) parasitism genes.

2. Material and methods

2.1. Nematodes

Meloidogyne incognita, line 48, was propagated in greenhouse cultures on tomato cultivar Moneymaker at 20-25°C. Eggs were harvested approximately 12 weeks after inoculation and isolated using 0.5% NaOCl-solution (Hussey & Barker, 1973). Second stage juveniles (J_2) were collected from eggs in water on a cotton wool filter, purified using 70% sucrose, and stored at –80°C until further processing.

2.2. RNA isolation

Total RNA was extracted from 100 000 frozen pre-parasitic J_2 using Trizol Reagent (Life Technologies, Grand Island). Following a chloroform extraction, the RNA was precipitated in isopropyl alcohol. The pellet was subsequently washed in 75% ethanol, and the remaining RNA was dissolved in an appropriate volume of sterile dimethyl pyrocarbonate-treated water. Analysis of the total RNA on denaturing agarose gel resulted in a smear from 50 to 3000 bp.

2.3. cDNA synthesis

The cDNA for the library was prepared using the Smart cDNA library construction system (Clontech, Palo Alto, CA, USA) with a few modifications. Briefly, 3 µl containing 50ng total RNA was transcribed into single strand cDNA using a Smart oligonucleotide, a modified oligo $d(T)_{30}$ anchor primer, and Superscript II reverse transcriptase (Life Technologies). The single strand cDNA was amplified in 23 cycles (long-distance) of PCR according to the manufacture's protocol (Clontech). The amplified cDNA was digested with *Sfi* I restriction enzyme, and fractionated in a Chroma-Spin-400 column (Clontech). Only the size fractions including cDNA ranging from 700 to 1500 bp were pooled, and subsequently ligated into the vector plasmid pMAK1.

2.4. Construction of library into plasmid pMAK I

The plasmid pMAK1 was derived from the plasmid pcDNA II (Invitrogen, San Diego, CA, USA). The *Eco* RI – *Bam* HI element in the multiple cloning site of

pcDNA II was replaced by a fragment, which includes two *Sfi* I restriction sites that allow for directionally cloning (Fig. 1). Briefly, two oligonucleotides 5'-AATTCGCTAGGCCATTATGGCCGCTAGGCC GCCTCGGCCGCTAG – 3' and 5'-GATCCTAGCGGCCGAGGCGGCCTAGCGGCCATA ATGGCCTAGCGA- 3' were annealed to construct a fragment that would produce two different overhangs (underlined) upon digestion with *Sfi* I restriction enzyme. Following propagation of the plasmid in *E. coli*, pMAK1 was digested with *Sfi* I, dephosphorylated using alkaline phosphatase (Life Technologies), and purified from Sea Plaque agarose gel using Glass MAX DNA Isolation Matrix System (Life Technologies). To construct a library the fractionated cDNA was directionally ligated in the *Sfi* IA restriction site at the 5' end (ATTAT) and *Sfi* IB restriction site at the 3' end (GCCTC) of pMAK I. The ligation mix was introduced into *E. coli* TOPO10 cells (Invitrogen) using electroporation, which resulted in 2.2 x 10^6 primary transformants on Luria-Bertani (LB) medium including ampicillin.

2.5. 5'- End cDNA sequencing

Approximately one thousand colonies directly following ligation and transformation were randomly picked from the plates for single pass sequencing at the 5' end of the library inserts (Incyte Pharmaceuticals, Palo Alto, CA, USA). Either the T7 promoter primer (5'-TAATACGACTCACTATAGGG-3') or the Universe M13 forward primer (5'-GTAAAACGACGGCCAG-3') was used for sequencing using the dye terminator chemistry.

2.6. Sequence analysis / EST characterisation

Vector sequences were automatically trimmed from the raw DNA sequences. Batches of EST sequences were analysed using Basic Local Alignment Search Tool (BLAST; Altschul *et al*., 1994) of the 'BLAST client' (blastcl3) server at the National Centre of Biotechnology Information (NCBI). Initially, each sequence was translated in six reading frames, and all reading frames were compared with published sequences using the BLASTX algorithm at Genbank. Sequences that produced no significant or poor homology were also compared with nucleotide databases at Genbank (February, 2000) using the BLASTN. To evaluate the redundancy of each EST sequence to all other isolated sequences we analysed our local *M. incognita* EST database with a 'Stand-alone BLAST' search engine (Blastall) downloaded from NCBI.

3. Results and Discussion

3.1. Quality of the library

The average insert size of the cDNA library that is used in this paper was 1.1 kb (range of 300 bp to 3.0 kb). The average length of the 1096 single read sequences was 650 bp. Only 2.5% of the library plasmids did not contain an insert and 14% of the sequences failed to meet our quality criteria. For the evaluation of redundancy in our local EST database two nucleotide sequences were considered to be similar or highly homologous if the bit score S' in the BlastN algorithm was larger than 200 (~E-value < 1 * e^{-50}). According to this stringent threshold the percentage of clones appearing only once or without highly conserved homologues in the data set was 78.0 %.

3.2. Global analysis of the sequence results

Single pass sequencing of randomly chosen colonies resulted in nucleotide sequences of 914 primary transcripts of *M. incognita* that were used to search

databases for homologues. Similarities were determined using BLAST algorithms, and ESTs were subsequently annotated and grouped by putative function (Table 1). The grouping is based on the top 'hit'. A similarity was considered as a 'hit' when the obtained BLAST score had an E-value less than $1x10^{-5}$, which means that 37.4% of predicted proteins had significant similarity to known sequences deposited in various databases. Out of these, approximately 7.8% of the predicted proteins – so called undefined homologues – have significant similarity to sequences with poor definitions only.

A substantial number of ESTs resulted in E-values higher than $1x10^{-1}$, and those are considered to encode novel translation products (33.9%), unique to this data set. A total of 398 ESTs resulted in a hit (E-value$\leq 1x10^{-1}$) with deposited sequences from nematode origins, which is 65.8% of all the significant similarities.

3.3. Parasitism related genes

Our main focus is aimed at cDNAs encoding proteins that are involved in the plant-nematode interaction. For many ESTs that are either poorly characterized ($10^{-5} \leq$ E-value $\leq 10^{-1}$) or produce open reading frames without significant matches in databases (E-value $> 10^{-1}$) it is more difficult to determine whether the corresponding gene is involved in parasitism. These genes require additional information regarding their function. For others the function of homologues may be indicative for a role in plant parasitism.

Products of two genes, *msp-1* (*Meloidogyne secretory protein 1* in Ding *et al*., 2000) and a *gp-sec2* homologue (*G. pallida secretory protein 2* in Prior *et al*., 1998), are secreted by plant-parasitic nematodes at relatively high levels. We found three ESTs (*MD0906*, *MD0676*, and *MD0432*) similar to the *msp-1* gene of *M. incognita*. Homologues of *msp1* have previously been described as venom allergen antigens in other nematodes (Hawdon *et al*., 1999; Schallig *et al*., 1997). Despite its presence in parasitic stages of *M. incognita* a function in plant-parasitism is not evident (Ding *et al*., 2000).

ESTs (*MD0814*, *MD0596*, *MD0421*, *MD0351*, *MD0254*, and *MD0298*) showing significant homology with gp-*sec2* in *G. pallida* (Prior *et al*., 1998) also appeared at a relatively high abundance in the dataset. Homologues of this transcript are characterized in several other animal-parasitic nematodes (Trenholme *et al*., 1994; Tree *et al*., 1995). Moreover, a high similarity of these ESTs with an ABA-1 allergen of *Ascaris lumbricoides* (McSharry *et al*., 1999) and *Ov-20* in *O. volvulus* (Tree *et al*., 1995) suggests retinol- and fatty acid- binding activities for this gene. Retinol deficiency in animals results in an impaired immune response to parasitic nematodes (Carman *et al*., 1992) and similarly vitamin A deficiency contributes to the pathogenesis of animal-parasitic nematodes (Rodger, 1962).

Several ESTs showed homology with genes encoding cell wall degrading enzymes produced in the oesophageal glands of nematodes. A large gene family encoding β-1,4-endoglucanases appears to be present in *M. incognita* (M. Rosso, Pers. Comm.). This observation is confirmed by four distinct ESTs (*MD0118*, *MD0139*, *MD0340*, and *MD0369*) with homology (59 to 98% identity) to *eng-1* (Genbank accession number AF100549) previously cloned from *M. incognita* (Rosso *et al*., 1999). Furthermore, *MD0915* identifies a novel gene encoding a β-1,4-endoxylanase (unpublished), which is another type of cell wall degrading enzyme. *MD0790* is homologous to the *cbp-1* from *M. incognita*. *Cbp-1* is characterized by a cellulose binding-domain and despite the absence of any enzyme activity it is expected to play a role in parasitism of root knot nematodes (Ding *et al*., 1998).

A novel chitinase gene is tagged by *MD0774* (Fig. 2). Chitinases (EC 3.2.1.14) have been characterised in bacteria, fungi and animal-parasitic nematodes such as *B. malayi* (Fuhrman *et al*., 1992), *Acanthocheilonema viteae* (Adam *et al*., 1996), and *O. volvulus* (Harrison *et al*., 1999). Studies showing a reaction of murine antibody with the cuticle of post-infective L3 of *O. volvulus* (Harrison *et al*., 1999) implies that it is secreted via the hypodermis. Wu *et al*. (1996) concluded that chitinases expressed in infective stages of filarial nematodes may play a role in moulting during post-infective development. In plant parasitic nematodes chitin is present in the eggshell only (Bird & Self, 1995), therefore it is anticipated that chitinase in *M. incognita* is involved in hatching of the juveniles. However, derivatives of chitin may a function as signal molecules in plants-microbe interactions (Mathesius *et al*., 1998). The chitinase fragment tagged by *MD0774* contains the conserved glutamic acid of class II chitinases (glycosyl hydrolase family 18), which acts as proton donor in the active site of the enzyme (Fig. 2).

Hydroxyl-3-methylglutaryl CoA reductase activity (HMGR; *MD0756*) has been localized in oesophageal glands of *M. incognita* and is believed to be the key enzyme for sterol synthesis (Bleve-Zacheo & Melillo, 1997). It is suggested that HMGR activity in giant cells is related to the high rate of sterol biosynthesis required to sustain the active demand of sterols for nematodes (Chitwood & Lusby, 1991; Sijmons *et al*., 1994). HMGR secreted by the nematode may regulate the de-alkylation of phytosterol into sterols to satisfy the extensive feeding requirements of the developing nematode (Bleve-Zacheo & Melillo, 1997).

Diverse antioxidant proteins as peroxiredoxin (*MD0522* and *MD0716*), catalase (*MD0897*, *MD0884*, and *MD0672*), glutathione peroxidase (*MD0641*, *MD0352*, and *MD0334*), and thioredoxin peroxidase (*MD0127* and *MD0137*) were tagged by several ESTs. Antioxidant enzymes have been identified in many helminths (Nathan *et al*., 1979; Callahan *et al*., 1988), and were shown to be one of the major surface-associated molecules that may shield the parasites by inactivating toxic products produced by host phagocytes. Recently, thioredoxin peroxidases (TPx) are described as a new class of anti-oxidant enzymes (Lu *et al*., 1998) and it is strongly suggested that this is probably the major H_2O_2-metabolizing system in filarial nematodes that enhance defence against the host immune response or limit damage from host inflammatory cells. A similar protective function could be envisioned for plant-parasitic nematodes to counteract the active oxidants released by host-plants. TPx genes have been found in vertebrates, fungi, plants, bacteria (Rhee *et al*., 1994) and also were cloned and characterized from the nematodes *Dirofilaria immitis* (Klimowski *et al*., 1997), *O. volvulus* (Lu *et al*., 1998; Chandrashekar *et al*. 1998), *B. malayi* (Ghosh *et al*., 1998) and *G. rostochiensis* (Robertson *et al*., 2000).

MD0736 identifies a cysteine proteinase with homology to a *Haemonchus contortus* proteinase (Skuce *et al*., 1999). Cysteine proteinase has been intensively studied in animal parasitic nematodes as *Ostertagia ostertagi* (Pratt *et al*., 1992) and *Ancylostoma caninum* (Harrop *et al*., 1995). They are also abundantly expressed in the intestine of *C. elegans* (Ray & McKerrow, 1992). Similarly, two cysteine proteinase genes (*hgcp-I* and *hgcp-II*) have been cloned from the plant parasitic nematode *Heterodera glycines* (Urwin *et al*., 1997). Specific protease inhibitors expressed as transgenes in hairy roots of host plants resulted in a reduced fecundity of feeding soybean cyst nematodes (Urwin *et al*., 1995).

3.4. Discussion

The success of an EST project largely depends on the quality of the cDNA library that is used for random sequencing, which is determined by the average insert

size, the percentage of full-length clones, the redundancy in the library, and the number of plasmids that carry no insert. The average transcript size in *C. elegans* is predicted to be 1.33 kb. If the same holds true for *M. incognita* then this cDNA library is in favour of a good representation of its genes. For reasons of large differences in cloning efficiencies most of the small transcripts have been excluded from the library (see Material and methods).

The 5' end of mRNA is usually more informative as compared to the 3' end, because sequences are generally more conserved at the 5' end. In addition, the signal sequences that target proteins to be secreted into the plant are generally also located at the 5'end, which is a crucial feature of candidate parasitism genes (Davis *et al*., 2000; Qin *et al.*, 2000). In conventional methods reverse-transcriptase frequently terminates before transcribing the complete mRNA sequence rendering many clones in a cDNA library incomplete. This is particularly true with long mRNAs primed with oligo-dT or if the mRNA molecule contains abundant secondary structures. To improve the percentage of full-length clones in cDNA libraries oligonucleotide primers based on a spliced-leader (SL) sequence have been used to amplify full-length cDNA only from the first-strand synthesis products (Williams *et al*., 1999). The number of mRNA species preceded by a spliced-leader (SL) sequence is estimated to be 70 percent for *C. elegans* (Blumenthal *et al*., 1997) and more than 80 percent for *Ascaris lumbricoides* (Nilsen, 1993). Messenger RNAs carrying a SL sequence have been isolated from *M. incognita* too, however, it is unclear if this counts for the majority of the transcripts. Some of the parasitism related genes that have been cloned to date are not preceded by a SL sequence (Rosso *et al*., 1999). Therefore, we have used the SMART-oligonucleotide system to essentially obtain the same effect as libraries made of SL amplified cDNA, a higher percentage of full-length clones, without any possible bias (Barnes, 1994; Chenchik *et al*., 1998).

These percentages The percentage of the EST with significant or no similarity with the known sequences deposited in various databases are in accordance with previous reports on other nematode EST projects (Willliams *et al*., 1999). ESTs with $1x10^{-5} \leq$ E-value $\leq 1x10^{-1}$ were considered to have less significant homologies (28.7%), and require further sequence analysis using more sophisticated algorithms (*e.g.*, PSI-BLAST). This percentage includes 3.8% of the predicted proteins that align to some extent with undefined homologues and a subset of 11.4% that have some sequence similarity with other database accessions that have poor descriptions.

According to their the putative functions all sequences with BLAST-score probabilities of $1x10^{-1}$ and less were categorized in 9 groups (Table 1) leaving 45% of the ESTs ungrouped. ESTs encoding proteins involved in the categories 'Metabolic processes' (12.6%), 'Gene expression' (9.8%), and 'Structure and muscle' (7.3%) are most abundantly represented in the cDNA library. These three categories contain many house keeping genes that have conserved homologues in many unrelated organisms and for which high expression levels, and therefore many ESTs, can be expected. These three categories are the main sources of redundancy of the cDNA library. Although our clustering method may be considered as arbitrary, as the same EST may be assigned to more than one group, the catalogue still reveals relatively reliable proportions of the genes expressed in pre-parasitic second stage juveniles of *M. incognita*.

3.5. Pioneering' sequences

The most challenging ESTs (33.9 %) are the ones for which no homologues were found in the public databases. Based on our experience with the potato cyst

nematode *Globodera rostochiensis* many genes potentially related to parasitism are pioneering sequences. A number of analytical steps may help to assign a function to pioneering gene sequences.

First, it is most likely that the majority of parasitism related genes encode secreted proteins (Williamson & Hussey, 1996). Single-pass 5' sequences may include the N-terminus of the encoded proteins, which allows for prediction of a signal peptide for secretion. The latest release of Signal-P at http://www.cbs.dtu.dk/services/SignalP-2.0/ has improved capabilities to discriminate between signal peptides and uncleaved signal anchors. Combined with computer software that searches for transmembrane domains a good overall prediction for a protein to be secreted is possible. A small number of secretory proteins lack a typical hydrophobic signal peptide for translocation via the classical secretory pathway. In these cases secreted proteins require the interaction with a helper-protein - usually ATP-binding-cassette (ABC)-transport proteins (Kuchler & Thorner, 1992). Examples of this mechanism of secretion have been found in both prokaryotic and eukaryotic organisms. Several secreted proteins of *O. volvulus* infective larvae, reported initially as a host protective antigen, lack a classical N-terminal signal peptide (Abdel-Wahab *et al*., 1996).

Secondly, large scale *in situ* hybridisation procedures would provide valuable data on unique EST sequences. A large scale *in situ* hybridisation procedure was developed for *C. elegans* by Tabara *et al*. (1996). Similar high-throughput methods to obtain spatial expression patterns are being developed in our laboratories at the moment.

Thirdly, a novel high-throughput RNA fingerprinting based strategy named cDNA-AFLP has been recently applied to obtain temporal expression data for unknown genes of the plant parasitic nematode *G. rostochiensis* (Qin *et al*., 2000). The biology of the potato cyst nematode especially during hatching lends itself perfectly to a differential display procedure like cDNA-AFLP. Despite more experimental difficulties a similar succession of distinct phases in the transition from preparasitic to parasitic juveniles of *M. incognita* should be amenable to analysis with cDNA-AFLP too.

Alternatively, introducing double-strand RNA to disrupt gene activity may be an ideal contribute to assess the function of genes in plant parasitic nematodes. This strategy has been shown to be useful for RNA interference in *C. elegans* (Fire *et al*., 1998) and the effects have persisted well into the next generation. No RNA interference mutants of plant parasitic nematodes have been reported yet, which may be the consequence of their more complex mode of reproduction and obligatory parasitic nature.

4. Concluding remarks

In conclusion, this small pilot EST sequencing project has produced EST tags for virtually all parasitism related genes that have been cloned from *M. incognita* at present. The efficiency of EST projects, which produce sequence data for thousands of genes in months time without any difficult pre-selections of mRNA pools, makes random sequencing of good quality cDNA libraries a superior method to identify candidates for parasitism related genes. Hence, this approach can be successfully applied to other economically important nematode species. For each of the candidates with interesting homologies herein further expression analysis or biological tests will have to demonstrate their role in parasitism. At the time this paper was prepared an EST sequencing project dealing with various parasitic nematode species was initiated

by the St. Louis Genome Sequencing Centre and Hinxton Sanger Centre (McCarter *et al*., 2000). This promising initiative will reveal the presence of thousands of interesting genes in nematodes for which nematologists will have to provide a biological understanding to assess their relative importance in the plant-nematode interaction.

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Table 1. A subdivision of the conceptual translations of 914 EST sequences of *M. incognita* preparasitic J2. The subdivision is based on the most significant homologues according to the BLASTX E-values. The total 'number of ESTs' is broken down into two fractions – the number of ESTs ('high') that have E-values < $1x10^{-1}$, and the number of ESTs ('low') that resulted in E-values between $1x10^{-5}$ and $1x10^{-1}$. Percentages between brackets indicate the relative numbers of ESTs as a ratio of the total number of 914 ESTs.

	Category	Description and examples	Number of ECTc	Similarity	
				high	*low*
1	Structural and muscle	Cytoskeletal and muscle proteins (e. g. myosin, actin, calponin)	67 (7.35%)	52 (5.7%)	15 (1.6%)
2	Enzymes and Metabolic	Proteins involved in diverse metabolic processes (e. g. GAPDH, GPD, fatty CoA ligase, catalase)	115 (12.6%)	86 (9.4%)	29 (3.2%)
3	Gene expression And protein synthesis	Proteins involved in transcription and translation (e.g. transcription factor, translation elongation factor, ribosomal proteins)	90 (9.8%)	54 (5.9%)	36 (3.9%)
4	Cell cycle	Proteins involved in cell division and DNA replication (e.g. cyclin, mitogen inducible gene, DNA topoisomerase, centrin)	11 (1.2%)	3 (0.3%)	8 (0.9%)
5	Transport	Membrane transporters and lipid transport proteins (e. g. clathrin heavy chain, axonal transport protein, transportin)	13 (1.4%)	10 (1.1%)	3 (0.3%)
6	Neuron function	Proteins involved in neuronfunction (e. g. neurofilament protein, synaptic vesicle protein, FMRF neuropeptide)	11 (1.2%)	9 (1%)	2 (0.2%)
7	Protein domains	Proteins defined by specific domains and repeats (e. g. C2 domain, Ca2+ binding protein, lipoprotein-binding prot.)	59 (6.5%)	35 (3.8%)	24 (2.6%)
8	Candidate parasitism Genes	Nematode-host interaction specific (e. g. cellulases, chitinase, xylanase, SEC2, CBP 1, Ov T1, Ov 20, cysteine proteinase)	28 (3.1%)	22 (2.4%)	6 (0.7%)
9	Undefined homologues	*C. elegans, B. malayi, H. sapiens* ESTs (e. g. yk8c7.5, proteins in chrom. III)	106 (11.6%)	71 (7.8%)	35 (3.8%)
10	Unknown	No matching	414 (45.2%)	310 (33.9%)[1]	104 (11.4%)[2]

[1] ESTs with E-values > $1x10^{-1}$ in BLASTX. These EST are unique to this data set.
[2] These ESTs have E-values of $1x10^{-5} \leq E \leq 1x10^{-1}$ in BLASTX, however, the aligning sequences have poor definition lines.

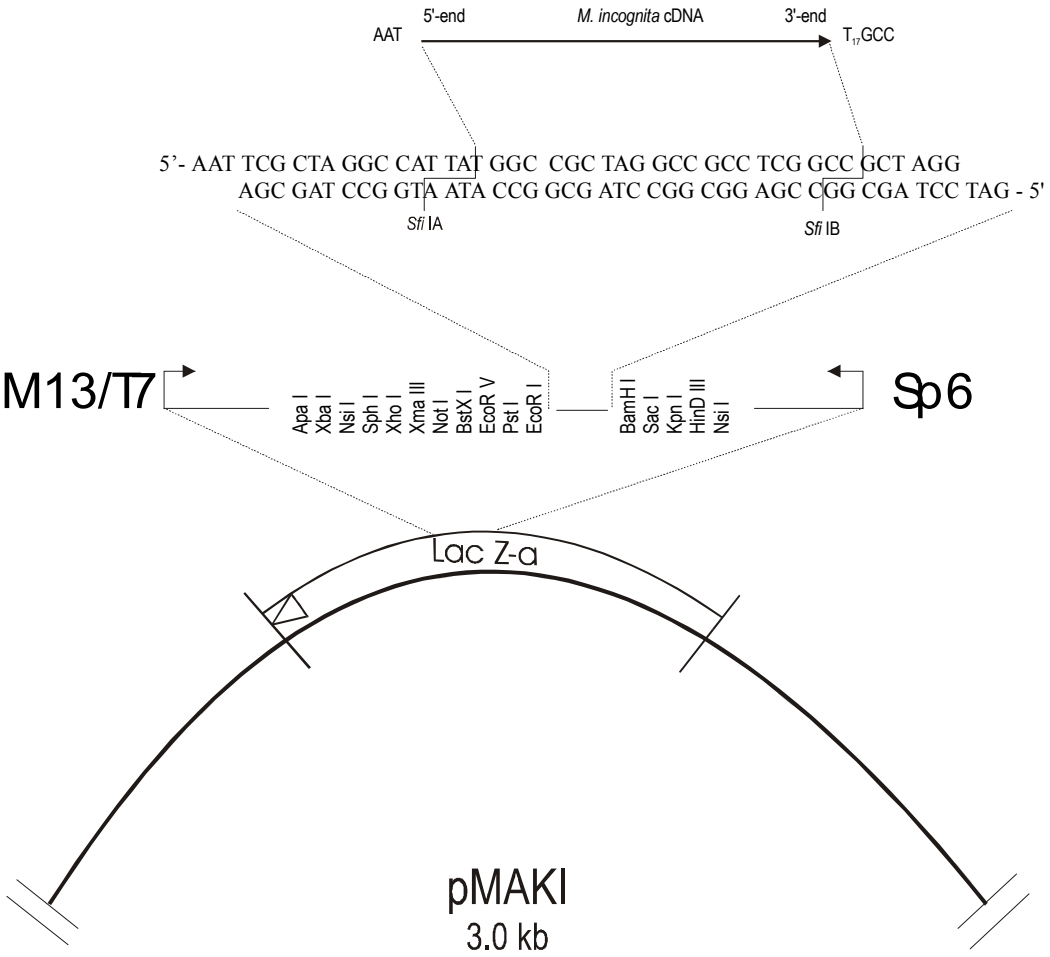

Figure 1. Construction of the cDNA library in the *Sfi* I restriction sites of the multiple cloning site of plasmid pMAK I.

```
AE003477        CYYTNWSQYRVKIGKFVPEDIPADLCTHIIFAFGWLKKNKLS-SYESNDETKDNV-PGLY 184
AF250997        CYYTNWAQYREGEGKFLPENIPNGLCTHILYAFAKVDELGDSKAFEWNDEDSEWS-KGMY 85
Q11174          CYFTNWAQYRQGRAKFVPEDYTPGLCTHILFAFGWMNADYTVRAYDPADLPNDWAGEGMY 116
Mi_MD0774       CYFTNWAIYRSGRAKFAPEDYAPGLCTHIFYAFAYFNESFEAYAIDPNDLPNDSDPLGQY 74
                **:***: **   .** **: . .*****::**. ..      : :  *  .:    * *

AE003477        ERMMTLKKANPKLKILLALGGWSFG--TQKFKDMSSTRYTRQTFVYSAIPFLRKRGFDGL 242
AF250997        SGVTKLKETNPELKILLSYGGYNFG--SAIFTEIAKSAQKTERFIKSAIEFLRKNNFDGF 143
Q11174          RRVNKLKVTDTQLKTLLSFGGWSFG--TALFQGMAASSASRKVFIDSAITFVRTWGFDGI 174
Mi_MD0774       ARVVALKKYDPNLKFVMSFGGWTFSTTTTLFQNMTSSKQNRGKFIKSSIAFIKKHGFDGI 134
                  :  **  :.:** ::: **:.*.  :  *  :: :  .   *: *:* *::. .***:

AE003477        DMDWEYPKGSDDKKNFVLLLKELREAFEAEAQELKKPRLLLSAAVPVGPDNIRGGYDVPA 302
AF250997        DFDWEYPLG--VAKEHAKLVEAMKSAFVEEAKTSGKQRLLLTAAVSAGKETIDGSYDVES 201
Q11174          DIDWEYPSGATDMANYVALVKELKAACESEAGSTGKDRLLVTAAVAAGPATIDAGYDIPN 234
Mi_MD0774       DLDIEYPDS---KENFNLLLQEFHLASCNEK--NVKTKLIITAAVAAGIDIVKNSYDIAT 189
                *:* *** .     :.  *:: :: *   *     * :*:::***..*   :  .**:

AE003477        IASYLDFINLMAYDFHGKWERETGHNAPLYAPSTDSEW--- 340
AF250997        LGKNFDLLFLMSYDLHGSWEKNVDLHGKLRPTKGEVSGI-- 240
Q11174          LAPNFDFILLMSYDFFGAWASLVGFNSPLYATTELPAEW-- 273
Mi_MD0774       MAKYVDFINLMTYDFHITSENKTGYNSPLRSKGLFEYYRCW 230
                :.  .*:: **:**:.      .. :. * .
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Figure 2. Amino acid sequences of chitinases of various origins aligned using the algorithm Clustal W, version 1.8 (Thompson *et al*., 1994). Only fragments of accession with significant homology to MD0774 of *M. incognita* are shown. The putative proton donor – a glutamic acid (E) – in the active site of the chitinases is in bolded font. Two chitinases of nematode origin (*C. elegans*, swiss-prot accession Q11174, *Wuchereria bancrofti*, genbank accession AF250997) and one chitinase sequence of arthropod origin (*Drosophila melanogaster*, genbank accession AE003477) are included in the alignment. Asterisk, identical or conserved resiudes in all sequences of the alignment; colon, conserved substitutions; single dots, semiconserved substitutions.