Genes and parasitism in plant pathogenic nematodes

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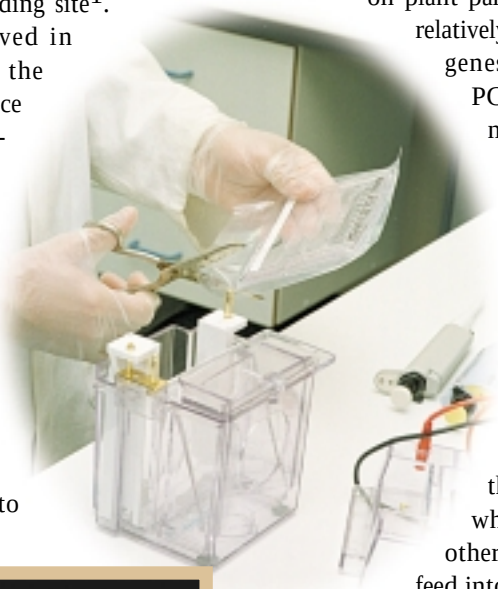
Plant parasitic nematodes cause serious damage to crops in the UK and throughout the world. Our work is aimed at understanding the proteins produced by parasitic nematodes that allow them to parasitise plants. In particular, we are investigating the nature and function of nematode secretions. Secretions of plant parasitic nematodes have been implicated in many aspects of the host-parasite interaction. For example, secreted cellulases are thought to be important in migration of the nematode through the root prior to the induction of the feeding site[1]. Secretions may then be involved in induction and maintenance of the syncytium, although direct evidence for this is still lacking. The nematodes spend a large proportion of their life cycles embedded within an organism possessing a battery of chemical and physical defences, and evidence is emerging that nematodes secrete proteins which help to protect them from these defences[2]. For these reasons, our studies are focused on analysing secretions of the potato cyst nematode (PCN).



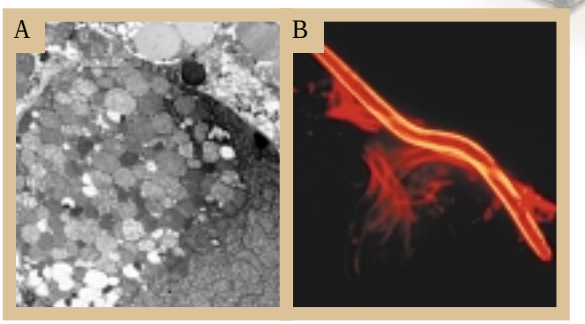


Figure 1 1. Genes encoding secreted proteins are likely to be abundant in a representative cDNA library made from PCN J2s. The gland cells of PCN are large and full of secretory granules (1a) and secreted proteins are produced in abundance from the J2s (1b). In figure 1a some of the secretory granules are arrowed and in figure 1b secreted protein is stained gold/red using an antibody raised against a recombinant PCN secreted protein. Note how the secretory material is present on and shed from the surface of the nematode.

One of the major problems in working with PCN is that, like many parasites, it is an extremely awkward experimental organism. It is an obligate endoparasite which means that many of the most interesting developmental stages are inaccessible and no *in vitro* culture systems exist. Furthermore, PCN is extremely small (to allow it to live inside plant roots) which means that it is not possible to work directly with the tissues of interest (in this case the gland cells). These problems have meant that molecular biological studies on plant parasitic nematodes have progressed relatively slowly. Until recently only three genes encoding secreted proteins of PCN had been identified. An alternative approach was clearly required.

Expressed sequence tag (EST) analysis offers a rapid and cost effective route to the discovery of novel genes. ESTs are single pass sequences of cDNA clones selected at random from a cDNA library. ESTs provide a background of information about the genes expressed in an organism, which can be extremely useful for other molecular biological projects to feed into. Over 25,000 ESTs from nematode parasites of animals are present in databases and have provided a valuable resource for researchers working with these parasites. Although obviously less selective than more direct approaches (such as library screening or differential screening), it is possible to target an EST project at genes expressed at the onset of parasitism which may be important in the host parasite interaction by using a cDNA library constructed from the hatched infectious stage juvenile (J2). Changes occur in J2s during hatching indicative of the transition to a parasitic mode of existence. These changes include activation of transcription in the gland cells as well as behavioural changes and changes in gene expression. It is also feasible that an insight into the molecules secreted by PCN may also be gained using ESTs – mRNAs encoding secreted proteins are likely to be abundant in a representative

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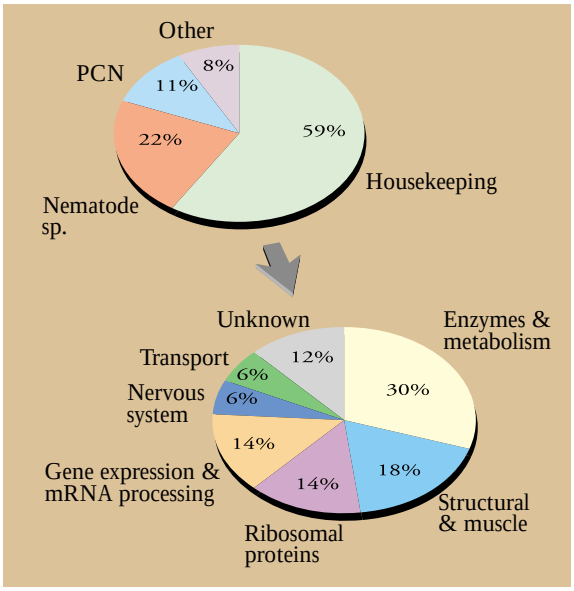

Figure 2 Classification of genes identified from the PCN EST dataset.

cDNA library (as explained in Fig. 1) and therefore likely to form a high proportion of the ESTs. For these reasons, we performed a small scale EST project using cDNA libraries made from J2s of *G. rostochiensis* and *G. pallida*. 1000 sequences were obtained in total. Having achieved our goal of identifying secreted proteins from these nematodes, our work is now aimed at identifying the most important genes and characterising the function of the proteins encoded by these genes. In this article, our work in this area is reviewed.

We first attempted to subdivide the ESTs into meaningful categories. Housekeeping genes were defined as those producing matches in the databases with genes from nematodes and other organisms. Fifty nine per cent of our ESTs fell into this category and were considered as likely to play a role in normal cell or whole body metabolism. Twenty two per cent of the ESTs only produced matches against genes from other nematodes and were thus classified as nematode specific genes. Eleven per cent of the ESTs gave no matches against any genes in any databases, or only produced matches against non-nematode genes (see below). These ESTs were classified as PCN specific genes. The remaining 8% of sequences (other genes) were sequences generated from bacterial or vector DNA fragments, or from sequence reads too short to use for meaningful database searching and were not considered to be of further interest. These figures are summarised in Fig. 2.

The genes classified as housekeeping genes were analysed in more detail. Thirty per cent of the housekeeping genes were enzymes and other proteins involved in basic cellular metabolism, 18% were structural or muscle proteins, 14% were ribosomal proteins and another 14% were proteins involved in the control of gene expression such as transcription factors or splicing proteins. Approximately 6% of the ESTs encoded proteins involved in transport (such as membrane transporters, fatty acid binding proteins) and slightly fewer were categorised as being involved in the functioning of the nervous system. The remainder of the housekeeping genes did not fit easily into any of these categories or have not yet had a precise function defined, despite being present in a wide range of organisms. One of the stated aims of the EST project was to identify genes encoding secreted proteins. It was possible to identify a signal sequence[3] in the predicted amino acid sequences of approximately 10% of the ESTs sequenced. Homologues of proteins known to be secreted by animal parasites and by plant parasitic nematodes were identified.

Having isolated a variety of genes, the next challenge is to identify those likely to be important in the host parasite interaction and to try to assign function to these, and the other, genes. It is relatively simple to assign a function to some genes; particularly those classified as housekeeping genes. These genes often produce matches against genes whose function has been previously defined from another organism. However, there are examples of genes in this category which may be adapted for a role in the parasitic process and which may therefore require further analysis. One example of this type of gene is thioredoxin peroxidase. Thioredoxin peroxidase normally functions as an enzyme which metabolises hydroperoxides generated internally by an organism's metabolic processes. However, our previous work had identified the protein encoded by this gene as being present on the surface of PCN J2s after hatching (See Figure 1b). Further work showed that this protein specifically breaks down hydrogen peroxide. Recent work has demonstrated that hydrogen peroxide is produced by plants as part of the defence response against nematode attack[4]. It seems likely therefore that, as in animal parasitic nematodes, thioredoxin peroxidase is secreted from PCN so that it can protect the nematode from host defence responses.

Many of the genes in the EST dataset produce matches only against other nematode genes for which no functional information is available, or do not

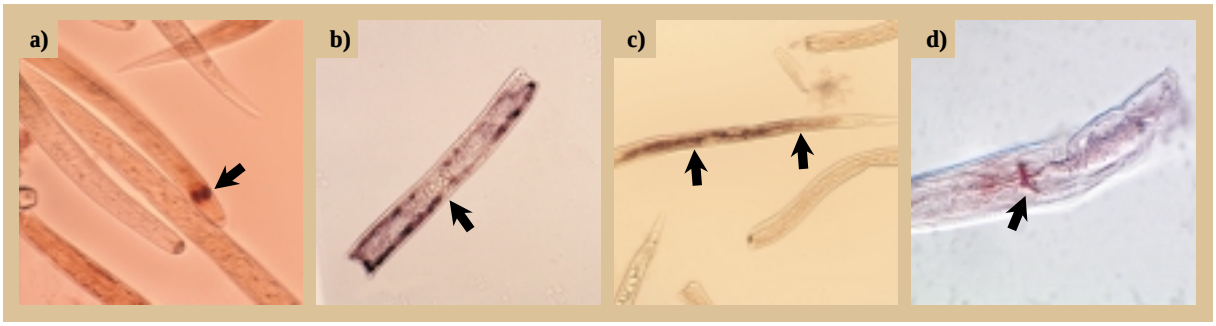

Figure 3 *In situ* hybridisation of DNA probes to *G. rostochiensis* J2s. A purple/brown deposit indicates a positive reaction (arrows). Genes expressed in the amphids (3a), the hypodermis (3b), the gut (3c) and the nervous system (3d) are present in the EST dataset.

match any genes in any of the databases. Naturally, predicting the function of these genes is a rather more difficult proposition. Computer predictions can, in some cases, be used to identify genes of potential interest on which it might be productive to focus further studies. An examination of the spatial expression patterns of a gene can often provide information about a potential function for the gene product. Performing such analysis on a relatively large scale is therefore one method of screening large numbers of sequences of unknown function in order to determine which may be worthy of further analysis. We are currently using this method of analysis to focus our studies on proteins encoded by genes in our PCN EST dataset that may be important in the host parasite interaction of PCN. First the ESTs are analysed to determine which could encode proteins with a predicted signal sequence. We then use *in situ* hybridisation to examine spatial expression patterns of the chosen genes. *In situ* hybridisation is performed by using non-radioactively labelled DNA probes that are amplified from the clones used to generate the original ESTs. J2s of PCN are fixed, cut into 3 to 4 pieces (to allow probe to enter) and incubated with labelled probe. Following extensive washing to remove unbound or non-specifically bound probe, the remaining probe, hybridised to its corresponding mRNA, is detected using a secondary antibody coupled to alkaline phosphatase. A colorimetric reaction allows the spatial distribution of the mRNA in the nematode to be visualised. Our studies are targeted at genes expressed in tissues from which they could be secreted from the nematode to the external environment, such as the oesophageal gland cells, hypodermis and amphidial sense organs. Using *in situ* hybridisation has enabled us to find genes encoding secreted proteins expressed in many of these tissues (Fig. 3).

Once genes of interest are identified, detailed functional analysis on the proteins they encode can be undertaken. Where a clear biochemical function is indicated (e.g. as for the thioredoxin peroxidase or other enzymes), assays can be performed with recombinant proteins. In many cases this is not possible and it may often be desirable to examine the function of a gene product *in vivo* rather than in a test tube. We are currently trying to develop systems to allow this sort of analysis in plant parasitic nematodes. In particular, we are attempting to develop methods of introducing double stranded RNA (dsRNA) into J2s of PCN. dsRNA has been shown to trigger sequence specific gene silencing in a wide range of organisms, including the extensively studied nematode *Caenorhabditis elegans*[5]. Considerable obstacles to using dsRNA inhibition (dsRNAi) in plant parasitic nematodes remain – in particular it is proving extremely difficult to introduce the dsRNA into the nematodes. If it is possible to overcome such problems, the potential offered by using dsRNAi in combination with the techniques described above is enormous. It will be feasible to characterise the functions of proteins with critical roles in the parasitic process of plant parasitic nematodes. As well as having scientific merit, this exercise will have practical benefits in allowing identification of important parasite proteins against which novel control methods can be targeted.

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