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Forum article

MOLECULAR APPROACHES IN PLANT NEMATOLOGY

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Summary – Molecular biology techniques are being applied to an increasing number of problems in plant nematology. This article first reviews the progress made in studies on diagnostics and genetic variation using molecular biological techniques. It then goes on to consider how such techniques are currently being applied in studies on the fundamental biology of plant parasitic nematodes and their interactions with their hosts. This part of the review is split into sections on gene cloning (DNA and protein based approaches) and analysis of gene expression. Finally, potential areas of importance for future work are considered. An effort has been made throughout to explain the theory behind the many techniques used by molecular biologists in an attempt to make them more accessible to the non-specialist.

Résumé – ***Approches moléculaires en phytonématologie*** – Les techniques de biologie moléculaire sont utilisées pour l'étude d'un nombre croissant de problèmes concernant la phytonématologie. Dans cet article, sont d'abord passées en revue les études utilisant les techniques de biologie moléculaire et portant sur le diagnostic et la variabilité génétique. Cela conduit ensuite à examiner comment ces techniques sont actuellement appliquées aux recherches sur la biologie fondamentale des nématodes phytoparasites et leurs interactions avec leurs hôtes. Cette dernière partie de l'article est divisée en sections traitant du clonage des gènes (basé sur l'ADN et les protéines) et l'analyse de l'expression génétique. Pour terminer sont passés en revue les champs potentiels des travaux du futur. Les auteurs se sont efforcés d'expliquer la théorie sous-tendant les nombreuses techniques utilisées par les biologistes moléculaires de façon à essayer de rendre celles-ci plus accessibles au non spécialiste.

Key-words : nematode, molecular biology, DNA, protein.

Recent years have seen a rapid increase in the application of the various techniques of molecular biology to problems in plant nematology. However, our discipline has lagged behind many others, most notably animal parasitology, in realising the potential benefits of molecular biology. This can be explained partly by the difficulties most plant nematologists face in accumulating sufficient biological material to undertake basic molecular cloning. The development of new techniques, particularly the polymerase chain reaction (PCR) and the refinement of existing techniques, have allowed this obstacle to be overcome. Progress has since been made at a rapid and occasionally bewildering rate as ever more people begin to use molecular biological techniques. This is illustrated by the number of molecular biology presentations at the meetings of the European Society of Nematology in recent years : At a meeting in Uppsala in 1988 only one presentation which could be described as being on molecular biology was made (Burrows, 1988). At the most recent meeting (1994) in Gent, however, there were nearly 40 such presentations and even half a dozen sessions devoted to molecular biology work (Evans, 1995).

Despite, or perhaps because of this rapid growth rate, many workers remain unaware of the range of problems which can be addressed by molecular biology. Whilst most people will be aware of the ways in which molecular techniques have been used for diagnostic purposes, fewer will be aware of the different methods available to isolate genes, to study their expression patterns and thus answer questions about the basic biology of organisms. This review aims to address this problem. It is divided into two main sections; genetic variation and diagnostics, and gene cloning. In each of these sections the various techniques which can be used to study relevant problems are reviewed with examples given, where possible, of such studies using nematodes. Where nematological examples are not given, studies which illustrate the power of the technique under discussion are chosen. This review makes no attempt to cover the vast amount of literature generated by the *Caenorhabditis elegans* research community other than where relevant techniques have first been applied using this animal. Many of the classical genetical and mutagenesis techniques used by the majority of these workers are in any case not applicable to plant parasitic nematodes. For a review of the progress made using *C. elegans* as a model metazoan one need only look at the most recent genetic map of *C. elegans* or at one of the text books written on the subject. Furthermore, this review only covers techniques

applicable to the nematode side of the host parasite relationship, those applicable to studies on nematodes themselves. Excellent reviews of the work carried out on the changes induced in plants by nematodes have been published recently (e.g. Gheysen & van Montagu, 1995).

Genetic variation and diagnostics

This section considers the application of molecular biological techniques to problems of genetic variation and classical taxonomy. Two principal approaches are available. Firstly the use of " random " techniques which aim to sample the entire genome of the animals or populations of interest and which are mostly used in studies of genetic variation. Secondly " non-random " techniques which target defined regions of the genome such as particular genes or gene products and are most frequently used in identification to species or sub-species level. The defined region of the genome may be the ribosomal genes, the mitochondrial genome or a conserved nuclear gene. Whilst analysis of these regions may allow information about taxonomic relationships to be gathered, focusing on one region of the genome imposes certain limitations on the experimenter. There may, for example, be insufficient genetic variation in some regions to give the resolution required. Furthermore, such analysis does not allow polymorphisms which may be genetically or functionally linked to biological phenomena to be identified. For this purpose, a random approach, where genetic variation throughout the genome is sampled, may be more helpful.

Molecular techniques were applied early to one of the major problems confronted by plant nematologists, namely the need to rapidly and reliably identify morphologically similar animals. Molecular biological techniques have allowed differences in protein composition, enzyme structure and activity and DNA sequences to be used as tools to aid the taxonomist. Such techniques generally have high discriminatory powers, are often inexpensive and can be designed in such a way as to be simple to use, even for the non specialist.

Protein electrophoresis was the first of these techniques to be applied in nematology. At the simplest level proteins from related species can be separated on polyacrylamide, starch or agarose gels on the basis of different molecular masses. Differences in banding patterns between related species or populations may be observed and used as taxonomic markers or as a means to calculate levels of similarity. Such techniques have long been applied to a range of nematode species and were applied very early to the study of *Heterodera rostochiensis* (Trudgill & Carpenter, 1971). Observing total protein profiles, however, has limited value for the study of most nematode species as it provides a wealth of information but is often difficult to interpret. Difficulties in obtaining consistent and clear banding patterns from nematode samples, perhaps due to variation in the condition of the sample used, have been improved by using techniques such as isoelectric focusing (IEF) where proteins are separated on the basis of their charge and resolve into sharp bands. This technique overcomes some of the variation observed when using other methods but it still provides a large amount of data that is difficult to interpret.

The application of enzyme staining techniques to proteins separated on the basis of molecular weight or by I EF limits the number of bands observed and provides an easier means of checking the reliability of bands. As the isozymes are the products of expressed genes they have the advantage of giving genetic and allelic information that is valuable in genetic studies both in terms of inheritance and population genetic structure and is of direct interest in the study of genetic variation. Specific differences in isozyme patterns are of value as diagnostic tools. A disadvantage of this approach is that these techniques examine gene products which may be life stage specific or influenced by environmental factors.

Such studies have now been applied to an enormous range of nematodes including *Globodera, Meloidogyne* and *Pratylenchus* species (Bergé & Dalmasso, 1975; Fox & Atkinson, 1984; Ibrahim *et al.,* 1995). An important advantage of the use of isoenzymes is that statistical analysis can be applied to the results. The presence or absence of isoenzyme bands can be scored and the subsequent statistical analysis can be used to compare the degree of similarity of a number of species.

Many studies have been carried out on *Globodera* species using isoenzyme techniques. Some of these (e.g. Fleming & Marks, 1983) have focused on the development of a cheap and user friendly method for routine identification of *Globodera* species. However, these studies, and a number of others using a wide variety of enzyme stains, have found variation in enzyme profiles between and within these amphimictic species (Fox & Atkinson, 1984; Marks & Fleming, 1985). Such variations was sometimes found to be correlated with pathotype. However, variation between different populations of the same pathotype was also found, limiting the use of isoenzyme techniques for routine pathotype recognition.

In contrast, the use of esterase phenotypes in the taxonomy of *Meloidogyne* species has become widely accepted as a routine technique, mainly because the esterase phenotype of adult females of *Meloidogyne* species seems remarkably stable. It has been shown to be unaffected by time of hatch, age of females, age of juveniles when infecting the root and age of the host plant. Females grown on different cultivars of tomato with differing susceptibilities to infection do not show significantly different esterase phenotypes (Fargette, 1987 *a*). Stable interspecific differences in esterase phenotypes are observed and some race specific characters have been found (Fargette, 1987 *b*). The limited variation observed using a range of techniques in esterase phenotypes of

Meloidogyne, many of which multiply clonally, as compared to those of *Globodera* species, reflects the overall pattern of genetic variation in these genera using a range of techniques. Such differences are thought to be correlated with the differing modes of reproduction of these nematodes.

Separation of proteins using firstly I EF and secondly separation by molecular weight (2D electrophoresis) has also been applied to the study of *Globodera* species (Bakker & Gommers, 1982) where the data was interpreted in terms of alleles. Thus population differences could be described in terms of gene frequencies. Whilst this technique provides the greatest resolution it is expensive and gel analysis and interpretation are a major challenge. Given the discriminatory powers and ease of application of isoenzyme studies, it seems that 2D electrophoresis is more likely to be useful in functional studies on nematodes than as a diagnostic tool.

Whilst isoenzyme studies allow a greater degree of resolution than total protein staining, antibodies can be used to identify directly the presence or absence of an epitope. Despite early acknowledgement of the potential of antibodies as tools in nematode characterisation (Bird, 1964), it was not until the development of monoclonal antibody technology that antibodies to specific epitopes became available. A range of methods are available for raising antibodies and for carrying out diagnostic tests (Curran & Robinson, 1993). Species specific monoclonal antibodies are now available against *Meloidogyne incognita* (Jones *et al.,* 1988) and *Globodera pallida* (Bakker *et al.,* 1988). Antibody-based systems still offer the greatest potential for quantitative analysis of nematode samples (Davies & Carter, 1995) although no such system is yet available.

Direct examination of the genetic material, or DNA analysis, is potentially the most powerful method of nematode diagnosis. With antibody-based systems and indeed all systems based on protein it is possible that the protein examined may not be present in all stages of the nematode. Thus, a monoclonal antibody which can successfully identify an invasive stage juvenile may be useless if a sample consists solely of adult stage nematodes. Furthermore, expression of a protein can be influenced by unforeseen environmental factors which may cast doubts on the accuracy of any protein-based system. Identification based on DNA potentially samples the entire genome rather than the small portion expressed in any given nematode stage, and is therefore more likely to reveal species specific or even population specific variation.

The earliest analyses of nematode DNA used analysis of fragments of DNA produced by digestion of total genomic DNA (Curran *et al.,* 1985) with restriction enzymes (enzymes which recognise specific DNA sequences and cut the DNA at these sites). Digestion of highly repetitive sequences in the genomes of many organisms generates relatively large amounts of pieces of DNA of specific size which can be separated on agarose gels to give distinct banding patterns. This method has been used to separate various *Meloidogyne* species (Curran *et al.,* 1986) and *G. rostochiensis* from *G. pallida* (Burrows & Boffey, 1986). However, this approach is time consuming, requires substantial amounts of DNA, is not particularly sensitive and exploits only a small proportion of the genetic variation present in the genome. Consequently it does not fulfil many of the requirements of a commercially exploitable or routinely useful nematode identification system.

An extension of this technique examines Restriction Fragment Length Polymorphisms (RFLPs) and has been used in some of the earliest work examining intraspecific nematode genetic variation. Total genomic DNA is digested with a restriction enzyme, separated on a gel and blotted on to a membrane (this process is termed Southern blotting after its inventor). A radioactively labelled probe is then hybridised with the DNA on the membrane. The probe is specifically chosen by the user and can be almost any fragment of cloned or previously isolated DNA. Whether the probe will hybridise depends on its degree of homology with the DNA under examination. Differences in the banding pattern obtained reflect differences in the distribution of recognition sites for the restriction enzyme used in the original digest. These differences can then be used to estimate genetic variation. Some RFLPs are consistently associated with a biological trait, such as resistance to a pathogen and can be considered to be linked to the gene associated with the biological phenomenon. Most uses of RFLPs in nematology have been for purely taxonomic purposes but one of the earliest applications was the analysis of genetic variation between different populations of *G. pallida* (Schnick *et al.,* 1990; Phillips *et al.,* 1992). A range of probes from small insert genomic libraries were used in both these studies to demonstrate genetic differences within *G. pallida.* However, it proved difficult to correlate these polymorphisms with virulence or to produce consistently pathotype specific probes.

Another approach for obtaining diagnostic tools has been to produce a specific DNA probe (usually cloned into a plasmid vector) which specifically hybridises to the DNA of a particular nematode species. The most popular method of achieving this has been to produce a small insert genomic library from the nematode species in question and to screen it with the digested genomic DNA of a second, related, nematode species. Clones which are not recognised by the DNA of the related species are likely to contain sequences specific to the nematode used for library construction. This can be confirmed using dot blot techniques and at this stage the candidate clone can also be probed with the DNA of other more distantly related species to confirm that it is truly species specific. This strategy has been used to produce cloned DNA fragments which differentiate *G. pallida* and *G. rostochiensis* (Burrows & Perry, 1988)

and a probe used for identification of *Ditylenchus dipsaci* (Palmer *et al.*, 1991). Because the root knot nematodes are so much more homogeneous than the cyst nematodes it has proved more difficult to produce species specific probes to the various members of the genus *Meloidogyne* than to *Globodera* species. Hence, a similar but more refined method has been used to produce a probe which specifically recognises *M. incognita* (Chacon *et al.*, 1991). Initially, a probe which recognised *M. incognita* more strongly than *M. javanica* and *M. arenaria* was isolated using a similar approach to that described above. This cloned fragment of DNA was then digested into smaller fragments each of which was then tested for its ability to hybridise with the DNA of various *Meloidogyne* species. Most of the fragments cross reacted with the DNA from all species tested. One fragment however specifically hybridised to the DNA of *M. incognita* and could therefore be used as a species specific probe. A short probe such as this, once sequenced, can also be chemically synthesised rather than cloned.

Most of these techniques including RFLPs have been used extensively for genetical studies but suffer from several limitations; they require relatively large quantities of DNA; they are technically complicated to perform and require the use of radioisotopes. For these reasons techniques based on the Polymerase Chain Reaction (PCR) are proving more useful in studies on genetic variation. This procedure amplifies specific parts of the genome, is simpler to perform and requires only nanogram quantities of DNA. Short oligonucleotide primers of known sequence are mixed with the target or template DNA where they bind to homologous regions. A polymerase enzyme duplicates the DNA between primer binding sites. The process is repeated a number of times with the DNA being amplified each time thus producing large amounts of a specific product from a small initial quantity. The product or products of the PCR reaction are separated on agarose gels and visualised by staining with ethidium bromide or, prior to this, they can be digested with restriction enzymes to examine RFLPs within the product.

This technique has been applied to amplifying highly repeated sequences in the nematode genome for diagnostic purposes. This approach yields highly sensitive assays since some DNA sequences are repeated many thousands of time in the genome. The two most popular repeated regions used for taxonomic and diagnostic purposes are ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA).

The genes coding for ribosomal RNA are arranged in cukaryotes in tandem repeats. Although some variation in gene arrangement occurs the structure of tandem repeats is fairly well conserved. Each repeat consists of three coding regions which are separated by two internal transcribed spacers (ITS). Each repeat is separated by an external transcribed spacer and by an intergenic spacer (Fig. 1). The rDNA is useful for diagnostic purposes as it is repeated many hundreds of times in the genome (thus increasing sensitivity) and because the spacer regions tend to be variable between species. PCR primers can be made which allow amplification of one or even both of the ITS regions; this is facilitated by the fact that the rRNA coding regions are fairly conserved between species. The amplified ITS regions can then be examined by restriction digestion or by cloning and sequencing, and differences between species or populations can be sought. ITS restriction fragment length polymorphisms (RFLPs) have been used as taxonomic indicators for the *Xiphinema americanum* group (Vrain *et al.*, 1992) whilst direct analysis of ITS sequence has been used to examine phylogenetic relationships between members of the genus *Heterodera* (Ferris *et al.*, 1993). In studies on insects, it has been shown that analysis of ribosomal ITS and IGS regions may provide intraspecific markers and that even biotype specific patterns of variation may be found (Birch *et al.*, 1994). Furthermore, in a preliminary report, differences in the variation levels within the ITS regions of South American and European populations of *Globodera* species have been described (Fleming & Powers, 1995). Thus use of rDNA can be seen to provide an excellent tool for phylogenetic and ecological studies.

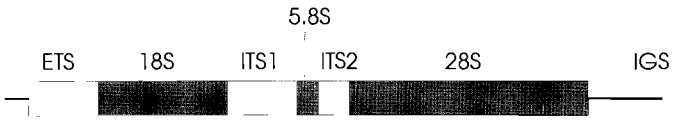

Fig. 1. *rDNA array in eukaryotes showing positions of rRNA genes and spacer regions.*

Restriction fragment analysis of mitochondrial DNA (mtDNA) and sequencing of specific regions of the mitochondrial genome following amplification by PCR are currently the methods of choice for many studies to identify variation at the population level. There are several reasons for this : there are many copies of the mitochondrial genome in each cell which increases the sensitivity of any assay based on mtDNA, the small size of the mtDNA genome and conservation of its gene content amongst metazoans. It is almost exclusively maternally inherited; this is of value in studies on introductions of organisms into new environments. Most importantly it has been estimated that mtDNA evolves five to ten times faster than single copy nuclear genes in primates (Brown *et al.*, 1979) and although the concept of a uniform rate of evolution for mtDNA is now considered redundant (Vawter & Brown, 1986) it does seem likely that the mtDNA of plant parasitic nematodes evolves faster than its nuclear genome as is the case in *C. elegans* (Kelly & Wilson, 1991). All these reasons coupled with the lack of segregation and recombination within the mtDNA means that the mitochondrial genome contains complete and unambiguous informa-

tion and has consequently led to a considerable effort being put into analysis of the mtDNA of plant parasitic nematodes.

There are a number of approaches used to analyse mtDNA depending on the application. Five major *Meloidogyne* species, for example, were identified by amplification of a region of the mitochondrial genome encoding cytochrome b and 16S rRNA genes and RFLP analysis of the PCR product (Powers & Harris, 1993). The use of PCR in this strategy enabled identification from single juveniles. An alternative approach is to isolate a species specific mtDNA probe (e.g. Besal *et al.,* 1988). Using this method, *Heterodera glycines* could be unambiguously distinguished from *H. leuceilyma, H. weissi* and *G. virginae.* Furthermore, *H. trifolii* was characterised by weak hybridisation. This approach enabled precise detection of the target DNA in the presence of unfamiliar microbial and plant host nucleic acid and may therefore be suitable for field testing. Mitochondrial DNA analysis has been shown to be a powerful tool for the investigation of genetic variation at the subspecies level. Two DNA probes made from regions of repetitive mtDNA have been used to distinguish all host races of *M. incognita, M. hapla* and *M. arenaria* (Okimoto *et al.,* 1991). This method provides an alternative to lengthy and cumbersome host specificity testing procedures used traditionally for the identification of races of *Meloidogyne* species.

Similar techniques have been used to assess genetic diversity at the population level. Genomic digests and hybridisation with total mtDNA probes have been used to reveal RFLP variation between populations of *G. pallida* (Phillips *et al.,* 1992). At present this work is being extended to sequencing informative regions of the mitochondrial genome to establish levels of genetic diversity in this parasite.

The PCR-based techniques described above use targetted areas of the genome but other PCR approaches randomly sample the genome. Examples of such techniques, which are used to generate molecular markers are RAPD PCR (RAPD - Randomly Amplified Polymorphic DNAs) (Williams *et al.,* 1990) and arbitrarily primed PCR (Welsh & McClelland, 1990). RAPDs are generated by using a single short oligonucleotide primer which binds to many regions in the genome in a PCR reaction. The primer is usually around ten nucleotides long, of random sequence, approximately 50 % GC rich and lacking any internal inverted repeats. The products of the PCR reaction – usually five to ten amplification products are obtained, separated on agarose gels and visualised by staining with ethidium bromide. The presence or absence of a PCR band is scored. Polymorphisms between samples arise from either changes in the primer binding sequence (e.g. point mutations) which prevent stable association between primer and template or changes in the template which prevent successful amplification (e.g. inversions, deletions or insertions). RAPD analysis has been used at its simplest level in studies on *G. rostochiensis* and *G. pallida* aimed at estimating genetic variation between and within populations of these nematodes (Folkertsma *et al.,* 1994). In this study RAPDs were shown to separate populations of *G. rostochiensis* into groups correlating with pathotype designations. However, this proved impossible with *G. pallida* populations. It has still proved impossible to develop meaningful lineage (i.e. pathotype) specific markers for PCN using RAPD analysis. This is due to the limitations of the pathotyping system and also the inherent genetic heterozygosity found within this group of nematodes. Studies on the much more homozygous *Meloidogyne* group have been more productive in this respect (Cenis, 1993).

The greatest potential of RAPD analysis is in obtaining markers linked to genes of interest. Although not used yet in nematological studies, combining RAPD analysis with the use of NILs (near isogenic lines) has been the most productive way of isolating markers linked to a trait of interest (Waugh & Powell, 1992). NILs are generated by repeatedly backcrossing with selection for the trait of interest after each round of crossing. One example may be repeated back-crossing a resistant genotype with a susceptible genotype and at each stage selecting the resistant offspring. After a number of backcrosses the offspring are essentially genetically identical at all loci except for the region surrounding the trait under selection. There is then an extremely high probability that any polymorphisms identified between the susceptible parent and the resistant NIL are located close to the gene of interest. Martin *et al.* (1991) used this approach to isolate three markers tightly linked to the *pto* gene in tomato, which confers resistance to *Pseudomonas.* Attempts to apply this approach to nematodes have been rare : repeated backcrossing of many nematodes rapidly reduces their fecundity. Bakker *et al.* (1993) however are attempting to identify markers linked to virulence genes in PCN by studying the inheritance of the gene that confers virulence to the H1 resistance gene. They first confirmed a gene for gene relationship (Janssen *et al.,* 1991) and subsequently by crossing virulent and avirulent lines and repeatedly backcrossing to the avirulent line have produced a range of virulent lines with a largely avirulent background. RAPD markers associated with virulence are now being sought. Additionally they expect this approach to allow the production of a linkage map. In another study, Pastrik *et al.* (1995) have used RAPDs to analyse a population of *G. pallida* selected for increased virulence to resistance derived from *Solanum vernei.* Comparison of this selected population with an avirulent population revealed two RAPD products associated with virulent lines. Furthermore, after cloning, this product was shown by hybridisation studies to bind only to the DNA of " virulent " populations, suggesting it is tightly linked to a gene involved in virulence.

Assuming virulence has a genetic basis, such studies and others using emerging marker technologies such as simple sequence repeats (SSRs) and amplified fragment length polymorphisms (AFLPs) are likely to become more common in future as groups begin to unravel the molecular mechanisms behind the diversity observed in many groups of nematodes.

Gene cloning

Gene cloning can be defined as the application of molecular techniques to the study of the function of an organism. Genes are cloned for several reasons. Cloning the gene coding for a protein is the simplest way in which it is possible, albeit indirectly, to examine the structure and function of that protein. In the absence of a suitable antibody the only way to examine expression patterns of a gene is through DNA based techniques which require knowledge of the sequence of a gene. To answer questions about how a gene is regulated it is clearly necessary to begin by cloning the gene of interest. A range of techniques and approaches are available for the isolation of genes. These include DNA based approaches such as homology cloning and protein based approaches in which, for example, antibodies may be used as a tool for the isolation of a gene.

DNA-BASED APPROACHES

DNA libraries are valuable tools for the isolation of genes. A cDNA library is made by cloning all the *expressed* genes from a particular tissue or nematode stage whilst a genomic library is made by digesting the entire genome, coding and non coding regions, and cloning it all in small fragments. Genes can be isolated from such libraries by screening them using a related gene or the same gene from a different organism as a probe. A cDNA library is screened if coding regions are the main interest. If the entire gene is to be examined a genomic library is screened. A genomic library is also chosen if the time of expression of the target gene is not known. The main advantage of the library screening approach is its simple and direct nature. Disadvantages include the relatively large quantities of DNA or RNA required for construction of a representative DNA library (although this problem may be overcome by the use of an amplification step in the protocol; Bertioli *et al.,* 1994) and the fact that the probe used to screen the library may not be sufficiently homologous to the target gene to allow successful screening.

This approach has been used in a number of studies on nematodes. Two examples are studies on molecules important in cuticle structure : collagens and cuticulins.

Collagens are structural proteins found in all metazoan phyla and are characterized by their triple helical structure. This structure is given to the molecules by regions of the molecules composed of the (Gly-X-Y)n repeat in which Gly is always glycine and the X and Y are frequently proline and hydroxyproline. It was the fact that this region is so conserved and makes up such a large part of the collagen molecule that allowed a probe made from chicken collagen to be used to isolate the collagen genes from *C. elegans* (Kramer *et al.,* 1982). First a southern blot of *C. elegans* genomic DNA was carried out with the chicken collagen gene to confirm that the probe was sufficiently homologous to the target to bind successfully. This also allowed an estimate of the size of the collagen gene family of *C. elegans* to be made. A genomic library was then screened in order to obtain clones containing *C. elegans* collagen genes. Two genes, *col-1* and *col-2,* were obtained in this way. These genes could then be used as probes themselves, in order to isolate further members of this gene family.

More familiar to plant nematologists is the use of genes cloned from *C. elegans* as probes for the isolation of the corresponding genes from plant parasitic nematodes. The isolation of the gene coding for cuticulin from *Meloidogyne artiellia* is one example of the application of this strategy (De Luca *et al.,* 1994). Cuticulins are insoluble non-collagenous components of the nematode cuticle first described from *Ascaris lumbricoides* (Fujimoto & Kanaya, 1973). Two forms of this gene, *cut-1* and *cut-2* were subsequently isolated from *C. elegans.* A probe made from the *C. elegans cut-1* gene was used to screen a genomic DNA library made from *M. artiellia.* Analysis showed that the genes from the two organisms were 75 % homologous at the amino acid level and that they had similar promoter regions. Homology was also observed between the promoter regions of the *cut* genes and the promoter regions of two other cuticle genes from *C. elegans, col-2* and *col-6.* However, the *cut-1* genes from *C. elegans* and *M. artiellia* were found to have entirely different genomic organisation. This work demonstrates how a great deal of information about a gene can be gathered fairly simply by library screening if a suitable probe is available. It should be noted however, that collagens and cuticulins are ancient and well conserved genes, some of which are thought to have evolved before nematodes became a separate phylogenetic group. Other, less well conserved genes, may be more difficult to isolate using this approach. In this case a PCR based approach which focuses on regions of high homology and effectively ignores regions which may be more variable may be more productive.

The development of the polymerase chain reaction immediately overcame one of the major obstacles facing nematologists wishing to carry out molecular biological studies, i.e., the need for relatively large amounts of starting material. Using PCR it is routinely possible to start an experiment with a few nanograms of nucleic acid. PCR is also used as a tool for cloning genes. The products of a PCR reaction are determined by the primers used in the reaction. By designing primers which would amplify a portion of the gene of interest from a

related species one can hope to amplify the same portion of the gene from another species. The DNA sequence of the gene need not be known : it is possible to design primers for the isolation of a gene fragment given only an amino acid sequence. This approach is useful since when amino acid sequences of a gene from different species or of related genes from a gene family are compared regions of high homology, conserved regions, are often observed. These regions are often important to the structure or function of a protein; essential to the identity of the molecule. Degenerate PCR primers (made from amino acid sequences with variation at several nucleotide residues to account for the degenerate nature of the genetic code) designed to recognise the DNA coding for these regions can be made and employed in a PCR reaction. Thus the gene is probed only at the regions where high homology is likely to be found, rather than across the whole gene as for a library screen. Furthermore, the resulting PCR product can then be used as a probe to isolate the rest of the gene from a DNA library with a greater chance of success since the probe is, by definition, 100 % homologous to the target.

Although this approach has been used infrequently with plant parasitic nematodes it is currently being used to isolate steroid hormone receptors (Jones *et al.,* 1995) and for studies on the mitochondrial cytochrome *b* gene (Armstrong *et al.,* 1995) from *Globodera* and *Heterodera* species. The approach was pioneered by Libert *et al.* (1989) in studies on the thyrotropin receptors of mammals. These neurotransmitter receptors are members of a superfamily of genes, the G-protein coupled or seven transmembrane domain receptors. These genes code for molecules throughout the living world with a diverse range of functions including mating factor receptors in yeast, the visual pigment opsin and neurotransmitter receptors throughout the animal kingdom. When the amino acid sequences of the various members of this group are compared seven conserved regions are observed containing predominantly hydrophobic amino acid residues. These regions of the molecules are thought to reside in cell membranes. Libert *et al.* (1989) designed degenerate PCR primers which recognised the DNA coding for several of these regions which were then used in PCR reactions. The resulting PCR products were cloned, sequenced and the sequences were compared to those of known receptors. In this way 40 gene fragments were isolated which had significant homology to known receptors. Subsequent library screenings using some of these fragments as probes yielded a number of full length novel receptor sequences, including putative ß-adrenergic and serotonin receptors.

This method will doubtless be used more and more for the isolation of genes from plant parasitic nematodes given its requirements for minimal starting material and the ever increasing repertoire of genes being cloned from *C. elegans*.

Protein-based approaches

There are a number of methods for isolating genes starting from proteins when nothing is known about the DNA sequence of the gene of interest. One uses an expression library and antibodies as a tool for gene isolation while the other relies on direct analysis of the amino acid sequence of a protein.

Antibodies are invaluable tools for the molecular biologist. With an antibody it is possible to examine the spatial expression of the molecule of interest using microscopical techniques (often the criterion used in the screening of the antibody in the first place). The temporal expression pattern can also be examined by western blotting using proteins extracted from tissues of different ages. The same technique also facilitates the gathering of preliminary information (molecular weight, pI) about the molecule. Various staining methods may also be applied to determine whether or not the molecule is glycosylated. Finally, the antibody can be used to isolate the gene coding for the protein from an expression library. This, however, is not necessarily a simple matter. When making an expression library genes are cloned directionally into a vector/host system which will allow transcription and translation of the gene, usually as a fusion protein, in the presence of a chosen stimulant. In order to achieve expression the gene must be in the correct reading frame for translation to occur and if expression occurs the product must be non-toxic to the host cells. Further problems may be encountered during screening. There is no guarantee that the protein will be folded in the host cells as it would be in its natual environment and it is unlikely to be glycosylated as it may usually be. Both these factors may influence the ability of the antibody to bind normally to the molecule.

Despite these potential problems, expression libraries are regular sources of novel genes and have even been so for plant nematologists. One example is the recent isolation of a molecule produced in the subventral gland cells (SVG) of *M. incognita* (Ray *et al.,* 1994). Initially an antibody was selected on the basis of its ability to specifically recognise an epitope in the SVG. Electron microscopical studies using immunogold techniques were used to demonstrate that the antibody recognised an epitope in the secretory granules of the gland cell, suggesting that the molecule was secreted and may have a role in the host-parasite relationship. Western blots were used to estimate the molecular weight of the molecule and an expression library was constructed and screened. A cDNA clone was isolated from the expression library, sequenced and used to screen a genomic library. The sequence of the gene suggested that the antibody recognised a molecule important in intracellular transport, possibly in moving the secretory granules around the SVG cell. Analysis of the genomic organisation of the gene was also carried out. Thus, starting with an antibody it has been possible to isolate a full length gene

sequence and hence a deduced amino acid sequence. Furthermore, the structure of the gene has been examined and investigations into its regulation by promoter analysis are now feasible.

Occasions may arise where a protein may be of potential interest but no antibody is available for screening an expression library. Comparisons of 2-D gels may, for example, reveal unique protein spots worthy of further investigation. Whilst one course of action may be to cut out the protein and use it to raise an antibody, another method of obtaining a handle on the gene is to sequence the N-terminal of the protein directly and to use this information to access a clone in a cDNA or genomic library. Only a limited number of amino acid residues of a protein can be sequenced in this way but it is often possible to obtain more sequence by specifically cleaving the protein in one or two places thus generating one or two more N terminals for sequencing. Amino acid sequencing is traditionally carried out by someone else who you send a suitable quantity of protein to, usually fixed on to a membrane sold for this purpose. Two methods of protein sequencing are used : Edmann degradation and ladder sequencing. Edmann degradation is the more extensively used technique and generates up to a dozen amino acids of sequence from a few micrograms of protein. Ladder sequencing is potentially more sensitive and may therefore give a longer sequence from less protein. This technique is still being developed however, and is rarely used on anything other than small polypeptides. Protein sequencing requires fairly large amounts of pure protein, often difficult to isolate from plant parasitic nematodes. For this reason little work of this nature has been carried out on nematode proteins. However, the technique has recently been applied to the study of the interaction between *Heterodera schachtii* and *Arabidopsis thaliana* (Grundler *et al.*, 1995; Schmidt *et al.*, 1995). Proteins of root extracts from *A. thaliana* were separated by 2-D gel electrophoresis. Comparisons of gels obtained from infected and uninfected roots were made and several spots unique to infected roots were identified. One of these was also identified by an antibody previously shown to bind specifically to feedings sites. Protein cleavage followed by sequencing revealed that the parts of this molecule sequenced had significant homology to the cyanogenic ß-glycosidases of various other plant species. On the basis of the sequences obtained it is now possible for these workers to design degenerate oligonucleotides for library screening or PCR in order to determine the full length sequence of this molecule and understand its function in the host parasite relationship. These examples and strategies show how genes can be isolated and show some of the ways in which the genes are selected for study. In some cases the selection of the gene to be studied may also lead to its isolation.

Analysis of differentially expressed genes

Differentially expressed genes are those genes whose expression is limited to a particular tissue or stage. They are often of interest as they effectively confer identity to a stage or tissue and allow a particular function to be carried out. The methods used for analysis and isolation of differentially expressed genes have always been some of the most technically challenging and confusing in molecular biology. In some ways the simplest way of examining differential gene expression is to compare two dimensional protein gels from two samples. This however leaves one with the task of isolating the gene from a protein spot which is a far from trivial problem. A similar system for separation of mRNA from two samples would be a wonderful tool : it is far easier to go from mRNA to gene than from protein to gene. However, no such system exists and molecular biologists are left to choose from three main approaches for isolating differentially expressed genes : differential screening of cDNA libraries, construction of subtracted libraries or methods based on differential display.

Differential library screening is, in principle, the simplest approach to the problem. A cDNA library made from the tissue of interest is screened with probes made from the mRNA of another tissue. Clones which do not hybridise with the mRNA of the other tissue may be specific to the tissue of interest and may be worthy of further investigation.

This approach allows the library to be screened with probes from a range of tissues in order to narrow down the number of genes to be examined and thus increase the chances of isolating a gene specific to the tissue of interest.

Often, it is enough to isolate genes specific to one tissue compared to another, and to remove the genes responsible for the everyday functioning of all cells : the housekeeping genes. An example (as discussed below) may be the desire to isolate syncitium specific genes. In this case it is sufficient to compare the syncitium with root tissue, removing all housekeeping genes, all genes which make a cell a root cell and leaving behind only genes which are specific to or up regulated in the syncitium. A more productive approach however, may be to construct a subtracted library, enriched in syncitium specific clones and to screen that differentially.

The principle behind a subtracted library is that a library is made from the tissue of interest (the target) with genes expressed in another tissue (the driver) removed beforehand. A number of methods to achieve this exist. In one, first strand cDNA is made from the mRNA of the driver tissue and a label, such as biotin, is incorporated. A vast excess of this cDNA is then hybridized with mRNA from the target tissue. Since the mRNA and cDNA are opposite strands, sequences common to both will hybridise to one another. These hybridised sequences and any unhybridised (single

stranded) driver cDNA can be removed using avidin, which specifically binds to the biotin tag. The remaining mRNA can then be made into a target specific library which is screened differentially as above.

These approaches have been used by a number of groups attempting to isolate genes specifically expressed in the syncitia or giant cells induced by plant parasitic nematodes. Gurr *et al.* (1991) used a differential screening method to isolate a gene expressed in the giant cells induced in potato by *G. rostochiensis* but declined to reveal the nature of this gene. More success was obtained using the subtracted library approach outlined above modified by the addition of a PCR amplification step in order to overcome the fact that only a minute quantity of mRNA can be isolated from syncitia (Wilson *et al.*, 1994). This method yielded 58 clones for further analysis. While this, and other, methods may allow a range of genes up regulated in giant cells or syncitia to be isolated and may also allow genetically engineered resistant plants to be produced, it seems unlikely that it will yield a great deal of information on how feeding sites are induced by nematodes. The primary changes in plant gene expression responsible for the induction of feeding cell formation need only be brief and clearly will occur very soon after invasion, well before the feeding site is established enough for it to be seen and its RNA collected. If the mechanisms by which nematodes induce feeding sites are to be studied it is likely that studies on nematodes themselves, on what they produce to induce the changes, will be more productive.

Recently a number of methods for the isolation of differentially expressed genes which do not require library construction have been developed. These methods all rely on comparison of randomly amplified mRNAs from two tissues, although they differ slightly in how they achieve this. The first such method, differential display, was reported in Liang and Pardee (1992). In this technique a small percentage of the genes expressed in the tissues to be compared are first converted to cDNA. This cDNA is then subjected to PCR using small randomly chosen primers. The PCR products obtained from the tissues are run on a high resolution gel and those specific to the tissue of interest are isolated and cloned. The main advantages of this method are that no library construction is needed, it is PCR based, which means only small quantities of tissue are required and several tissues can be compared with one another at the same time. The main disadvantages are doubts about the reproducibility and the difficulties many workers encounter in cloning the PCR products obtained. Large numbers of DNA fragments are produced using the amplification conditions required in differential display reactions; isolating the product of interest for cloning is often extremely difficult. No published studies of the use of this technique in nematology exist. It is however being used to isolate genes from syncitia (Bertioli *et al.*, 1995) and to isolate genes expressed in response to hatching stimuli in *G. rostochiensis* (Jones & Perry, 1995).

A modification of this method which may help overcome some of the problems encountered was recently published (Solokov & Prockop, 1994). In this method cDNA is made from all of the mRNA of the tissues of interest using oligo dT or random priming as normal. Arbitrary primers, of a length designed to amplify only a small number of fragments, are then used in a PCR reaction and the products separated on agarose gels. A large number of PCR cycles are used in order to amplify sufficient PCR product for visualisation on agarose gels and direct cloning of differentially amplified bands. Since this technique uses longer primers and produces far fewer products it may take more primer combinations and reactions to isolate a differentially expressed gene. It may be that the genes isolated are easier to clone and more reproducibly amplified however. As yet no reports of success using this technique are published.

Analysis of gene expression

The analysis of when or where in the body a gene is expressed can often give an excellent indication of its function. It may also be necessary to demonstrate spatially specific expression to prove the suspected function of a gene (see examples below). Four methods of examining gene expression patterns are discussed below; northern blotting, RNAse protection assay (RPA), reverse transcriptase - PCR (RT-PCR) and *in situ* hybridisation.

Northern blotting has traditionally been the method of choice for the examination of patterns of gene expression. To carry out a northern blot mRNA from the tissues being examined is separated on a denaturing (usually a formaldehyde) gel. This separated RNA is then transferred to a membrane by any one of a number of methods and is probed with the antisense strand of the gene of interest. The presence of a band in a lane indicates the presence of the mRNA coding for the gene in the original tissue sample and therefore the expression of the gene. No band, no expression (or a degraded RNA sample - positive controls are essential given the ease with which RNA is broken down). This technique has been used routinely in most molecular labs in the world for many years. Ray *et al.* (1994) have used it in a nematological context to examine the expression of the gene coding for a SVG protein of *M. incognita* and found expression in a variety of stages including the J2 and adult female. Northern blots can also be quantitative as long as certain requirements are met; the stronger the band on the blot, the greater the amount of message present in the original sample. However, northern blotting has certain requirements which make it unsuitable for many nematological applications, particularly the requirement for large quantities (several micrograms) of mRNA. Furthermore, it is clearly impossible to dissect

individual tissues from most nematode species in order to examine which part of the body gene expression is occurring in. For this reason the techniques outlined below may be more applicable to nematological studies, although most have yet to be applied.

The RNAse protection assay (RPA) is a direct alternative to the northern blot and is reportedly far more sensitive. In this technique a labelled probe is hybridised directly with mRNA from the tissues of interest. An RNAse which specifically degrades single stranded nucleic acid molecules is then added. Unbound probe and all unrecognised mRNA are degraded and can be removed. All that remains is the double stranded probe-target complex. This can be run on an acrylamide gel and detected as a band, the same size as the original probe. As in a northern blot, the presence of the band indicates gene expression, the absence suggests no gene expression. An advantage of this approach is that as long as the probes are of different size, the expression patterns of several genes can be examined at the same time with the same mRNA sample. This is clearly advantageous if mRNA is in short supply as it often is when working with plant parasitic nematodes.

If even greater sensitivity is required PCR can be incorporated into the protocol. In an RT-PCR reaction very small quantities of mRNA are required and several genes can be searched for at the same time, provided the PCR primers chosen are designed to give products of different lengths. Both these features make it a useful technique for nematologists. The first step is to convert mRNA into cDNA using an enzyme called reverse transcriptase. The first level of selectivity can take place at this stage by using a gene specific primer as the primer for reverse transcription. The products of the RT reaction are then subjected to amplification by PCR with two gene specific primers (one of which is usually the same primer used in the RT reaction). A radioactive label such as ^{32}P dATP is usually included in the reaction to increase the sensitivity. In some cases one of the primers is end labelled in order to cut down on the number of non specific PCR products displayed.

Few examples of the application of this technique to plant nematology exist but the power of the technique has been demonstrated in studies on the analysis of expression of different members of the same multigene family (Simpson *et al.*, 1993). *Phaseolus vulgaris* contains a number of genes coding for the photosynthetic enzyme ribulose 1,5 bisphosphate carboxylase/oxygenase (rubisco). Three of these genes are identical in their coding sequences but have different 3′ untranslated sequences which allows their expression to be regulated differently. To examine the expression patterns of these three genes a primer recognising part of the coding sequence (of all three genes) was produced and end labelled. This was then used in an RT reaction, the products of which were used in combination with three different primers designed to bind to each to the three untranslated regions of the three rubisco genes in a PCR reaction. These primers were designed such that the PCR products from each gene were of slightly different size. The presence or absence of each gene could then be determined by the presence of absence of a band of known size after electrophoresis. RT-PCR with end labelled primers allows transcript detection in a single reaction and different transcripts can be detected in the same reaction. Only minute quantities of RNA are required making this technique suitable for many nematological applications. However, given that nematode dissection is more or less impossible for plant parasites, *in situ* hybridisation is required to examine the spatial expression of genes in these nematodes.

In situ hybridisation combines the skills and techniques of histology and molecular biology. A labelled probe is hybridised to the mRNA preserved in its subcellular localisation in the tissue of interest. Wherever the probe binds and the label is seen it can be concluded that the gene of interest is being expressed. To carry out *in situ* hybridisation the nematode tissue is first fixed (to retain the mRNA in its true location) and then permeabilised (to allow the relatively large DNA/RNA probe access to the targets). A labelled probe is then produced and allowed to permeate the nematode tissue. Washing removes unbound probe and the label is then detected by one of a number of methods. The main advantages of this technique, from a nematological point of view, are that it allows examination of spatial expression patterns and that whole nematodes or nematode sections can be used depending on the level of localisation required : it is even possible to carry out *in situ* hybridisation and to examine the tissue under the electron microscope, providing a suitable label is chosen. The main disadvantage is that it is extremely technically challenging and may take many months to master as a technique. The combination of molecular and microscopical skills required are rare; this is perhaps one area which collaboration between molecular biologists and microscopists may be fruitful. *In situ* hybridisation studies on any nematodes other than *C. elegans* are rare, partly due to the difficulties of using the technique and partly because very few genes have been cloned from plant parasitic nematodes to examine the expression of.

The power of *in situ* hybridisation and of the use of other molecular techniques is demonstrated excellently by two papers describing the isolation of olfactory receptors from the channel catfish and the subsequent use of these receptor genes to elucidate the mechanisms by which olfactory information is encoded and processed by the brain (Ngai *et al.*, 1993 *a, b*). From previous studies (Buck & Axel, 1991) it was known that the olfactory receptors were members of the seven transmembrane domain superfamily of receptors; several had been cloned from mammals. A PCR cloning approach was first used to isolate the homologs of these genes from the catfish. The genomic organisation of the recep-

tors and the size of the gene family was then determined by Southern blotting. In order to confirm the genes isolated coded for olfactory receptors and not some other receptors (e.g. neurotransmitter receptors) it was necessary to demonstrate that they were expressed solely in olfactory tissues. This was achieved using RNAse protection assays on mRNA from a variety of tissues. However, it was the use of *in situ* hybridisation, which allowed the detection of transcripts in single identified cells, which allowed the most useful biological information to be obtained. One of the most important questions in chemoreception has been the need to know how olfactory systems recognise and distinguish many thousands of odours. One hypothesis has been that any given sensory neuron expresses only one receptor type and that discrimination of odours by the brain is achieved by detection of which nerve is stimulated (nerve X firing means odorant A is present, nerve Y means odorant B etc.). Given the ability to detect which neuron is expressing which receptor and to detect how many receptors are being expressed in each neuron using *in situ* hybridisation with the cloned receptors as probes, it was possible to answer this question. Experiments with a number of probes, coupled with the knowledge of the size of the olfactory receptor gene family, showed that each neuron did indeed express only one receptor type and that the hypothesis about olfactory information processing was correct.

These studies demonstrate the power of molecular biology to answer biological questions of fundamental importance. It is in this type of study that molecular biological techniques are most useful and can provide information that other techniques can not.

Future directions

In this section some of the techniques and directions which may be important in the future are discussed. It is clear that the wealth of information from the *C. elegans* project will be of increasing use to plant nematologists. Sequences of genes cloned from this organism will be used as tools to isolate the corresponding genes from plant parasites. However, this approach will be of limited use in the isolation of genes involved in plant parasitism. It seems likely that there will also be a " trickle down " of techniques from *C. elegans* researchers to plant nematologists, as has already been the case for *in situ* hybridisation protocols. Methods developed for one nematode are often easily amended for use with another. There are some cases where this has not been so. Genetical studies using mutants have not been of much use to plant nematologists, as nearly all interesting mutants would be lethal to plant parasites given their highly specialised parasitic life cycles. This will continue to be so unless an artificial feeding mechanism for plant parasitic nematodes is developed. This sort of feeding mechanism would also allow far more biochemical studies to be carried out on plant parasitic nematodes than is currently possible, since great numbers of nematodes could be grown and many more enzymes purified and bioassayed. The absence of biochemical studies on plant parasitic nematodes has seriously hampered our ability to understand their biology. It seems extremely likely that as more sensitive biochemical techniques for working with smaller quantities of biological material are developed in the future, biochemical studies on plant parasitic nematodes will become of major importance.

Another technique which has been of great assistance to molecular biologists has been the ability to produce transformed organisms. Transformation of nematodes has only previously been achieved using *C. elegans*. However, there has not yet been a trickle down of this technique to plant nematologists. Several features of *C. elegans* make it amenable to such studies; it has a short life cycle, reproduces sexually and asexually, it can be maintained on bacterial plates and can be grown in large quantities. Its structure and cell lineages are known in minute detail at the electron microscope level. All of these features facilitated the development and exploitation of a transformation system for this nematode. Since then a range of gene constructs have been developed for transformation including *lac Z* fusions (Fire *et al.*, 1990) and more recently a set of vectors incorporating the green fluorescent protein (gfp). The most productive approach has been " promoter trapping " (Hope, 1991), in which small inserts of genomic DNA are cloned 5′ of a reporter gene in a plasmid vector. Nematodes are transformed with these plasmids by microinjection into the gonad of an adult nematode. Expression of the reporter is achieved in a spatially or temporally specific manner only when the genomic fragment contains a suitable promoter. From this point it is possible to isolate the gene which the promoter normally controls.

Transformation of plant parasitic nematodes is likely to be a much more difficult process than transformation of *C. elegans*. It seems unlikely that a system identical to that used in *C. elegans* will be feasible for plant parasites. Selectable markers, to prove that the nematode is still transformed, are likely to be difficult to find for plant parasites. Several groups are starting work in this area however, using a range of methods on a range of nematodes. It seems probable at the moment that a transient transformation system, where one makes no attempt to maintain transformed nematode lines, is most likely to yield results with plant parasitic nematodes. Such a transformation system would make it possible to isolate genes and study the function of gene products which are presently inaccessible using existing methods.

When used properly to answer the right questions molecular techniques are the most powerful available. They will almost certainly lead to the production of a range of transgenic plants resistant to nematodes in the next few years. They will also allow us to understand the function of nematodes in detail unimagined a few years ago.

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