A comparison of the efficiency of differential display and cDNA-AFLPs as tools for the isolation of differentially expressed parasite genes

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Summary – A range of novel PCR-based techniques for the isolation of differentially expressed genes have recently been described for the isolation of stage-specific or host-responsive parasite genes. We have been using two such techniques, differential display and cDNA-AFLPs, in studies on gene expression changes during hatching and subsequent development of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. In this paper, we compare the efficiency of these two techniques for the isolation of such genes. cDNA-AFLPs were found to be an extremely efficient method of isolating differentially expressed parasite genes while, in contrast, differential display was of limited value. Problems associated with the techniques and their use with plant parasitic nematodes are discussed.

Résumé – *Comparaison de l'efficacité de l'affichage différentiel et du cDNA-AFPs comme outils permettant l'isolement de gènes parasitaires à expression différentielle* - Une série de nouvelles techniques, fondées sur la PCR, pour l'isolement des gènes à expression différentielle ont été récemment décrites pour l'isolement de gènes parasitaires dont la spécificité est liée au stade du parasite ou à la réaction de l'hôte. Nous avons utilisé deux de ces techniques, l'affichage différentiel et le cDNA-AFLPs, pour l'étude des changements de l'expression des gènes durant l'éclosion et le développement ultérieur des nématodes à kystes de la pomme de terre, *Globodera rostochiensis* et *G. pallida*. Dans cet article, nous comparons l'efficacité de ces deux techniques pour l'isolement de tels gènes. Il a été constaté que la technique cDNA-AFLPs constitue une méthode extrêmement efficace pour l'isolement des gènes parasitaires à expression différentielle, tandis que au contraire l'affichage différentiel n'avait qu'une valeur limitée. Les problèmes associés à ces techniques et leur utilisation chez les nématodes parasites des plantes sont discutées.

Key words: cDNA-AFLP, differential display, gene expression, potato cyst nematode.

Differentially expressed genes are those whose expression is limited to a particular tissue or stage or whose expression patterns are changed after a stimulus is received by the organism. They are of interest as they often have important functional roles in the stage or tissue in which they are expressed. In order to isolate genes of potential importance in the pathogenesis of plant parasitic nematodes, we have been carrying out work aimed at the isolation of genes expressed specifically in invasive (second) stage juveniles (J2s) of the potato cyst nematode (PCN) *Globodera pallida*. We have also been carrying out experiments designed to isolate genes from J2s of *G. rostochiensis* whose expression patterns change during the hatching process (Jones & Perry, 1995). Such changes in gene expression may be involved in the transition to a parasitic mode of existence.

The methods traditionally used to isolate differentially expressed genes are amongst the most technically challenging in molecular biology. They include subtractive hybridisation, construction of subtracted libraries and differential screening of cDNA libraries

(reviewed by Jones *et al.*, 1997). Many of these techniques require large quantities of biological material to have a realistic chance of working successfully, limiting their effectiveness in studies on plant parasitic nematodes.

A number of PCR-based techniques have recently been described which potentially simplify the isolation of differentially expressed genes. These include differential display of mRNA (Liang & Pardee, 1992) and, more recently, cDNA-AFLPs (Bachem *et al.*, 1996). Such techniques theoretically have great potential for use in parasitological studies as, in contrast to the more established techniques, they generally require only small amounts of starting biological material.

Differential display is a well publicised technique which has been applied to a range of biological systems. In differential display, a small subset of the mRNAs present in a series of tissues is first converted to cDNA. This cDNA is then used as a template in a series of PCR reactions using small, randomly chosen primers. Comparison of the reaction products from each tissue on a high resolution gel allows differences

to be identified. Bands present in one lane and absent in another can be cut out and cloned for further analysis. Although the technique appears straightforward, practical problems and many modifications of the technique have been described (*e.g.,* Solokov & Prockop, 1994; Yeatman & Mao, 1995; Zhang *et al.,*1996). Differential display appears to be biased towards abundant mRNAs (Bertioli *et al.,* 1995) and the short primers and low annealing temperatures used in the reaction can give rise to high levels of non-specific background products leading to problems in cloning and analysis of the PCR products. Furthermore, since the products are often short and since the nature of the technique is such that one primer in the reaction binds to the poly-A tail of the mRNA, many of the products displayed are from 3' untranslated regions of genes. Such regions are difficult to characterise by database searches or by northern blotting (Bachem *et al.,* 1996). Thus, although simple and elegant in principle, it is often extremely difficult to obtain useful information using differential display.

Recently, cDNA-AFLPs, another PCR-based technique for the isolation of differentially expressed genes was described (Bachem *et al.,* 1996). This protocol, which is based on the AFLP technique developed by Vos *et al.* (1995), is more complex than differential display and is summarised in Fig. 1. AFLP technology is based on the selective amplification of a subset of DNA fragments using PCR. The template DNA is digested with restriction enzymes and double stranded DNA adapters are ligated to the ends of this digested DNA. The sequence of the adapters and adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. Selective amplification of a subset of these PCR products is then achieved by repeating the PCR procedure on the amplified DNA using the same primers but with 3' extensions of one, two or three bases. Only fragments in which the nucleotides flanking the restriction site match those chosen at the 3' end of the primer will be amplified. Products generated from a range of tissues or developmental stages can be compared and differences identified. Bands of interest, present in one lane but absent from others, can be cut out of the gel and cloned for further analysis.

Few studies have described the use of differential display on nematode tissues and no work has been published in which cDNA-AFLPs have been applied to nematological problems. In this paper we describe our experiments using both techniques to investigate changes in gene expression during the life cycle of the potato cyst nematodes *G. rostochiensis* and *G. pallida.* The effectiveness of each technique at generating probes from differentially expressed genes is estimated and the utility of these probes as tools for further iso-lating and characterising the genes from which they are produced is assessed.

Materials and methods

BIOLOGICAL MATERIAL

Cysts of *G. rostochiensis* (Ro1) and *G. pallida* (Pa 2/3) were from single generations. They were grown on appropriate potato cultivars in pots and stored dry at room temperature (approx. 20°C) after extraction. Potato root diffusate (PRD) was obtained by the method of Fenwick (1949) from 10-week-old potato plants (cv. Désirée) grown in sterilised loam pot cultures in a glasshouse. PRD was stored in polythene bottles at 4°C until required, when it was diluted 1 in 4 with sterile distilled water (SDW). The studies on changes in gene expression occurring before, during and after hatching used nematodes at different stages in the hatching process:

Dry cysts: Cysts were rolled to ensure no contaminating material was present.

Cysts stimulated for 3 days: Water soaked cysts, obtained by placing dry cysts in SDW for 5 days, were placed in PRD for 3 days and then rinsed in several changes of SDW to remove hatched J2s.

Hatched J2s: Clean cysts were soaked in SDW for 5 days as above, rinsed in several changes of SDW and placed in diluted PRD. Hatched J2s were collected after 24 h or after longer than 3 days in PRD and rinsed in several changes of SDW.

In other experiments adult females were used. These were obtained by inoculating potatoes in pots with approximately fifteen cysts. After 8 weeks the potato roots were examined for white females which, if present, were removed manually and immediately frozen on dry ice. Adult females, and all other nematode stages used, were stored at −70°C until needed.

DIFFERENTIAL DISPLAY

Total RNA was first extracted by crushing cysts or J2s under liquid nitrogen in a mortar and pestle under ten volumes of solution A (4 M guanidine isothiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 100 mM ß-mercaptoethanol). After the powder had thawed, insoluble debris was removed by centrifugation at 10 000 rpm for 10 min. RNA was purified by acid phenol extraction followed by several rounds of precipitation in isopropanol. After the final precipitation, the pellet was washed thoroughly in 70% ethanol, dried and resuspended in an appropriate volume of DEPC treated water (Sambrook *et al.,* 1989). The integrity of the RNA was checked by running a small aliquot on a formaldehyde gel (Sambrook *et al,* 1989) and checking for the presence of the ribosomal RNA bands. The RNA was quantified by comparisons of ribosomal band intensities with those of RNA

5'———— GAAUUC ——————————UUAA ————— AAAAAAAAAA 3'   mRNA

Reverse Transcriptase
Oligo dT primer   cDNA Synthesis
DNA Polymerase I

5'———— GAATTC ————————————TTAA ———— AAAAAAAAAA 3'   Double stranded cDNA
3'———— CTTAAG ————————————AATT ———— TTTTTTTTTT 5'

EcoRI
MseI   Digestion of template cDNA - only a subset
                              of the cDNA will contain the restriction sites

AATTC ————————————T
G ————————————AAT

TA   Ligate adapters to each end of the DNA

EcoRI Adapter   .TTAA   MseI adapter
                 T4 DNA Ligase

AATTC ————————————TTA   cDNA with adapter sequences attached to each end
TTAAG ————————————AAT

Taq DNA polymerase   Preamplification
Primers binding to adapter sequences

AATTC ————————————TTA   Multiple copies of cDNA with adapter sequences at each end
TTAAG ————————————AAT

Primers
Taq DNA polymerase   Selective amplification

TTAAGXX

AATTC ————————————TTA
TTAAG ————————————AAT

XXTTA

Fig. 1. *Schematic diagram of the cDNA-AFLP procedure (PCR products generated where XX bind to homologous sequences in template DNA; separate products on polyacrylamide gel for analysis).*

molecular weight standards or by UV spectrophoto-metry in a Genequant spectrophotometer (Pharmacia). The RNA was treated with DNASe I (Life Technologies) to remove any contaminating genomic DNA. cDNA was then synthesised using Superscript II Reverse Transcriptase (Life Technologies) according to the manufacturers instructions. The primer used for reverse transcription was the oligonucleotide $T_{12}VC$, where V is a degenerate position representing any nucleotide except T. After cDNA synthesis the sample was treated with RNASe H for 30 min at 37°C followed by an incubation at 95°C for 10 min. The sample was then centrifuged briefly and stored at –20°C until use.

Differential displays were set up essentially according to the method of Liang and Pardee (1992). PCR reactions were set up containing 1X Taq buffer, 1.5 mM $MgCl_2$, 20 μM dNTPs, 1 μM $T_{12}VC$ primer, 1 μM random oligonucleotide, 1 μl cDNA and 1 unit Taq DNA polymerase (Promega). The random oligonucleotide used varied depending on the experiment being carried out but was always a ten mer from one of the Operon kits (Operon). A small amount (approx. 2.5 μCi) of alpha ^{32}P dATP (NEN) was also included in the reaction to allow visualisation of the reaction products. Forty cycles of synthesis were carried out with an annealing temperature of 42°C on a Hybaid Omnigene thermal cycler. Negative controls (PCR reactions containing no template, PCR reactions set up with cDNA synthesised in the absence of reverse transcriptase) were used throughout.

PCR products were separated on 7% polyacrylamide gels, containing urea and TBE, made and run according to standard protocols (Sambrook *et al.*, 1989). Gels were fixed in 10% acetic acid/10% methanol, rinsed in water and dried onto 3MM paper (Whatmann) and PCR products were then visualised by exposing the dried gels to X-ray film overnight.

cDNA-AFLPs

Messenger RNA was extracted from approximately 200 mg of nematode tissue using a Micro-Fast Track kit (Invitrogen) according to the manufacturers instructions. The mRNA was quantified by UV spectrophotometry or using DNA Dipsticks (Invitrogen). cDNA synthesis was carried out using a Riboclone cDNA synthesis kit (Promega). First strand synthesis was primed with an oligo dT primer and followed directly by second strand replacement synthesis using RNASe H and DNA polymerase I (Gubler & Hoffman, 1983). The resulting double stranded cDNA was extracted with phenol/chloroform, ethanol precipitated, resuspended in 20 μl of SDW and stored at –20°C until use.

Aliquots (500 ng) of the cDNA were then subjected to the standard AFLP template production (Vos *et al.*,

1995). The cDNA was first digested with EcoRI and MseI (Pharmacia) for 1 h at 37°C. Next, a solution containing 5 pmol EcoRI adapters, 50 pmol MseI adapters, one unit T4 DNA ligase and an appropriate ligase buffer was added and the incubation was continued for a further 3 h at 37°C.

After inactivation of the ligase at 65°C for 10 min the primary template was stored at -20°C until use. Fifteen cycles of preamplification (94°C: 30s, 56°C: 30s, 72°C: 1 min) were carried out using 1.5 μl of the primary template and preamplification primers designed to bind to the ligated adapters (below). Aliquots of the preamplification reactions were run on a 1.5% agarose gel to verify the presence of a smear of DNA between 100 bp and 1000 bp before proceeding. Following preamplification the reactions were diluted with T0.1E buffer (10 mM Tris, 0.1 mM EDTA) to a final volume of 75 μl. Selective amplification reaction was carried out using amplification primers (below) one of which was previously end labelled with ^{33}P dATP using standard protocols (Sambrook *et al.*, 1989). The products of the reactions were separated on 7% polyacrylamide gels, containing urea and TBE, made and run according to standard protocols (Sambrook *et al.*, 1989). Gels were fixed in 10% acetic acid/10% methanol, rinsed in water and dried onto 3MM paper (Whatmann) and PCR products were then visualised by exposing the dried gels to X-ray film overnight.

CLONING AND FURTHER ANALYSIS OF DIFFERENTIALLY EXPRESSED GENE FRAGMENTS

Some apparently differentially expressed gene fragments generated by differential display or cDNA-AFLPs were cloned for further analysis. Bands of interest were cut out of dried gels and placed in 20 μl elution buffer (0.5M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS) for 4 h at 37°C. One μl of the fluid was then used as a template in PCR reaction carried out under the same conditions and with the same primer combination used in the reaction which generated the product of interest. The reamplified PCR product was run on an agarose gel, cut out and purified using an easimer kit (Scotlab) and finally cloned into the pCR-Script SK+ vector using the pCR-Script cloning kit (Stratagene). Manufacturers instructions for these kits were followed throughout. Subsequent screening and analysis was carried out using standard protocols (Sambrook *et al.*, 1989). Sequencing was carried out on an ABI 373 Stretch automatic sequencer using an Applied Biosystems PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit.

NORTHERN BLOTTING

Digoxigenin labelled probes were generated using a random priming kit (Boehringer, Mannheim) from gel purified inserts digested out of plasmid clones. For cDNA-AFLP products probes were also generated directly from purified and reamplified products prior to cloning. This was possible as the higher annealing temperatures and longer primers used in this technique prevented background products from being generated in the reamplification reactions. 250 ng of mRNA was separated on a 1% formaldehyde gel and transferred to Hybond N+ nylon membrane by capillary transfer. Prehybridisation, hybridisation, washing and detection of bound probes were carried out using standard protocols (Sambrook *et al.*, 1989).

PRIMERS AND ADAPTERS

The sequences of primers and adapters used in this study are:

    OPG 2: GGCACTGAGG
    OPG 6: GTGCCTAACC
    E19: GACTGCGTACCAATTCGA
    E181: GACTGCGTACCAATTCCTA
    M307: GATGAGTCCTGAGTAATCAG
    M44: GATGAGTCCTGAGTAAATC
    M68: GATGAGTCCTGAGTAAGCC
    EcoRI adapter:
        CTCGTAGACTGCGTACC
                CTGACGCATGGTTAA
    Msel adapter:
        GACGATGAGTCCTGAG
                TACTCAGGACTCAT

Results

DIFFERENTIAL DISPLAYS

Differential displays gave reproducible banding patterns when comparing gene expression in *G. rostochiensis* before and after hatching. Many gene fragments were displayed whose expression patterns appeared to change during the hatching process (Fig. 2). Bands were amplified from J2s, both 24 h and several days after hatching, which were not amplified from dry cysts or cysts soaked for 3 days in PRD (Fig. 2B). Examples of genes apparently down regulated during the hatching process were also found (Fig. 2A). Attempts to further characterise the genes represented by these fragments proved unsuccessful. After cloning and sequencing of many fragments of DNA eluted from isolated bands no significant matches to known genes were found in database searches. Many of the fragments isolated were extremely AT rich and contained no open reading frames which gave matches from the database. This problem was accentuated as many of the fragments
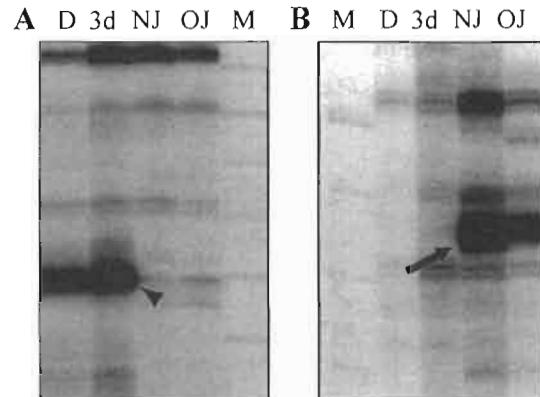
Fig. 2. *Differential display gels comparing hatched and unhatched potato* Globodera *nematodes. A: Bands representing genes apparently down-regulated during the hatching process (arrowhead) are observed; B: Bands representing genes up-regulated in hatched nematodes (arrow) are also observed (Products were generated using primers dTVC and OPG 6 [A] and OPG 2 [B]; M = marker, D = dry cysts; 3d = water soaked cysts placed in PRD for 3 days: NJ = J2s hatched within previous 24h; OJ = J2s hatched up to 3 days previously).*
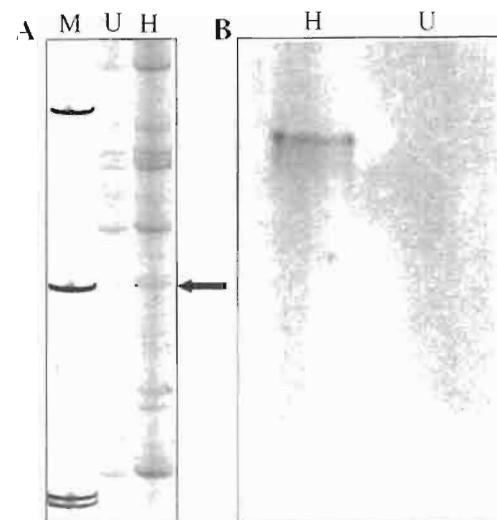
Fig. 3. *Gene fragment (arrow) isolated using cDNA-AFLPs which is expressed specifically in hatched J2s of* Globodera rostochiensis. *A: cDNA-AFLP gel showing a band produced specifically in reactions containing cDNA from hatched J2s. The DNA fragment was generated using primers E181 and M307; B: The band was cut out of the gel, the DNA eluted and used as a template to generate a probe which was subsequently used in a northern blot (M = marker; U = unhatched J2s; H = hatched J2s).*
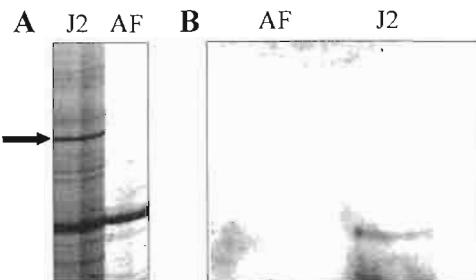
Fig. 4. Gene fragment (arrow) isolated using cDNA-AFLPS expressed specifically in J2s of Globodera pallida. A: cDNA-AFLP gel showing a band produced specifically in reactions containing cDNA from J2s. The DNA fragment was generated using primers E19 and M68. B: Northern blot carried out using a probe generated from this fragment of DNA (AF = adult females).

Fig. 5. A: cDNA-AFLP gel showing a band (arrow) produced at very high levels in reactions using cDNA from adult females but produced at a much lower level from reactions containing cDNA from J2s; the DNA fragment was generated using primers E19 and M44; B: Northern blot using a probe generated from this fragment of DNA suggests the gene is expressed at similar levels in both tissues (AF = adult females).

isolated were extremely short (>200 bp) making meaningful comparisons difficult. It was also clear from the cloning and sequencing experiments that each band on a differential display gel often contained more than one species of DNA of the same size, further complicating analysis. A more serious failure of the differential display technique was that none of the fragments generated ever hybridised to bands on northern blots (not shown), making it impossible to verify whether the fragments were generated from genes which are truly differentially expressed.

CDNA-AFLPs

Like differential displays, cDNA-AFLPs generated highly reproducible banding patterns (Figs 3A, 4A, 5A). Differences in banding patterns were observed between hatched and unhatched J2s of *G. rostochiensis* (Fig. 3A) and between J2s and adult females of *G. pallida* (Fig. 4A). Further analysis of the gene fragments obtained using cDNA-AFLPs was much more productive than for differential display. All fragments tested recognised messages on northern blots, although not all the fragments tested recognised differentially expressed genes. However, the ease with which it was possible to isolate genuinely differentially expressed genes contrasted markedly with the problems encountered using differential display. Genes expressed specifically in second stage juveniles were detected (Fig. 4B). Genes were also isolated which are expressed only after hatching when compared to unhatched juveniles (Fig. 3B). Quantitative differences on cDNA-AFLP gels (Fig. 5A) were not usually reflected on northern blots (Fig. 5B).

Preliminary sequence analysis was carried out on fragments generated using cDNA-AFLPs. Use of the TESTCODE program suggested the fragments were

generated from coding regions of genes. Open reading frames were present through all gene fragments tested, although since some of the fragments sequenced were short (approx 250 bp) this is not unexpected. Sequence homology searches carried out using the BLAST search facility available through the ExPASy home page* showed that some of the fragments obtained had significant homology to known nematode genes.

Predictably, the best matches were found when searches were carried out using the longer fragments of sequence. The constitutively expressed gene fragment shown in Fig. 5, for example, which is almost 350 bp in length showed good similarity to a *Caenorhabditis elegans* vacuolar ATPase. Similarly, the J2-specific fragment (Fig. 4) which is 450 bp in length showed similarity to a number of RNA binding proteins from a range of species, including *C. elegans*. Searches using shorter fragments were less informative. The hatched nematode specific fragment shown in Fig. 3 which was just 180 bp in length, for example, gave no informative matches from searches although sequences generated from the *C. elegans* sequencing project were detected.

* (http://expasy.hcuge.ch/www/tools.html)

Nothing is known yet of the functional significance of any of the sequence matches found. Further characterisation of these genes is currently underway; a cDNA library is being screened with some of the probes. Once full length genes have been isolated it will be more feasible to examine the functional roles of these genes and the significance of their expression patterns. Details of this work will be published elsewhere once it is completed.

Discussion

Although AFLPs and RAPDs have been compared as tools for plant breeding (*e.g.*, Becker *et al.*, 1995; Powell *et al.*, 1997) this is the first work we are aware of which compares the merits of differential display with those of cDNA-AFLPs. Our results show cDNA-AFLPs are a potentially powerful tool for the isolation of differentially expressed genes from parasitic nematodes. All cDNA-AFLP fragments tested to date (those shown here and others not reported) recognised messages on northern blots and 50% recognised genes which were differentially expressed. In contrast no messages, constitutively or differentially expressed, were recognised on northern blots by probes generated using differential display. Although some workers have reported success in using differential display, our findings are in common with those of many others who have encountered problems using this technique (*e.g.*, Bertioli *et al.*, 1995). The existence of such problems is demonstrated further by the large number of papers describing modifications of the technique and the consequent evolution of the technique; when initially described it was claimed that differentially expressed fragments simply had to be cut out of gels and cloned or labelled directly for use on northern blots (Liang & Pardee, 1992). The most recent paper, involving one of the same authors (Zhang *et al.*, 1996), suggests making mini-libraries from DNA eluted from excised fragments, amplifying each of the cloned inserts, dot-blotting these onto duplicate membranes, and screening these dot blots with labelled cDNA from the two tissues of interest in order to identify which clones contain gene fragments from differentially expressed genes. While a feasible operation, this procedure is far removed from the simple and elegant technique described in the original paper!

Analysis of the problems associated with differential display suggests that most arise due to the use of short primers and low annealing temperatures. This gives rise to high non-specific background levels and thermodynamically favours amplification from abundant messages, even where a number of mismatches are present between primer and template, rather than amplification from rare messages where primers and template match exactly. In the cDNA-AFLP technique, highly stringent PCR conditions· are used,

facilitated by the ligation of adapters which serve as primer sites during amplification, making the technique less prone to these problems. Furthermore, unlike differential displays, cDNA-AFLPs do not require the use of a poly-T primer in the amplification reaction making it more likely to generate products from coding regions of genes. Therefore, subsequent analysis and characterisation of the fragments generated is a far simpler prospect. These advantages suggest that cDNA-AFLPs will be the method of choice in future studies on isolation of differentially expressed parasite genes.

References

BACHEM, C. W. B., VAN DER HOEVEN, R. S., DE BRUIJN, S. M., VREUGDENHIL, D., ZABEAU, M. & VISSER, R. G. F. (1996). Visualisation of differential gene expression using a novel method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development. *Plant J.*, 9: 745-753.

BECKER, J., VOS, P., KUIPER, M., SALAMANI, F. & HEUN, M. (1995). Combined mapping of AFLP and RFLP markers in barley. *Molec. Genes Genetics*, 249: 65-73.

BERTIOLI, D. J., SCHLICHTER, U. H. A., ADAMS, M. J., BURROWS, P. R., STEINBISS, H. & ANTONIW, J. F. (1995). An analysis of differential display shows a strong bias towards high copy number mRNAs. *Nucl. Acids Res.*, 23: 4520-4523.

FENWICK, D. W. (1949). lnvestigations on the emergence of larvae from cysts of the potato root eelworm *Heterodera rostochiensis*. 1. Technique and variability. *J. Helminthol.*, 23: 157-170.

GUBLER, U. & HOFFMAN, B. J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene*, 25, 263.

JONES, J. T., PHILLIPS, M. S. & ARMSTRONG, M. A. (1997). Molecular approaches in plant nematology. *Fundam. appl. Nematol.*, 20: 1-14.

JONES, J. T. & PERRY, R. N. (1995). Analysis of gene expression during the hatching process in the potato cyst nematode *Globodera rostochiensis* using the differential display technique. *Nematologica*, 41: 313. [Abstr.]

LIANG, P. & PARDEE, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*, 257: 967-971.

POWELL, W., MORGANTE, M., ANDRE, C., HANAFIJ, M., VOGEL, J., TINGEY, S. & RAFALSKI, A. (1997). The utility of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molec. Breed., (in press)*.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular cloning, a laboratory manual, 2nd Ed.*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 1745 p.

SOLOKOV, B. P. & PROCKOP, D. J. (1994). A rapid and simple PCR-based method for isolation of cDNAs from differentially expressed genes. *Nucl. Acids Res.*, 22: 4009-4015.

VOS, P., HOGERS, R., BLEEKER, M., REIJANS, M., VAN DE LEE, T., HORNES, M., FRIJTERS, A., POT, J., PELEMAN, J., KUIPER, M. & ZABEAU, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucl. Acids Res.*, 23: 4407-4414.

YEATMAN, T. J. & MAO, W. (1995). Identification of a differentially expressed message associated with colon cancer liver metastasis using an improved method of differential display. *Nucl. Acids Res.*, 23: 4007-4008.

ZHANG, H., ZHANG, R. & LIANG, P. (1996). Differential screening of gene expression difference enriched by differential display. *Nucl. Acids Res.*, 24: 2454-2455.