

DIFFERENTIAL DISPLAY: A NOVEL PCR-BASED METHOD FOR GENE ISOLATION AND CLONING¹

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ABSTRACT - Differential Display (DDRT-PCR) is a powerful technique for analyzing differences in gene expression. DDRT-PCR has the following essential steps: total RNA isolation and purification, cDNA synthesis from mRNAs, PCR amplification of cDNAs, visualization of PCR products, reamplification and cloning of the differentially expressed PCR products, confirmation by northern blot, sequencing the confirmed clones, and finally cDNA library screening to isolate the genes of interest. After its invention in 1992, a number of modifications have been introduced to optimize the technique and specifically to reduce the major problem of "false positives". Since understanding of specific gene expression patterns that regulate developmental and stress responses is a major concern of molecular biology, DDRT-PCR has become a very popular molecular technique during the past decade.

Additional index terms: flooding, gene expression, mRNA differential display.

DIFFERENTIAL DISPLAY: UM NOVO MÉTODO DE PCR PARA ISOLAMENTO E CLONAGEM DE GENE.

RESUMO - Differential Display (DDRT-PCR) é uma poderosa técnica utilizada para análise de diferenças na expressão de genes. DDRT-PCR engloba as seguintes etapas: isolamento e purificação de RNA total, síntese de cDNA, PCR, visualização dos produtos de PCR, reamplificação e clonagem dos fragmentos de interesse (diferencialmente expressos), confirmação por análise de "northern blot", sequenciamento dos clones confirmados e, finalmente, seleção em bibliotecas de cDNA para isolamento do gene de interesse. Após seu desenvolvimento em 1992, DDRT-PCR tem sofrido uma série de modificações com o propósito de melhorar e otimizar a técnica principalmente no que se refere a eliminação ou diminuição de "falsos positivos". Visto que o entendimento do padrão de expressão gênica e à comparação de genes diferencialmente expressos em vários tecidos são de fundamental importância nas diferentes áreas da biologia molecular, DDRT-PCR tem se tornado, dentro de seus propósitos, uma das ferramentas mais eficientes e práticas da última década.

Termos adicionais para indexação: alagamento, expressão gênica, mRNA differential display.

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INTRODUCTION

Identification of differentially expressed genes in various cells or under different conditions is one of the main areas of molecular biology. Until 1992, subtractive hybridization was the only method that could isolate differentially expressed genes. Although subtractive hybridization is a reliable method, it is tedious, time consuming and difficult to perform. It also requires large amounts of mRNA that can be limited in many situations. In 1992, Liang and Pardee developed a new PCR-based technique called Differential Display (DDRT-PCR) (Liang & Pardee, 1992). This technique focused on detecting differentially expressed genes among nearly 15000 individual mRNA sequences in mammalian cells. It was first described to compare messages that differ between normal and tumorigenic cells.

The method is based on the detection of the differentially expressed cDNAs from two or more samples that are separated on adjacent lanes of sequencing gels. The differentially expressed bands can readily be cloned and used as probes in Northern or Southern (DNA) blots and to isolate genes from cDNA or genomic libraries. Compared to the subtractive hybridization method, DDRT-PCR is simpler, quicker and more sensitive. However, false-positive results can generate a large number of spurious sequences that do not represent differential expressed genes. A number of technical modifications have been introduced to reduce the problem of false positives and to increase the reproducibility of the technique (Liang & Pardee, 1995a; Huang *et al.*, 1996; Jones *et al.* 1997; Zhao *et al.* 1995; Doss, 1996; Pfeffer *et al.* 1995; Averboukh *et al.*, 1996; Chen & Peck, 1996; Callard *et al.* 1994)). Modifications that allowed the display of longer cDNAs have also been reported (Averboukh *et al.*, 1996). Therefore, with properly designed primers and controls, DDRT-PCR could produce results that truly reflect gene expression patterns of different tissues. Recently a comprehensive text containing all the procedure involving this technique, as well as additional information on materials, equipment, reagents and suppliers was published by Collona-Romano *et al.* (1998).

METHOD

In this technique (Fig. 1) mRNAs are first reverse transcribed into cDNA using each of the four sets of generate 3'-two-base anchored poly-T primers ($T_{11}MN$), where M can be G, A or C and N is G, A, T, or C. The use of these primers allows the sampling of mRNA into 12 fractions, which should represent, in theory, 1/12 of the total mRNA population expressed in a particular tissue.

The cDNAs are then amplified by the Polymerase Chain Reaction (PCR) using the same 3' anchored primer and an arbitrary decamer. The DDRT-PCR products are separated on sequencing gels and visualized either by autoradiography or silver staining (Fig. 2). The pieces of

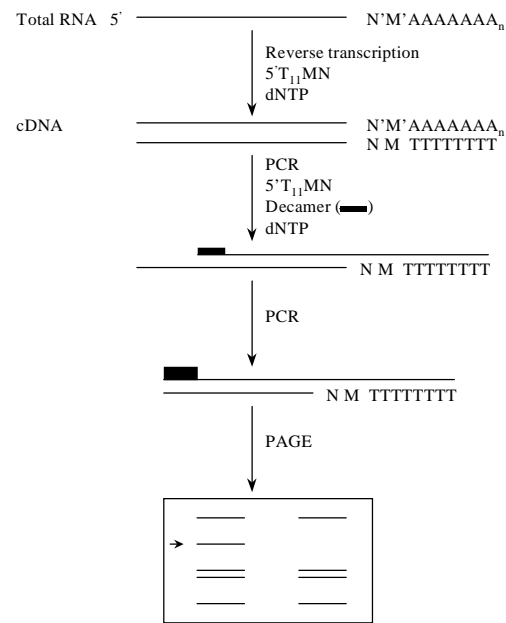


FIGURE 1 - Schematic representation of DDRT-PCR. $T_{11}MN$, degenerate oligo (dT) primer; M indicates A, C or G (degenerate); N can be A, C, G or T. (adapted from Liang & Pardee, 1995b). Arrowhead indicates a differentially expressed fragment.

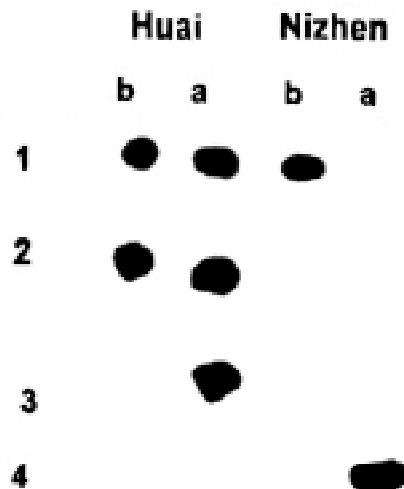


FIGURE 2 - Non-radioisotopic differential display by silver staining. Total RNA (1 μ g) was reverse transcribed using the protocol supplied by Promega. The derived cDNA was used as template in RT-PCR as described by Liang & Pardee (1994). The reaction contained one of each arbitrary decamer (AGTCCGCTGC, CGTCGGGCCT) in combination with one of the degenerated anchored oligo dT primer set ($T_{11}NM$, where M can be G, A, or C and N can be G, A, T, or C). RT-PCR of the cDNA from Huai 849 and Nizhen roots before (b) and after (a) 72 h of submergence using two random decamers and four sets of degenerated anchored oligo (dT) primers produced over 960 bands on the 5% polyacrylamide 8 M urea sequencing gels, and forty three of the bands (see arrowheads) showed differentially expressed patterns (Alves & Vantoi, 1997).

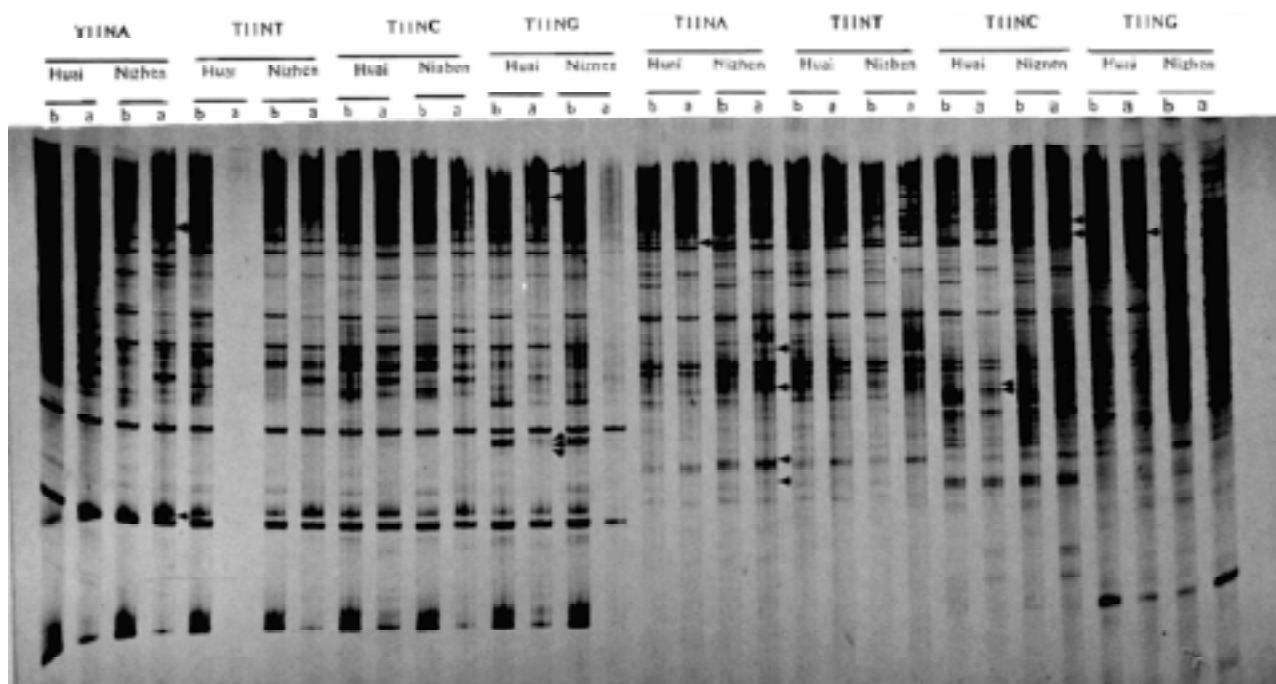


FIGURE 3 - Northern analysis of differential display clones. b and a: before and after 3 days of flooding, respectively; 1, 2, 3, 4: class of gene expression (Alves & Vantoai, 1997).

acrylamide bands corresponding to the differentially expressed PCR products are cut from the gel, reamplified with the same primers and used as probes in northern analysis (Fig. 3). The sequences that reproducibly express the differential pattern are cloned, characterized by restriction mapping and sequenced.

DISCUSSION

In the five years since its introduction, DDRT-PCR has been widely used to identify and analyse the expression patterns of previously uncharacterized genes in many different species. In 1996 the Medline database listed several hundred publications describing genes identified using DDRT-PCR. A great deal of research have focused on understanding the different aspects of developmentally regulated genes that are differentially expressed under stressful conditions in plants (Sharma & Davis, 1995; Knaap & Kende, 1995; Tsengh *et al.*, 1995; Momiyama *et al.*, 1995; Wilkinson *et al.*, 1995; Tieman & Handa, 1996; Alves & Vantoai, 1997; Paiva, 1997). Since this technique is very efficient, a large number of differentially expressed genes can usually be identified in a given condition. For example, using this technique, Mathews and Heinz (1998) reported that over 50 genes were identified in soybean roots in response

to cyst nematode infection. However, the role of these genes in the resistance or susceptibility to cyst nematode remains unknown. Alves and Vantoai (1997) cloned 15 bands which reproducibly expressed the differential pattern in Northern analysis (Fig. 3). Of these, eight clones with unique restriction maps were sequenced. A Genbank search showed no significant homology between six of the eight sequences to known genes. The others two clones showed over 90% homology with a shaggy kinase gene. In *Arabidopsis*, one of the important functions of this gene is the regulation of the DNA binding activity of a transcription factor (Bianchi *et al.*, 1994). Its role in the expression of flooding tolerance in plants remains to be worked out.

While gene identification has received a great deal of attention, the application of molecular biology to agriculture or medicine requires an understanding of gene function which will remain the focus of molecular biology during the next decade.

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