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Rapid selection and classification of positive clones generated by mRNA differential display

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Differential display of mRNA (DDRT–PCR) is a recently developed technique (1,2) which allows the identification and molecular cloning of genes differentially expressed at two states in a given tissue. Using specifically designed 3′-anchored oligo(dT) primers, the mRNA populations of the two states are divided into subpopulations and reverse transcribed. Then, fragments of the two cDNA populations are amplified using the same set of 3′-anchored primers in combination with short, arbitrarily chosen 5′-primers, and the PCR products are separated on denaturing polyacrylamide gels. Bands with differential appearance in DDRT–PCR reactions from the two states of the tissue, the 'differentials', are candidates for cloning and further analysis.

One of the problems intrinsic to the published method is the rather high rate of 'false positives' among cloned differentials, which cannot be confirmed by Northern blot analysis (3). In part, this could be attributed to factors such as bad PCR tubes (4), degraded RNA or contaminating DNA. One major additional problem, however, is the fact that a single band on the gel can be composed of more than one cDNA fragment. Even though there are reports that one band represents only one gene (5), it becomes increasingly evident in the literature that single bands displayed on the gel are additive products of distinct cDNA fragments (3,6). Some of these cDNAs may be derived from differentially expressed genes, others from constitutively expressed genes. For further characterization, it is of interest to select only the clones that are derived from differentially expressed genes. To differentiate between the two classes of genes, Li *et al.* (3) proposed to screen cDNAs generated by DDRT–PCR by Northern blot affinity capturing. This method is applicable only when large quantities of mRNA are available. An alternative approach for selecting clones containing differentially expressed cDNAs consists of spotting plasmid DNA of several clones obtained from one differential onto a nylon membrane which then is hybridized to a portion of the original differential eluted from the polyacrylamide gel (6). This method may work well if the bulk of the radioactivity in the original differential is derived from the actual differentially expressed gene. However, it will fail if the differential is a composite of several constitutively expressed genes, or if one constitutively expressed gene contributes predominantly to the band. To circumvent this problem, we have developed a new method in which the original DDRT–PCR reactions from control RNA and the RNA source of interest are hybridized to several individual clones obtained from one differential. By comparing the hybridization patterns, we were able to distinguish between clones containing cDNA fragments from constitutively expressed genes and clones containing cDNA fragments from differentially expressed genes.

We use DDRT–PCR to identify genes which are differentially expressed during mycorrhizal colonization of bean roots. Total RNA from mycorrhizal and non-mycorrhizal control roots was isolated by a hot phenol method and DNase treated using the MessageClean Kit (GenHunter Corporation). Differential display with one-base anchored primers flanked at the 5′-end by an *Hin*dIII restriction site (7), was performed according to the manufacturer's instructions (RNAimage Kit, GenHunter Corporation) with 30 cycles of PCR, with a final concentration of 3′-primer of 0.25 μM, and with 1 μCi [α-^{33}P]dATP (DuPont NEN, 2000 Ci/mmol). Bands of interest were cut out from the polyacrylamide gel, and the eluted and reamplified cDNA fragments cloned into the pCR-TRAP vector (GenHunter Corporation) according to the manufacturer's instructions. Tetracycline-resistant colonies were screened for plasmids with inserts by PCR using Lgh and Rgh primers (GenHunter Corporation), and six to ten colonies containing inserts of correct size were chosen for further analysis. To differentiate between colonies containing cDNA inserts of constitutively expressed genes and those containing cDNA inserts of differentially expressed genes, inserts were amplified using the Lgh and Rgh primer combination and examined by DNA slot blot analysis (8). Approximately 100 ng of denatured PCR product each was applied in duplicates to a nylon membrane. After baking for 2 h at 80°C in a vacuum oven, the membranes were equilibrated in 6× SSC, and prehybridized for at least 1 h at 60°C in 6× SSC, 5× Denhardt's solution (8), 0.5% SDS and 100 μg/ml denatured salmon sperm DNA (Sigma D-1626). For hybridization, one duplicate membrane was hybridized to the PCR products from control samples, the other one to the PCR products from treated samples. For this purpose, 15 μl of the original PCR reaction performed in the presence of [α-^{33}P]dATP was denatured and added to a fresh aliquot of hybridization solution. For best results, PCR reactions from two to three reactions containing the differential of interest were combined. Hybridization was done overnight at 60°C and the membranes were washed three times for 30 min each in 0.2× SSC, 0.2% SDS at 60°C. The membranes then were exposed for 4–8 h to a BioRad GS-250 Molecular Imager high sensitivity screen and the hybridization patterns compared.

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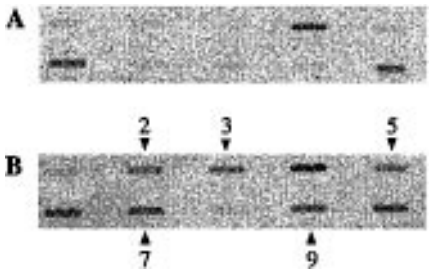

Figure 1. Selection of clones with cDNA inserts from differentially expressed genes by reverse Northern blot analysis utilizing DDRT–PCR products as hybridization probes. 100 ng of PCR amplified inserts of 10 individual *E.coli* colonies obtained by transformation with the differential Myc 4 (H-T_{11}C and H-AP3) were immobilized in duplicate sets onto nylon membranes and hybridized with the original ^{33}P PCR product from control roots (**A**) or from mycorrhizal roots (**B**). The order of inserts is from left to right starting with number 1 and ending with number 10. Clones with differentially expressed cDNAs are indicated by arrows.

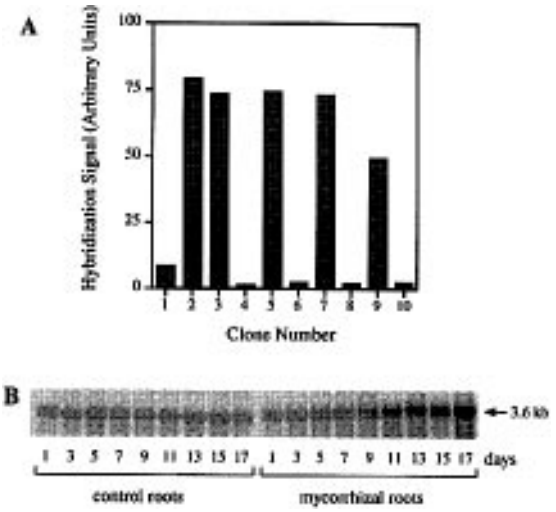

Figure 2. (**A**) Classification of clones with cDNA inserts from differentially expressed genes. H-T_{11}C and H-AP3 PCR product of clone pMyc 4.7 was labelled with [α-^{33}P]dATP and hybridized to a membrane containing the cDNA inserts of clones pMyc 4.1–pMyc 4.10. Hybridization signals were quantified using a BioRad GS-250 Molecular Imager. (**B**) Northern blot analysis of the Myc 4.7 probe. Two membranes, one containing 10 µg/lane RNA from non-mycorrhizal control roots, the other one 10 µg/lane RNA from mycorrhizal roots, were hybridized simultaneously with [α-^{32}P]dATP labelled Myc 4.7 (1×10^6 c.p.m./ml). Days indicate time after inoculation with fungus or corresponding control treatment.

Ten bacterial colonies obtained from the differential Myc 4 (primer combination H-T_{11}C and H-AP3) were screened for plasmids with cDNA inserts of differentially expressed genes. As shown in Figure 1, half of the colonies contained cDNA inserts of genes which were up-regulated in mycorrhizal roots, the other five cDNAs were from genes equally expressed in control and mycorrhizal roots. To determine whether the up-regulated sequences were homologous, the insert of pMyc 4.7, one of the plasmids containing a differentially expressed cDNA, was amplified using the original PCR primer combination (H-T_{11}C and H-AP3), labelled with a random primed DNA labelling kit (Boehringer Mannheim), and hybridized to a membrane that had been charged with Lgh and Rgh PCR products of all other pMyc 4 clones. As shown in Figure 2A, Myc 4.7 hybridized to the PCR products of all clones containing differentially expressed cDNAs, indicating that these sequences are highly homologous. Differential expression of this gene during mycorrhization of bean roots was also verified by Northern blot analysis (Fig. 2B).

When our screening method was applied to eight cloned differentials identified by DDRT–PCR, in three cases all clones tested contained differentially expressed cDNAs, in three cases cloning of the differential revealed a mixed population of constitutively and differentially expressed cDNAs, and in two cases none of the clones tested proved differential (data not shown). Representative positive clones were tested as probes in Northern hybridizations. Five of the six cDNAs were confirmed to be differentially expressed, with two of them representing low abundant messages, and one cDNA probe failed to detect any signal in Northern blots (data not shown).

In conclusion, the two-step method presented allows a fast identification and classification of clones with differentially expressed cDNAs derived from the mRNA differential display. This helps to cut down the number of false positives generated by DDRT–PCR, making this method even more appealing for the rapid identification and characterization of differentially expressed genes.

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