Protocols

Improved Screening of cDNAs Generated by mRNA Differential Display Enables the Selection of True Positives and the Isolation of Weakly Expressed Messages

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Abstract: The high percentage of false positives generated by differential display (as high as 85%) has previously limited the potential of the method. This report describes an efficient methodology that enables false positives to be discarded prior to cloning, via reverse Northern analysis. This first step of the screening also allows the detection of putative low abundance differential clones. Following cloning, a second reverse Northern combined with partial DNA sequencing and RT-PCR detection allows isolation of all differential cDNAs including very low abundance clones. Use of the sequential screening procedure described here led to the isolation of novel tomato genes responding to the plant hormone ethylene while minimising labor and materials input.

Introduction

Differential display (DDRT-PCR), recently developed by Liang and Pardee (Liang & Pardee, 1992; Liang et al., 1993), is a powerful tool

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Abbreviations: DDRT-PCR, differential display reverse transcription-polymerase chain reaction; SDS, sodium dodecylsulfate; SSC, 0.15 M sodium chloride + 15 mM sodium citrate.

for analysing altered gene expression. However, among the putative differentially expressed clones isolated, a high proportion prove to be false positive which represents a real obstacle to exploiting the full potential of the method.

Most false positives arise from the DDRT-PCR step and it is therefore vital that an efficient methodology is adopted to identify and discard false positives at an early step of the differential screening procedure. Northern blot affinity capturing which has been used to identify true positives (Li et al., 1994), relies on the use of one Northern blot for each clone and is therefore impractical when only a small amount of RNA is available or when hundreds of bands are to be analysed. Another approach n aliquot of the DDRT-PCR product as a probe in reverse Northern blot analysis to screen clones for true positives (Vögeli-Lange et al., 1996). This method requires cloning of all bands including false positives and uses DDRT-PCR products as probes which already contain reverse transcription and PCR-borne false differentials. The use of total cDNA probes described by Mou et al. (1994), represents a real improvement over other methods. Zhang et al. (1996) recently described a method in which labeled cDNAs from the RNA samples are used as probes in colony hybridization to differentiate between true and false positives. Once again this method requires the cloning of all differential bands including false positives. In addition, as the screening procedure is based solely on the use of total cDNA probes this method is not sufficiently sensitive to detect low abundance clones. Finally, the use of the colony lift technique is likely to lead to uneven loading of bacterial DNA on the duplicate filters resulting in artefactual differential signals upon hybridization.

In this paper we describe an efficient and time-saving methodology where most false positives are eliminated via reverse Northern analysis prior to the cloning step. In addition, within this first screening step, putative low abundance differential clones are identified. Following cloning, a second reverse Northern combined with partial DNA sequencing and RT-PCR detection allow the isolation of true differential cDNAs including very low abundance clones. The sequential screening procedure described was used to isolate novel tomato genes responding to short term treatment with the plant hormone ethylene.

Materials and Methods

Differential display and reamplification of cDNA fragments

Late immature green tomato fruit (*Lycopersicon esculentum* Mill.), were treated with exogenous ethylene (50 μL.l-1) for either 15 minutes or 5 hours. Total RNA was extracted from both ethylene treated and untreated tomato fruits (Hamilton et al., 1990). The differential display step was performed as previously described (Liang & Pardee, 1992; Liang et al., 1993), using four anchored oligo(dT) primers in combination with twenty 10-mers arbitrary primers. Differential display bands of interest were cut from the non-fixed polyacrylamide gel, eluted in 100 μL TE buffer by boiling for 5 min and then incubated for 6 h at 37 °C. The eluted solution was dried and resuspended in 10 μL of water. A 2 μL aliquot was used to PCR re-amplify the cDNA fragments in a 50 μL reaction mixture under the same conditions as used for the differential display PCR.

Reverse Northerns and cloning

Aliquots of the reamplified fragments (20 μL) were run in duplicate on the same 1.4% agarose gel allowing identical transfer onto a Nylon membrane (GeneScreen Plus, Dupont). Both membrane duplicates were prehybridized for 3 hours at 65 °C in 5· Denhardt's solution, 5· SSC, 0.5% SDS and 100 mg/ml denatured salmon sperm DNA. The duplicate membranes were then hybridized overnight, one to a total cDNA probe from ethylene treated tomato fruit and the other to a total cDNA probe from the untreated sample. The probes were synthesized for 1 hour at 37 °C by reverse transcription of 5 μg of total RNA from each sample using 50 μCi [α–32P]dCTP (Amersham), 0.5 μg of oligo(dT)21 primer, 30 Units of RNAsin (Promega), and 20 U of M-MuLV reverse transcriptase (Stratagene). After hybridization, membranes were washed twice for 20 min at 65 °C in 2· SSC, 1% SDS and once for 15 min at 65 °C in 0.5· SSC, 0.1% SDS, and finally autoradiographed for 24 hours at –80 °C.

The cDNA fragments of interest were cloned into a pGEM® -T vector according to the manufacturer's protocol (Promega). Following transformation, thirteen recombinant colonies issued from each DD band were picked at random, transferred to new agar plates and grown for 16 hours in order to obtain sufficient DNA for subsequent experiments.

Bacterial lysate was then obtained by boiling half of each amplified colony in 10 μL of water for 10 min. Following removing of bacterial debris, by 5 min centrifugation the supernatant was ready for use in: (i) PCR amplification of the cDNA insert for the second reverse Northern blot (1 μL of 1/6 dilution), (ii) partial sequencing (2.5 μL), and (iii) cDNA probe synthesis (1 μL of 1/6 dilution).

Partial sequencing

The partial DNA sequencing was performed using the colony lysates directly as a source of DNA template. Samples corresponding to true differential were sequenced with the ThermosequenaseTM cycle sequencing kit (Amersham) according to the supplier's instructions, except that $\frac{1}{4}$ of the volume indicated in the original protocol was used, and only one ddNTP (G, A, T, or C) was incorporated in the termination step. The reaction was carried out in the presence of [α–35S]dATP using M13 universal primer. The following cycling parameters were used: 40 cycles of [95 °C, 20 s; 60 °C, 30 s] were applied to the labeling step and 40 cycles of [95 °C, 30 s; 72 °C, 1 min] to the termination step.

Northern blot and RT-PCR analysis

Northern blots were carried out as previously described (Ausubel et al., 1987) using 25 μg of total RNA from untreated fruit and from fruit treated with ethylene for 15 min or 5 hours. The probes were synthesized by PCR amplification using insert specific primers and either plasmids or colony lysate as DNA template in the presence of 10 μM dNTP and 50 μCi [α–32P]dCTP.

The RT-PCR analysis was carried out as described previously (Lasserre et al., 1996) except that, after the reverse transcription step, the resulting products were used as templates for the amplification of the cDNA clone of interest using 2 μM of its specific primers, and 2 μM of tomato ubiquitin (*Ubi3*) specific primers for the internal control (the later added to the reaction mixture after two cycles in order to be amplified in a non-saturated manner). The PCR products were separated on a 1.4% agarose gel, transfered to a nylon membrane and hybridized with a mixture of equal cpm of the cDNA and *Ubi3* probes. The labelled cDNA probes were synthesized by PCR using the clone or *Ubi3* specific primers and either plasmids or colony lysate as DNA template in the presence of 200 μM dNTP and 10 μCi [α–32P]dCTP. The membranes

were washed according to the manufacturer's instructions (GeneScreen Plus, Dupont) and exposed to Amersham Hyperfilm-MP at –80 °C for 60 min.

Results and Discussion

Selection of true differential bands prior to cloning

The differential display step was conducted as previously described (Liang & Pardee, 1992; Liang et al., 1993) using total RNA extracted from late immature green tomato fruit (*Lycopersicon esculentum* Mill.) treated or not with exogenous ethylene (50 μL.l-1 for either 15 minutes or 5 hours). Differential display bands of interest were cut and eluted from the polyacrylamide gel and PCR re-amplified using the same primers as in the display step. In the first screening, a reverse Northern blot was used to discriminate between true and false positives prior to cloning. An aliquot of the re-amplified PCR product was run in duplicate on an agarose gel (Fig. 1A) with the remainder stored for subsequent cloning should the bands prove to be true positives. Following identical transfer of the bands, one of the pair of duplicate membranes was hybridized to [32P]-labelled total cDNAs from ethylene-treated tomato fruit and the other to total cDNAs from untreated fruit. When the hybridization patterns corresponding to ethylene treated (+) and untreated tissues (–) were compared (Fig. 1B), most of the isolated cDNA fragments displayed expression independent of ethylene treatment. Out of the total of approximately one thousand bands isolated from the polyacrylamide gels, an equal signal, signifying a false positive, was observed in 85% of cases. While these false positives were subsequently discarded, it should be pointed out that an inherent problem with DDRT-PCR is that differential bands may be masked by bands corresponding to more abundant but constitutively expressed mRNAs. We could not exclude therefore, that some of the bands shown to be false positives in Fig. 1B contained a mixture of weakly expressed differential cDNAs and more abundant constitutive clones.

Interestingly, although all tracks on the gel displayed a visible band upon ethidium bromide staining (Fig. 1A), in several cases the bands failed to hybridize to the probes in either of the duplicates (Fig. 1B open arrowheads). In previous studies, these bands have been either

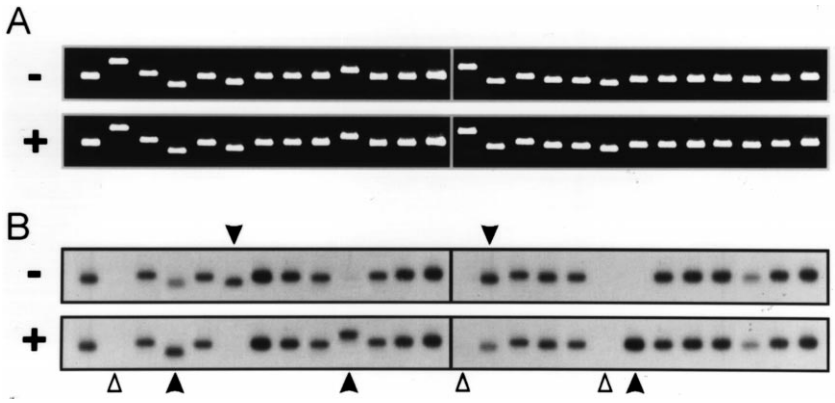

Fig 1. **Elimination of false positive bands prior to cloning.** (A) Differential bands eluted from the polyacrylamide gel were re-amplified. Equal amounts of the reamplified fragments were run in duplicate (– and +) on the same agarose gel and displayed following ethidium bromide staining. (B) Reverse Northern screening. The cDNA fragments were transferred onto a Nylon membrane. The duplicate membranes were then hybridized overnight, one to a total cDNA probe from ethylene treated tomato fruit (+), and the other to a total cDNA probe from the untreated sample (–). The closed arrowheads indicate differential bands and open arrowheads show bands with no expression signal.

disregarded (Mou et al., 1994) or considered as contaminating genomic DNA and discarded (Zhang et al., 1996). However, given the limited sensitivity of the reverse Northern technique, we considered that, among these bands, some may have corresponded to weakly expressed genes. Therefore, both these bands and those identified as differential were subsequently cloned into the pGEM® -T vector (Promega).

Elimination of constitutively expressed cDNAs contaminating the cloned fragments

While the overwhelming majority of false positives were discarded in the first screening, many bands excised from the polyacrylamide gel contain heterogenous cDNA sequences corresponding to a mixture of differentially and constitutively expressed genes (Bauer et al., 1993). In order to eliminate constitutively expressed cDNAs contaminating the selected bands, a second reverse Northern analysis was performed using PCR products of the cloned fragments. Because of the limited sensitivity of the reverse Northern, bands that failed to show any signal

in figure 1B were not re-screened at this step. For all other cloned bands, identified as differential, bacterial colonies were screened via a second reverse Northern as described in the materials and methods. In Fig. 2, we present the results of the further analysis of one of the many DD bands that proved to be positive after the first reverse Northern. In the example illustrated in Fig. 2A, out of thirteen colonies, only seven contained cDNA fragments corresponding to differentially expressed genes. The remaining colonies were either from genes equally expressed in both tissues or from genes whose expression was undetectable.

Identification of independent sequences by partial sequencing

The second reverse Northern identified true differential clones, however, we could not exclude that the seven positive clones, corresponding to one DD band, shown in Fig. 2A corresponded to heterogenous population of cDNAs (Bauer et al., 1993). Therefore, a partial sequencing of cDNA inserts was performed to identify independent sequences among the seven differential clones. This sequencing-based screening was performed directly from colony lysates using a primer flanking the polylinker region (M13), thus there was no need for plasmid extraction or PCR amplification. As shown in Fig. 2B, the 7 differential clones corresponded to two different cDNA populations. Colonies 1 and 7 share the same sequence and colonies 4, 6, 8, 11, and 13 belong to the same cDNA specie. As two band patterns can correspond to the same insert ligated in two different orientations in the plasmid, however, the existence of two different cDNA populations was confirmed by performing a second partial sequencing using another primer located at the opposite side of the polylinker region (data not shown). Even though this interesting finding concerns only few bands ($< 10\%$), it indicates the importance of this step for detecting these differential clones that would otherwise have been missed.

Ethylene-regulated expression of cloned cDNAs

Once the clones had satisfied all of the above criteria, further characterization of their expression patterns could proceed. The differential expression of most isolated ethylene-responsive clones (ER), has been confirmed by Northern analysis. A representative example of the pattern of expression obtained is given in Fig. 3A which shows up-regulated

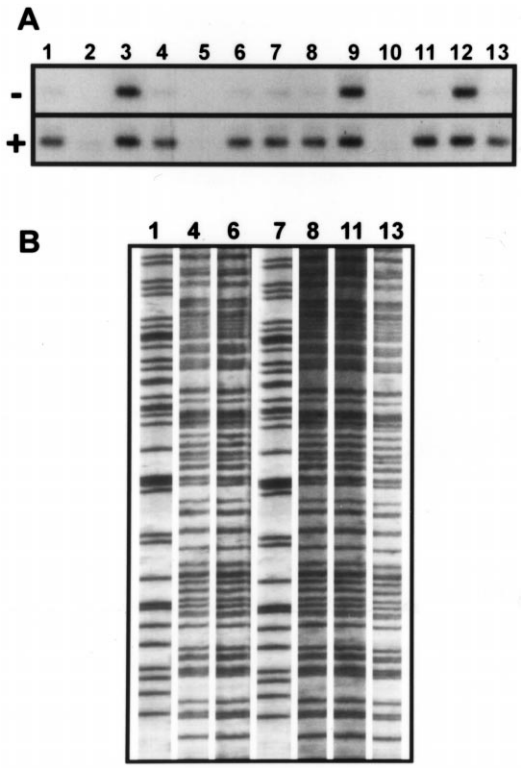

Fig 2. **Selection of colonies containing differentially-expressed inserts.** (A) Elimination of constitutively expressed cDNAs contaminating the cloned fragments. Fragments showing a differential signal in Fig. 1 were cloned and the cDNA inserts corresponding to one DD band were PCR-amplified from single colonies. The PCR products were then processed for analysis by reverse Northern as indicated in Fig. 1. (B) Identification of independent sequences by partial sequencing. The partial DNA sequencing of the true differential clones (colonies 1, 4, 6, 7, 8, 11, 13) was performed using the colony lysates directly as a source of DNA template. The patterns represent the sequencing reactions performed with only ddGTP in the termination step.

expression for ER35 and down-regulation for ER21 upon hormone treatment.

Cloned bands that failed to show any signal in the first reverse Northern (Fig. 1B open arrowheads) were screened by partial sequencing in order to determine the number of DNA species they contain. As we assumed that these clones may correspond to rare mRNA their expres-

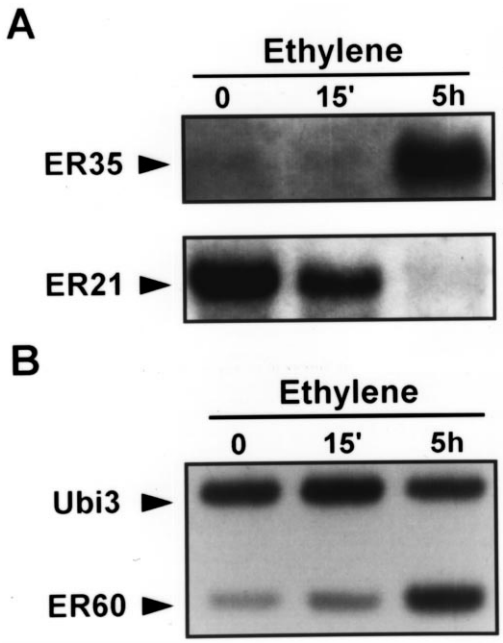

Fig 3. **Ethylene-regulated expression of cloned cDNAs.** (A) Northern blot analysis of positive clones ER21 and ER35. Northern blots were carried out using 25 μg of total RNA from untreated fruit (0) and from fruit treated with ethylene for 15 min (15′) or 5 hours (5 h). (B) RT-PCR detection of ER60 expression. Total RNA from untreated fruit (0) and from fruit treated with ethylene for 15 min (15′) or 5 hours (5 h) were reverse transcribed in the presence of an oligo-d(T)21 primer. The PCR amplification was performed using ER60 cDNA specific primers. As an internal control, the endogenous tomato ubiquitin cDNA (Ubi3) was amplified concomitantly with ER60 by adding to the PCR reaction Ubi3 specific primers. The PCR products were separated on a 1.4% agarose gel, transfered to a nylon membrane and hybridized with a mixture of equal cpm of ER60 and Ubi3 probes. The membranes were exposed to Amersham Hyperfilm-MP at $-80\ ^{\circ}$C for 60 min.

sion pattern in response to ethylene was analyzed by RT-PCR. Figure 3B shows the ethylene regulated expression of the ER60 clone. ER60 is a representative of this low abundance group whose expression could not be detected in the first reverse Northern. The RT-PCR detection was scaled by using ubiquitin as an internal standard during the PCR amplification (Fig. 3B).

In summary, while previous data has clearly shown that DDRT-PCR bands correspond to differentially expressed mRNAs, the generation of false positives has limited the capacity of researchers to conduct extensive searches for differentially expressed genes. The methodology described here opens the way for mRNA differential display to be applied in comprehensive studies of altered gene expression. The overall advantages of the sequential screening procedure described here are: (i) the ability to efficiently discard the majority of false positives prior to cloning thus avoiding considerable waste in cloning and characterizing artefactual bands, and (ii) the detection and isolation of low abundance messages that may have been missed in previous protocols. This latter point could be of prime importance since transcripts corresponding to regulatory genes are often rare.

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