Global gene expression profiling (GGEP) plays a pivotal role in biological research. We developed an improved GGEP method called “robust ordered mRNA differential display (RoDD)” by combining mRNA differential display (DD) and complementary DNA amplified fragment length polymorphisms (cDNA-AFLP) using elaborately designed primers and a poly(dT:A) replacement technique. Redundancy was minimized by bead-based isolation and coverage was improved by using restriction enzymes that recognized 4-bp sites. This method offers the common virtues of gel-based methods along with the reliability of cDNA-AFLP. The most significant advantage of RoDD over current gel-based methods is greatly improved coverage and minimized redundancy.

Global gene expression profiling (GGEP) provides a valuable tool for investigating genes and transcripts on a genome-wide scale. Principally, GGEP strategies fall into three categories: (i) sequencing-based, (ii) hybridization-based, and (iii) gel-based (1). Although global sequencing and microarrays are state-of-the-art, gel-based strategies remain useful for their simplicity, straightforwardness, and universality (2,3).

mRNA differential display (DD) is a traditional gel-based method that amplifies single-stranded cDNA using the HindIII-7-nt-arbitrary primers (4). It is polymorphic in anchoring the 3′-untranslated region (5). However, the primers are so short that DD suffers effects of low stringency PCR conditions including non-specific amplifications and false-positive results; additionally, the arbitrary-7-nt-dependent primer leads to high redundancy (6).

Global gene expression profiling (GGEP) is a traditional gel-based method that combines DD and cDNA-AFLP (Figure 1). The procedure begins by introducing a 5′-biotin-[protection bases]-[AcuI site]-3′ sequence 16 bp upstream of the poly(dT:A) boundary of the cDNA, followed by detecting length polymorphisms of the 3′-restriction fragments using a bead-based cDNA-AFLP protocol that relies on Apol [CTGAAG(N)₆]

and a frequent cutter such as MspI (C’CGG). This method offers the reliability of cDNA-AFLP using adapter-specific primers and high stringency PCR conditions along with the polymorphic nature of DD through anchoring of the 3′-untranslated region. Because the artificial AcuI sites allow full coverage of 3′ ends of mRNAs, the final coverage is dependent on the orderly presence of a 4-bp restriction site in the 5′ ends. The biotin aids in minimizing redundancy through streptavidin-coated magnetic beads. The MspI site

was designed as protection bases to help eliminate the artificial heels when necessary. This method was named “robust ordered mRNA differential display” (RoDD).

A validation experiment was performed. Total RNA was isolated from the leaves and roots of one-week-old rice seedlings (Oryza sativa L.) with TRIZol Reagent (Life Technologies, Carlsbad, CA, USA), followed by purification and quantity and quality assessment as described (4). Single-stranded cDNA was synthesized with PrimerScript Reverse Transcriptase (Takara, Dalian, China) using 500 ng total RNA and 100 pmol 5′-biotin-MspI-AcuI-T₆-V primers (bmaTᵥ⁶V, 5′-biotin-GAGTC-GGAATTTTTTTTTTTTTTTTTTIV, V are anchoring bases composed of A, G and C). Double-stranded cDNA was synthesized by nick translation using RNase H, DNA polymerase I, and Escherichia coli ligase (Takara) and purified using AxyPrep PCR Clean-up kit (Axygen, Union City, CA, USA), followed by digestion using 10 U MspI (New England Biolabs, Ipswich, MA,

to the bead-based cDNA-AFLP protocol, (14) but this technique has not been widely applied.

Here we report an improved GGEP method that combines DD and cDNA-AFLP (Figure 1). The procedure begins by introducing a 5′-biotin-[protection bases]-[AcuI site]-3′ sequence 16 bp upstream of the poly(dT:A) boundary of the cDNA, followed by detecting length polymorphisms of the 3′-restriction fragments using a bead-based cDNA-AFLP protocol that relies on Apol [CTGAAG(N)₆]

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USA) and ligation to 50 pmol MspI adapter (Table 1) using 200 U T4 ligase (New England Biolabs). The biotinylated fragments were trapped by 50 μL activated streptavidin-coated magnetic beads (Prome ga, Madison, WI, USA) in a magnetic field in 1 × STEX buffer (1.0 M NaCl, 10.0 mM Tris-HCl pH 8.0, 1.0 mM EDTA, 1% Triton X-100) and washed with 1 × STEX buffer to remove the redundant fragments, followed by digestion using 5 U AcuI (New England Biolabs) to release the target fragments from the bead-immobilized biotinylated ends, with a 5′-AA or 5′-NN dinucleotide overhang retained. The 5′-NN overhangs were derived from the existing AcuI sites in some restriction fragments. The released fragments (in supernatant) were divided into two aliquots and marked PTT and PNN, followed by ligation to 50 pmol PTT adapter and PNN adapters (N is the mixture of A, G, C and T; Table 1) using 100 U T4 ligase. The solution was diluted 5-fold with distilled water and used as the templates for pre-amplification with the primer combination of P+0/M+0, followed by selective amplifications as described (7). The selective primer combinations for the PTT and PNN templates are P+VN/M+NN and P+NN (excluding P+TT)/M+NN (Table 1), respectively. Amplified fragments were separated by 4% denaturing PAGE, visualized by silver staining (15), and recorded with a charge-coupled device (CCD) camera. The band patterns were analyzed with BandScan 4.30 software (Glyko, Novato, CA, USA).

The results showed that RoDD has high coverage and good reliability. There were 2128 and 1628 transcript-derived fragments (TDFs) obtained using 32 PTT adapter and 30 PNN primer combinations, respectively (Figure 2). Assuming that 192 PTT (12 × 16 = 192) and 240 PNN (16 × 15 = 240) primer combinations were completely used, 1.28 × 10^4 and 1.30 × 10^4 transcripts could be covered, respectively, summing up to 2.58 × 10^4. There were 920 (31.46%) and 832 (29.34%) differentially expressed transcripts could be covered, respectively, summing up to 2.58 × 10^4. There were 920 (31.46%) and 832 (29.34%) differentially expressed transcripts could be covered, respectively, summing up to 2.58 × 10^4. Therefore, 920 (31.46%) and 832 (29.34%) differentially expressed transcripts could be covered, respectively, summing up to 2.58 × 10^4.

Table 1. Primers and adapters involved in the RoDD method

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′ to 3′)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>bmaT16V</td>
<td>biotin-GAGTCCTAGTTTTTTTTTTTTTTVV</td>
<td>Mixture; for RT-PCR</td>
</tr>
<tr>
<td>MspI adapter</td>
<td>M-F: GATGATGAGTTCTGGAA M-R: CTTCAGAAGCAT</td>
<td>PCR template preparation</td>
</tr>
<tr>
<td>PTT adapter</td>
<td>PTT-F: CTCGTAGACTGCGAACCTTT PTT-R: GGTGCGAGTC</td>
<td>PCR template preparation</td>
</tr>
<tr>
<td>PNN adapters</td>
<td>PNN-F mixture: CTCGTAGACTGCGAACACCTTTNN PNN-R: AAGGTCCAGTCC</td>
<td>Mixture; for PCR template preparation</td>
</tr>
<tr>
<td>P+0</td>
<td>CTCGTAGACTGCGAACCTTT</td>
<td>Pre-amplification</td>
</tr>
<tr>
<td>M+0</td>
<td>GATGATGAGTTCTGGAAACGG</td>
<td>Pre-amplification</td>
</tr>
<tr>
<td>P+VN</td>
<td>GTAGCTGCGACACCTTVN</td>
<td>Selective amplification</td>
</tr>
<tr>
<td>P+NN</td>
<td>GTAGCTGCGACACCTTNN</td>
<td>Selective amplification</td>
</tr>
<tr>
<td>M+NN</td>
<td>GATGATGAGTTCTGGAAACGG</td>
<td>Selective amplification</td>
</tr>
</tbody>
</table>

bmaT16V represents 5′-biotin-Myl-AcuI-T16V-3′. AcuI is an asymmetrically cutting enzyme with a cleavage site 16 bases downstream of its recognition site. The adapters and primers are divided into two series, M (MspI) and P (AcuI). V represents A, G, and/or C, and N represents A, G, C, and/or T. The protruding ends of the adapters are underlined. The NN in the P+NN excludes TT.
A miniature assay confirmed the reliability of RoDD (Supplementary Figure S2); another validation assay indicated that RoDD is applicable to rice, Arabidopsis thaliana, and mouse, and that RoDD has many advantages over closely related methods (Supplementary Figure S3). A draft mathematical model (Supplementary Figure S4) and an in silico simulation assay revealed that RoDD has >80% coverage on human and rice transcripts (Table 2), which is higher than currently used gel-based methods (Supplementary Table S2).

The RoDD method has many advantages, including simplicity, straightforwardness, universality, reliability, high coverage and low redundancy. Furthermore, there is room for enhancement: the LI-COR DNA sequencers and infrared dye detection technology can improve accuracy and sensitivity and enable automation; many frequent cutters are available to increase coverage; and the assay may be modified into a high-throughput sequencing version. Thus, this method holds promise for global gene expression profiling in a variety of cells and tissues.

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Competing interests
The authors declare no competing interests.

References

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