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A Rapid and Efficient Method for the Isolation of Differentially Expressed Genes: Simplified Differential Display

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The differential screening method and the subtraction method generally have been used to allow identification and molecular cloning of the genes that are differentially expressed. However, these methods are not suitable for the detection of genes whose expression level is extremely low (*e. g.* transcription factor). In order to isolate and clone these genes, the differential display (DD) method using arbitrarily primed RT-PCR has been developed [1, 2]. Advantages of the DD method are as followed: (1) genes that are expressed at low levels can be detected. (2) the minimal quantities of RNA are required. (3) more than two samples can be readily compared. (4) direct cloning of cDNA can be achieved. DD method is useful, but it is laborious and time-consuming and provides DNA fragments of only 100-600 bp. Yoshida *et al.* (1994) developed a rapid and simple method for nonradioactive DD, the simplified DD (SDD) [3].

Schematic figure of SDD is shown in **Fig. 1.** In SDD, fully degenerate random hexamers are used as primers for reverse transcription of mRNA. After removal of the random hexamers, PCR amplification with a single arbitrarily sequenced 10- or 12-mer primer (designed for RAPD analysis) are performed. The PCR products with 10-20 distinct bands are easily separated by agarose gel and detected by ethidium bromide. The cDNA fragment of interest in the gel is recovered and directly used in cloning and the further analysis. In SDD, cDNAs of a subpopulation of more internal mRNAs are obtained as compared with DD, because only a single arbitrary primer, instead of 3′-anchored oligo(dT) primer plus an arbitrary primer, is used for amplification by PCR. In addition, cDNA fragments of up to 2000 bp are usually obtained in SDD. Thus, SDD has an advantage to identify genes through the sequences because it provides a longer and internal sequence of a gene. The number of distinct bands displayed in a gel is, however, much lower in the SDD system. In order to display more bands, we improved the SDD method. In this paper, we describe the refined method of SDD. The difference between the original DD method that was described by Liang and Pardee [1] and the SDD method that is described in this paper is summarized in **Table 1.**

1. *Preparation of RNA*

Total RNA from plant tissues is extracted by the

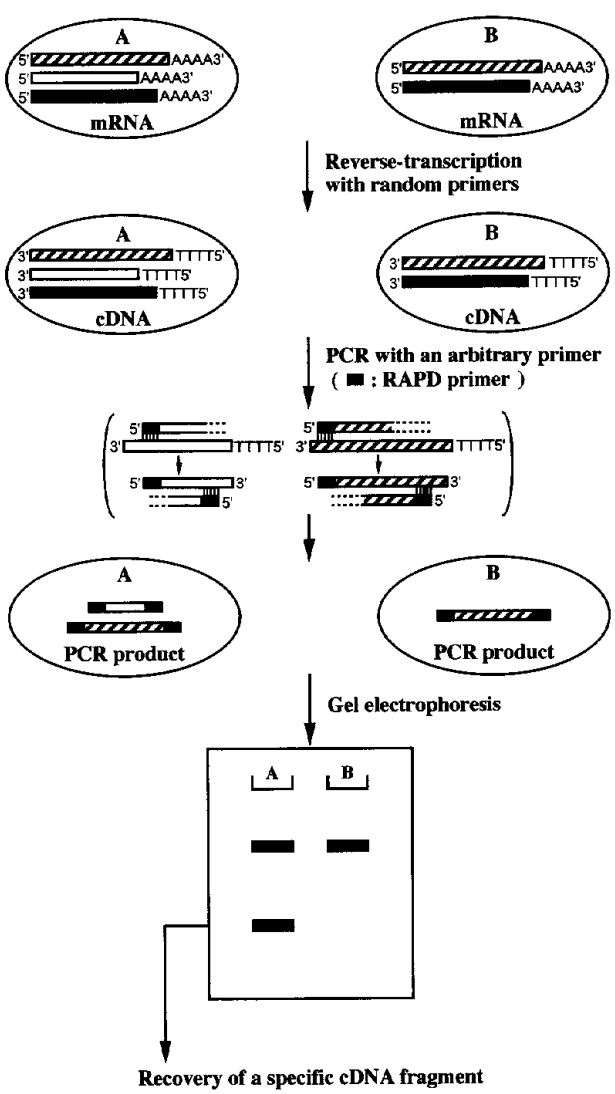

Fig. 1 Schematic representation of the simplified differential display protocol.

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Table 1.
Comparison of the original DD method and the simplified DD method.

Procedure	Original DD method	Simplified DD method
RNA	total RNA	poly(A)$^+$-RNA
Primer for synthesis of cDNA	3′-anchored oligo(dT)	random primers (6 mer)
Primer for amplification by PCR	arbitrary primer (10 mer) and 3′-anchored oligo(dT)	arbitrary primer (10 or 12 mer)
Electrophoresis	denaturing polyacrylamide gel for sequencing	nondenaturing polyacrylamide gel (16 cm×16 cm; thickness: 2 mm)
Detection	autoradiogram	SYBR™ Green I Nucleic Acid Gel Stain
Number of bands	50~100	20~40
Size of bands	0.1~0.6 kb	0.1~2.0 kb
Necessity of reamplification of DNA fragments	yes	(usually) no

standard guanidine thiocyanate/CsCl method [4] or by the SDS-phenol method [4], and precipitated with lithium chloride [5]. RNA extraction kits such as Isogen (Nippon Gene, Tokyo) are also commercially available. Poly(A)$^+$-RNA is prepared using an mRNA isolation kit, such as Oligotex-dT30 super (Takara Shuzo, Kyoto). The quality of the total RNA must be checked by electrophoresis through gels containing formaldehyde.

2. *Synthesis of cDNA using random primers*

(1) Incubate poly(A)$^+$-RNA (0.5 μg) at 65°C for 10 min.
(2) Chill the tube in iced water.
(3) Add the following reagents to the tube;

Reverse transcriptase buffer (10×)	2.5μl
10mM dNTPs mixture	2.2μl
160μM random primers [6]	5.0μl
100U/μl RNase inhibitor	1.0μl
Water	to 23.0μl

Add

20U/μl AMV reverse transcriptase	2.0μl

(4) Incubate the reaction tube at 42°C for 1 hr.
(5) Heat the tube at 95°C for 5 min.
(6) Remove the random primers by ultrafiltration with spin-column such as Suprec-02 (Takara Shuzo).
(7) Adjust the final volume to 120μl with sterilized water.

3. *Amplification by PCR*

The second-strand cDNA synthesis is carried out by PCR using an arbitrarily chosen RAPD primer.

(1) Add reagents to a reaction tube for PCR in the following;

cDNA (8-10 ng)	2.0μl
PCR buffer (10×)	5.0μl
1mM dNTPs mixture	10.0μl
100pmol/μl RAPD primer (10- or 12-mer)	1.0μl
Water	to 49.0μl

Add

5U/μl *Taq* DNA polymerase	1.0μl

(2) Heat the reaction tube at 92°C for 5 min.
(3) Amplify as follows [7]:
denaturation at 92°C for 1 min.
annealing at 35°C for 1 min.
polymerization at 72°C for 2 min.
cycle 35~40 times
The final polymerization step is prolonged to 5 min.

4. *Gel electrophoresis and recovery of cDNA fragments*

In order to improve the resolving power of gels and to detect a number of low-abundance PCR products, we use a nondenaturing polyacrylamide gel for separation and SYBR™ Green I Nucleic Acid Gel Stain (Molecular Probes, Inc., USA) for staining. The sensitivity of SYBR™ Green I Nucleic Acid Gel Stain is several times as great as ethidium bromide using standard 300 nm transilluminator (**Fig. 2**).

(1) Separate the PCR products by electrophoresis in a 4 % nondenaturing polyacrylamide gel poured in 1×TBE (0.9 M Tris-borate and 2 mM EDTA, pH 8.0). Size and thickness of the glass plate that we used were 16 cm×16 cm and 2 mm, respectively.
(2) Run the gel at voltage of 1-8 V/cm.
(3) Dilute SYBR™ Green I Stain 1: 10000 in a pH 7.0-8.5 buffer (*e.g.* 1×TBE) in a clear plastic polypropylene container.
(4) Stain the gel with the diluted SYBR™ Green I Stain for at least 30 min.
(5) Illuminate the stained gel using a 300 nm ultraviolet transilluminator (**Fig. 2**).
(6) Photograph the gel using a SYBR Green Gel Stain Photographic Filter.
(7) Cut out the specific cDNA fragment of interest from the polyacrylamide gel.
(8) Recover the cDNA from the gel slice with the

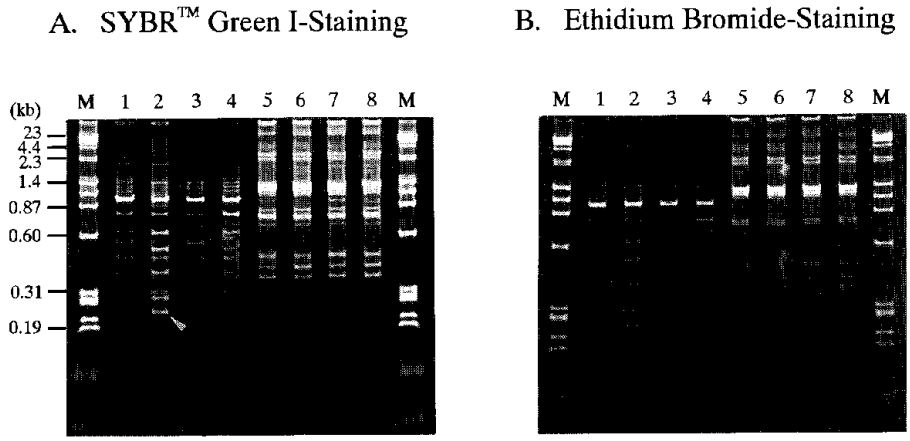

Fig. 2 Electrophoretic patterns of SDD stained with SYBR™ Green I Nucleic Acid Gel Stain (panel A) and ethidium bromide (panel B).
Amplification by PCR of cDNA from pistils 1 day before flowering (lanes 1 and 5), pistils at flowering (lanes 2 and 6), pistils at 2 to 4 hours after flowering (lanes 3 and 7), and ovaries 1 day after flowering (lanes 4 and 8) of rice. PCR was carried out using RAPD primers OPA7 (lanes 1-4) and OPA17 (lanes 5-8) (Operon Technologies; Alameda, CA, USA). M indicates marker fragments (a mixture of a *Hin*dIII digest of λDNA and a *Hae*III digest of ϕX 174 RF DNA). The numbers given on the left indicate sizes of fragments in kilobasepairs (kb). The fragment indicated by an arrowhead was isolated and was used for further analysis (see **Fig. 3**).

electroelution method [8, 9] .

(9) Extract the cDNA fragment once with TE-saturated phenol and once with chloroform.

(10) Precipitate it with ethanol.

5. *Cloning of specific cDNA fragments*

In most cases, the individual band is composed of several species. Thus, cloning of cDNA fragments is performed before sequencing analysis and expression analysis. The electroeluted cDNA fragments are directly cloned into the pCRII vector with the TA Cloning System Kit purchased from Invitrogen (San Diego, CA, USA) [10] .

6. *Confirmation of the specific expression of the isolated genes*

In order to investigate the expression pattern of the transcripts corresponding to the PCR-fragment derived clone with the display pattern, Northern blot analysis is necessary. The isolated genes identified using DD, however, are expected to express only at low levels and are not easily detected by Northern blot analysis. In this case, to raise the sensitivity of detection, amplification by RT-PCR should be carried out. For RT-PCR analysis, mRNA is prepared and cDNA is synthesized according to the methods 1 and 2, respectively. After removal of the random primers, an internal primer set that correspond to the authentic sequences of the gene of interest is added and PCR amplification is carried out. An example of the result of RT-PCR is shown in **Fig. 3.**

7. *Troubleshooting*

(1) Amplification by PCR using an arbitrary primer did not completely occur.

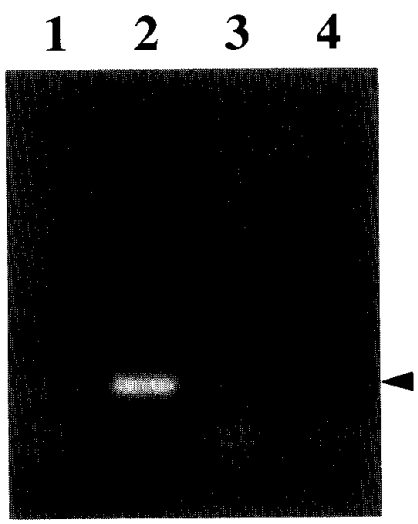

Fig. 3 RT-PCR assay of a specific band detected by SDD. The sequence of a cloned flowering pistil-specific cDNA (band indicated by an arrowhead in **Fig. 2**) was determined and the internal primer set of 18 bases was designed for RT-PCR analysis. RT-PCR was performed with cDNA templates prepared from pistils 1 day before flowering (lane 1), pistils at flowering (lane 2), pistils at 2 to 4 hours after flowering (lane 3), and ovaries 1 day after flowering (lane 4) of rice.

PCR products could not be obtained or the band patterns were extremely different among samples. This problem is almost always caused by the low quality of RNA samples. Another possibility is that synthesis of cDNA was not successfully completed. Isolation of RNA and synthesis of cDNA should be performed again.

(2) Cloning efficiency was very low.

When PCR product was recovered from the gel slice, it is possible that phenol was not completely removed. The following ligation reaction is highly inhibited by the contaminating phenol. The interface and organic phase must be discarded.

(3) False positive fragments were cloned.

The most serious problem in the differential display method is that the false positive fragments are frequently observed. In order to minimize the false positive bands, the following contrivances are necessary.

(i) If it is possible that PCR products were derived from genomic DNA, DNase I digestion of RNA samples should be performed.

(ii) When putative candidate bands appear, the PCR amplification experiments should be repeated and the reproducibility checked.

(iii) More than two related RNA populations or cDNA templates should be prepared, such as a diluted series of cDNAs and a series of RNA samples, *e.g.* different timing of sampling and different concentration of the chemical used in the treatment. Multiple display using these samples may be a powerful tool to eliminate false positive bands.

(iv) If the band of interest is faint, the entire sample should be concentrated by ethanol precipitation and subjected to electrophoresis. Comparison among concentrated samples on the display gel clarify the specificity of the bands of interest.

(v) Most individual bands include several fragments that originate from different transcripts. It is necessary to check at least five independent cDNA clones by sequencing or cross hybridization among the clones. The clone that occupies the majority may be a true positive clone.

References and Notes

[1] Liang, P., Pardee, A. B., 1992. Science, **257**: 967-971.

[2] Welsh, J., Chada, K., Dalal, S. S., Cheng, R., Ralph, D., McClelland, M., 1992. Nucleic Acids Res., **20**: 4965-4970.

[3] Yoshida, K. T., Naito, S., Takeda, G., 1994. Plant Cell Physiol., **35**: 1003-1009.

[4] Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K. (eds), 1991. Current Protocols in Molecular Biology, Greene Publishing Associates & Wiley-Interscience, NY.

[5] Watanabe, A., Price, C. A., 1982. Proc. Natl. Acad. Sci. USA, **79**: 6304-6308.

[6] Oligo(dT) primer instead of random primers is also available in the reaction of reverse transcription.

[7] The thermal cycler that we used was a GeneAmp PCR System (Perkin Elmer, USA) or a THERMO PROCESSOR TR-100 (TAITEC). We reproducibly obtained the same bands from either thermal cycler.

[8] Sambrook, J., Fritsch, E. F., Maniatis, T., 1989. "Molecular Cloning: A laboratory manual, 2nd edn." Cold Spring Harbor Press, Cold Spring Harbor, NY.

[9] The "crush and soak" method that originally described by Maxam and Gilbert (1977) [11] is also available for isolation of DNA fragments from a polyacrylamide gel. This method has been described in detail in Molecular Cloning: A laboratory manual [8].

[10] If necessary, cDNA fragments are reamplified by PCR and then are cloned into a plasmid vector.

[11] Maxam, A. M., Gilbert, W., 1977. Proc. Natl. Acad. Sci. USA, **74**: 560-564.