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Systematic Screening of Mutants of Rice by Sequencing Retrotransposon-Insertion Sites

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Rice genome research is progressing very rapidly [1] and a large number of genes have already been discovered by large scale sequencing of cDNAs. Although sequence data on genes are accumulating very rapidly, information about the functions of these genes is very limited. Based on their sequence similarity to other genes with known functions, the biochemical functions of only about 25 % of the genes have been predicted. In addition, the biological functions of the genes are totally unknown. The next important challenge is to determine the function of each gene.

Gene inactivation is a powerful tool for determining gene function. Among the gene inactivation strategies, insertion mutagenesis using transposable elements seems most suitable for a systematic functional analysis of a large number of genes. In rice, endogenous active retrotransposons have recently been found [2] and the most active, *Tos17*, was characterized in detail. *Tos17* is silent under normal conditions and becomes active under tissue culture conditions. Five to thirty transposed *Tos17* copies were found in each regenerated plant. Since *Tos17* becomes inactive in regenerated plants, mutations induced by *Tos17* insertion are fixed and inherited stably in the next generations. *Tos17*-induced mutants are being used for forward (transposon-tagging) and reverse genetic studies [2, 3]. Because *Tos17* induces mutations at a high frequency and at the same time tags the causative genes molecularly, they can be cloned using *Tos17* as a probe (transposon tagging). For reverse genetic studies, two strategies are employed. One is PCR-screening of mutants of the gene of interest. Mutants are identified in a large mutant population by PCR using *Tos17*- and gene-specific primers. Phenotypes shown by identified mutants can be studied to determine the biological function of the gene. Another important strategy is random sequencing of mutated genes by isolating the sequences flanking the transposed *Tos17*. As reported previously [2], mutants of interesting genes were identified by analyzing only eight sequences flanking *Tos17* insertions, suggesting many mutants of important genes should be identified by large scale sequencing of the flanking sequences. In the previous study, inverse-PCR was used to amplify the flanking sequences and the amplified sequences were sequenced after cloning. In this issue, we will describe a simpler procedure suitable for large scale sequencing. In this procedure, PCR methods such as thermal asymmetric interlaced (TAIL)-PCR [4, 5] and suppression PCR [6] were adopted and PCR products were directly sequenced using automated DNA sequencer. Sequences obtained were subjected to a similarity search using the BLAST algorithm. By using this method, mutants of different classes of genes, such as genes for transcription factors, genes involved in the signal transduction, genes with similarity to disease-resistance genes, and genes involved in metabolism, have been identified (Miyao *et al.* unpublished).

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1. Amplification of *Tos17*-flanking sequences using thermal asymmetric interlaced (TAIL) PCR

1. Primer
   (1) Specific primers of *Tos17*
   Tos17 - **tail2** AGTCGCTGATTTCTTCACCAAGG
   Tos17 - **tail3** GAGAGCATCATCGGTTACATCTTCTC
   Tos17 - **tail4** ATCCACCTTGAGTTTGAAGGG
   Tos17 - **tail5** CATCGGATGTCCAGTCCATTG
   (2) Degenerate primers
   AD1 NGTCGA(G/C)(A/T)GANA(A/T)GAA
   AD2 GTNCGA(G/C)(A/T)CANA(A/T)GTT
   AD3 (A/T)GTGNAG(A/T)ANCANAGA
2. Amplification of flanking sequences
   (1) 1st PCR
   The reaction mixture of 20 μl contains:
   30 ng template DNA
   1×LA PCR buffer (Mg free)

2.5 mM $MgCl_2$
0.2 mM dNTP
5 % DMSO
0.4 μM *Tos17*-tail2 primer
4 μM ADx (AD1 or AD2 or AD3) primer
1 unit LA *Taq* polymerase (TaKaRa, 5 units /μl)

Thermal condition
94°C 1min, 95°C 1min, 1 cycle
94°C 1min, 65°C 1min, 72°C 3 min, 5 cycles
94°C 1min, 25°C 6min, ramp to 72°C in 6min, 72°C 3 min, 1 cycle
94°C 30 sec, 68°C 1 min, 72°C 3 min,
94°C 30 sec, 68°C 1 min, 72°C 3 min,
94°C 30 sec, 44°C 1 min, 72°C 3 min, 15 cycles
72°C 5 min, 1 cycle

(2) 2nd PCR
The reaction mixture of 20μl contains:
1 μl of 50 fold diluted 1st PCR product
1×r*Taq* buffer
2.5 mM $MgCl_2$
0.2 mM dNTP
5 % DMSO
0.4 μM *Tos17*-tail3 primer
4 μM ADx primer (same primer used in the 1st reaction)
1 unit r*Taq* DNA polymerase (Toyobo 5 units/μl)

Thermal condition
94°C 30 sec, 65°C 1 min, 72°C 3 min,
94°C 30 sec, 65°C 1 min, 72°C 3 min,
94°C 30 sec, 44°C 1 min, 72°C 3 min, 12 cycles
72°C 5 min, 1 cycle

(3) 3rd PCR
The reaction mixture of 50 μl contains:
1 μl of 20 fold diluted 2nd PCR product
1×r*Taq* buffer
2.5 mM $MgCl_2$
0.2 mM dNTP
5 % DMSO
0.4 μM *Tos17*-tail4 primer
4 μM ADx primer (same primer used in the 1st reaction)
2.5 units r*Taq* DNA polymerase

Thermal condition
94°C 30 sec, 65°C 1 min, 72°C 3 min,
94°C 30 sec, 65°C 1 min, 72°C 3 min,
94°C 30 sec, 44°C 1 min, 72°C 3 min, 10 cycles
72°C 5 min, 1 cycle

2. Amplification of *Tos17*-flanking sequences using suppression PCR

A special adaptor is ligated to the ends of DNA fragments generated with blunt end producing enzymes. Specific primers which anneal to the *Tos17* terminal sequence and nested adaptor primers which have parts of the adaptor sequence are used for the suppression PCR. The phosphorylated blunt ends of the adaptor are ligated to all ends of digested DNA (**Fig. 1-A**). Because an amino group at the 3′-end of the short adaptor strand (AD-R) blocks extension of the strand where the adaptor primer (AP1) anneal, the adaptor primer (AP1) can not work as a primer at both ends of adaptor ligated DNA. Only products primed from the specific primer are extended to the end of adaptor (**Fig. 1-B**). These products can be amplified with the adaptor primer and the specific primer in subsequent cycles (**Fig. 1-C**). The adaptor has recognition sites of *Not* I and *Srf* I /*Sma* I. Therefore, the amplified fragment can be easily cloned into commonly used vectors. Since the suppression PCR amplifies sequences from the annealing

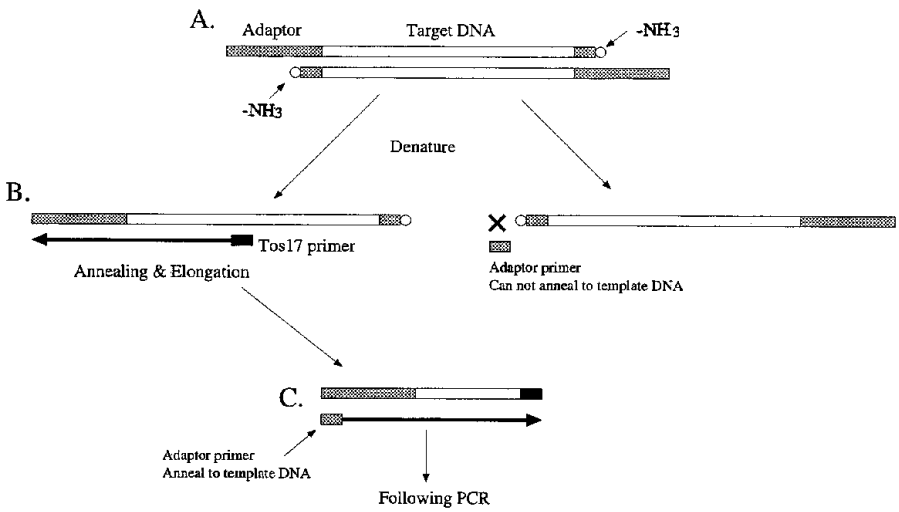

Fig. 1 Scheme of suppression PCR.

site of a specific primer to the recognition site of restriction enzyme, this method is applicable to flanking sequences which are difficult to amplify by the TAIL-PCR method.

1. Adapter and primers
   (1) Adaptor
   AD-F CTAATACGACTCACTATAGG-GCTCGAGCGGCCGCCCGGGCAGGT
   AD-R P-ACCTGCCC-NH_3
   (2) Adaptor primers
   AP1 GGATCCTAATACGACTCACTA-TAGGGC
   AP2 AATAGGGCTCGAGCGGC

2. Digestion with restriction enzyme and adaptor ligation
   (1) Digest DNA with blunt end-producing enzyme (*Dra*I, *Eco*RV, *Sca*I, etc.) for several hours. The reaction mixture of 5 μl contains:
   50 ng Genomic DNA
   1×Restriction enzyme buffer
   10 units Restriction enzyme
   (2) Add the following reagents into the reaction mixture. Ligate at 16°C for several hours.
   5 μM AD-F
   5 μM AD-R
   1×ligation buffer
   0.5 mM ATP
   1 unit T4 DNA ligase

3. Amplification for walking
   (1) Dilute the reaction mixture with TE up to 200 μl.
   (2) First PCR reaction mixture of 20 μl contains:
   1 μl of 10 fold diluted ligation mixture
   1×r*Taq* buffer
   2.5 mM $MgCl_2$
   0.2 mM dNTP
   1 μM *Tos17*-tail3 primer
   1 μM AP1 primer
   2 units r*Taq* DNA polymerase
   (3) PCR condition with the "Hot start method" is as follows:
   94°C 1 min, 1 cycle
   94°C 1 min, 68°C 6 min, 35 cycles
   68°C 15 min, 1 cycle
   (4) Second PCR reaction mixture of 50 μl contains:
   1 μl of 100 fold diluted reaction mixture
   1×r*Taq* buffer
   2.5 mM $MgCl_2$
   0.2 mM dNTP
   1 μM *Tos17*-tail4 primer
   1 μM AP2 primer
   5 units r*Taq* DNA polymerase
   (5) PCR condition is the same as the first PCR condition except there are 30 thermal cycles.

3. Isolation of amplified fragments and direct sequencing

1. Separate DNA fragments by electrophoresis with 1% agarose gel in TAE buffer (one typical example is shown in **Fig. 2**).
2. Isolate each gel piece containing amplified DNA fragments into 1.5 ml tube.
3. Add 1 ml of Wizard PCR preps DNA purification resin (Promega) and melt the gel at 65°C for 20 min.
4. Filtrate the melted gel solution with special column for Wizard system.
5. Wash the resin with 2 ml of 80% isopropanol and then dry up the resin.
6. Add 40 μl of hot distilled water (70°C) to the resin. Set the column into new 1.5 ml tube.
7. Spin the tube at 12000 rpm for 20 sec.
8. Collect the eluate.
9. Dye-terminator sequence reaction:
   11 μl of eluted DNA solution
   1 μl of *Tos17*-tail5 primer (3.3 μM)

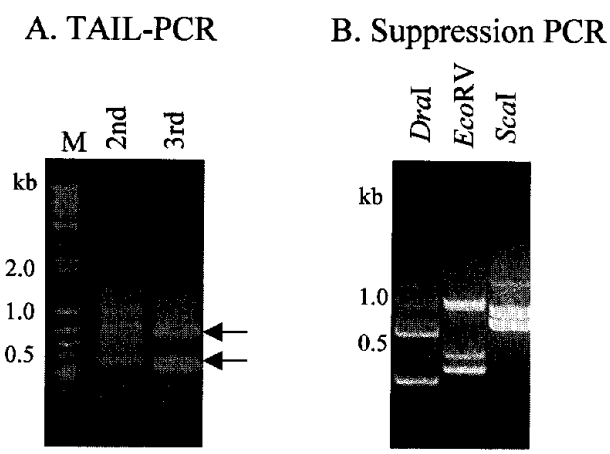

Fig. 2 Amplification products generated by TAIL-PCR (A) and suppression PCR (B) method. (A) Lane M contains DNA size markers (*Hin*dIII digests of λDNA and *Hinc*II digests of ϕX174 DNA). Lanes 2nd and 3rd are products of the 2nd and the 3rd TAIL-PCR, respectively. Products from the 3rd reaction indicated by arrows are smaller than those from the 2nd reaction, because the nested primer was used in the 3rd reaction. (B) Lanes *Dra*I, *Eco*RV, and *Sca*I are products of suppression PCR using corresponding enzymes.

8 μl of Dye terminator cycle sequencing FS ready reaction premix (Applied Biosystems)

Thermal condition
96°C 2 min, 1 cycle
96°C 10 sec, 50°C 5 sec, 60°C 4 min, 25 cycles

10. Add 74 μl of ethanol.
11. Spin at 15,000 rpm for 15 min.
12. Remove the supernatant.
13. Dissolve the pellet in 6 μl of formamide-EDTA (500 μl formamide and 100 μl of 100 mM EDTA, pH 8.0).
14. Denature for 2 min at 95°C.
15. Chill on ice.
16. Apply to the sequencer.

4. Troubleshooting

1. PCR products show smear in the agarose gel
   (1) Reduce the template DNA concentration on 1st PCR.
   (2) Further dilute the 1st and/or 2nd PCR products.
   (3) Set higher annealing temperature on high-stringency cycles (for TAIL-PCR).
   (4) Set shorter time for extension step.
2. Product is not amplified
   (1) Increase the template DNA concentration on 1st PCR.
   (2) Change to other AD primers (for TAIL-PCR).
   (3) Change to other restriction enzymes (for suppression PCR).
   (4) Set lower annealing temperature on low and/or high-stringency cycles (for TAIL-PCR).

References

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