Using rice TN67 T-DNA insertional mutagenesis flanking sequence for rice functional genomics research

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Abstract

The Adaptor ligation PCR (AL-PCR) and Inverse PCR (IPCR) were used to analyze the flanking sequence of rice TN67 T-DNA mutant lines. Those mutant lines were using activation tagging vectors (pTAG-8) which contain octamer 35S enhancer sequences and can be inserted more than one copy in the rice genomic. The AL-PCR and IPCR methods were found much useful in this research and hundreds of fragment sequences had been obtained. We aligned those fragment sequences with the original sequences existed in the T-DNA border and results showed 261fragment sequences had been confirmed existing the flanking sequence. The T-DNA flanking sequences were browsing by BLAST in rice genome annotation database-TRIM (Taiwan Rice Insertional Mutants Database, http://trim.sinica.edu.tw) to find out possible genes for functional genomic research. We chose two mutant lines, M0066706 and M0042958, to demonstrate the results. The phenotype of M0066706 is as follow: dark green leaf, thick culms, semidwarf, late heading date, and big seed. The phenotype of M0042958 is the plant height higher than 170 cm. After browsed by BLAST, we found the T-DNA was inserted in chromosome 6 and 3 respectively. The putative activation gene of M0066706 is hypothetical protein which is similar to retrotransposon protein; while the putative activation gene of M0042958 is unknown protein which is similar to the receptor protein kinase.

Material & Method

Material: Totally 832 lines of rice TN67 T-DNA insertion mutagenesis were used.

Methods: The Inverse PCR (IPCR) and Adaptor ligation PCR (AL-PCR) methods were applied to isolate the flanking sequences of T-DNA.

Experiment procedure:

1.Genomic DNA was isolated using an improved CTAB method. The T-DNA left border (LB) or right border (RB) flanking regions were rescued using two modified AL-PCR and IPCR methods.

2. The IPCR method was used to isolate the flanking sequences of T-DNA. One microgram of genomic DNA was digested with 10 units of EcoR I in 50ul for 10h. After the enzymes were heat inactivated, the samples were ethanol precipitated and dissolved in 30ul of ligation buffer, then ligated at 25˚C for 12 h, using 1 unit of T4 DNA ligase. Nested PCR was performed to amplify the flanking sequence. For the first PCR, approximately 1/50 of the ligated DNA and 5ul of each primer were incubated in 25ul of a reaction solution containing dNTPs and 0.1 unit of Taq polymerase. A 0.1ul aliquot of the first PCR product was used for the second PCR template. The PCR products were separated on a 2% (w/v) agarose gel for electrophoresis. Distinct DNA bands amplified were cut off and recovered for direct sequencing (Fig.1).

3. AL-PCR was carried out by a modify PCR walking protocol to rescued the T-DNA flanking regions. This method consisted of three steps: digestion of genomic DNA using blunt end restriction enzyme and ligation of an asymmetrical adaptor, PCR amplification using two primers specific for the T-DNA and the adaptor respectively, and a successive PCR using two nested specific primers. All the products of the second round PCR were loaded to 2.0% agarose gel for electrophoresis. Distinct DNA bands amplified were cut off and recovered for direct sequencing (Fig.2).

4. Those rice genome DNA sequences were analyses by the Taiwan Rice Insertional Mutants Database (TRIM, http://trim.sinica.edu.tw).

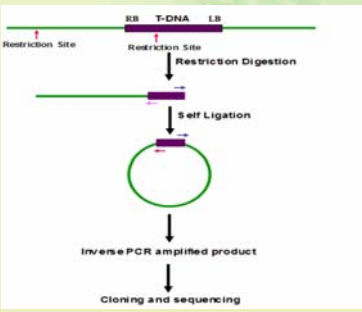


Figure 1.Schematic representation of Inverse PCR.
This involves restriction digestion of genomic DNA from mutant plant with an appropriate enzyme that cuts (preferably cuts once within the T-DNA) followed by self-ligation. The circularized ligation products are used for PCR amplification using appropriate primers from the T-DNA region. The flanking plant DNA is represented by line (green). The appropriate primers (forward and reverse) are indicated by blue and red arrows.
Modified from The mutagenesis in Arabidopsis: a tool for functional genomics. Electronic Journal of Biotechnology(2005) Vol.8 No.1

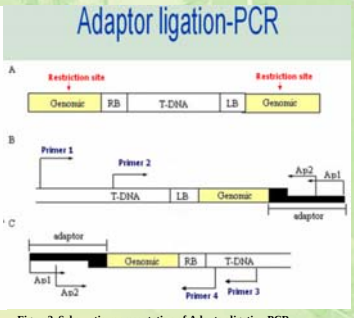


Figure2. Schematic representation of Adaptor ligation PCR.
A. Restriction digestion. B,C. Specific adaptor molecules are ligated to the ends of the restricted DNA fragments carrying part of the insert. The restricted fragments are end filled and subjected to PCR using primers specific to the adaptor region and nested primers specific to T-DNA. The flanking plant DNA region is represented by yellow box genomic, the Adaptor molecules are shown in black box. The adaptor specific primer is shown in AP1,AP2. The T-DNA specific nested primers are indicated by B (Primer1,Primer2), C (Primer3, Primer4).

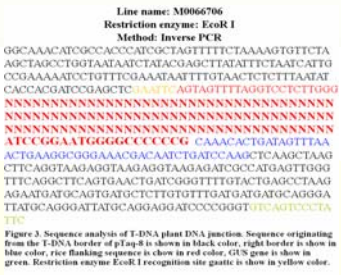


Figure 3. Sequence analysis of T-DNA plant DNA junction. Sequence originating from the T-DNA border of pTaq-8 is shown in black color, right border is show in blue color, rice flanking sequence is chow in red color, GUS gene is show in green. Restriction enzyme EcoR I recognition site gaattc is show in yellow color.

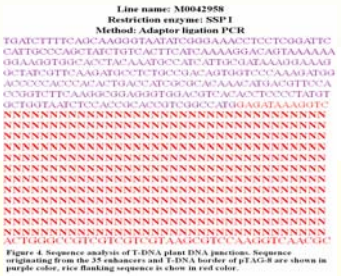
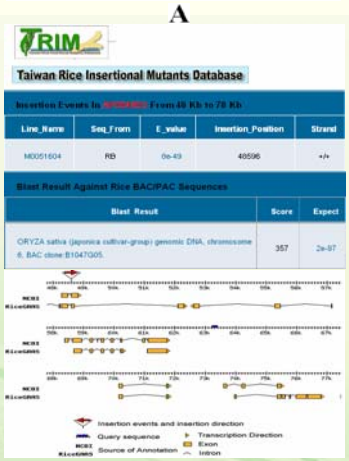


Figure 4. Sequence analysis of T-DNA plant DNA junctions. Sequence originating from the 35 enhancers and T-DNA border of pTAG-8 are shown in purple color, rice flanking sequence is chow in red color.



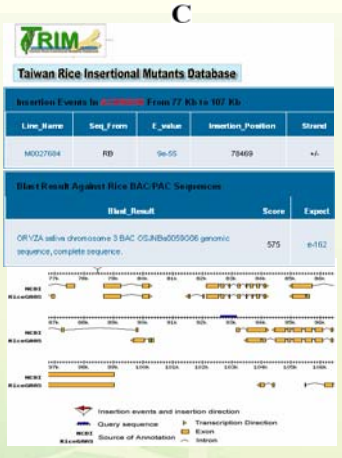


Figure 5.The T-DNA mutant lines M0066706 (Fig. A, Fig. C) and M0042958 (Fig. B, Fig. D) were used to demonstrate the results. The rice genome annotation data for predicted and expressed genes were downloaded form the TRIM (Taiwan Rice Insertional Mutants Database http://trim.sinica.edu.tw). Fig. A, Insertion events and insertion direction of the M0066706 on the exon and it also contain three transcription direction next to the exon at 10kb. Fig. B, The putative activation gene of M0066706 is hypothetical protein which is similar to retrotransposon protein. Fig. C, Insertion events and insertion direction of the M0066706 and it also contain four transcription direction next to the downstream at 10kb. Fig. D, M0042958 is unknown protein which is similar to the receptor protein kinase.

Discussion

The results showed that the Adaptor ligation PCR (AL-PCR) and Inverse PCR (IPCR) were able to analyze the flanking sequence of rice TN67 T-DNA mutant lines in our lab.. Compare there two methods we found AL-PCR could obtained stable products while inverse PCR could obtained right border and left border flanking sequences at the same time. Totally 261 franking were generated in this research. Those results had been pasted in the TRIM to compare the similarity from the existent rice genomic database and predicated the putative function of these sequences. Two outcomes are showed as fellow : The upstream flanking sequence of M0066706 is similar to the sequence of rice genomic located at exon area of chromosome No. 6 which may be the putative gene of the retrotransposon protein. In this consequence, the plant becomes dark green leaf, thick culms, semi-dwarf, late heading date, and big seed. The downstream flanking sequence of M0042958 is similar to the sequence of rice genomic located at exon nearby area of chromosome No.3 which may be the putative gene of protein receptor protein kinase. We also found the plant turn to be giant high (over 170 cm) (Fig. 6). This result indicated that the T-DNA maybe insert in the upstream of this receptor protein kinase gene and the enhancers in the T-DNA promoted gene to over expression.

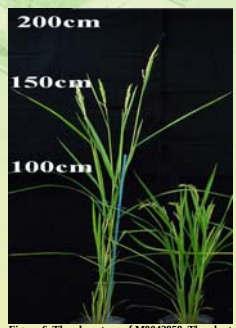


Figure 6. The phenotype of M0042958. The plant height of M0042958 is higher then 170 cm.
*Left: M0042958; Right: wide type.

Result

Totally 832 accessions, we propagated in this year, genomic DNA were extract from each of them. The AL-PCR and IPCR were used to isolate their flanking sequence separated. The results showed 689 fragments were generated from PCR. We aligned those fragment sequences with the original sequences existed in the T-DNA border. Among them, 261 fragment sequences had been confirmed exist the flanking sequence. The T-DNA flanking sequences were compared with rice genome annotation database-TRIM (Taiwan Rice Insertional Mutants Database, http://trim.sinica.edu.tw) to find out putative genes for functional genomic research. Sequence of fragment generated from M0066706 by IPCR was show as Fig. 3. Totally 650 base of the flanking sequence was generated by this method. Sequence of fragment generated from M0042958 by AL-PCR was show as Fig. 4. Totally 870 base of the flanking sequence was generated by this method. Those sequences were browsing by BLAST in TRIM database. The result is show as Fig. 5.

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