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# Molecular Analysis of Rice Plants Harboring an *Ac/Ds* Transposable Element-Mediated Gene Trapping System

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**Abstract**

In rice, limited efforts have been made to identify genes by the use of insertional mutagens, especially heterologous transposons such as the maize *Ac/Ds*. We constructed *Ac* and gene trap *Ds* vectors and introduced them into the rice genome by *Agrobacterium*-mediated transformation. In this report, rice plants that contained single and simple insertions of T-DNA were analyzed in order to evaluate the gene-tagging efficiency. The 3′ end of *Ds* was examined for putative splicing donor sites. As observed in maize, three splice donor sites were identified at the 3′ end of the *Ds* in rice. Nearly 80% of *Ds* elements were excised from the original T-DNA sites, when *Ac* cDNA was expressed under a CaMV 35S promoter. Repetitive ratoon culturing was performed to induce new transpositions of *Ds* in new plants derived from cuttings. About 30% of the plants carried at least one *Ds* that underwent secondary transposition in the later cultures. 8% of transposed *Ds* elements expressed GUS in various tissues of rice panicles. With cloned DNA adjacent to *Ds*, the genomic complexities of the insertion sites were examined by Southern hybridization. Half of the *Ds* insertion sites showed simple hybridization patterns which could be easily utilized to locate the *Ds*. Our data demonstrate that the *Ac/Ds* mediated gene trap system could prove an excellent tool for the analysis of functions of genes in rice. We discuss genetic strategies that could be employed in a large scale mutagenesis using a heterologous *Ac/Ds* family in rice.

Among cereal species, rice has the smallest genome size (around 430 megabases), which is just over three times the *Arabidopsis* genome size. The physical structure of the rice genome has been characterized in detail at the molecular level, especially during the course of developing physical maps using large DNA fragments cloned in YAC and BAC libraries. International efforts have begun to sequence the whole rice genome. Along with genes identified by expressed sequence tags, a vast amount of data on rice genes will accumulate from genome sequencing projects. In the near future, emphasis will shift towards the assessment of the functions of sequenced genes. A major tool in dissecting the functionality of the genomes of maize and *Arabidopsis* are insertional mutagens, such as transposable elements or T-DNA. Due to detailed knowledge about its transposition requirements, *Ds* has been successfully utilized in enhancer/gene trap vehicles in *Arabidopsis*. Enhancer/gene traps are advanced versions of the conventional transposon-mediated insertional mutagenesis. Trap systems have been successfully operated to identify genes in *Drosophila*, mouse, and yeast. An enhancer trap transposon harbors a weak minimal promoter fused to the open reading frame of a reporter gene and a genetic marker. Upon insertion near or at a host gene, the weak or minimal promoter will be *cis*-activated by enhancer elements in the host gene. The *P* element promoter and The 35S CaMV minimal promoter have been commonly used with this system in *Drosophila* and plants, respectively. Reporter genes usually encode β-galactosidase (*lacZ*) for animals and β-glucuronidase (*gusA*) for plants. A gene trap contains multiple splicing acceptor sites fused to the coding region of the reporter gene. A fusion protein of the reporter gene with the N-terminal portion of a host gene will be produced when the element is inserted into either an exon or an intron of the host gene in the same transcriptional orientation.

*Genetic components for a gene trap system in rice*

The *Ac/Ds* mediated gene trap systems consisted of three genetic components, they were *Ac*, trap *Ds*, and a counter-selection marker. *Ac* cDNA was used for a transposase source that was under the control of a CaMV 35S promoter. Gene trap vehicles carrying an intron with alternative splice acceptors in front of a reporter gene greatly enhance the efficiency of gene detection. In our study, two different introns were utilized to construct a gene trap *Ds*. These introns did not carry splicing donor sites. One intron was the same one as had been used in *Arabidopsis* (CSHL-gDs). The intron was a modified form of the fourth intron of the *Arabidopsis* GPA1 (the alpha subunit of G-protein) gene. The other one was developed after substantial modification of the maize *Adh1* intron III (Acc-gDs). Introns fused to the GUS gene were placed 222 bp from the 3′ end of the *Ds* element, while a *bar* expression cassette was inserted into a *Bst*XI site, 1000 bases from the 5′ end of *Ds*. A modified bacterial cytochrome P450 gene was used as the counter-selection marker in this system. The cytochrome P450 can be used for negative selection not only in tobacco but also in rice (the data not shown).

*Analysis of splicing products of gene trap Ds in rice*

In a gene trap *Ds*, GUS is expressed as fusion protein whose N-terminal peptide is from a host gene. Therefore, alternative multiple splicing donor/acceptor sites are essential to guarantee a transcript that carries a GUS coding region in frame with the 5′ end of the host gene. In developing trap systems for rice, it is important to define the splicing donor sites at the *Ds* ends and to evaluate the utilization efficiencies of the splicing donor/acceptor sites. T-DNA vector was used for *in vivo* assay of splicing between the 3′ end of *Ds* and a GUS coding region. The 3′ end of CSHL-gDs including the GUS gene was under the control of a 35S promoter. There were 4 putative splicing donor sites at the 3′ end of *Ds*. The data showed that the second splicing donor site at the 3′ end of *Ds* was, if at all, rarely utilized in rice

*Analysis of transgenic plants*

Cytochrome P450, *Ac*, and one of either CSHL-gDs or Acc-gDs were cloned into T-DNA vector pSB11. The two different T-DNA vectors were introduced *via Agrobacteria* into a Japonica type Korean rice cultivar, Dong Jin. A *bar* gene inside *Ds* was used as a selection marker. A PPT (phosphinothricin) resistance-based selection method was established for the production of transgenic rice plants. After Southern analysis, three lines that carried a single or two copies of T-DNA insert were selected. One (#12-19) carried one copy of T-DNA and part of *Ds*. Another (#13-2) carried two copies of T-DNA insert. The third line (#13-21) carried one copy of T-DNA. Transgenic line #12-19 contained CSHL-gDs, while #13-2 and #13-21 had Acc-gDs. The total number of mature T1 plants obtained from #12-19, #13-2, and #13-21 were 77, 100, and 46, respectively. These T1 transgenic plants were maintained in a greenhouse through multiple rounds of ratoon culture.

Since excised *Ds* is not always reinserted into a genome, we described the mobility of *Ds* in terms of two separate activities, excision and reinsertion, in individual transgenic plants. Depending on the mobility of *Ds* at the original site, *Eco*RI digestion of genomic DNA would generate uniquely sized DNA fragments. The excision and reinsertion events of *Ds* in a total of 221 T1 plants were analyzed by Southern hybridization. Genomic DNA was extracted from flag leaves of flowering plants. #12-9 and #13-2 exhibited highly diverse polymorphism with the GUS-hybridizing DNA. In contrast, all 47 plants of #13-21 showed only 4 RFLPs. Therefore, *Ds* in #13-21 must have transposed before the emergence of plantlets from calli. In summary, around 80% of *Ds* elements that had been introduced with the T-DNA had been mobilized in T1 mature plants. Most of the *Ds* elements in many T1 plants were transmitted to the next generation.

*Examining the mobility of Ds through repetitive cuttings of T1 plants*

In rice, when culms are cut 5-15 cm above the ground, new plant bodies develop from young tillers that had emerged at the time of the cutting. Theoretically, if *Ds* mobilization is triggered after the tillering of plants, new primary transpositions can be expected to appear in subsequent ratoon cultures. In this study, the mobility of *Ds* was assessed by comparing the first (original) plants with plants of the third ratoon culture. The third plants must have developed from tillers that were not yet initiated at the time the original plants were cut out. Sixty-eight plants were randomly selected from line #12-19 and 13-2, for Southern analysis Overall, around 18% of the *Ds* elements of the first plants were mobilized in later cultures. Completely new *Ds* polymorphism was never detected in the plants examined. These data suggest that the primary transposition of *Ds* in the original plants took place before tillers had differentiated. Most of the new *Ds* sites in the third plants came from secondary transpositions of *Ds*.

*Genomic complexities of Ds insertion sites*

TAIL-PCR (thermal asymmetrical interlaced polymerase chain reaction) had been employed for the cloning of flanking DNA of T-DNA or *Ds*, mainly in *Arabidopsis*. In our study, we modified the PCR conditions such as to enhance the amplification efficiency of rice genomic target DNA. Each of four different arbitrary primers (AD2-5) was paired with primers specific to either the 5′ or 3′ end of *Ds* in order to amplify the insertion sites through a series of PCR reactions. *Ds*-adjacent DNA was identified by a size difference between the products of the 2nd and 3rd PCR. Overall, using both Ds5 and Ds3 primers, 94% of the samples were shown to produce at least one *Ds* insertion site by TAIL-PCR. Cloning efficiency could be further improved by using additional arbitrary primers.

The genomic complexities of the *Ds* insertion sites were judged based on the hybridization patterns of Southern blots. The results should provide the information on how efficiently PCR clones could be used to locate *Ds* loci and to identify genes at or near insertion sites. Simple structures and low copy DNA at *Ds* loci greatly facilitate chromosomal mapping and molecular analysis. DNA clones from 44 *Ds* loci were used as probes on *Eco*RI or *Xho*I cut genomic DNA. Among them, 13 clones (30%) detected one or two discrete hybridization bands. Nine clones (20%) showed two or three strong hybridization signals with or without one or two weak ones. Therefore, a total of 22 genomic clones (50%) detected low copy DNA. The rest exhibited either a highly repetitive DNA pattern or smearing (5 and 9 clones, respectively). The data, therefore, indicate that many *Ds* elements were inserted into genomic loci of simple structure.

*GUS expression mediated by gene trap Ds.*

The GUS coding region of a gene trap that is expressed by a host has the characteristics of a dominant trait. To evaluate the efficiency of gene identification *via* GUS expression, floral organs from 121 T1 plants carrying only transposed *Ds* were subjected to GUS staining. GUS staining was performed with panicles at two different developmental stages. The first experiment was conducted with fully mature panicles of original T1 plants. The second stainings were done on immature panicles of plants that were newly grown after a first ratoon culture. These young panicles were still wrapped in the upper leaves. Pants showing unique and consistent GUS patterns in both experiments were counted in order to calculate the gene trap efficiency. Nineteen plants (15.7%) showed consistent GUS patterns in floral organs. Since on average two copies of *Ds* were present per plant, about 7.9% of total transposed *Ds* showed GUS staining in floral organs. This number may be an under-estimation of the host genes that express GUS of the trap *Ds*, because some *Ds* may not be at the same locus in the second culture. In *Arabidopsis*, around 26% of transposants carrying gene trap *Ds* exhibited GUS staining in whole seedlings, leaves, or flowers [16]. Therefore, it can be argued that the efficiency of gene identification *via* GUS staining is comparable between rice and *Arabidopsis*. Figure 8 shows examples of panicle organs expressing GUS.

## Discussion

Multimeric *Ds* could induce chromosomal rearrangement and substantially reduce the transposition frequency. To maximize the frequency of simple insertions of *Ds*, *Agrobacterium*-mediated transformation is a better method than direct DNA transfer procedures, such as PEG-mediated transfection and particle bombardment, that frequently lead to multicopy insertions at a single locus. The high frequencies of *Ds* transposition observed in this study could be due to early and strong expression of *Ac* cDNA. Similar observations were made in rice when genomic *Ac* DNA was expressed by the same 35S promoter . Scofield *et al.* (1993) found that stronger promoters such as the 35S resulted in large (early) sectors of transposition and few later events. Even though the timing of *Ds* transposition differed between rice lines, around 80% of *Ds* exhibited primary transposition and 18% of *Ds* elements were mobilized into secondary transposition in newly developed shoots. We do not have any explanation of why the secondary transposition of *Ds* was that much less active. Izawa *et al.* (1997) made the similar observation that many early transposition events were followed by a loss of activity in later generations. However, as suggested previously, if *Ds* becomes mobilized after the tillering stage, multiple rounds of cuttings might generate a new (primary) transposition from the original *Ds* locus.

High efficiency of gene detection by gene trap *Ds* should be due not only to the installment of introns in front of a reporter gene but also the presence of splicing sites at the end of *Ds*. As in maize, rice uses three of the four putative splicing donor sites at the 3′ end of *Ds* sites. All the three donor sites in rice are each in a different frame. However, between the 3′ end of *Ds* and the last splicing donor site are two putative stop codons, TAG and TAA. These stop codons could lower the frequency of functional GUS

protein being expressed. The fact that 15.7% of plants showed GUS expression in floral organs demonstrates that the *Ac/Ds* mediated gene trap systems can be powerful tools used in identifying rice genes. Along with the development of an effective gene detection system in rice, we have adapted a PCR technique to clone about 94% of the insertion sites. Rapid cloning of insertion sites greatly facilitates the mapping of *Ds* loci. This is a very important step for local mutagenesis, since *Ds* can be re-mobilized by re-introduction of transposase. Also, each insert can be used as a molecular marker for positional cloning of a known mutation (for review, see Martienssen, 1998). In particular, a mutation that has been mapped as close and between two insertion sites of *Ds* can be easily cloned by chromosome walking or *via* short-range transposition.

Our data clearly demonstrate that *Ac/Ds* mediated gene identification can be performed in rice as effectively as in maize or *Arabidopsis*. Since around 80% of trap *Ds* is mobilized in T1 plants carrying *Ac*, another way to develop a large number of transposant lines might be to conduct massive pollinations between *Ds* and *Ac* separate lines to generate F1 seeds. In the F2 generation, the cytochrome P450 gene can be used to select unlinked transpositions of *Ds* and to eliminate *Ac* and *Ds* at original T-DNA loci. Successful application of a negative selection marker has been demonstrated in *Arabidopsis*. Overall, this study has provided molecular and genetic information that should be very informative for the establishment of effective gene tagging systems in rice.

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