

## Transposon Insertional Mutagenesis in Rice<sup>1,2</sup>

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Transposon mutagenesis systems are being developed in rice to address gene functions using forward and reverse genetics strategies. Although the well-characterized maize (*Zea mays*) transposons seem an obvious choice to develop efficient transposon-tagging systems for gene knockouts and gene detection, heterologous transposons have not yet become a genome-wide system for saturation mutagenesis in rice (*Oryza sativa*). Our contributions as a multinational European initiative in developing heterologous transposon mutagenesis strategies for functional genomics in rice are described here.

The endogenous retrotransposon *Tos17* (Hirochika, 1997) has been effectively used to make knockouts of rice genes in a reverse genetics approach, but has a high frequency of untagged mutations, probably due to somaclonal variation associated with the tissue culture-based retrotransposition generation. Recent reports on transposition of *Ac-Ds* heterologous systems in rice have shown their activity and potential use as effective insertional mutagens (Izawa et al., 1997; Chin et al., 1999; Enoki et al., 1999; Nakagawa et al., 2000). The autonomous *Ac* has been shown to maintain a high rate of transposition, although *Ds* elements might undergo silencing in later generations. The use of both systems in reverse genetic approaches has been reported to be potentially efficient.

One principle we sought to employ was to generate rice genotypes with multiple transposons that could effectively saturate the genome with a fewer number of plants. Transformation of T-DNA-bearing transposons normally generates a few copies; thus, transposon amplification was used to increase copy number.

### KNOCKOUT MUTATION MACHINE

We developed a green fluorescent protein excision assay that allowed the identification of transposon excision events. In a construct with *Ac* containing a double cauliflower mosaic virus-35S enhancer adjacent to the autonomous *Ac* promoter (Fig. 1), all transformants generated showed very early transposition. About one-half of the lines also showed amplification of the *Ac* copy number in the first cell after transformation. This phenomenon could be attributable to the influence of the 35S enhancer on the adjacent endogenous *Ac* promoter, inducing a high level of transposase expression, in combination with the transposition of *Ac* during replication (Greco et al., 2001). This allowed the generation of lines containing multiple transposons (approximately four from a single copy T-DNA line) that could generate an average of one to two new inserts per progeny giving a frequency of 15% to 50% of independent transpositions in the next generation ( $T_1$ ).

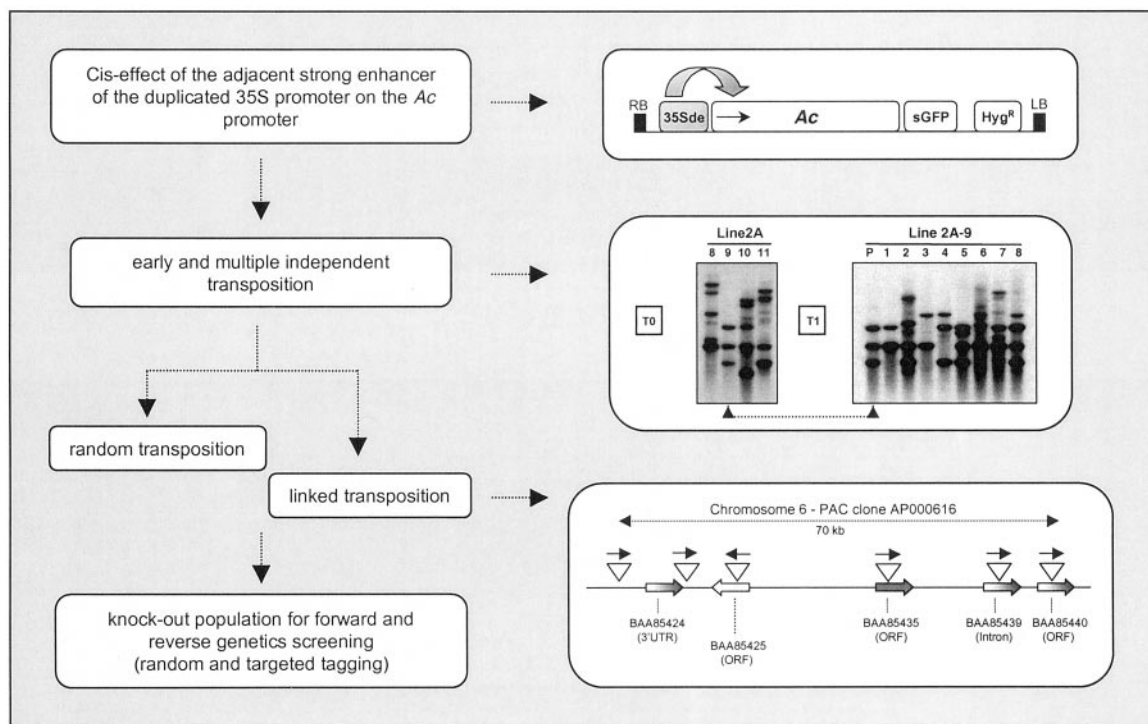
Transposition of *Ac* to a linked position in the genome, applicable for targeted tagging, was demonstrated by the recovery of a set of six insertions in a 70-Kb interval of sequenced DNA on chromosome 6. Unlinked transposition was also observed by segregation of the T-DNA locus from the *Ac* insertions. The isolation of *Ac*-flanking genomic sequences revealed a preferential insertion in protein-coding sequences, as shown by the presence of four times more insertions in genes than randomly expected. This confirms the earlier results of *Ac* insertional preference in rice, in which 4% of the inserts were observed to be in sequences homologous to expressed sequence tags (Enoki et al., 1999), and suggests that the preferential transposon insertions in genes could be a valuable asset for generating mutants in rice.

Multiple transposon lines and gene insertional specificity enable generation of a tagging population requiring a reduced number of lines to reach saturation. The propagation of these genotypes for three to four generations can generate a population of plants containing four or more *Ac* inserts at different positions in the genome. Using 25,000 lines for three to four generations would generate about 100,000 inser-

<sup>1</sup> This research was funded in the participating labs by the European Union (project no. BIO4 CT 972132, "Transposon Mutagenesis in Rice").

<sup>2</sup> This paper is dedicated to the loving memory of our colleague, Dr. J. Harry C. Hoge, who passed away during the course of this research.

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**Figure 1.** Generation of a knockout population using multiple copy *Ac* lines. The construct used to introduce *Ac* in rice conferring early transposition. The Southern blot represents different regenerants ( $T_0$ ) from a single copy line (2A) and the  $T_1$  progeny of plant 2A-9, showing transposon amplification and active transposition of the multiple copies. Shown below is local transposition in genes (thick arrows) on chromosome 6, as revealed by sequencing *Ac* insertion sites (triangles) from Line 2A.

tions that are suitable for identifying knockouts for forward as well as reverse genetic strategies. Due to the ability of *Ac* to autonomously transpose, identified knockout insertions will be unstable. For insertions in the coding region of genes, *Ac* excision can produce footprints that can lead to generation of mutant and revertant alleles. These mutant and revertant alleles derived from an identical genetic background can be used to assess the phenotypic effect of the gene mutation (stable), helping reduce the effect of background mutations.

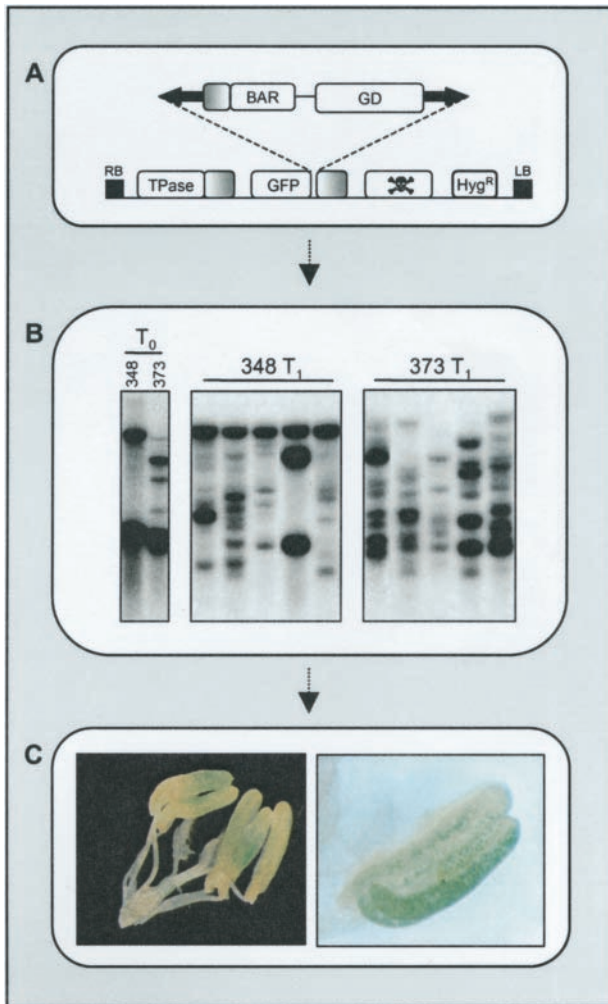
The scope of knockout mutations is limited because the majority of genes display no obvious phenotype, probably due to functional redundancy in which one or more other homologous loci can substitute for the same function. A way to circumvent this is the sequential disruption of redundant genes in an individual genotype that might ultimately reveal a mutant phenotype and uncover the gene function.

#### GENE DETECTION STRATEGY

Gene detection strategies have been developed to address the function of genes that do not directly reveal a knockout phenotype. One way is expression detection that can make use of inserts containing reporter gene constructs such as enhancer traps (ETs) or gene traps, whose expression depends on tran-

scriptional regulatory sequences of the adjacent host gene. Another way is by creating misexpression mutants, such as activation tags (ATs) that might reveal a gain-of-function phenotype.

Constructs were made with the aim of generating populations of transposon inserts for knockout mutagenesis as well as gene detection using ETs and ATs. The general structure of the ET and AT constructs is outlined in Figure 2A. The *Ds* mobile transposon contains a phosphinothricin acetyltransferase (BAR) gene conferring resistance to the herbicide Basta with a GUS reporter gene (with minimal promoter) in the ET constructs or a multiple cauliflower mosaic virus-35S enhancer in the AT constructs. The T-DNA contains an immobile *Ac* transposase under control of a strong promoter and a negative selection marker to allow selection of transposase-free segregants in a later phase. Using a combination of these greenhouse/field-selectable markers, progeny of single locus transformants can be used to identify stable transposants, where the *Ds*-BAR transposes from the T-DNA to unlinked positions. From the ET and AT lines generated in cv Nipponbare, about 50% show transpositional activity. The most active single locus lines containing multiple transpositions (Fig. 2B) are being propagated to advanced generations to make a transposon insertion library.



**Figure 2.** Gene detection strategy. A, Example of a two-component *Ac/Ds* construct in which the mobile *Ds* transposon contains the sequences for gene detection (GD) and a transposition marker (BAR), and the immobile T-DNA contains the *Ac* transposase source (TPase), an excision marker (GFP), a negative selection marker, and a selectable marker for transformation ( $\text{Hyg}^R$ ). B, Example of two single T-DNA copy ET lines, which display amplification of *Ds* copy number in the  $T_0$  and  $T_1$  generations. C, Example of pollen-specific  $\beta$ -glucuronidase (GUS) staining in anthers of a mature rice flower, identified in a line containing an *Ac/Ds* ET construct.

The *Ds*-ET inserts contain a minimal promoter that can initiate transcription upstream of the GUS marker gene upon insertion near enhancers of host genes in the genome, thus displaying the expression pattern of the adjacent gene (Fig. 2C). Such patterns will help identify the adjacent plant gene on the basis of its expression. Gene detection systems in rice have been developed by Chin et al. (1999) using a gene trap *Ds* construct containing a promoter-less GUS reporter gene with upstream splice acceptor sites, which revealed a frequency of detection of gene expression of trapped genes comparable to Arabidopsis. In a gene trap, expression of the reporter gene occurs only upon *Ds* insertion in the correct orienta-

tion within a gene. On the other hand, ETs are subjected to less constraint and therefore have a higher probability of detecting expression patterns, making their use especially effective for identification of gene functions in reverse genetics strategies.

Although the frequency of *Ds* transposition in early generations appears to be high, inhibition of transposition is reported in later generations (Izawa et al., 1997). Although this might happen in some lines, active lines have been identified that still show good transposition activity in the  $T_2/F_3$  generation (Nakagawa et al., 2000; R. Greco and A. Pereira, unpublished data), suggesting that *Ds* inactivation may not be a general phenomenon. Transposon populations of stable *Ds* insertions sufficient for genome saturation can be generated in a few generations starting from a minimal number of active multiple transposons lines. Assuming preferential transposition of *Ds* in genes as described for *Ac*, a collection of around 100,000 inserts would be adequate to ensure the tagging of almost every gene. Such a population could be produced in three generations after transformation and seed multiplication from 10 starter lines, considering a 20% transposition rate per insertion, four inserts per plant, and a 20% frequency of transposition to unlinked positions. With a concerted international effort, the transposon libraries can be produced and made available to rice researchers worldwide.

Received November 16, 2000; returned for revision December 11, 2000; accepted December 21, 2000.

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