ORIGINAL PAPER

Narayana M. Upadhyaya · Qian-Hao Zhu
Xue-Rong Zhou · Andrew L. Eamens
Mohammad S. Hoque · Kerrie Ramm
Ramannee Shivakkumar · Kathryn F. Smith
Shu-Ting Pan · Suzhi Li · Kefan Peng
Song J. Kim · Elizabeth S. Dennis

Dissociation (*Ds*) constructs, mapped *Ds* launch pads and a transiently-expressed transposase system suitable for localized insertional mutagenesis in rice

Abstract We have developed a transiently-expressed transposase (TET)-mediated *Dissociation* (*D*s) insertional mutagenesis system for generating stable insertion lines in rice which will allow localized mutagenesis of a chromosomal region. In this system, a *Ds* containing T-DNA construct was used to produce *Ds* launch pad lines. Callus tissues, from single-copy *Ds*/T-DNA lines, were then transiently infected with *Agrobacterium* harbouring an immobile *Ac* (*iAc*) construct, also containing a green fluorescent protein gene (*sgfpS65T*) as the visual marker. We have regenerated stable *Ds* insertion lines at a frequency of 9–13% using selection for *Ds* excision and GFP counter selection against *iAc* and nearly half of them were unique insertion lines. Double transformants (*iAc*/*Ds*) were also obtained and their progeny yielded ~10% stable insertion lines following excision and visual marker screening with 50% redundancy. In general, more than 50% of the *Ds* reinsertions were within 1 cM of the launch pad. We have produced a large number of single-copy *Ds*/T-DNA launch pads distributed over the rice chromosomes and have further refined the *Ds*/T-DNA construct to enrich for "clean" single-copy T-DNA insertions. The availability of single copy "clean" *Ds*/T-DNA launch pads will facilitate chromosomal region-directed insertion mutagenesis. This system provides an opportunity for distribution of gene tagging tasks among collaborating laboratories on the basis of chromosomal locations.

Communicated by E. Guiderdoni

N. M. Upadhyaya (✉) · Q.-H. Zhu · X.-R. Zhou
A. L. Eamens · M. S. Hoque · K. Ramm · R. Shivakkumar
K. F. Smith · S.-T. Pan · S. Li · K. Peng · S. J. Kim · E. S. Dennis
CSIRO Plant Industry, GPO Box 1600, 2601,
Canberra, ACT, Australia
E-mail: Narayana.upadhyaya@csiro.au
Tel.: +61-2-62465491
Fax: +61-2-62465000

Introduction

Following the completion of the genome sequences of Arabidopsis (The Arabidopsis Genome Initiative 2000) and rice (Goff et al. 2002; Yu et al. 2002; International Rice Genome Sequencing Project 2005), the international focus now is to identify the specific functions of the predicted ~30,000 plant genes. Significant advances have been made in rice, a model for cereals including the complete physical map of the *japonica* rice cv. Nipponbare (Chen et al. 2002), 200,000 publicly available rice expressed sequence tags (ESTs) and ~28,000 full-length cDNAs (Kikuchi et al. 2003). Various bioinformatics, gene mining and gene prediction tools are being developed to improve the sequence annotations. Genome-wide techniques such as microarrays are helping to group genes involved in specific pathways, developmental stages or tissues. Nevertheless, assigning a function to each plant gene is a slow, tedious and often difficult process.

One way of determining gene function is to isolate mutants for each gene and study the effects of those mutations on the plant. Site directed mutagenesis using homologous recombination in plants is still at its infancy (Hanin et al. 2001; Terada et al. 2002). Specific gene expression knockouts by RNA interference (RNAi) or virus-induced gene silencing (VIGS) can be used (for reviews see Wesley et al. 2001; Lu et al. 2003). Another way of producing mutants is random insertion mutagenesis. Retrotransposons (*Tos17*), T-DNA, *iAc*/*Ds* and *En*/*I* are being used as insertion mutagens (for a

review see Hirochika et al. 2004). Nearly 50,000 *Tos17* insertion lines, each containing ∼10 copies of *Tos17* have been generated (Hirochika 2001; Hirochika et al. 2004). However, only 3% of visible mutants could be traced back to a *Tos17* insertion suggesting difficulties in using this method for forward genetics. A sizable population of T-DNA insertion lines have been generated (An et al. 2003, 2005; Chen et al. 2003; Sallaud et al. 2004). Although these resources will be useful for reverse genetics, there are limitations in obtaining T-DNA insertions into smaller genes such as single exon genes which may account for up to 40% of the genes in rice (http://www.btn.genomics.org.cn:8080/rice/annotation.php).

The two-component *iAc/Ds* (Chin et al. 1999) or *En/I* (Greco et al. 2004) system provides an advantage over T-DNA system. The transposon-based insertion mutagens can be remobilized to produce new insertion lines in order to target genes in a specific chromosomal region, e.g. corresponding to a mapped quantitative trait loci (QTL). The *iAc/Ds*-based gene and enhancer trap systems work in rice yielding 5–10% unique stable insertion lines (Chin et al. 1999; Nakagawa et al. 2000; Greco et al. 2001, 2004; Upadhyaya et al. 2002, 2003; Ito et al. 2004; Kim et al. 2004; Kolesnik et al. 2004). The *Ds* re-insertions linked to the original location of *Ds* within the T-DNA (the *Ds* launch pad) varied from 36 to 67% with the majority being within 1 cM of the *Ds* launch pad (Upadhyaya et al. 2002, unpublished; Kim et al. 2004).

Most of the T-DNA and transposon (*Ds* or *I*) constructs used as insertional mutagens have been modified to act as gene traps, thus, in addition to producing gene knockouts, they provide gene expression data as measured by the trap reporter activity. Gene trapping efficiencies of ∼6% have been reported for these constructs (see Hirochika et al. 2004). The efficiency of T-DNA gene trapping depends on the frequency of "clean" T-DNA insertions, i.e. insertions devoid of direct or inverted T-DNA repeats or of the incorporation of vector backbone (VB) sequences derived from outside the T-DNA borders (Sallaud et al. 2004; Upadhyaya et al. unpublished). A "clean" *Ds* containing T-DNA is also essential for satisfactory mobilization of *Ds*.

Here we report a novel method of producing stable *Ds* insertion lines using a transiently-expressed transposase (TET) system. We have developed constructs suited for high efficiency insertional mutagenesis in general, and the TET system in particular. By super-infecting callus tissue from single-copy *Ds*/T-DNA lines, having both *Ds* excision and reinsertion markers, with *Agrobacterium* harbouring *iAc* constructs containing a visual marker s*gfpS65T*, we have been able to regenerate stable *Ds* insertion lines at a frequency of ∼5%, in addition to *iAc/Ds* double transformants. Application of mapped single-copy *Ds*/T-DNA launch pads, produced using these constructs, in efficient chromosomal region-directed insertion mutagenesis is discussed.

Materials and methods

Construction of gene trap *Ds* and *iAc* vectors

The various cloning intermediates involved in the construction of different gene trap *Ds*/T-DNA constructs (Fig. 1) can be obtained upon request from the authors. Sources of various components of these constructs are binary VB, T-DNA borders and selectable marker constructs—part of pCAMBIA1300 (http://www.cambia.org/main/r_et_camvec.htm) or pWBVec8 (Wang et al. 1998) or pMNDRBBin1 (Lu et al. 2001); *Ds3* and GPA1 intron—pSK200 (Kumar and Narayanan 1997); *bla* and ColE Ori—pSP72 (Promega Corporation, Madison, USA); *Ds5*—a 378 bp end region of *Ds5′* from pEU334BN (Eamens et al. 2004); s*gfpS65*—kindly supplied by J. Sheen (Chiu et al. 1996); *Barnase*—PCR amplified from pMT416 (Hartley 1988) as described previously (Eamens et al. 2004), CaMV35S P-*hph(i)*-*nos*T—from pWBVec8 (Wang et al. 1998).

The constructs pUR224NA (GenBank Acc. No. DQ225746) and pUR224NB (GenBank Acc. No. DQ225747) essentially have the *Ds* gene trap cassette from pSK200 [unidirectional gene trap *Ds* with GPA1 intron-triple splice acceptor-*uidA* as trap reporter, *bla* and pBR322 *ori* as the flanking sequence tag (FST) recovery cassette, and 2′ promoter-*nptII*-*ocs*T as *Ds* tracer] inserted between the CaMV35S promoter and *bar*-*ocs*T cassette (placed after T-DNA right border) so that *bar* gene can serve as *Ds* excision marker. In pUR224NA, the CaMV35S promoter is upstream of the *Ds3′* end which harbours gene trap reporter and in pUR224NB it is upstream of the *Ds5′* end. Both constructs have CaMV35S P-*hph(i)*-*nos*T selectable marker gene cassette placed before the T-DNA left border.

The constructs pNU393A1 (GenBank Acc. No. DQ225748) and pNU393B2 (GenBank Acc. No. DQ225749) have bidirectional gene trap *Ds* cassette (*Ds3′*-GPA1-SA-*uidA*-*nos*T and *Ds5′*-GPA1-SA-*eyfp*-*nos*T), also containing FST recovery cassette and CaMV35S P-*bar*-*ocs*T as the transformation selection marker and *Ds* reinsertion marker. The *Ds* cassette (*D5′–Ds3′* orientation) is placed between the CaMV35S promoter and *hph(i)*-*tml*T cassette so that *hph* gene can serve as *Ds* excision marker. In pNU393A1, the entire construct is oriented as LB-promoter-*DsG*-excision marker-RB where as in pNU393B2 it is oriented as RB-promoter-*DsG*-excision marker-LB. The second pBR322 origin of replication was removed from both binary vector constructs to increase the efficiency of FST recovery by plasmid rescue. The *Hin*dIII restriction enzyme (RE) recognition site adjacent to the LB in pNU393B2 was found to be absent due to a 4 bp deletion (possibly happened during the end-filling step of cloning).

The latest construct pNU435 (GenBank Acc. No. DQ225750) has the promoter-*DsG*-excision marker cassette of pNU393A1. A specially designed cassette,

maize ubiquitin promoter-first exon-modified intron (with LB repeat sequences incorporated)-intron interrupted *barnase-nos*T was placed to serve as VB counter selector and dormant gene activator. A promoterless intron interrupted *barnase-nos*T cassette was placed next to RB (before the promoter-*DsG*-excision marker cassette) to serve as T-DNA direct repeat (RB–LB–RB–LB) counter selector and T-DNA gene trap counter selector. This construct also does not have any pBR322 *ori* in the VB.

The Ubi1P-*sgfpS65T-nos*T cassette from pSK200 was excised as *Spe*I/*Pme*I fragment, end-filled and ligated with pSK300 linearized with *Pme*I (just inside RB border) in direct orientation to produce pKU352NA (GenBank Acc. No. DQ225751). pCAMBIA1300 was digested with *Xho*I/*Eco*RI, end-filled and the vector part was self-ligated. A *Sac*I/*Sac*II fragment from the resulting plasmid (LB region) was used to replace a *Sac*I/*Sac*II fragment (containing *iAc*-CaMV35SP-*hph*-CamV35S T-LB) of pKU352NA. The *iAc* fragment (*Sac*I fragment) was then incorporated to the resulting plasmid to produce pNU400 (GenBank Acc. No. DQ225752), which is another version of transposase construct having only *egfpS65T* as visual marker without any selectable marker.

Standard procedures were used for restriction endonuclease digestion, restriction fragment elution and purification, end-filling, ligation and bacterial transformation. *E. coli* strains DH5α (Hanahan 1983) or XL Blue MRF' (Stratagene, La Jolla, California, USA) were used. Where required, the insert orientation following cloning was determined by sequencing or restriction endonuclease analysis.

Transformation

The steps involved in the callus induction from mature seeds of rice cv. Nipponbare, *Agrobacterium*-mediated transformation of embryogenic calli and subsequent regeneration of transformant lines have all been previously described (Upadhyaya et al. 2000). pUR224NA and pUR224NB transformant calli were selected with hygromycin (conferred by the CaMV35S promoter-driven *hph* chimeric gene), whereas pNU393A1 and pNU393B2 transformants were selected with Basta (conferred by CaMV35S promoter-driven *bar* gene). In both the cases T-DNA copy number was determined by Southern blot hybridization of appropriately digested DNA with radioactively labelled *hph* probe as described previously (Upadhyaya et al. 2002). Regenerants were grown under controlled greenhouse conditions of $25 \pm 3°C$ and natural light conditions.

Transiently-expressed transposase system

Calli containing single-copy *Ds*/T-DNA were selected based on Southern blot and FST rescued (plasmid rescue or TAIL PCR). A portion of the primary transgenic callus tissues were used to regenerate plants and produce seeds for subsequent use. Introduction of the *iAc* constructs was achieved by super transformation as described previously (Upadhyaya et al. 2002). A transient GFP expression gave a measure of number of host cells infected with *Agrobacterium* which could also express transposase transiently. Subsequent selection pressure for *Ds* and its excision allowed proliferation of callus cells with transposed *Ds*. The *bar* gene was used as *Ds* excision marker for lines derived from the use of pNR224NA or pNR224NB and pKU352NA. Hygromycin resistance gene (*hph*) was used as excision marker and *bar* as reinsertion marker for lines derived from the use of pNU393A1 or pNU393B1 and pNU400. In both the cases GFP served as a visual marker for *iAc* to separate putative stable insertion lines (GFP⁻) from possible double transformants (GFP⁺).

Transgene analysis

Genomic DNA was extracted from *Ds*/T-DNA transformants using the PureGene (Gentra Systems Inc., Minneapolis, MN, USA) total nucleic acid isolation kit according to the manufacturer's instructions. Following the initial selection and separation based on hygromycin and/or Basta selection and GFP visualization, respective transgenes were confirmed by PCR (see supplementary Table 1 for primer list) and/or Southern blot hybridization.

Analysis of reporter gene expression

Segments of callus, leaf and root tips of regenerated plantlets and root, leaf, spikelet, mature seed and germinating T_1 seed samples of primary transformants were used to screen for reporter gene expression. Screening for sGFPS65T and eYFP expressions was conducted as described previously (Upadhyaya et al. 2002) using special emission and barrier filters (Kurup et al. 2005). Leica MZ FLIII microscope with GFP2 (480/40 nm excision filter and 510 nm barrier filter) and YFP (510/20 nm excision filter and 560/40 nm barrier filter) was used to visualize GFP and YFP expressions. Screening for GUS expression using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) was done according to Jefferson et al. (1987).

Recovery and analysis of rice *Ds*/T-DNA flanking sequences

The recovery of sequences flanking *Ds*/T-DNA gene trap insertions was carried out using the built-in plasmid rescue system by digesting 5 μg of genomic DNA with 10 u of appropriate restriction endonucleases (supplementary Table 2) according to Upadhyaya et al. (2002).

A pUR224NA

pUR224NB

B pNU393B2

pNU393A1

C pNU435

D pKU352NA

E pNU400
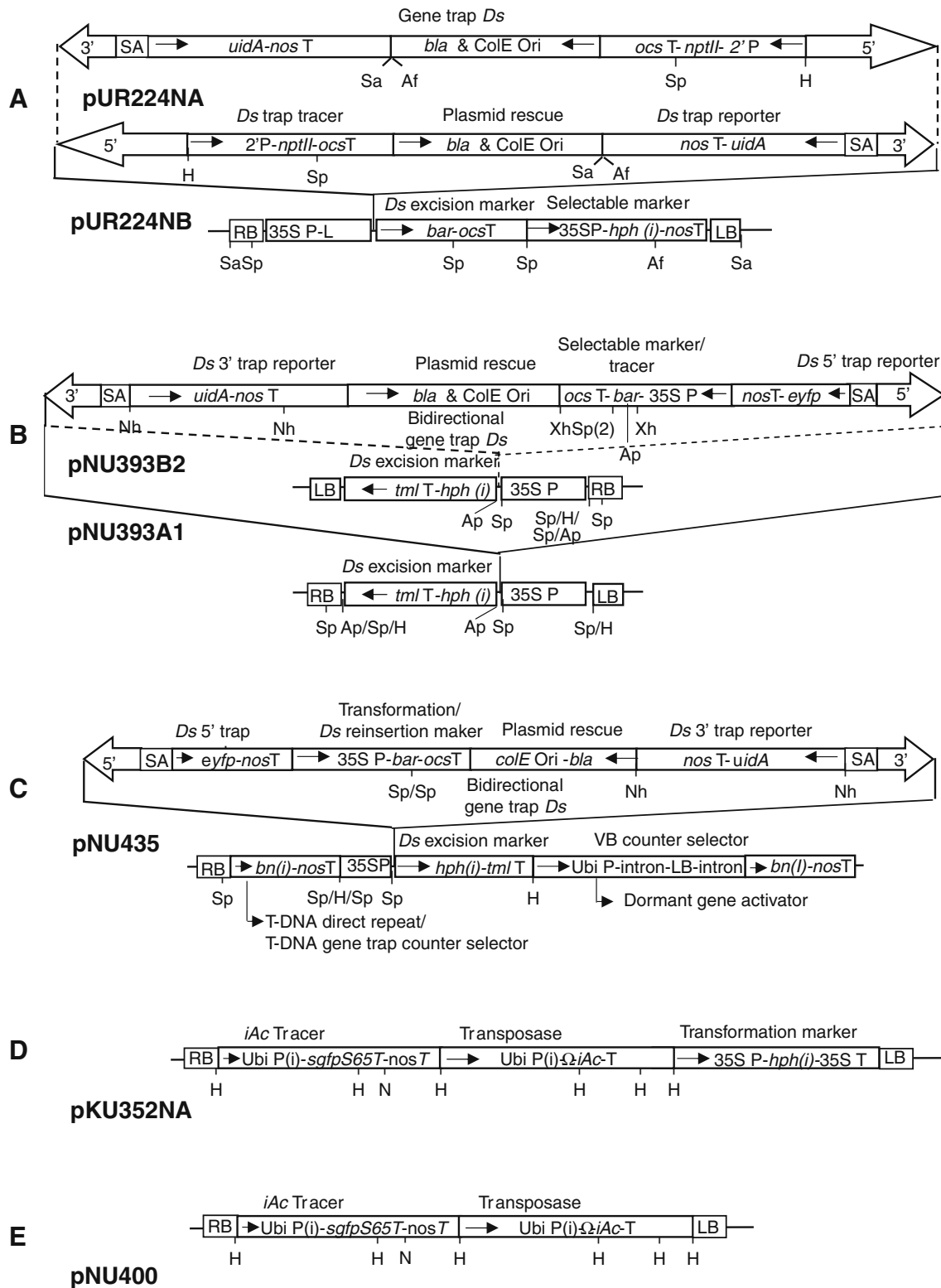
Rescued plasmids showing the correct construct-derived "footprint" following cleavage with restriction endonuclease (*Apa*LI) were sequenced using the appropriate primers and reagents from the ABI Prism BigDye termination cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Programs from the University of Wisconsin Genetics Computer Group (GCG) were used for initial flanking sequence analysis (Devereux et al. 1984). All sequences were BLAST searched against publicly

◄

available sequences from GenBank (http://www.ncbi.nlm.nih.gov/), China Rice GD (http://www.btn.genomics.org.cn:8080/rice/) or the Rice Genome Whole Annotated Database (http://www.tigr-blast.tigr.org/euk-blast/index.cgi?project = osa1) at The Institute for Genome Research (TIGR) to map the FSTs in the rice genome.

TAIL PCR was performed to rescue the RB flanking sequences of some pUR224NA/NB transformants and all pNU393A1/B2 transformants according to previously reported protocol (Liu et al. 1995) with minor modifications. The primers used in the primary, secondary and tertiary PCR reactions were RB-TAIL1 (5′-GCTGATAGTGACCTTAGGCGAC-3′), RB-TAIL2 (5′-CGTTGCGGTTCTGTCAGTTCC-3′) and RBnest5 (5′-ATCAGATTGTCGTTTCCCGC-3′). They were used with one of the two arbitrary degenerate primers AD2 (5′-STTGNTASTNCTNTGC-3′) and AD5 (5′-RCAGNTGWTNGTNCTG-3′), respectively. The secondary and tertiary PCR reactions were loaded on 1% agarose gel side by side. Specific bands with expected size difference were excised from the gel and eluted using UltraClean™ 15 agarose gel DNA purification kit

(Cambio, Cambridge, UK) according to the manufacture's instructions. The purified PCR products were then re-amplified using the primer combinations used in the tertiary PCR before sequencing.

Availability of the materials reported in this paper

Flanking sequence tag information on the single-copy LPs reported in this paper has been submitted to NCBI (GenBank Acc. Nos. DU711663–DU711806). A regularly updated list of mapped single-copy LPs will be posted at our project website http://www.pi.csiro.au/fgrttpub. These lines will be available for distribution under a normal biological material transfer agreement with CSIRO Plant Industry. Our wish is that people receiving one or more of these lines will keep the imported stock for at least 10 years and will provide the lines to others in their home country upon request from CSIRO Plant Industry.

Results

Constructs for production of *Ds* launch pads and *iAc*

Three *Ds*/T-DNA constructs and two *iAc* constructs developed in this study are shown in Fig. 1.

Ds/T-DNA construct with bar excision marker

A CaMV35S promoter driven *bar* gene cassette (Lu et al. 2001) was used in constructs pUR224NA and pUR224NB (Fig. 1a) as the *Ds* excision marker by placing the gene trap *Ds* (*DsG*) cassette between the promoter and the *bar* gene. The preferred orientation of the unidirectional *DsG* cassette is as in pUR224NB where the *Ds* 5′ end is facing the CaMV35S promoter, thus avoiding *cis*-activation of the gene trap reporter *uidA*. The gene trap *Ds* cassette in these constructs was from pSK200 (Kumar and Narayanan 1997; Upadhyaya et al. 2002) which has *nptII* as the *Ds* reinsertion marker.

Table 1 Resources produced so far using the two new *Ds*-T-DNA constructs suited for targeted localized insertional mutagenesis in rice cv. Nipponbare

Construct	pUR224NA/NB	pNU393A1/B2
Ds/T-DNA lines (*Ds* launch pads)	106	656
Single copy launch pads	24	263
Mapped launch pads	10	64
Mutagenic lines[a]	1,009	868
Screening stock (g)	6,240	> 2,639
Putative stable insertion (PSI) lines	3,400	3,500

[a]Mutagenic lines are either a by-product of the TET system or produced by crossing with a single locus *iAc* lines (TT196-1-2 and TT196-9-2)

The hygromycin resistance gene cassette [CaMV35S P-*hph*(*i*)-*nos*T] as a transformation marker (Lu et al. 2001) was placed near the LB end. The positioning of this selectable marker at the LB end was to avoid selection of transformants with truncated T-DNAs (An et al. 2003; Eamens et al. 2004; Sallaud et al. 2004).

Bidirectional gene trap Ds/*T-DNA* construct with *hph* excision marker

The constructs pNU393A1 and pNU393B2 (Fig. 1b) have a previously proven (Upadhyaya et al. 2002; Eamens et al. 2004) bidirectional gene trap *Ds* cassette (*Ds*3′-GPA1-SA-*uidA*-*nos*T and *Ds*5′-GPA1-SA-*eyfp*-*nos*T). It also contains CaMV35S P-*bar*-*ocs*T cassette (Lu et al. 2001) as the transformation selection marker and *Ds* reinsertion marker, and the FST recovery cassette (plasmid rescue system). A previously tested CaMV35S promoter driven intron-interrupted *hph*-*tml*T gene cassette (Wang et al. 1998) was used as the *Ds* excision marker by placing the *DsG* cassette (*Ds*5′–*Ds*3′ orientation) between the promoter and the *hph*(*i*) gene cassette. Constructs (pNU393A1 and pNU393B2) with either orientation of the entire cassette (promoter-*DsG*-excision marker) within the T-DNA borders were used in this study. The second pBR322 origin of replication sequence was removed from both the binary vector constructs to increase the efficiency of FST recovery by plasmid rescue (Upadhyaya et al. 2002; Eamens et al. 2004).

Further improvement of Ds/*T-DNA* construct design

Further construct improvements were made to increase the frequency of selecting single-copy clean T-DNA insertion lines. In the construct pNU435 (Fig. 1c), a maize ubiquitin promoter (with its own first exon and the intron and LB sequence incorporated in the intronic region) driven, intron-interrupted *barnase* serves as a VB counter selector as transformed cell lines with VB-containing T-DNA inserts will be eliminated by the activity of *barnase* gene. Moreover, with a clean T-DNA insert the ubiquitin promoter near the LB has the potential to act as a dormant gene activator.

A second copy of promoterless intron interrupted *barnase*-*nos*T [*bn*(*i*)-*nos*T] cassette placed within the T-DNA next to RB of the construct pNU435 (before the promoter-*DsG*-excision marker cassette) has the potential to serve as T-DNA direct repeat (RB–LB–RB–LB) counter selector and as a T-DNA gene trap counter selector. A T-DNA direct repeat transgene will have the strong ubiquitin promoter upstream of the RB side copy of the *barnase* gene and the resulting cell lines will be eliminated by the *barnase* gene activity. Insertion of this *Ds*/T-DNA within a gene with high constitutive expression has the potential to form an active gene-*barnase* fusion thus counter selection of such gene traps. pNU435 does not contain pBR322 *ori* in the VB.

iAc *constructs*

Two versions of *iAc* constructs were produced and used in this study (Fig. 1d, e). A maize ubiquitin promoter driven (also containing the first exon and intron and the Ω) *iAc* (Kumar and Narayanan 1997; Upadhyaya et al. 2002) was used in these constructs. In the construct pKU352NA, a Ubi1P-*sgfpS65T*-*nos*T cassette is present to serve as a visual marker for *iAc* as we and others have shown that this expression cassette works very well in rice (Upadhyaya et al. 2002; Kolesnik et al. 2004). Consequently, this can be used effectively as a counter selector for *iAc* in the TET system of generating stable insertion lines. Another version of the *iAc* construct (pNU400) was made by removing the selectable marker gene cassette (CaMV35S P-*hph*-CaMV35S T) to make it compatible with the pNU393A1, pNU393B2 and pNU435 *Ds*/T-DNA constructs which have *hph* as excision marker.

Production and mapping of Ds/*T-DNA* launch pads in rice

A large number of selectable marker resistant callus lines were produced with different *Ds*/T-DNA constructs (Table 1). We produced 106 lines using the *Ds*/T-DNA constructs with *bar* as *Ds* excision marker and *nptII* as *Ds* reinsertion (tracer) marker (pUR224NA/NB), and 656 lines using the *Ds*/T-DNA constructs with *hph* as the *Ds* excision marker and *bar* as *Ds* reinsertion marker (pNU393A1/B2).

Southern blot hybridization of *Ds*/T-DNA lines (see examples in Fig. 2a) indicated that 24 out of 106 lines from pUR224NA/NB and 263 out of 656 lines from pNU393A1/B2 were single-copy lines, respectively (Table 1) which accounted for 23 and 40% of the total number of lines generated, consistent with previous reports (Jeon et al. 2000; Upadhyaya et al. 2002; An et al. 2003, 2005; Kim et al. 2003; Eamens et al. 2004; Ryu et al. 2004; Sallaud et al. 2004). We have obtained T-DNA FSTs from 13 and 88 lines, respectively, from these two sets of lines. FST rescue seemed to work better with pNU393A1/B2 lines than that with pUR224NA/NB lines, presumably because of the absence of a second pBR322 *ori* in the VB of constructs pNU393A1/B2 as when two copies of *ori* one of them tends to become inactive by forced mutation (Upadhyaya et al. 2002; Eamens et al. 2004). A complete list of mapped single-copy *Ds*/T-DNA launch pads is presented in Table 2. The chromosomal locations of these launch pads are graphically represented in Fig. 3. We have launch pads evenly distributed in chromosome 1, the short arm of chromosome 3 and the long arms of chromosomes 4, 5 and 10. We are yet to obtain launch pads in short arms of chromosomes 2, 9 and 10. T-DNA insertion preference for chromosomes 1, 2, 3 and 6 and avoidance of chromosomes 9, 10 and 12 has been reported (An et al. 2005). We are obtaining FST information for the

Table 2 Mapped single-copy *Ds*/T-DNA launch pads suitable for targeted regional *Ds* insertion mutagenesis using transiently-expressed transposase system

FST name	Line	GenBank Acc. No.	Mapping BAC/PAC clone Acc. No.	Chromosome	cM
ZHU1450	TT214-1-1	DU711709	AP002526	1	9.5
RS751	TT209-24-1	DU711688	AP003052	1	28.9
RS600	TT209-32-1	DU711736	AP003052	1	28.9
RS730	TT205-4-1	DU711742	AP003103	1	52.7
CRS872	TT202-1-11	DU711722	AP003535	1	63.9
CRS856	TT197-8-3	DU711668	AP003444	1	72.8
ZHU1275	TT219-3-2	DU711800	AP003342	1	87.1
RS1020	TT300-29-2	DU711673	AP003330	1	98
RS994	TT300-29-1	DU711770	AP003330	1	98
RS773	TT212-5-2	DU711744	AP003328	1	101.8
RS985	TT219-21-4	DU711769	AP002819	1	102.3
ZHU1646	TT49-49-1[a]	DU711716	AP002839	1	103.7
CRS865	TT49-162-1[a]	DU711670	AP003725	1	106.2
ZHU1414	TT209-42-1	DU711805	AP003410	1	123.2
RS860	TT301-21-1	DU711696	AP003076	1	129
RS880	TT296-1-1	DU711764	AP003076	1	129
RS967	TT328-28-1	DU711759	AP003290	1	136.6
RS908	TT209-41-1	DU711768	AP003299	1	136.6
ZHU2004	TT49-101-1[a]	DU711717	AP003241	1	143.2
ZHU1281	TT273-14-1	DU711801	AP003415	1	144.5
RS914	TT240-2-4	DU711698	AP003611	1	146.4
ZHU1237	TT139-13-1	DU711796	AP003431	1	151
ZHU1634	TT49-44-1[a]	DU711712	AP007203	2	105.8
ZHU2010	TT49-124-1[a]	DU711718	AP007203	2	105.8
CRS863	TT293-5-1	DU711669	AP005798	2	71.3
RS896	TT302-5-1	DU711766	AP005825	2	122.8
RS833	TT293-5-1	DU711694	AP004098	2	126.4
ZHU1459	TT332-37-2	DU711710	AP005303	2	151.6
RS559	TT162-38-1	DU711682	AP004026	2	156.3
RS309	TT139-4-1	DU711677	AC105733	3	1.1
rs345	TT74-3-1[a]	DU711719	AC105733	3	1.1
RS978	TT139-23-1	DU711774	AC107225	3	16.8
RS352	TT74-105-1[a]	DU711678	AC107225	3	16.8
RS378	TT139-26-1	DU711681	AC107225	3	16.8
RS816	TT300-67-1	DU711748	AC126223	3	18.4
RS754	TT253-10-2	DU711743	AC125471	3	21.5
CRS833	TT202-2-1	DU711667	AC125471	3	21.5
CRS808	TT140-1-1	DU711666	AC125471	3	21.5
CRS869	TT202-2-6	DU711671	AC125471	3	21.5
RS879	TT300-50-1	DU711763	AC105928	3	27.9
RS614	TT209-27-1	DU711738	AC136136	3	36.9
ZHU1301	TT329-5-1	DU711707	AC125495	3	40.3
ZHU1304	TT332-17-1	DU711803	AC118668	3	47.7
RS897	TT300-61-2	DU711767	AC117988	3	136.5
RS783	TT203-26-2	DU711690	AC120506	3	161.7
RS731	TT205-12-1	DU711685	BX569685	4	19.6
RS965	TT328-18-1	DU711758	AL606989	4	16.7
RS980	TT216-14-2	DU711775	AL606989	4	16.7
CRS526	TT203-6-1	DU711664	AL606994	4	18.3
ZHU1319	TT219-13-2	DU711754	AL662999	4	23.3
ZHU1641	TT49-160-1[a]	DU711714	AL606458	4	62.6
ZHU1642	TT49-162-1[a]	DU711715	AL606458	4	62.6
CRS873	TT202-1-10	DU711773	AL606998	4	64.5
CRS530	TT205-4-1	DU711665	AL731579	4	99.3
CC472	TT49-73-1[a]	DU711663	AL607004	4	99.3
ZHU1232	TT139-42-2	DU711702	AL607004	4	99.3
RS806	TT301-20-2	DU711692	AL606454	4	111.3
RS1024	TT301-21-1	DU711675	AL606454	4	111.3
RS360	TT140-1-1	DU711728	AL606999	4	120.3
RS780	TT293-10-1	DU711746	AL606651	4	129.1
RS346	TT74-88-1[a]	DU711725	AC152969	5	NA
RS906	TT203-29-1[a]	DU711697	AC136222	5	70.5
RS894	TT297-13-1	DU711765	AC136222	5	70.5
RS729	TT203-33-3	DU711684	AC135429	5	80.7
CRS822	TT218-14-7	DU711721	AC117265	5	94.5
ZHU1259	TT214-65-5	DU711799	AC117265	5	94.5

Table 2 (Contd.)

FST name	Line	GenBank Acc. No.	Mapping BAC/PAC clone Acc. No.	Chromosome	cM
RS1021	TT300-48-1	DU711674	AC108500	5	94.5
RS996	TT300-48-1	DU711771	AC108500	5	94.5
RS779	TT293-11-1	DU711745	AC136492	5	111
RS875	TT217-41-1	DU711761	AC104709	5	112.4
RS998	TT301-11-2	DU711772	AC120988	5	117.9
ZHU1270	TT216-13-3	DU711704	AC137608	5	120.6
RS1028	TT318-24-1	DU711676	AP004993	6	51
ZHU1252	TT203-5-2	DU711703	AP004810	6	70.9
RS972	TT203-26-1	DU711700	AP004810	6	70.9
ZHU1130	TT332-24-1	DU711750	AP005453	6	100.8
ZHU1295	TT326-17-1	DU711802	AP004797	6	110.6
RS343	TT74-1-1[a]	DU711724	AP004324	6	117
RS953	TT293-2-1	DU711699	AP004377	7	NA
RS987	TT293-2-1	DU711701	AP004377	7	NA
ZHU1324	TT273-14-2	DU711755	AP004382	7	46.5
RS952	TT209-14-2	DU711757	AP005786	7	75.6
ZHU1254	TT203-22-1	DU711798	AP005786	7	75.6
RS749	TT209-5-1	DU711687	AP005542	8	20.2
ZHU1305	TT332-51-1	DU711804	AP005531	8	38.8
ZHU1448	TT209-11-2	DU711708	AP004703	8	80.7
ZHU1632	TT49-36-1[a]	DU711711	AP003914	8	114.4
RS633	TT219-35-1	DU711739	AP006057	9	NA
RS810	TT300-3-2	DU711693	AP005575	9	60.8
RS877	TT300-3-2	DU711762	AP005575	9	60.8
RS598	TT209-27-1	DU711735	AE016959	10	NA
RS872	TT203-11-2	DU711760	AE016959	10	NA
ZHU1271	TT217-32-1	DU711705	AE016959	10	NA
ZHU1250	TT203-1-2	DU711797	AE016959	10	NA
ZHU1288	TT318-28-1	DU711706	BX000497	11	1.4
RS822	TT205-10-1	DU711749	AC128643	11	19
RS599	TT209-27-1	DU711683	AC125783	11	111.8
RS752	TT217-35-1	DU711689	AC112658	11	117
RS747	TT205-2-2	DU711686	BX569688	12	NA
RS725	TT203-9-2	DU711740	BX000503	12	0
ZHU1420	TT330-15-1	DU711806	AL844880	12	95.1

NA not available

[a]These are produced using pUR224 series constructs while others are produced using pNU393 series constructs. Future updates will be published at http://www.pi.csiro.au/fgrttpub

remainder of the single-copy lines and the updated list will be posted at http://www.pi.csiro.au/fgrttpub.

For producing mutagenic lines by crossing we have several single-copy *iAc* lines (Fig. 2b) with the construct pNU400 which has *gfp* as a visual maker but no selection marker.

Ds transposition mediated by transiently-expressed transposase

Ds transposition by TET was demonstrated by introduction of *iAc* into single-copy *Ds*/T-DNA (pUR224NA or pUR224NB) callus lines. For the first set of experiments the selection for *Ds* excision was delayed until 10–12 days following standard co-cultivation and washing but in later experiments selection was applied 3–5 days after co-cultivation. It was important to maintain hygromycin selection throughout. Results of the first 13 super-transformation sessions with nine different *Ds*/T-DNA lines are summarized in Table 3. The number of putative stable insertion (PSI) lines (GFP⁻, hyg^R and Bialaphos^R) and the putative double transformant (GFP⁺, Hyg^R) regenerants varied from session to session. In total, out of 230 lines generated (982 plants) 64 lines yielded one or more PSI lines (89 plants), the remainder were *iAc*/*Ds* double transformants.

Subsequently, we used single-copy *Ds*/T-DNA T_0 or T_1 callus lines of the constructs pNU393A1 and pNU393B2 for the TET system of generating PSIs as they have a *Ds* excision marker (*hph*) better suited for selection at the callus stage and have a *Ds* reinsertion marker (*bar*) better suited for selection at the seedling stage. An overall strategy for generating PSI lines from these launch pads either by the TET system or by double transformant progeny screening is presented in Fig. 4. Among the 1,013 plants generated so far with the TET system 132 were PSIs and the rest were double transformants giving a PSI frequency of ~13% (data not shown). The maximum expected redundancy is 50% as no more than two PSI plants were retained per independent callus line.
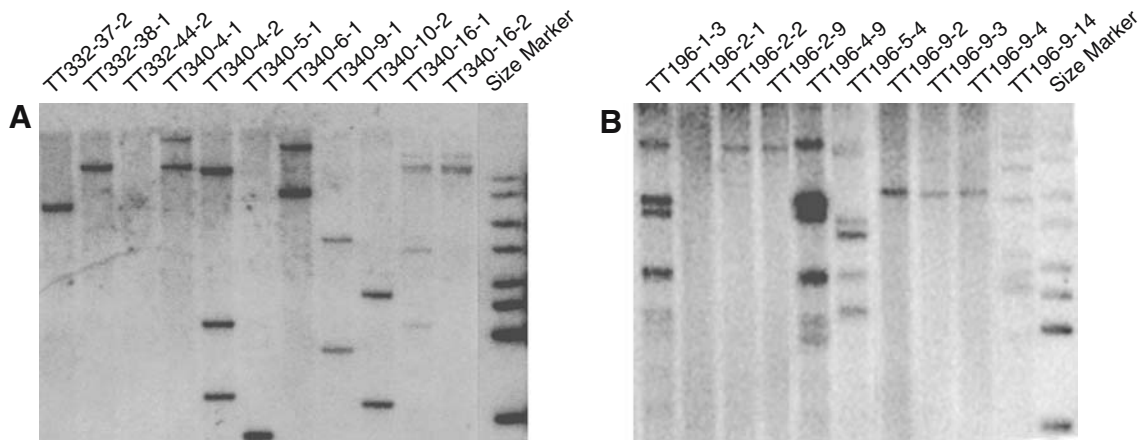
Fig. 2 Determination of copy number of *Ds*/T-DNA or *iAc*/T-DNA in primary transgenic lines. **a** *Apa*I digested genomic DNA isolated from transgenic lines transformed with the *Ds*/T-DNA construct pNU393B2 and hybridized with radioactively-labelled *hph* gene sequences; **b** *Not*I digested genomic DNA, isolated from different transgenic lines transformed with the *iAc* construct pNU400, probed with radioactively-labelled *iAc* sequences

Identification of PSIs in the progeny of double transformants

All the *iAc*/*Ds* double transformants obtained by super transformation of pUR224NA/NB lines were grown to maturity and DtT$_1$ seeds collected for further screening for PSI. We have optimized the screening strategy as follows: (a) GFP scoring with germinating seeds and separate mutagenic (*iAc*$^+$) and non-mutagenic (*iAc*$^-$) seeds, (b) hygromycin spraying of seedlings to separate LP$^+$ (hyg^R) and LP$^-$ (hyg^s) plants, (c) Basta spray to hyg^R seedlings to select PSIs linked to LP (eliminating intact *Ds*/T-DNA which could be escapes from DtT$_0$ selection or empty LPs) and (d) PCR analyses of hyg^S seedlings for transposed *Ds*. We have collected 6,000 g of seeds from about 1,000 mutagenic lines and identified ∼3,400 PSIs (Table 1) among ∼30,000 seeds screened.

We are now concentrating on the T$_1$ progeny of double transformants (DtT$_1$) derived from the super transformation of proven single-copy pNU393A1/B2 construct lines. A total of 868 DtT$_0$ plants have yielded 2,639 g of seeds. We have identified ∼3,500 PSI lines from among ∼30,000 DtT$_1$ seeds screened by the strategy outlined in Fig. 4. We have observed considerable variability in DtT$_1$ populations derived from different DtT$_0$ lines with respect to level of somatic transposition
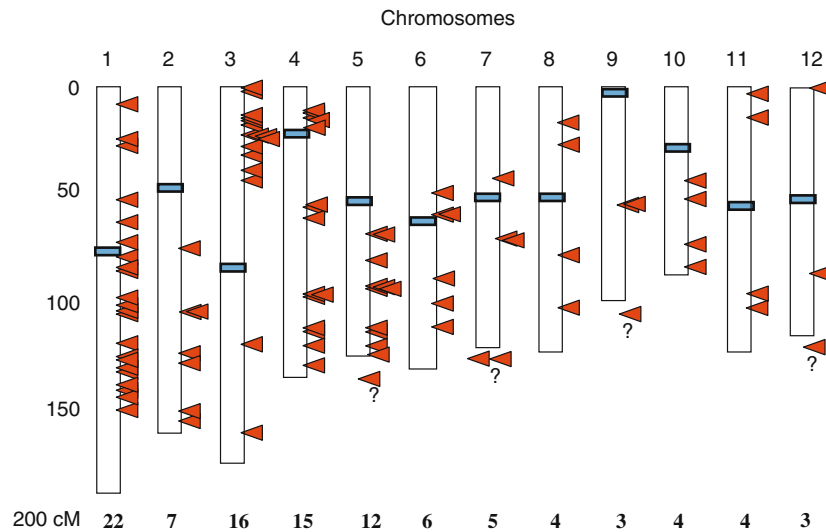

Fig. 3 Mapped single-copy *Ds*/T-DNA launch pads—graphical representation. The BAC/PAC clones corresponding to the FST sequences of single-copy *Ds*/T-DNA lines were identified by BLAST searching the public rice genome sequences (NCBI). Corresponding mapping (indicated by *shaded triangle*) in the rice chromosomes (centromere position is indicated by *shaded rectangle*) was based on the whole genome annotation database of The Institute of Genomic Research (TIGR, http://www.tigr.org/tdb/e2k1/osa1/irgsp.shtml). Regularly updated information of CSIRO *Ds*/T-DNA launch pads will be available at http://www.pi.csiro.aau/fgrttpub. These lines will be available for distribution under a normal biological material transfer agreement with CSIRO Plant Industry

Table 3 Putative stable insertion lines and double transformant lines produced in selected transiently-expressed transposase experiments

Experiment	Ds/T-DNA LP lines[a]	Number of lines (plants) generated	Double transformant lines (plants)	PSI lines (plants)
TT65	TT49-73-1	36 (104)	31 (83)	10 (17)
TT75	TT49-34-1	2 (13)	2 (10)	0 (0)
TT76	TT49-73-1	73 (396)	66 (308)	13 (28)
TT77	TT49-54-1	8 (117)	7 (76)	0 (0)
TT78	TT49-150-1	5 (13)	5 (11)	1 (1)
TT79	TT49-162-1	6 (14)	6 (13)	4 (4)
TT80	TT49-73-1	16 (47)	13 (37)	9 (12)
TT81	TT49-49-1	16 (69)	14 (56)	0 (0)
TT82	TT49-101-1	19 (107)	18 (83)	0 (0)
TT87	TT49-73-1	35 (62)	25 (41)	26 (26)
TT89	TT49-150-1	4 (12)	3 (8)	0 (0)
TT90	TT49-151-1	8 (24)	7 (18)	1 (1)
TT107	TT74-41-1	2 (4)	2 (2)	0 (0)
Total	9	230 (982)	199 (746)	64 (89)

[a] Single-copy Ds/T-DNA (pUR224 series) lines were super transformed with *Agrobacterium* harbouring *iAc* construct pKU352 also containing the visual marker *sgfp*S65T

(as measured by the frequency of GUS spots and sectors in shoot and roots) and frequency of PSIs (Table 4 and Fig. 5). We did find a few lines with no GFP expression (iAc^-) in the progeny (presumably chimeric primary transformants) and a few lines showing all GFP^+ progeny (presumably due to multi-copy *iAc*). We are yet to look at the efficacy of the bidirectional gene trap feature incorporated in these constructs. Although it was technically challenging to distinguish sGFP and eYFP when both are expressing, we have managed to
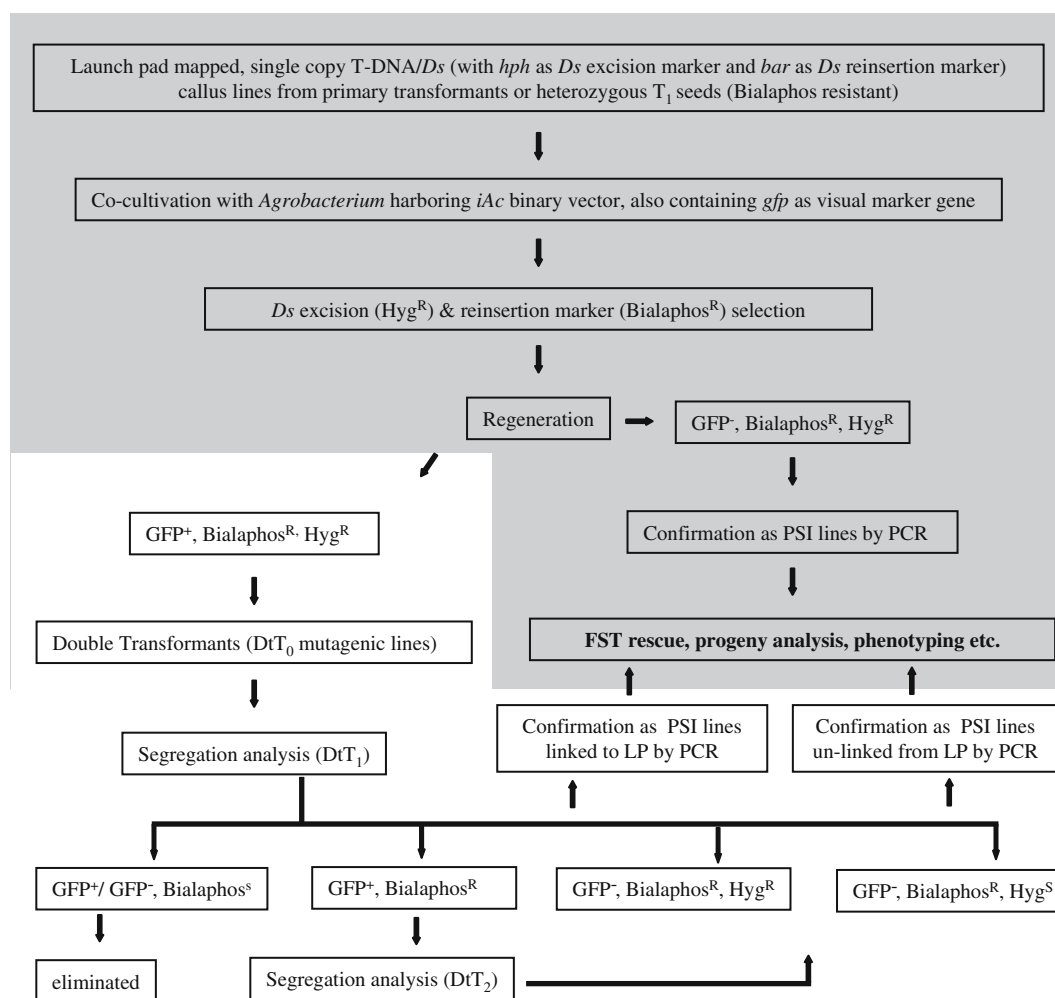

Fig. 4 Strategy for generating PSI lines using *Ds*/T-DNA launch pads either by the TET system (*shaded*) or by double transformant progeny screening (*not shaded*)

Table 4 Transposition behaviour in the progeny of new mutagenic iAc/Ds double transformants (pNU400/pNU393) derived from super transformation

Ds/T-DNA line (LP)	DtT$_0$ line	DtT$_1$ tested	DtT$_1$ GFP$^+$	Ungerminated	Level of somatic transposition (GUS spots/sectors)	Doublenulls (GFP-Basta^S)	Ac^-/Ds^+ (GFP$^-$/Basta^R)	PSI segregants (GFP$^-$, Basta^R) Linked to LP (hph^+)	PSI segregants (GFP$^-$, Basta^R) Unlinked from LP (hph^-)
TT139-28-1 (Chr 4)	TT305-1-8	55	50	0	++	2	3	3	0
	TT305-4-3	47	38	1	+++	1	7	7	0
	TT305-2-8	16	11	0	+++	1	4	4	0
	TT305-6-7	100	81	12	+	13	4	3	1
	TT312-2-1	24	20	0	++	0	4	4	0
	TT321-1-1	100	77	0	++++	17	6	6	0
	TT321-4-2	100	82	1	−	12	5[a]	3	1
	TT321-5-2	100	0	ND	ND	0	0	NA	NA
	TT321-6-1	100	77	7	++	6	10	9	1
	TT321-8-4	100	0	ND	ND	0	0	NA	NA
	TT321-10-1	100	76	0	++	16	6	5	1
	TT321-12-3	100	50	0	−	21	29	29	0
TT139-29-1 (Chr 5)	TT306-3-5	100	73	0	+	11	16	16	0
TT139-4-1 (Chr 3)	TT309-1-2	100	94	4	−	0	2	1	1
	TT309-4-1	66	60	1	+	0	5[a]	3	1
TT139-42-1 (Chr 4)	TT178-2-2	26	0	ND	ND	0	0	NA	NA
	TT307-5-8	100	94	1	++	2	3	3	0
	TT307-6-6	76	56	13	−	5	6	6	0
	TT307-7-1	80	59	6	+++	8	7	7	0
	TT314-1-1	33	26	0	+	7	0	NA	NA
	TT314-2-5	31	21	1	+++	8	1	1	0
	TT314-3-2	100	100	ND	+++	0	0	NA	NA
	TT322-1-1	59	48	8	+	0	3	2	1
TT203-22-2 (Chr 6)	TT322-5-1	35	26	5	++	1	3	3	0
	TT335-3-3	39	0	ND	ND	0	0	NA	NA
TT203-5-2 (Chr 6)	TT351-9-4	100	74	ND	++++	2	20	20	0
TT205-10[b] (Chr 11)	TT244-1-5	23	23	ND	+	0	0	NA	NA
	TT244-4-3	100	71	3	+	6	20	19	1
TT215-3-3 (ND)	TT335-5-1	14	9	3	++++	0	2	2	0
	TT336-4-4	100	73	5	++++	7	15	15	0
	TT336-10-2	100	80	6	−	5	8	6	2
	TT347-3-3	100	96	3	++	0	1	1	0
	TT347-5-1	100	94	6	+	0	0	NA	NA
	Total	2,059[b]	1,491[b]	86[b]		151	190	169	10

NA not applicable, *ND* not done

[a] One or more PSIs were non-viable under normal germination conditions

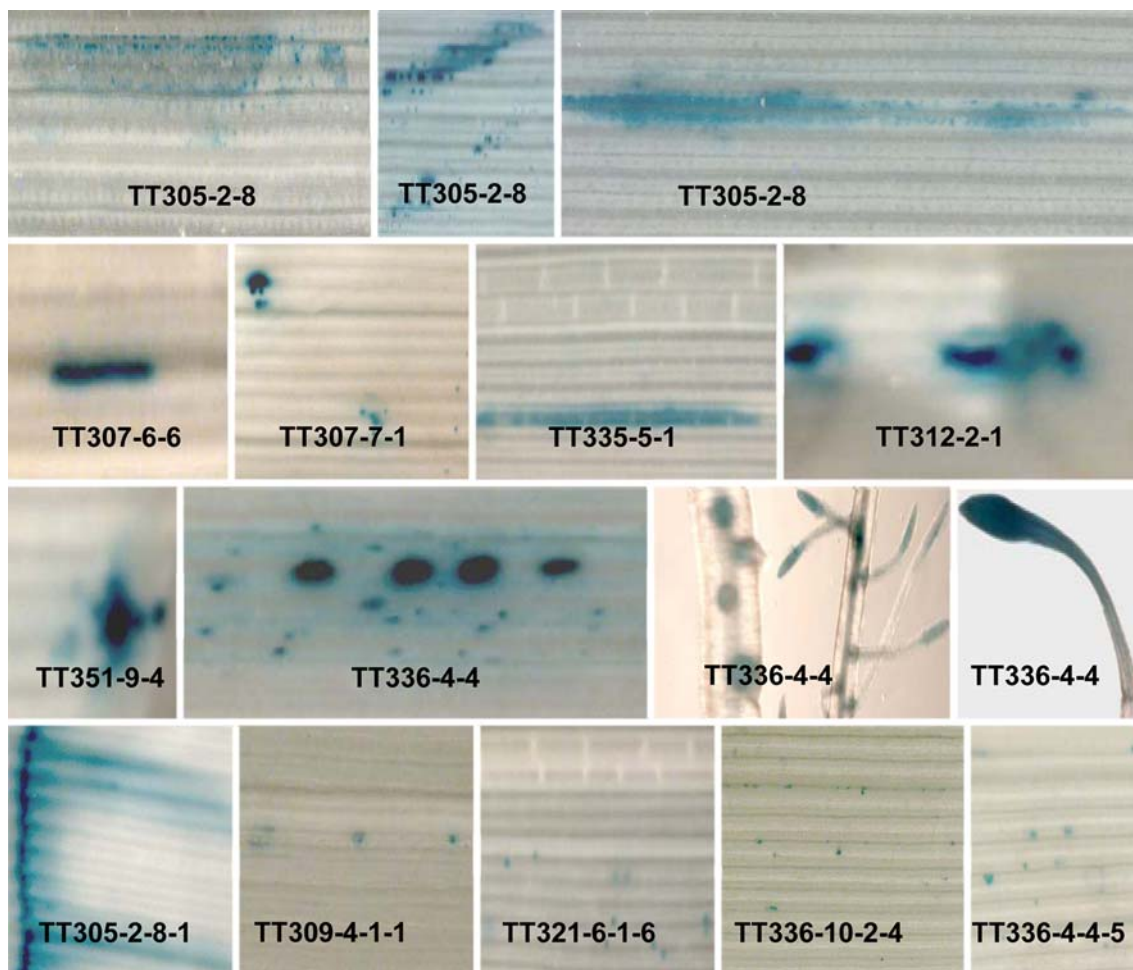[b] Lines not showing none or all GFP were not included

Fig. 5 GUS expression patterns in selected mutagenic (double transformants) lines and PSI lines derived from the super transformation of pNU393B2 lines with *iAc*

distinguish sGFP and eYFP in few double transformants with suitable emission and barrier filter especially when GFP expression was low to medium.

Transposition behaviour of Ds *from selected* Ds/*T-DNA launch pads*

Transposition behaviour of *Ds* from three launch pads—a pSK200 construct derived launch pad in chromosome 7, a pUR224NA construct derived launch pad in chromosome 4 and a pNU393B2 derived launch pad in chromosome 5—is presented in Table 5. *Ds* insertions into the same chromosome as the launch pad varied from 40 to 56%. The pNU393B2 construct derived launch pad in chromosome 5 yielded the highest rate of intra-chromosomal transposition and > 70% of such transpositions were within 1 cM region flanking the LP. Another set of analyses with nine different launch pads derived from the construct pNU393B2 showed that linked transpositions could be as high as 95%, as out of 179 germinated PSIs 169 were linked to the launch pad (Table 4). We followed a secondary transposition from a

PSI mapping to a chromosome 5 BAC clone (GenBank Acc. No. AC108500) linked to the LP which is represented in Fig. 6. Eleven of the 17 lines had the retransposed *Ds* in the same chromosome (Chr 5) with four mapping to the same BAC as the original PSI.

Discussion

A novel TET system of producing stable *Ds* insertion lines was developed using constructs specially designed for efficient screening of *Ds* transposants linked to the initial *Ds*/T-DNA launch pads. We have produced large number of *Ds*/T-DNA launch pads and show that these launch pads can be used in efficient chromosomal region-directed insertion mutagenesis in rice.

It is well-established fact that the two-element *iAc/Ds* system works very well in rice (Chin et al. 1999; Nakagawa et al. 2000; Greco et al. 2001, 2004; Upadhyaya et al. 2002, 2003; Kim et al. 2004; Kolesnik et al. 2004). We and others have previously shown that the *Ds* transpositions linked to the original launch pad varies from 36 to 67% (Upadhyaya et al. 2002; Kim et al. 2004)

Table 5 Transposition behaviour of *Ds* in rice

Ds launch pad	Distribution of transposed *Ds* (in PSI lines from specified T-DNA/*Ds* launch pad) on 12 rice chromosomes												% on the LP Chr	% within 1 cM (on LP Chr)
	1	2	3	4	5	6	7	8	9	10	11	12		
TT2-10-1 (Chr 7, 99.6 cM)	7	5	7	1	3	5	42	9	2	4	2	2	47	19
TT49-73-1 (Chr 4, 99.3 cM)	11	9	25	63	4	6	9	11	5	6	4	3	40	32
TT139-14-1 (Chr 5, 0 cM)	1	0	3	1	14	0	1	2	1	1	0	1	56	71

which is consistent with reports both from maize—the original source of *Ds* and other heterologous systems. Most of the previously used *Ds* insertion mutagens have been the modified forms with a unidirectional gene trap facility, which provide additional gene expression data as measured by the trap reporter activity (see Hirochika et al. 2004). One way of increasing gene trapping efficiency is by having bidirectional gene traps (Eamens et al. 2004; Ryu et al. 2004). The two *Ds*/T-DNA constructs (pNU393A1/B2 and pNU435) described in this paper have this bidirectional gene trap facility.

Ds excision markers were incorporated in all the three *Ds*/T-DNA constructs to increase the screening efficiency for stable insertion lines in a TET system and in the progeny of mutagenic (*iAc*/*Ds*) lines. Excision markers in combination with *Ds* reinsertion markers are particularly useful for selection of *Ds* insertions linked to the *Ds*/T-DNA launch pad. Although the constructs pUR224NA/NB had 2′ promoter driven *nptII* as *Ds* reinsertion marker we later found out that this *nptII* expression cassette is not functional in rice (Upadhyaya et al. 2002) and we had to rely on *nptII* gene specific PCR while developing the TET system to detect the presence of *DsG*. The newer constructs pNU393A1/B2

and pNU435 have a previously tested CaMV35S promoter-driven intron-interrupted *hph* gene cassette as the excision marker. Again the choice was based on our experience that this cassette works well as a selectable marker in rice transformation using embryogenic calli as the target tissue (Upadhyaya et al. 2000). We found it particularly advantageous for selecting callus lines with *Ds* excision using the TET system.

Although the *bar* gene is not an ideal selectable marker at the callus stage of selection (at least in our hands) it is an excellent selectable marker at the plant stage and is the preferred positive selection marker for *Ds* tagging in rice (Chin et al. 1999; Kim et al. 2004; Kolesnik et al. 2004). A single spray of Basta can eliminate *Ds* null segregants, in this instance, lines without *DsG* reinsertion, thus increasing the screening efficiency. Constructs pNU393A1/B2 and pNU435 have a proven CaMV35 promoter driven *bar* as *Ds* reinsertion marker.

The efficacy of T-DNA gene trapping depends on the frequency of "clean" T-DNA insertions without any T-DNA repeats and VB sequences. T-DNA repeats and VB sequences are present in 30–60% of the existing insertion lines (Jeon et al. 2000; Upadhyaya et al. 2002; Kim et al. 2003; Eamens et al. 2004; Sallaud et al. 2004)
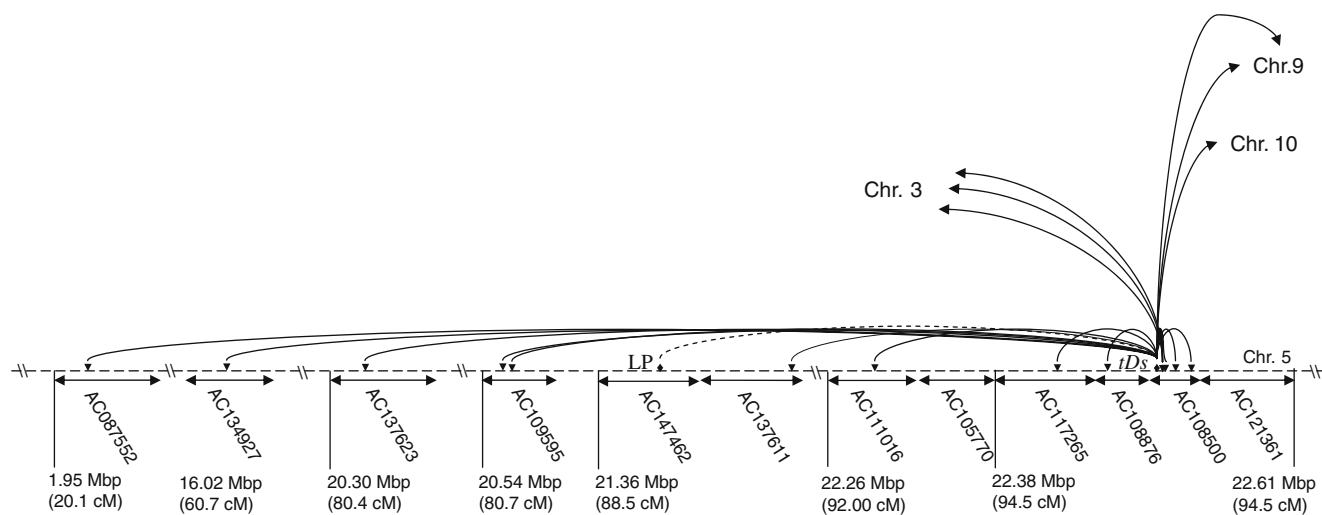

Fig. 6 Mapping of *Ds* in new PSI lines derived from a mutagenic line having a transposed *Ds* (*tDs*) linked to the original LP (indicated by *dotted line*). FST sequences rescued from different PSI lines were used to identify corresponding BAC clones by BLAST searching the National Centre for Biotechnology Information (NCBI) nucleotide sequence database (http://www.ncbi.nlm.nih.-gov/BLAST/) and the relative positions of the BAC clones were obtained from The Institute of Genomic Research (TIGR) BAC/PAC clone table available at http://www.tigr.org/tdb/e2k1/osa1/irgsp.shtml. GenBank accession numbers are presented along with their relative positions (physical in Mb and genetic in cM) in the TIGR chromosome 5 pseudomolecule (version 3)

and are prone to post-integration rearrangements and gross deletions. Furthermore, rescuing the FST from these lines is very difficult or in some cases impossible. In our bidirectional gene trap constructs (Eamens et al. 2004) we did utilize an intron-interrupted *barnase* gene as a counter selector for VB containing lines (Hanson et al. 1999). Although we saw a reduction in the number of VB containing *Ds*/T-DNA lines, ~27% lines still contained direct or inverted repeats of T-DNA. We have therefore further refined the construct design (pNU435) by placing two copies of intron-interrupted *barnase*—one in the VB immediately after ubiquitin promoter-LB sequence and the other immediately after the RB repeat sequence. This configuration has the capacity to counter-select transformants with either VB or direct repeat T-DNA concatermerized inserts. One additional advantage with pNU345 construct design is a potential to counter select *Ds*/T-DNA insertions in gene with high constitutive expression as explained before. We could not incorporate a counter selector for inverted repeat T-DNA insertions, a common feature in T-DNA integration. This could, however, be addressed by a novel binary vector design where direct and inverted RB–LB integrations will produce a hairpin RNA targeting the selectable marker gene *hph* (Chen et al. 2005).

During the process of *Agrobacterium*-mediated transformation single stranded T-DNA is chaperoned into the nucleus of the infected cell (Tzfira et al. 2003, 2004). Although the molecular mechanisms of T-DNA integration leading to stable transgenic events are yet to be fully unravelled, two models have been proposed: single-strand gap repair or double-strand break repair (Tinland 1996; Tzfira et al. 2004). From a close scrutiny of junctions of a large number of T-DNAs in our insertion lines we believe that a double-strand break repair via synthesis dependent strand annealing (SDSA) mechanism is one of the main pathways for T-DNA integration (Zhu et al. unpublished). Integration of T-DNA is most likely to be through a double stranded T-DNA intermediate (Tzfira et al. 2004).

Transient expression of introduced foreign DNA in target plant cells is a well-known phenomenon which occurs before any stable integration through illegitimate recombination or its breakdown by the plant surveillance system. A burst of transient expression of genes carried by the introduced T-DNA can be visualized by reporter gene expression within 48–72 h of co-cultivation (Yoshioka et al. 1996; Upadhyaya et al. unpublished). Using a *Ds*-interrupted *uidA* gene construct (excision reporter construct) transposase activity in stably transformed *Ac* transgenic lines has been measured (Takumi et al. 1999; Koprek et al. 2000). Excision of a *Ds*-like maize transposable element (*Ac* delta) by transient expression of transposase has been demonstrated in Petunia protoplasts (Houba-Herin et al. 1990). Encouraged by the above findings, we have developed a system where a TET is used to produce stable insertion lines in rice. For the TET system to work efficiently, it is essential to have single-copy *Ds*/T-DNA lines which are

equivalent to a heterozygous line. It is also important to select *Ds*/T-DNA lines with no gene trap reporter gene expression. None of the single-copy *Ds*/T-DNA lines showed any GUS activity in this study.

The main advantage of the TET system is that PSIs can be produced as primary transformants. In contrast, with the *iAc*/*Ds* crossing system the first available screening population is F$_2$. Selection for *Ds* excision (hygromycin or PPT depending on the construct used) is applied after callus co-cultivation with *Agrobacterium* harbouring *iAc* which also contains GFP as a visual marker. In theory, any resulting excision marker resistant and GFP$^-$ (subsequently confirmed by *iAc* specific PCR) callus lines would be PSIs (unless resulting callus lines are chimeric). We have confirmed the authenticity of these PSIs by FST rescue and later by segregation analyses. A previous work in tomato on the use of *hph* gene as a *Ds* excision marker (Rudenko et al. 1994) did show phenotypic excision despite intact structure of the T-DNA (*Ds* in its original LP) when the selection pressure was low (30 mg/l). We have pre-tested several single copy *Ds*/T-DNA lines (containing *hph* as excision marker) for hygromycin resistance. Although 30 mg/l hygromycin B was not enough to kill rice calli 50 mg/l was good enough to suppress the callus growth efficiently. In tomato also a higher level of hygromycin (80 mg/l) was good enough to suppress any false positive results.

One disadvantage of the TET system is the tissue culture induced somaclonal variation which has been a major drawback with T-DNA insertional mutagenesis (An et al. 2005). We are addressing this by keeping the tissue culture phase to the bare minimum.

As with the screening for PSIs in the progeny of any double transformant lines (originated from single copy *Ds*/T-DNA lines), the main advantage here is easy sorting of PSIs linked to the original LP (GFP$^-$ and *iAc* PCR$^-$, *Ds* tracer/reinsertion marker^R and excision marker^R) and PSIs unlinked from the original LP (GFP$^-$ and *iAc* PCR$^-$, *Ds* tracer/reinsertion marker^R and excision marker^S). It should however be noted that if there were to be no retranspositions in any line one would get just a single linked PSI from that line (only thing happened is *iAc* segregation). In our latest constructs, the excision marker is *hph*. Hygromycin selection works very well with calli but hygromycin application does not kill the seedlings but can easily be scored for resistance and sensitivity. Because of this, any unlinked PSIs also can be easily identified. Again, if there were to be no retransposition one would get a single PSI from a line. For all these things final confirmation would be obtaining unique FSTs.

In our hand, use of GFP as a tracer for *iAc* is working very well with ~90% accuracy as confirmed by *iAc* specific PCR. Although any GFP silenced (or undetectably expressing) plants will initially be considered as PSIs, subsequent *iAc* specific PCR eliminates them. This holds good in screening the progeny plants of double transformants or crosses between *iAc* (with GFP) and *Ds*/T-DNA lines. Only problem in using both *yfp* (one of the gene trap reporter) and *gfp* (*iAc* visual reporter) is

distinguishing YFP expression from GFP expression. Although special filters can be used to differentiate these two it is very difficult to show the YFP expression superimposed in GFP background by photography (although with careful observation we can distinguish them by eye). For a large-scale use it would be better to use some other visual reporters such as DsRed.

Our strategy is to produce sizable numbers of single-copy *Ds*/T-DNA launch pads distributed all over the chromosomes so that subsequent localized or targeted insertional mutagenesis can be achieved by selecting *Ds* transposants linked to the original launch pad. Although published data indicate a great variability in the transposition behaviour of *Ds* in rice (Chin et al. 1999; Upadhyaya et al. 2002; Kolesnik et al. 2004), it is generally accepted that *Ds* tends to transpose to locations close to its original position (launch pad) in plant species tested so far (Muskett et al. 2003). Our study with PSI lines derived from three launch pads (one of each construct) also showed frequency of intra-chromosomal insertion ranging from 40 to 56%. Our pNU393B2 construct derived launch pads seemed to yield higher proportion of linked insertions (Table 4). These launch pads are in different chromosomes (Chr 3, Chr 4, Chr 5, Chr 6 and Chr 11) and our intention now is to use such mapped launch pads in trait targeted insertional mutagenesis.

In conclusion, we have produced a set of *Ds* and *iAc* constructs with features suited for targeted localized insertion mutagenesis, and using these constructs, we are producing a large number of mapped single-copy *Ds*/T-DNA launch pads. Using the callus tissue from some of these single-copy *Ds*/T-DNA launch pads we have shown that a TET can be used to produce stable insertion lines. The availability of a large number of such clean *Ds*/T-DNA launch pads located throughout the rice genome will facilitate chromosomal region-directed high throughput insertion mutagenesis. A similar approach has been reported for Arabidopsis (Muskett et al. 2003). Saturation insertional mutagenesis of the whole rice genome is a huge task and requires serious collaborations among rice researchers. Chromosomal region-wise distribution of gene tagging task among collaborating laboratories is feasible with such *Ds* launch pads.

References

An S, Park S, Jeong DH, Lee DY, Kang HG, Yu JH, Hur J, Kim SR, Kim YH, Lee M, Han S, Kim SJ, Yang J, Kim E, Wi SJ, Chung HS, Hong JP, Choe V, Lee HK, Choi JH, Nam J, Park PB, Park KY, Kim WT, Choe S, Lee CB, An G (2003) Generation and analysis of end sequence database for T-DNA tagging lines in rice. Plant Physiol 133:2040–2047

An G, Lee S, Kim SH, Kim SR (2005) Molecular genetics using T-DNA in rice. Plant Cell Physiol 46:14–22

Chen M, Presting G, Barbazuk WB, Goicoechea JL, Blackmon B, Fang G, Kim H, Frisch D, Yu Y, Sun S, Higingbottom S, Phimphilai J, Phimphilai D, Thurmond S, Gaudette B, Li P, Liu J, Hatfield J, Main D, Farrar K, Henderson C, Barnett L, Costa R, Williams B, Walser S, Atkins M, Hall C, Budiman MA, Tomkins JP, Luo M, Bancroft I, Salse J, Regad F, Mohapatra T, Singh NK, Tyagi AK, Soderlund C, Dean RA, Wing RA (2002) An integrated physical and genetic map of the rice genome. Plant Cell 14:3–545

Chen S, Jin W, Wang M, Zhang F, Zhou J, Jia Q, Wu Y, Liu F, Wu P (2003) Distribution and characterization of over 1000 T-DNA tags in rice genome. Plant J 36:105–113

Chen S, Helliwell CA, Wu L-M, Dennis ES, Upadhyaya NM, Zhang R, Waterhouse PM, Wang M-B (2005) A novel T-DNA vector design for selection of transgenic lines with simple transgene integration and stable transgene expression. Funct Plant Biol 32:671–681

Chin HG, Choe MS, Lee SH, Park SH, Koo JC, Kim NY, Lee JJ, Oh BG, Yi GH, Kim SC, Cho HC, Choi HC, Cho MJ, Han CD (1999) Molecular analysis of rice plants harboring an *Ac/Ds* transposable element-mediated gene trapping system. Plant J 19:615–623

Chiu W-l, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter for plants. Curr Biol 6:325–330

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12:387–395

Eamens AL, Blanchard CL, Dennis ES, Upadhyaya NM (2004) A bidirectional gene trap construct for T-DNA and *Ds* mediated insertional mutagenesis in rice (*Oryza sativa* L.). Plant Biotechnol J 2:367–380

Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H, Hadley D, Hutchison D, Martin C, Katagiri F, Lange BM, Moughamer T, Xia Y, Budworth P, Zhong J, Miguel T, Paszkowski U, Zhang S, Colbert M, Sun WL, Chen L, Cooper B, Park S, Wood TC, Mao L, Quail P, Wing R, Dean R, Yu Y, Zharkikh A, Shen R, Sahasrabudhe S, Thomas A, Cannings R, Gutin A, Pruss D, Reid J, Tavtigian S, Mitchell J, Eldredge G, Scholl T, Miller RM, Bhatnagar S, Adey N, Rubano T, Tusneem N, Robinson R, Feldhaus J, Macalma T, Oliphant A, Briggs S (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). Science 296:92–100

Greco R, Ouwerkerk PB, Sallaud C, Kohli A, Colombo L, Puigdomenech P, Guiderdoni E, Christou P, Hoge JH, Pereira A (2001) Transposon insertional mutagenesis in rice. Plant Physiol 125:1175–1177

Greco R, Ouwerkerk PB, Taal AJ, Sallaud C, Guiderdoni E, Meijer AH, Hoge JH, Pereira A (2004) Transcription and somatic transposition of the maize *En/Spm* transposon system in rice. Mol Genet Genomics 270:514–523

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557

Hanin M, Volrath S, Bogucki A, Briker M, Ward E, Paszkowski J (2001) Gene targeting in *Arabidopsis*. Plant J 28:671–677

Hanson B, Engler D, Moy Y, Newman B, Ralston E, Gutterson N (1999) A simple method to enrich an *Agrobacterium*-transformed population for plants containing only T-DNA sequences. Plant J 19:727–734

Hartley RW (1988) Barnase and barstar. Expression of its cloned inhibitor permits expression of a cloned ribonuclease. J Mol Biol 202:913–915

Hirochika H (2001) Contribution of the *Tos17* retrotransposon to rice functional genomics. Curr Opin Plant Biol 4:118–122

Hirochika H, Guiderdoni E, An G, Hsing YI, Eun MY, Han CD, Upadhyaya N, Ramachandran S, Zhang Q, Pereira A, Sundaresan V, Leung H (2004) Rice mutant resources for gene discovery. Plant Mol Biol 54:325–334

Houba-Herin N, Becker D, Post A, Larondelle Y, Starlinger P (1990) Excision of a *Ds*-like maize transposable element (*Ac*

delta) in a transient assay in *Petunia* is enhanced by a truncated coding region of the transposable element *Ac*. Mol Gen Genet 224:17–23

International Rice Genome Sequencing Project (2005) The map-based sequence of the rice genome. Nature 436:793–800

Ito Y, Eiguchi M, Kurata N (2004) Establishment of an enhancer trap system with *Ds* and GUS for functional genomics in rice. Mol Genet Genomics 271:639–650

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jeon JS, Lee S, Jung KH, Jun SH, Jeong DH, Lee J, Kim C, Jang S, Yang K, Nam J, An K, Han MJ, Sung RJ, Choi HS, Yu JH, Choi JH, Cho SY, Cha SS, Kim SI, An G (2000) T-DNA insertional mutagenesis for functional genomics in rice. Plant J 22:561–570

Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, Yazaki J, Ishikawa M, Yamada H, Ooka H, Hotta I, Kojima K, Namiki T, Ohneda E, Yahagi W, Suzuki K, Li CJ, Ohtsuki K, Shishiki T, Otomo Y, Murakami K, Iida Y, Sugano S, Fujimura T, Suzuki Y, Tsunoda Y, Kurosaki T, Kodama T, Masuda H, Kobayashi M, Xie Q, Lu M, Narikawa R, Sugiyama A, Mizuno K, Yokomizo S, Niikura J, Ikeda R, Ishibiki J, Kawamata M, Yoshimura A, Miura J, Kusumegi T, Oka M, Ryu R, Ueda M, Matsubara K, Kawai J, Carninci P, Adachi J, Aizawa K, Arakawa T, Fukuda S, Hara A, Hashizume W, Hayatsu N, Imotani K, Ishii Y, Itoh M, Kagawa I, Kondo S, Konno H, Miyazaki A, Osato N, Ota Y, Saito R, Sasaki D, Sato K, Shibata K, Shinagawa A, Shiraki T, Yoshino M, Hayashizaki Y, Yasunishi A (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice. Science 301:376–379

Kim SR, Lee J, Jun SH, Park S, Kang HG, Kwon S, An G (2003) Transgene structures in T-DNA-inserted rice plants. Plant Mol Biol 52:761–773

Kim CM, Piao HL, Park SJ, Chon NS, Je BI, Sun B, Park SH, Park JY, Lee EJ, Kim MJ, Chung WS, Lee KH, Lee YS, Lee JJ, Won YJ, Yi G, Nam MH, Cha YS, Yun DW, Eun MY, Han CD (2004) Rapid, large-scale generation of *Ds* transposant lines and analysis of the *Ds* insertion sites in rice. Plant J 39:252–263

Kolesnik T, Szeverenyi I, Bachmann D, Kumar CS, Jiang S, Ramamoorthy R, Cai M, Ma ZG, Sundaresan V, Ramachandran S (2004) Establishing an efficient *Ac/Ds* tagging system in rice: large-scale analysis of *Ds* flanking sequences. Plant J 37:301–314

Koprek T, McElroy D, Louwerse J, Williams-Carrier R, Lemaux PG (2000) An efficient method for dispersing *Ds* elements in the barley genome as a tool for determining gene function. Plant J 24:253–263

Kumar SC, Narayanan KK (1997) Gene and enhancer traps for isolating genetic regions from rice. Rice Biotechnol Quart 31:17–18

Kurup S, Runions J, Kohler U, Laplaze L, Hodge S, Haseloff J (2005) Marking cell lineages in living tissues. Plant J 42:444–453

Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 8:457–463

Lu HJ, Zhou X-R, Gong Z-X, Upadhyaya NM (2001) Generation of selectable marker-free transgenic rice using double right-border (DRB) binary vectors. Aust J Plant Physiol 28:241–248

Lu R, Martin-Hernandez AM, Peart JR, Malcuit I, Baulcombe DC (2003) Virus-induced gene silencing in plants. Methods 30:296–303

Muskett PR, Clissold L, Marocco A, Springer PS, Martienssen R, Dean C (2003) A resource of mapped dissociation launch pads for targeted insertional mutagenesis in the *Arabidopsis* genome. Plant Physiol 132:506–516

Nakagawa Y, Machida C, Machida Y, Toriyama K (2000) Frequency and pattern of transposition of the maize transposable element *Ds* in transgenic rice plants. Plant Cell Physiol 41:733–742

Rudenko GN, Nijkamp HJ, Hille J (1994) *Ds* read-out transcription in transgenic tomato plants. Mol Gen Genet 243:426–433

Ryu CH, You JH, Kang HG, Hur J, Kim YH, Han MJ, An K, Chung BC, Lee CH, An G (2004) Generation of T-DNA tagging lines with a bidirectional gene trap vector and the establishment of an insertion-site database. Plant Mol Biol 54:489–502

Sallaud C, Gay C, Larmande P, Bes M, Piffanelli P, Piegu B, Droc G, Regad F, Bourgeois E, Meynard D, Perin C, Sabau X, Ghesquiere A, Glaszmann JC, Delseny M, Guiderdoni E (2004) High throughput T-DNA insertion mutagenesis in rice: a first step towards in silico reverse genetics. Plant J 39:450–464

Takumi S, Murai K, Mori N, Nakamura C (1999) Variations in the maize *Ac* transposase transcript level and the *Ds* excision frequency in transgenic wheat callus lines. Genome 42:1234–1241

Terada R, Urawa H, Inagaki Y, Tsugane K, Iida S (2002) Efficient gene targeting by homologous recombination in rice. Nat Biotechnol 20:1030–1034

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796–815

Tinland B (1996) The integration of T-DNA into plant genomes. Trends Plant Sci 1:178–184

Tzfira T, Frankman LR, Vaidya M, Citovsky V (2003) Site-specific integration of *Agrobacterium tumefaciens* T-DNA via double-stranded intermediates. Plant Physiol 133:1011–1023

Tzfira T, Li J, Lacroix B, Citovsky V (2004) *Agrobacterium* T-DNA integration: molecules and models. Trends Genet 20:375–383

Upadhyaya NM, Surin B, Ramm K, Gaudron J, Schünmann PHD, Taylor W, Waterhouse PM, Wang M-B (2000) *Agrobacterium*-mediated transformation of Australian rice cultivars Jarrah and Amaroo using modified promoters and selectable markers. Aust J Plant Physiol 27:201–210

Upadhyaya NM, Zhou X-R, Ramm K, Zhu Q-H, Wu L, Eamens AL, Sivakumar R, Kato T, Yun D-W, Kumar S, Narayanan KK, Peacock WJ, Dennis ES (2002) An *iAc/Ds* gene and enhancer trapping system for insertional mutagenesis in rice. Funct Plant Biol 29:547–559

Upadhyaya NM, Zhu Q-H, Eamens AL, Dennis ES (2003) Rice gene machine: a vehicle for finding functions of cereal genes. Asia Pacific Biotechnol News 6:936–942

Wang MB, Cheng Z, Keese P, Graham MW, Larkin PJ, Waterhouse PM (1998) Comparison of the coat protein, movement protein and RNA polymerase gene sequences of Australian, Chinese, and American isolates of barely yellow dwarf virus transmitted by *Rhopalosiphum padi*. Arch Virol 143:1005–1013

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27:581–590

Yoshioka Y, Takahashi Y, Matsuoka K, Nakamura K, Koizumi J, Kojima M, Machida Y (1996) Analysis of virulence loci in *Agrobacterium tumefaciens* required for T-DNA transfer by the transient gene expression system. Plant Cell Physiol 37:782–789

Yu J, Hu S, Wang J, Wong GK, Li S, Liu B, Deng Y, Dai L, Zhou Y, Zhang X, Cao M, Liu J, Sun J, Tang J, Chen Y, Huang X, Lin W, Ye C, Tong W, Cong L, Geng J, Han Y, Li L, Li W, Hu G, Li J, Liu Z, Qi Q, Li T, Wang X, Lu H, Wu T, Zhu M, Ni P, Han H, Dong W, Ren X, Feng X, Cui P, Li X, Wang H, Xu X, Zhai W, Xu Z, Zhang J, He S, Xu J, Zhang K, Zheng X, Dong J, Zeng W, Tao L, Ye J, Tan J, Chen X, He J, Liu D, Tian W, Tian C, Xia H, Bao Q, Li G, Gao H, Cao T, Zhao W, Li P, Chen W, Zhang Y, Hu J, Liu S, Yang J, Zhang G, Xiong Y, Li Z, Mao L, Zhou C, Zhu Z, Chen R, Hao B, Zheng W, Chen S, Guo W, Tao M, Zhu L, Yuan L, Yang H (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *indica*). Science 296:79–92