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Transpositional behaviour of an *Ac/Ds* system for reverse genetics in rice

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Abstract A collection of transposon *Ac*/*Ds* enhancer trap lines is being developed in rice that will contribute to the development of a rice mutation machine for the functional analysis of rice genes. Molecular analyses revealed high transpositional activity in early generations, with 62% of the T_0 primary transformants and more than 90% of their T_1 progeny lines showing ongoing active transposition. About 10% of the lines displayed amplification of the *Ds* copy number. However, inactivation of *Ds* seemed to occur in about 70% of the T_2 families and in the T_3 generation. Southern blot analyses revealed a high frequency of germinal insertions inherited in the T_1 progeny plants, and transmitted preferentially over the many other somatic inserts to later generations. The sequencing of *Ds* flanking sites in subsets of T_1 plants indicated the independence of insertions in different T_1 families originating from the same T_0 line. Almost 80% of the insertion sites isolated showing homology to the sequenced genome, resided in genes or within a range at which neighbouring genes could be revealed by enhancer trapping. A strategy involving the propagation of a large number of T_0 and T_1 independent lines is being pursued to ensure the recovery of a maximum number of independent insertions in later generations. The inactive T_2 and T_3 lines produced will then provide a collection of stable insertions to be used in reverse genetics experiments. The preferential insertion of *Ds* in gene-rich regions and the use of lines containing multiple *Ds* transposons will enable the production of a large population of inserts in a smaller number of plants. Additional features provided by the presence of *lox* sites for site-specific recombination, or the use of different transposase sources and selectable markers, are discussed.

This report is dedicated to the loving memory of our colleague Dr J. Harry C. Hoge

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Introduction

Cereals are the staple food for more than 90% of the world population, and rice alone feeds more than three billion people. Due to its smaller genome size (Arumuganathan and Earle 1991), the availability of dense genetic and physical maps (Harushima et al. 1998; Chen et al. 2002), the ease of transformation (Hiei et al. 1994) and the extensive synteny shared with the other cereals (Gale and Devos 1998), rice has become a model system for monocot species (Izawa and Shimamoto 1996). Deciphering the function of all rice genes is an important step towards the identification of genes of key agronomic importance for the improvement of rice itself and of the other major staple cereals like maize, wheat and barley, in terms of yield and nutritional values.

The complete sequence of the rice genome is now available, thanks to public and private efforts. In the last years, draft genome sequences have been produced for Nipponbare (Barry 2001; Goff et al. 2002) and 93-11 (Yu et al. 2002), cultivars belonging to the two major rice subspecies *japonica* and *indica*, respectively. Concurrently, the International Rice Genome Sequencing Project (IRGSP) has published the complete sequence of Nipponbare chromosomes 1 and 4 (Feng et al. 2002; Sasaki et

al. 2002) and recently announced the release of a high-quality draft sequence of the whole rice genome (Buell 2002; http://rgp.dna.affrc.go.jp/rgp/Dec18_NEWS.html). Overall, some 32,000–55,000 rice genes have been predicted, many of them requiring experimental confirmation. Valuable information on the actual number of expressed genes can be obtained from the large EST sequencing projects that are being undertaken by several groups using different rice varieties (Yamamoto and Sasaki 1997; Bohnert et al. 2001; Reddy et al. 2002; Wu et al. 2002; Beijing Genomics Institute at http://btn.genomics.org.cn/rice/index.php). However, most of the genes identified will still lack a putative function. Unravelling their biological role will then be the next compelling and challenging step.

Among the several functional genomic approaches available (reviewed in Pereira 2000), insertional mutagenesis using T-DNA or transposons (retrotransposons or heterologous transposons) is considered to be a very promising tool for high-throughput gene function discovery in rice (reviewed in Jeon and An 2001). Several collections of rice lines containing the heterologous maize *Ac*/*Ds* transposon system are being established. Both the autonomous *Ac* element (Enoki et al. 1999; Greco et al. 2001b; Kohli et al. 2001) and a two component system, based on *Ds* transposition induced by an immobilised *Ac* transposase source (Izawa et al. 1997; Chin et al. 1999; Nakagawa et al. 2000; Upadhyaya et al. 2002), are being used. The latter collections have the advantage that the *Ds* insertions could be stabilised by segregation, allowing easier identification of tagged genes and the construction of insertional databases. In addition, gene and enhancer trapping features (reviewed in Springer 2000) were incorporated in the transposon vectors, to address the function of genes that would fail to display a visible knockout phenotype, therefore increasing the effectiveness of gene tagging (Chin et al. 1999; Upadhyaya et al. 2002). A *GUS* reporter gene with upstream splice acceptor sites (gene trap) or driven by a minimal promoter (enhancer trap), was placed in this case near the end of the *Ds* transposon. Its expression relies upon the presence of adjacent regulatory sequences, thus revealing the pattern of expression of neighbouring host genes.

Although the transposition rate of *Ac*/*Ds* in rice and the high frequency of germinal insertions support its use as an effective mutagenic agent, indications of inactivation of *Ds* in later generations have been reported (Izawa et al. 1997; Chin et al. 1999) that might hamper its efficiency for use in high-throughput functional genomics screenings. However, epigenetic inactivation of endogenous transposable elements is a naturally occurring phenomenon, not restricted to plants, associated with DNA methylation and most probably related to transposon control (reviewed in Martienssen and Colot 2001). Cyclic inactivation of *Ac* or *Ds* transposons was initially described in maize by Barbara McClintock (1959) and since then numerous studies have been carried out, but the molecular mechanisms underlying this process remain still largely unknown. In rice, Izawa et al. (1997) analysed the behaviour of *Ds* in progeny plants originating from a cross between lines carrying a CaMV 35S-immobilized *Ac* transposase (genomic clone) or a non-autonomous *Ds*, obtained by direct DNA transfer. A decrease in the frequencies of *Ds* transpositions was often observed already in the F_2 and, later, up to the F_5 generation. Among the *Ac*/*Ds* rice populations originated by Agrobacterium-mediated transformation experiments, Chin et al. (1999) employed a *Ds*-gene trap system with a CaMV 35S-*Ac* cDNA as a transposase source in the same T-DNA, and monitored transpositions only in T_0 primary transformants and after repetitive ratooning. Nevertheless they observed a decrease in the frequency of secondary transposition, with only 18% of *Ds* elements that showed primary transposition in the T_0 regenerants (80%) being re-mobilised in the third-ratoon generation. Other reports rely on the analysis of a restricted number of successive generations (Nakagawa et al. 2000; Upadhyaya et al. 2002), in which *Ds* still retains transpositional activity. Although more confirmatory data need to be gathered about the behaviour of *Ds* in advanced generations, loss of mobility seems to be an unquestionable fact that has to be taken into account in order to devise efficient tagging strategies using this transposon system.

In this paper we describe further advances in the development of an *Ac*-*Ds* enhancer trap collection by a multinational European Consortium (Greco et al. 2001a) and its evaluation as an efficient tagging tool, in terms of transposition behaviour over successive generations and in relation to gene tagging and enhancer trapping. Using these public resources, a strategy is proposed for the use of these lines for the development of a rice mutation machine for the functional analysis of all rice genes.

Materials and methods

Ac/Ds enhancer trap constructs

A diagram of the two enhancer trap constructs employed (ET1 and ET2) is shown in Fig. 1. The two constructs differ only for the presence in the latter of the sGFP excision marker.

T-DNA: the immobile transposase extending from the *Bst*NI site at position 939 of *Ac* (Genbank Accession X05424) till the end of the element, was obtained from a derivative of pKU2 (Baker et al. 1987). This promoter-less *Ac* fragment was fused to a 0.55-kb CaMV 35S promoter fragment derived from pDH51 (Pietrzak et al. 1986) and subsequently excised as a unique *Sal*I-*Not*I fragment. The *su1* gene was obtained from pSSU-SU12 (O'Keefe 1994) as a *Eco*RI-*Bam*HI (construct ET1) or *Eco*RI-*Sal*I (construct ET2) fragment. In the case of construct ET2, the mobile *Ds* was inserted in between a *Not*I-*Apa*I doubly enhanced CaMV 35S promoter and AMV leader (Sijmons et al. 1990), and a *Bam*HI-*Hin*dIII s*gfp*(*S65T*)-*nos* terminator (Chiu et al. 1996) fragment. In case of construct ET1, a fragment containing a CaMV 35S terminator and a *lox* site (Hoess et al. 1982) was used as a *Not*I-*Apa*I linker.

Mobile *Ds*: the 5′ terminus of *Ac* until the *Bal*I site at position 252 (*Ds* left junction) was fused to a intron-less maize Ubiquitin promoter-*bar*-*nos* terminator cassette, originally derived from pAHC25 (Christensen and Quail 1996), and was subsequently excised as a unique *Apa*I-*Spe*I fragment. A 0.94-kb fragment extending up to the *Bgl*II site in the first exon of the Ubiquitin promoter was used (Christensen et al. 1992). The 3′ terminus of *Ac* from the *Pac*I site at position 4,302 till the end (*Ds* right junction)

was fused to a 61-bp minimal CaMV 35S promoter fragment, a 48-bp oligonucleotide comprising a *lox* site, and a promoter-less *gus* reporter gene-*nos* terminator (Jefferson et al. 1987). The whole cassette was then excised as a single *Bam*HI-*Xba*I fragment.

The constructs were assembled by multi-point ligation in the binary vector pMOG22 (Zeneca-MOGEN, The Netherlands), containing the hygromycin phosphotranferase gene (*hpt*) for selection of plant transformants.

Plant transformation and growth conditions

Agrobacterium-mediated transformation of *Oryza sativa* ssp. *japonica* cv Nipponbare, plant regeneration and growth were performed as previously described (Greco et al. 2001b). Three transformation experiments were carried out, using construct ET1 (first) and construct ET2 (ET2-a: second; ET2-b: third) in *Agrobacterium tumefaciens* LBA4404. In the third experiment, DNA isolation from an aliquot of the cultured Agrobacterium strain containing the transposon construct was performed before co-cultivation, in order to assess the presence of an intact T-DNA in the binary vector.

Marker expression analysis

sGFP

sGFP expression in calli and transgenic plants containing construct ET2 was monitored as previously described (Greco et al. 2001b)

BAR

De-husked seeds were surface-sterilized (2 min in EtOH 100%, 1 h in NaOCl 5%+Triton X-100 0.1%, 6× rinse with H_20) and sown on 1×MS, 1% sucrose, 0.8% purified agar with 15 mg/l of phosphinothricin (Duchefa Biochemie, The Netherlands).

SU1

Greenhouse seedlings were sprayed with the pro-herbicide R7042 (DuPont, Wilmington, Del.) as described by Koprek et al. (1999).

GUS

Histochemical GUS staining was performed as described by Scarpella et al. (2000). Plant tissues were incubated in the staining solution for up to 2 days. Chlorophyll was then removed with 70% (w/v) ethanol and samples stored at 4°C.

Molecular analysis of *Ds* transposition

Genomic DNA isolations were performed from leaf samples collected either from in vitro young plantlets or from adult greenhouse plants, according to Pereira and Aarts (1998), in Eppendorf tubes or in 96 tube-racks after dry grinding using a Mixer Mill MM300 (RETSCH, Germany) with tungsten carbide beads. PCR analysis and Southern-blot hybridisation were then employed to analyse transposition of *Ds* at molecular level. For simplicity, only the analysis of plants containing construct ET2 is described in details.

PCR analysis

A preliminary PCR analysis was performed in order to select for transgenic lines with intact T-DNA, using primers for the *hpt* gene (HPT-for: *5′-AAAAGTTCGACAGCGTCTCCGACC-3′* and HPT-rev: *5′-TCTACACAGCCATCGGTCCAGACG-3′*), the immobile *Ac* transposase (Ac971-F: *5′-ACGACTCCATTCCTCAGATGACG-3′* and Ac1395-R: *5′-CTTGACTCGGATCTGTAGCTGTACC-3′*) and the *Ds* transposon (Ubi874-F: *5′-TAAATAGACACCCCCTCCA-CACC-3′* and BAR-R1: *5′-CAGGCTGAAGTCCAGCTGCCAG-3′*). For a preliminary screening of excision events, an Empty Donor Site (EDS) fragment of 0.6 kb is expected to be amplified in case of *Ds* excision, using primers in the 35S promoter (35S-for: *5′-ATCCCACTATCCTTCGCAAGACCC-3′*) and in the *sgfp* gene (sGFP-R2: *5′-GCTTGTCGGCCATGATATAGACG-3′*). In case of untransposed *Ds*, Full Donor Site fragments of 0.3 kb and 0.7 kb (left and right junction respectively) will be amplified, using the 35S- for primer in combination with the transposon specific primer *Ds150*-R (*5′-GTTTCCGTTTCCGTTTACCGTTTT-3′*) or the transposon primer *Ds4374*-F (*5′-GAACAAAAATACCGGTTCC-CGTCC-3′*) with sGFP-R2. About 20 ng of DNA and standard PCR conditions were used.

Southern blot analysis

About 3 μg of genomic DNA were digested with *Eco*RI and subjected to Southern blot analysis as previously described (Greco et al. 2001b). In order to monitor excision events, blots were hybridised with a *sgfp* probe, expecting to detect a 1.85-kb fragment in case of EDS and/or a 3.3-kb fragment in case of FDS (see Fig. 1). Transposed *Ds* elements were revealed after re-hybridisation of the same blots with a *gus* probe, as fragments with a size larger than 2.3 kb. Untransposed *Ds* will also be revealed as a 3.3-kb FDS band. The number of inserted copies of the original T-DNA was calculated as the number of *hpt* hybridising-fragments. In addition, the *bar* gene and an *Ac* internal fragment were used to confirm the presence of the *Ds* transposon and the *Ac* transposase respectively (data not shown).

Isolation of *Ds* flanking sites

Genomics sequences flanking the *Ds* insertions were isolated either by TAIL or Adapter PCR, and then compared to known sequences in public databases using the BLAST algorithm (Altschul et al. 1997).

TAIL PCR

TAIL PCR was performed according to Tsugeki et al. (1996) on 10 ng of genomic DNA. The *Ds* transposon primers and the arbitrary degenerate primers used were as reported in Liu and Whittier (1995) and Tsugeki et al. (1996). A re-PCR was generally performed before sequencing the amplified fragments, on agarose-picked tertiary products.

Adapter PCR

The adapter PCR was performed according to Tissier et al. (1999) on 400 ng of genomic DNA digested with *Bfa*I and ligated to the adapter. The *Bfa*I adapter primer was used in combination with *Ds* specific primers annealing to the right junction of the transposon (*Ds*3-1 to 4; Tsugeki et al. 1996). A biotinylated *Ds3*-1 primer was used in the first PCR, *Ds3*-2 in the nested PCR, *Ds3*-3 in the re-amplification of the eluted fragments and *Ds3*-4 for sequencing.

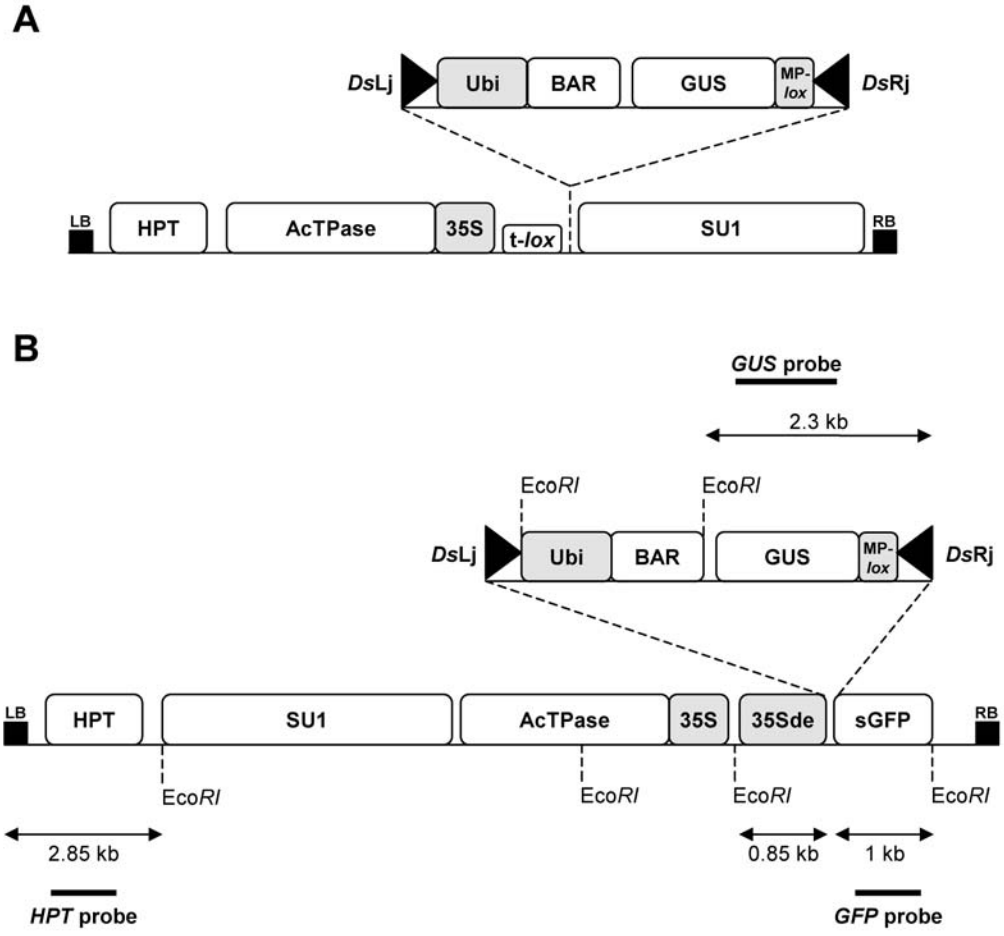

Fig. 1A, B Schematic representation of the enhancer trap constructs used. **A** Diagram of ET1. **B** Diagram of ET2, with the position of the *Eco*RI restriction sites and the probes used for Southern blot analysis. The *gfp* probe is expected to reveal a 1.85-kb hybridizing fragment in case of excision (EDS), or a 3.3-kb FDS fragment otherwise. Similarly, the *gus* probe will detect the 3.3-kb FDS fragment if present, or any transposed *Ds* as hybridizing bands bigger than 2.3 kb. The number of *hpt*-hybridising fragments (bigger than 2.85 kb) estimates the number of integrated T-DNA copies. *LB*, *RB*, left and right T-DNA borders; *HPT*, hygromycin phosphotransferase gene; *AcTPase*, immobilised *Ac* transposase; *35S*, CaMV 35S promoter; *35Sde*, doubly enhanced CaMV 35S promoter; *DsLJ*, *DsRJ*, left and right *Ds* ends; *Ubi*, maize Ubiquitin promoter; *MP-lox*, CaMV 35S minimal promoter and *lox* site; *t-lox*, CaMV 35S terminator and *lox* site

Results

Enhancer trap constructs

In the two-component *Ac*/*Ds* system employed, an immobilised *Ac* transposase driven by the CaMV 35S promoter was used to mobilise a *Ds* non-autonomous element. These two transposon components together with appropriate markers were placed in the same T-DNA vector, so that transposition could already occur directly after transformation in the transgenic calli and the regenerating T_0 plants, without need of further crossing.

Two enhancer trap constructs were made, namely ET1 and ET2 (Fig. 1). Both constructs bear the phosphinothricin acetyltransferase gene (*bar*; DeBlock et al. 1987) for resistance to the herbicide Basta as a marker for positive selection on the mobile *Ds* transposon. To select against the *Ac* transposase source, the cytochrome P450 gene (*su1*; O'Keefe 1994) from *Streptomyces griseolus*, which converts the pro-herbicide R7042 (DuPont, Wilmington, Del.) into a cytotoxic form, is present on the T-DNA. In construct ET2, the *Ds* transposon is inserted between the CaMV 35S promoter and the *sgfp* gene to monitor for excision events. Both constructs carry the *gus* reporter gene driven by a minimal CaMV 35S promoter on the mobile *Ds*, for enhancer trapping. Additionally, construct ET1 bears *lox* sequences for site-specific recombination (reviewed in Ow and Medberry 1995; Ow 1996) in the *Ds* transposon and the T-DNA. Upon crossing with lines expressing a Cre-recombinase, recombination can occur between two *lox* sites. Depending on their mutual orientation, chromosomal rearrangements like deletions, inversions or translocations can be induced.

Table 1 Summary of T_0 enhancer trap (ET) lines produced and analysed[a]

Generation	Construct[b]	Analysed	Entire T-DNA	%	Active	%	Single-copy
T_0	ET1	10 {26}	8 {23}	80 {88}	4 {9}	50 {39}	2
	ET2-a	26 {85}	9 {24}	35 {28}	3 {12}	33 {50}	1
	ET2-b	108 {108}	79 {79}	73	53 {53}	67	35
	ET total	144 {219}	96 {126}	67 {58}	60 {74}	62 {59}	38

[a] The numbers reported for each construct correspond to T_0 original lines and T_0 regenerants (brackets), respectively

Ac/Ds transposition activity in the T_0 primary transformants

Transgenic Nipponbare rice plants containing either constructs ET1 or ET2 were obtained from three independent Agrobacterium-mediated transformation experiments. In total about 1,000 T_0 plants were regenerated from 144 independent T_0 hygromycin-resistant calli (henceforth referred to as T_0 original lines), of which 219 were selected as a representative sample for molecular analysis. Plants were subjected to PCR and Southern hybridisation, to test initially for the presence of each of the components of the original T-DNA (see Materials and methods; data not shown), and then specifically for *Ds* excision, re-insertion and copy number. The results of the analyses are summarised in Table 1, with the constructs used and the transformation experiments described separately.

Figure 2 shows an example of Southern analysis done on a subset of 15 T_0 lines from the second transformation experiment with construct ET2 (only one regenerant per line was analysed in this case). One set of lines showed very early excision (lines 288, 348, 374 and 387), probably directly after transformation, as revealed by the presence of only EDS (Fig. 2A). Re-insertion of the excised *Ds* elements at different genomic positions is revealed after hybridisation with a *gus* probe (Fig. 2B). Among these lines, amplification of *Ds* copy number occurred in lines 348 and 374, since only one *hpt*-hybridising band corresponding to a single T-DNA copy is visible (Fig. 2C), while two *Ds* homologous fragments are detected (Fig. 2B). Another set of lines carried both EDS and FDS (323, 325, 373 and 383), indicating later or partial excision in some of the T-DNA copies. Among them, only 373 and 383 revealed *gus*-hybridising bands corresponding to transposed *Ds* elements (Fig. 2B), which implies a loss of the excised *Ds* in the other two lines. Finally, lines 276, 398 and 401 seemed transpositionally inactive, as revealed by the presence of only FDS after hybridisation with *sgfp* and *gus*, while another four lines underwent rearrangements (258, 274, 277 and 304).

Overall, the percentage of lines with a correct integration of the original T-DNA appeared to be dependent on the construct used, with a decrease of up to 35% in the first transformation experiment using construct ET2 (ET2-a; Table 1). The lack of complete T-DNA inserts in transformants with this construct turned out to be related to its instability in Agrobacterium. Therefore a pre-selection step was introduced in the next

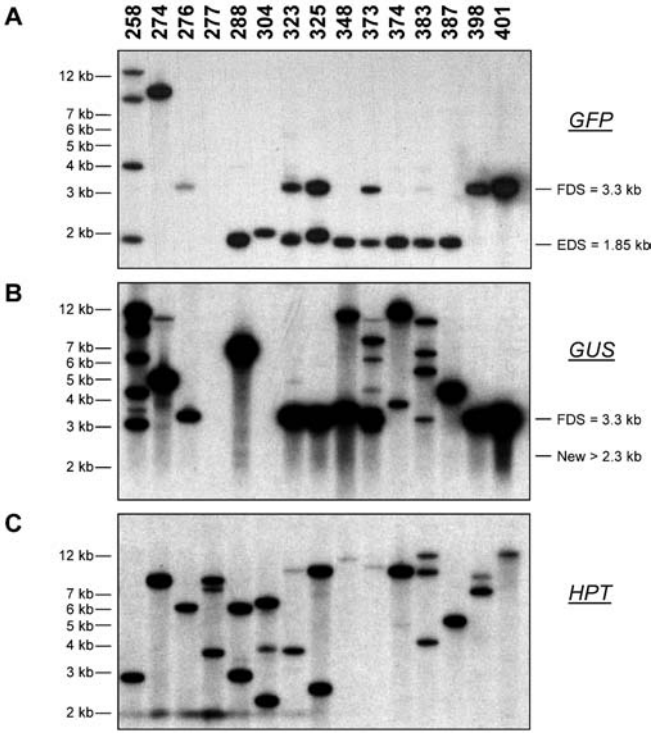

Fig. 2A–C Southern blot analysis of ET2-b enhancer trap lines in the T_0 generation. One T_0 regenerant per line is represented. **A** Hybridization with a*sgfp* probe detects excision events (*EDS*) and/or *FDS*. **B** Transposition was monitored by hybridisation with a *gus* probe. Newly transposed *Ds* (>2.3 kb) and/or *Ds* still-residing in the donor T-DNA (FDS) are revealed. **C** The number of T-DNA copies integrated in the rice genome is estimated by the number of fragments hybridising to an *hpt*-homologous probe

rice transformation experiment at the time of co-cultivation, to ensure that only Agrobacterium cultures with intact T-DNA were used during transformation. In this case, the proportion of lines with undamaged constructs could be raised to 73% (ET2-b; Table 1).

Of the T_0 enhancer trap lines that were not rearranged, 62% showed transpositional activity judged by the presence of *Ds* excision and re-insertion (Table 1). The timing of transposition varied among lines, but also among plants regenerated from the same line, and appeared to occur already in the transgenic calli or during the regeneration of the plantlets. Regenerants with the same pattern of transposition indicate excision events that took place early during callus development and before the formation of the regenerated shoots, while independent insertions result from later excisions in the individual

Table 2 Summary of activity of ET lines through successive generations[a]

Generation	Construct	Analysed	Exp. active	%	Active	%[b]
T_1	ET1	5 {11} [94]	3 {9} [79]	60 {82} [84]	2 {8} [52]	67 {89} [66]
	ET2-a	4 {12} [106]	1 {9} [85]	25 {75} [80]	1 {9} [59]	100 {100} [69]
	ET2-b	25 {67} [496]	20 {57} [449]	80 {85} [90]	19 {45} [214]	95 {79} [48]
	ET total	34 {90} [696]	24 {75} [613]	71 {83} [88]	22 {62} [325]	92 {83} [53]
T_2	ET1	2 {5} [9] (26)	All	100	1 {1} [1] (3)	50 {20} [11] (11)
	ET2-a	1 {8} [20] (104)	All	100	1 {5} [5] (7)	100 {62} [25] (7)
	ET2-b	12 {20} [70] (436)	All	100	9 {13} [26] (63)	75 {65} [37] (14)
	ET total	15 {33} [99] (566)	All	100	11 {19} [32] (73)	73 {58} [32] (13)

[a] The numbers reported for each construct and generation correspond to T_0 original lines, T_0 regenerants or T_1 families (curly brackets), T_1 progeny plants or T_2 families (square brackets), T_2 progeny plants (round brackets) respectively
[b] The percentage is calculated on the lines, families or plants expected active

regenerated plants. The percentage of lines showing complete excision (very early, probably at the time of transformation) at the time of the analysis amounted to approximately 55–60%.

On average, the number of integrated T-DNA copies in all the T_0 lines analysed varied between 1 to 3, with about 60% lines being single-copy and less than 5% having more than three copies (data not shown). In about 10–15% of the lines, amplification of *Ds* transposon copy number was observed, as revealed by multiple *Ds* insertions of the same intensity as a single-copy T-DNA/*Ds* insert.

As a preliminary indication for the occurrence of excision events, calli and regenerant plants were screened for GFP expression. GFP fluorescence is visualised as a result of excision of the *Ds* transposon from the T-DNA, which allows the CaMV 35S promoter to drive the expression of the *sgfp* gene. Of 74 lines displaying somatic or germinal excision, only 33 (45%) displayed GFP positive-sectors in calli growing on embryo-induction medium and hardly any GFP activity could be detected in the regenerating plantlets (data not shown). The unexpectedly low percentage of detectable green fluorescent sectors seemed to suggest a low activity of the *Ds* excision marker.

Ac/Ds transposition activity in T_1, T_2 and T_3 generations

To assess the utility of the *Ac/Ds* enhancer trap lines for tagging purposes, transposition activity was monitored in advanced generations. A total of 34 T_0 lines (90 T_0 regenerants) were selected and propagated in T_1, on the basis of their transposition behaviour. Among them, 24 showed active excision and transposition in T_0, while ten displayed only late somatic excision or were completely inactive (visualised as the presence of a strong FDS with faint or no *Ds* re-insertion bands on a Southern blot hybridised with a *gus* probe). In order to propagate only plants containing the *Ds* transposon, T_1 seeds were germinated in vitro on medium containing phosphinothricin (PPT) before transfer to the greenhouse. The selection allows the growth of progeny plants bearing *Ds* elements, either transposed or not excised. On the other hand, it will prevent the recovery of plants in which the *Ds* transposon is lost due to excision events not followed by re-insertion. A total of 696 T_1 BAR^+ plants were recovered and subjected to molecular analysis by PCR and Southern hybridisation, essentially as described for the T_0 generation.

On basis of the Southern analysis, a subset of 99 transpositionally active T_1 plants (originating from 15 T_0 lines and 33 T_1 families) were selected for further propagation in the T_2 generation. Unlike the previous generation, T_2 progeny seeds were directly sown in the greenhouse without selection for BAR resistance, so that plants lacking *Ds* were also included, together with plants lacking the *Ac*-transposase following segregation.

The results of the molecular analysis performed on the T_1 and T_2 lines are reported in Table 2. The occurrence of a newly transposed *Ds* in a plant was chosen as a criterion to indicate transpositional activity. Therefore, the number of plants with the newly transposed *Ds* is reported as the number of active plants. In general, lines that were inactive or showed only moderate activity in the T_0 generation (FDS with faint *gus*-hybridising bands) remained inactive or partially active also in T_1, independently from the construct used (data not shown).

On the other hand, about 92% of the lines considered active in T_0 (EDS or FDS+EDS) retained activity in T_1. Figures 3 and 4 illustrate the transposition behaviour of representative active ET2 lines over successive generations, analysed by Southern hybridisation with a *Ds* specific-probe (*gus*). In general, the insertions present in the T_0 parents were transmitted to the T_1 progenies, indicating their germinal nature (Figs. 3 and 4). Active transposition was evident in all the different T_1 progeny families, visualised as the presence of several additional independent *Ds* insertions within and among families. Plants showing a discrete number of bands of higher intensity, usually corresponded to plants in which the *Ac* transposase was lost by genetic segregation yielding stable *Ds* insertions and were not propagated further (i.e., plants 348-C-4, 374-D-1 and 7 in Fig. 4). In total, 83% of the T_1 families (53% of all the T_1 plants) analysed showed new transposed *Ds* inserts.

However, the transposition rate in the T_2 generation seemed to decrease considerably. Only about 32% of the

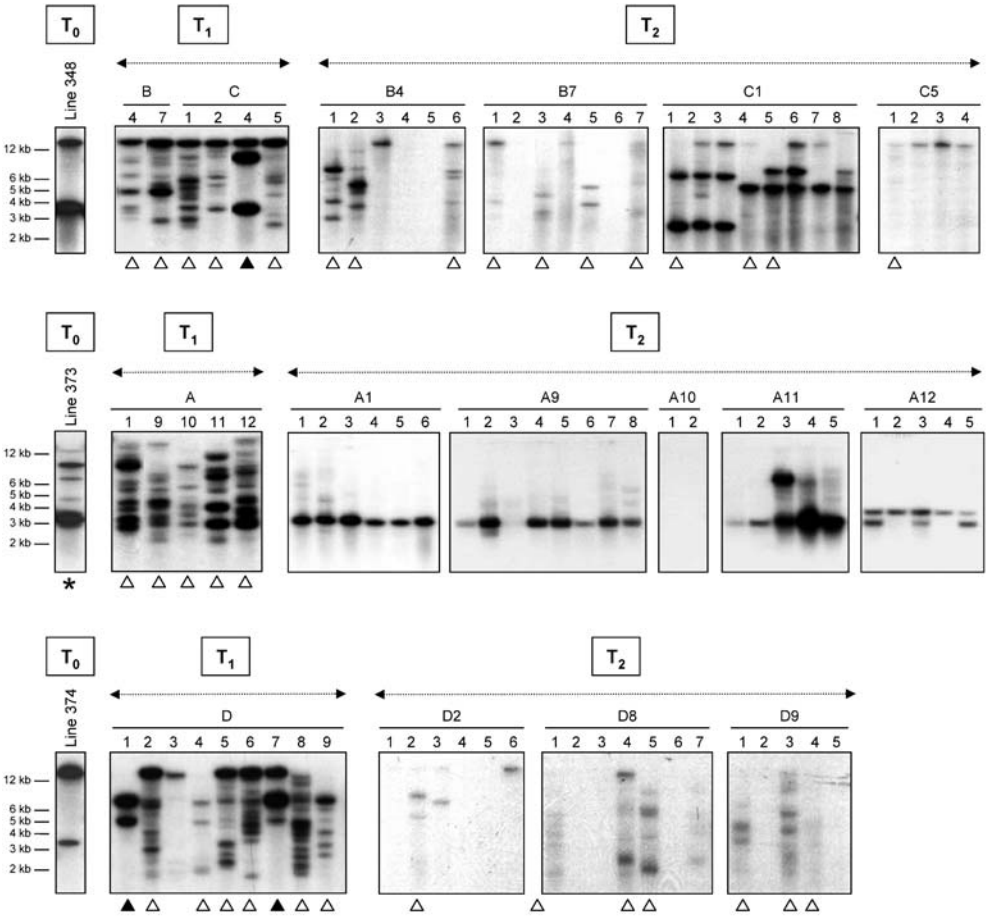

Fig. 3 *Ds* transposition behaviour in ET2 lines over three generations. Example of Southern analysis with a *gus* probe of representative T_1 and T_2 progeny plants from three single-copy ET2-b lines described in Fig. 2 (the T_0 pattern is also reported as a comparison). Only line 373 still displayed FDS in T_0, while full excision was detected in the next generation, as confirmed by hybridisation with a *gfp* probe (data not shown). Lines 348 and 374 revealed active independent transposition still occurring in T_2. On the other hand, line 373 represented a clear demonstration of loss of *Ds* mobility in T_2 and stabilisation of T_0 parental insertions. All the three lines displayed amplification of *Ds* copy number. Plants with new transposition events included in the calculations of frequencies are marked by an *open arrowhead*. Stable T_1 plants lacking the transposase are marked with a *filled arrowhead.* Plants with FDS are marked with an *asterisk*

T_2 families (58% of the original T_1 families or 13% of the T_2 progeny plants) that were propagated from the most active T_1 plants still displayed new transpositions (i.e., Fig. 3, lines 348 and 374). Surprisingly, in the lines exhibiting inhibition of transposition, only the original T_0 insertions seemed to be stably inherited among the many inserts from the T_1 parents, as exemplified by the T_2 progenies of line 373 (Fig. 3) or 13.7 G, H, I and L (Fig. 4, T_2 panel). This would imply that many of the *Ds* bands present in the T_1 parents represented somatic transposition events that were not inherited in the T_2 progeny. As a consequence, the T_2 progenies originating from the same T_1 family yielded T_2 plants harbouring mainly the same insertions, while propagation of several T_0 regenerants with independent transpositions resulted in the recovery of a higher number of T_2 families with different fixed insertions (Fig. 4).

The same behaviour was observed for a small set of T_3 progeny plants propagated from line 13.11 B (Fig. 4, T_3 panel). Overall, stabilisation of *Ds* inserts occurred in the T_2 for about 40% of the T_1 families analysed, which could be used for inheritance and segregation in later generations.

In several lines, amplification of the number of copies of the *Ds* transposon was observed, as exemplified by the three single-copy lines described in Fig. 3. On the other hand, inhibition of activity of the *Ds* transposon in later generations sometimes led to fixation of a lower number of *Ds* insertions, even in lines with an initial higher copy number (Fig. 4).

Enhancer trapping, GUS staining

In order to test the ability of the ET lines to generate GUS expression patterns, a set of active T_1 lines were stained with X-gluc. A total of 536 T_1 plants were tested for GUS staining patterns in roots and leaves. Only one positive plant was identified displaying somatic GUS expression in the leaves. On the other hand, GUS assays on young inflorescences and mature flowers revealed rachilla, stamen and carpel-specific staining in three plants out of 146 (2%) analysed so far (Fig. 5).

Genomic sequences flanking the transposon were isolated by TAIL PCR in order to identify the putative genes generating the GUS expression patterns identified. From one to several flanking sequences were isolated in each of the three plants, but no significant homologies were found after comparison to known sequences in public databases.

Inheritance of the GUS expression patterns identified was also checked, but unfortunately none of the *Ds*

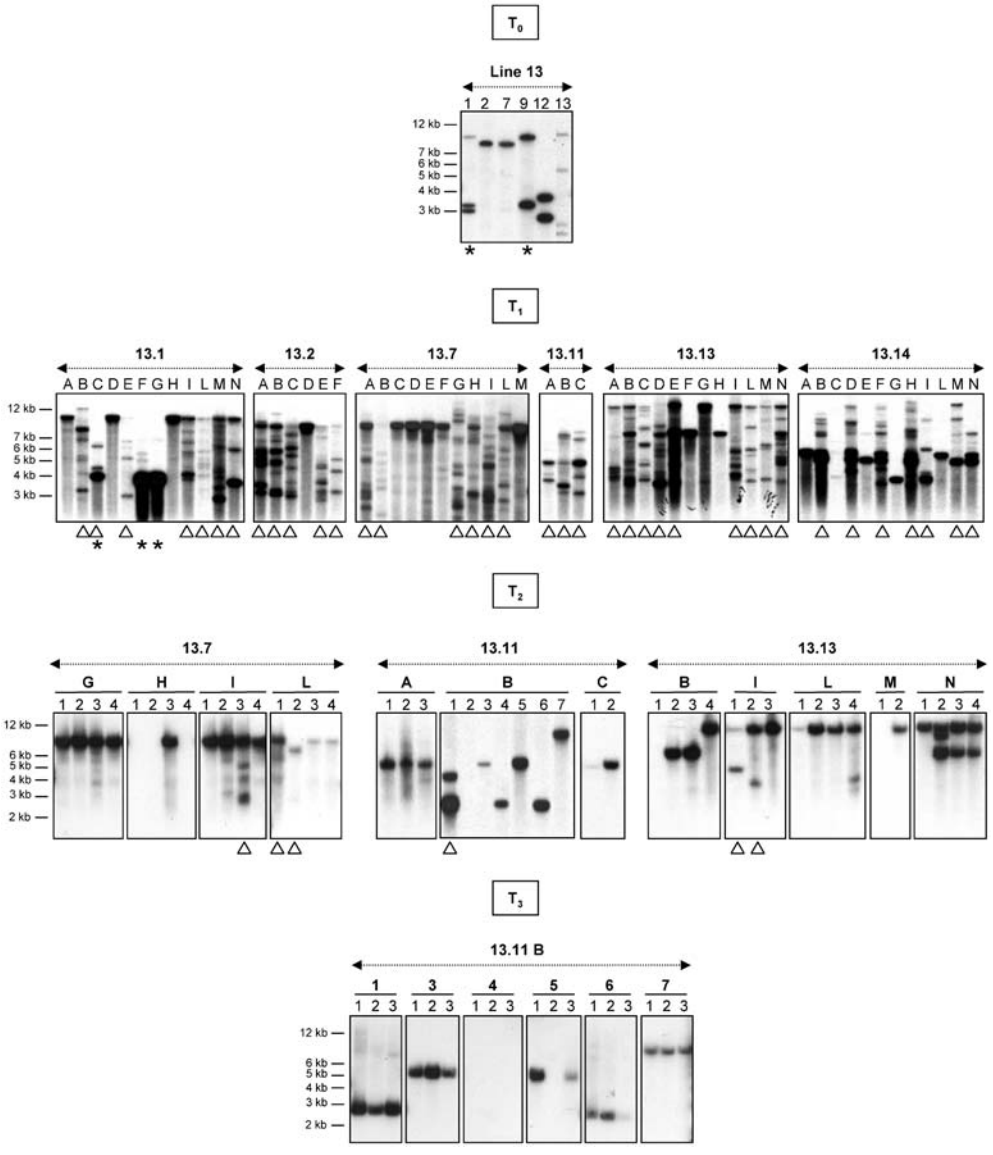

Fig. 4 *Ds* transposition behaviour in one ET2 line over four generations. Example of Southern analysis with a *gus* probe performed on T_0 regenerants and T_1 to T_3 progeny plants from one ET2-a line, containing three copies of the original T-DNA. Several hybridising fragments corresponding to a transposed *Ds* are revealed, with fainter bands indicating somatic transpositions. Stabilisation of independent insertions in different T_2 families is shown (i.e., 13.7, 13.11, 13.13). In case of later inactivation, independent stabilised insertions could be revealed within the same T_3 family (13.11 B). Plants still displaying FDS are present in T_0 and T_1 and are marked by an *asterisk*. An *open arrowhead* marks the plants with new transposition events included in the calculations of frequencies. Subsets of plants were selected in each generation for further propagation, according to transpositional activity and availability of greenhouse space, and representative samples are shown

insertions responsible for the staining was transmitted to the progeny.

Reverse genetics

The availability of the rice genome sequence promotes the development of reverse genetic strategies to find inserts in genes discovered by sequencing. To assess the suitability of the *Ac*/*Ds* transposon system to tag genes, genomic sequences flanking the *Ds* transposons (Insertion Tagged Sites, ITS) were isolated for three subsets of T_1 lines with active somatic transposition or with stable insertions, due to loss of the *Ac* transposase by genetic segregation. The pattern of *Ds* transposition analysed by Southern hybridisation of some of the selected plants can be visualised in Figs. 3 and 4. A summary of the significant homologies obtained after comparison of the flanking sequences obtained for known genes or proteins in public databases is shown in Table 3.

The ITSs were isolated using TAIL PCR and Adapter PCR (see Materials and methods). In general, TAIL PCR proved to be efficient in the case of plants with a single- or low-copy number of the transposon, as for the set of stabilised T_1 plants. When multiple *Ds* insertions containing plants were employed, techniques involving adapter-mediated PCR resulted in a higher percentage for recovery of good-quality sequences (data not shown).

A first subset of 17 active T_1 plants, derived from one original T_0 ET2-a line (line 13; Fig. 4), was analysed by Adapter PCR. In total 67 unique ITSs were obtained, accounting for an average number of 3.9 *Ds*-inserts per plant. A similarity search against public databases revealed that 46% of the flanking sites (31/67) showed significant nucleotide homology to rice genomic sequences spread over eight chromosomes. Among them, the percentage of insertions in transcribed regions

Table 3 Summary of Blast analyses of *Ds* flanking sequences (ITS) from T_1 ET2 plants

Item	T_1 lines		
	ET2-a	ET2-b	ET2-b
Total number of plants[a]	17 (1)	8 (4)	14 (9)
Transpositional status	Active	Active	Stable
Total number of ITS isolated	67	32	18
Average number of inserts / plant	3.9	4	1.3
ITS with homology to rice sequences[b]	31	14	12
Total number of rice chromosomes with ITS	8	5	4
ITS with homology to transcribed rice sequences[c]	16 (9)	10 (9)	7 (6)
Frequency of ITS in transcribed regions[d]	24% (52%)	31% (71%)	39% (58%)

[a] Within brackets the number of original T_0 lines
[b] The homologies reported refer to Blast searches against the "nt" or "nr" databases at NCBI. On the other hand, all the ITS gave homology to rice contigs when blasted against the rice database at the Beijing Genomics Institute
[c] The numbers refer to insertions in ORF and 5′ regulatory sequence, up to 0.5 kb upstream the ATG. Within brackets the number of ITS in ORF
[d] The percentage is calculated on the number of total ITS or on the number of ITS with homology to rice sequences (in brackets)

Table 4 Genomic sequences flanking *Ds* insertions (ITS) in T_1 progeny plants of ET2-b lines

Ds	Plant	ITS	Accession	Position	Chr.	Annotation	Upstream/Downstream[a]
Active	102-A2	A01	AAK55456	(blastx)		Putative reverse transcriptase	
		D05	AP002745	132,900	1	Put. endo-beta-1,4-glucanase	
		H06	AP002522	40,628	1	Putative polyprotein	
	102-A4	E02	AL732376	6,904	12	Intergenic	Ups: Trans. factor OSMYB2
		G08	AC082645	119,500	3	Intergenic	
		H07	AP004364	54,690	1	Hypothetical protein	
		H11	AP003247	73,898	1	Hypothetical protein	
	288-A2	H02	AL662987	87,077	4	Put. DNA helicase (Sen1-like)	
	373-A11	A02	AC103891	40,698	3	Intergenic	Ups: Putative RING zinc finger protein / Down: Putative protein kinase
	373-A12	A08	AP005178	133,618	7	Putative protein kinase Xa21	
		B12	BAB61155	(blastx)		Putative DnaJ protein	
		C08	AAK54295	(blastx)		Putative reverse transcriptase	
	374-D6	A04	AL928749	2,138	12	Intergenic	Ups: Hypothetical protein
		H03	AAK27805	(blastx)		Putative reverse transcriptase	
Stable	102-A8	C04	AP003793	99,943	1	Putative H+-pyrophosphatase	
	209-C3	10_21	AP003754	40,874	7	Put. glucuronosyl transferase	
	274-A6	C01	AL606648	132,513	4	UDP-galactase-4-epimerase	
	274-B2	A02	AL606694	97,261	4	Intergenic	Down: Hypothetical protein
	274-C7	D01	AP003052	108,460	1	Putative phosphoenolpyruvate carboxylase	
		E08	AP003413	19,641	1	Intergenic	
	348-B1	B09	Q9FWA4	(blastx)	3	Probable glycosyltransferase	
	348-C4	7_18	AP002521	8,562	1	Arginyl-tRNA synthetase	
	374-C2	8_19	AL606455	49,301	4	Intergenic	
	374-D7	8_17			Repeat		
	387-C2	10_19			Repeat		
		13_22	BAB87820	(blastx)		P450 [Triticum aestivum]	

[a] In case of *Ds* insertions in intergenic regions, the nearest genes predicted within a distance of 5 kb upstream (ups) or downstream (down) are reported

amounted to 52% (16/31) or 24% of the overall flanking sites isolated (16/67).

A second set of eight active T_1 plants (from four T_0 ET2-b lines) was analysed by TAIL PCR. Of 32 total ITSs isolated, ten represented insertions in codogenic regions out of 14, showing significant similarity to rice genomic sequences over six chromosomes (listed in Table 4), indicating that 31% of the total *Ds* insertions identified were in transcribed genes. The number of *Ds* inserts in these plants was also averaging four.

The last set of 14 stable T_1 plants (from nine original ET2-b lines) analysed by TAIL PCR yielded 18 total flanking-sites, with a decrease in the average number of *Ds* inserts per plant to 1.3. After comparison to public databases, about 39% (7/18) of the insertions could be assigned to transcribed genes. A list of the ITSs with

Fig. 5A–E GUS staining pattern in three T_1 enhancer trap lines with construct ET2. GUS assays were performed on flowers of T_1 progeny plants originating from line 13 (Fig. 4). GUS expression in line 13.4L was restricted to the rachilla of developing flowers (**A**). Anthers and pollen-specific expression was detected in lines 13.9D (**B**, **C**) and 13.14A (**D**), respectively. Some flowers in line 13.9 D showed also lodicules and carpel-specific staining (**E**)

significant homology to rice sequences (12/18) is presented in Table 4.

Discussion

In an effort to build a transposon population for functional genomics studies in rice, more than 1,000 transgenic lines containing *Ac*/*Ds* enhancer trap constructs were produced and analysed for activity. As problems in the continuous mobility of the *Ac*/*Ds* transposons in rice have been reported earlier, we assessed the behaviour of *Ds* over several generations to understand the different-parameters involved. The potential use of the lines generated for gene-tagging purposes was therefore evaluated by testing the transposition ability through three successive generations, the frequency of GUS staining patterns obtained and the nature of the insertion tagged sites generated.

Ds transposition behaviour

Excision occurred in about 80% of the intact T_0 lines analysed (data not shown), varying in time from very early at callus level or later during plant regeneration. Eighty percent of the excised *Ds* underwent re-integration at different genomic locations, accounting for about 62% of the T_0 plants displaying complete transposition. Early transpositions directly after transformation are likely attributable to the high levels of transposase induced by the CaMV 35S promoter, driving the immobilised *Ac*. This phenomenon was initially reported in Arabidopsis (Scofield et al. 1992; Swinburne et al. 1992) and later described in rice (Izawa et al. 1997; Chin et al. 1999), in case of the *Ds* excision promoted by either CaMV 35S-*Ac* transposase expressed from the cDNA or from the genomic clone. However, a higher frequency of early transpositions often resulted in a lower amount of independent insertions in Arabidopsis (Scofield et al. 1992; Swinburne et al. 1992). In our population, a high frequency of transmission of the T_0 parental *Ds* insertions (germinal) in the T_1 generation was observed, in agreement with previous reports (Chin et al. 1999). In some lines, the presence of the same parental band in plants belonging to the same T_1 family confirmed the occurrence of early transpositions in the To generation. Nevertheless, the percentage of plants within those T_1 families having also independent transpositions was high, suggesting the occurrence of additional secondary transpositions in the germ cells of the T_0 regenerants. Moreover, active transposition was still detectable in T_1, as indicated by the presence of numerous somatic insertions.

In spite of the high mobility of *Ds* observed in the T_1 plants, only about 30% of the T_2 families analysed included siblings showing new transpositions, which are being multiplied further. Considering the original T_1 families, about 40% underwent complete inactivation in the T_2. Surprisingly, most of the inactive T_2 plants stably inherited the original insertions present in the T_0 parent. Inhibition of transposition in later generations seemed to be associated with inactivation of the *bar* selectable marker. When applying PPT selection on T_2 and T_3 seeds, the recovery of resistant plants was much lower than expected from the normal genetic segregation ratios (Enckevort and Pereira, unpublished results). In agreement with previous reports (Schwartz and Dennis 1986; Wang et al. 1996), this would suggest the occurrence of methylation of the *Ds* as a cause for inhibition of transposition, though in this case methylation seems to spread over the whole transposon instead of being restricted to the terminal regions.

On the other hand, inactive T_2 and T_3 plants could be considered as a source of stabilised insertions to be used directly in reverse genetic experiments. The pattern of *Ds* transposition in the T_2 generation clearly indicated stabilisation of parental insertions in about 70% of the families analysed, with plants belonging to the same T_2 family having often the same *Ds* insert and different T_2 families having different *Ds* inserts. Given the prevalent inheritance of T_0 insertions, the probability to obtain T_2 progeny plants with different *Ds* inserts is then correlated to the amount of different T_0 primary regenerants, and therefore T_1 families that were propagated. In order to obtain a collection with the maximum number of independent stabilised insertions, an ample number of

lines in earlier generations (T_0 and T_1) are being propagated. T_2 and T_3 progenies will then be utilised as a source of stabilised lines.

Previous studies in maize demonstrated that silent *Ac* transposons could be re-activated following tissue culture (Peschke et al. 1987; Brettell and Dennis 1991). Accordingly, re-mobilisation of inactive *Ds* in rice was achieved in plants regenerated from cultured rice seeds containing inactive *Ds* and active *Ac* transposase (Izawa et al. 1997; Ki et al. 2002). Re-mobilisation of the *Ds* transposon appeared to be stage-specific, with a maximum of activity at the time of plantlet regeneration, and linked with demethylation of its terminal regions (Ki et al. 2002). At the same time, an increased level of *Ac* transposase-transcript was shown to occur in the regeneration stage with higher transpositional activity, suggesting a link between transposase activity and methylation of the *Ds*. The addition of a regeneration step via tissue culture as a way to reactivate transposon activity in a heterologous system proved to be successful also with *Ac*/*Ds* in *Brassica oleracea* (Mckenzie et al. 2002), and could then represent a useful strategy to obtain a high level of transposition for efficient insertional mutagenesis in rice.

Interestingly, the autonomous *Ac* element was shown to transpose efficiently in rice even in later generations (Izawa et al. 1997; Enoki et al. 1999; Greco et al. 2001b). However, once inactivated, the process appeared to be irreversible, in contrast with previous reports from maize (Brettell and Dennis 1991). This could suggest a different sensitivity (response) of the *Ac*/*Ds* transposon system to silencing (methylation) and subsequent inhibition of activity, when the *Ac* transposase is expressed under the control of its own promoter. If this is true, perhaps a transposase source consisting of an immobilised *Ac* retaining its own promoter, next to a strong enhancer, would provide an adequate level of transposition and be less prone to inactivation. Induction of early excision and transposition by a strong enhancer adjacent to the endogenous *Ac* promoter was previously demonstrated for a two-component *Ac*/*Ds* system in Arabidopsis (Balcells and Coupland 1994). Similarly, the CaMV 35S enhancer next to an autonomous *Ac* element also influences its transposition behaviour in rice (Greco et al. 2001b). Constructs based on this principle are currently under preparation.

Among the active lines produced, about 10% displayed amplification of *Ds* copy number. This phenomenon was initially described for *Ac* transposition in maize (Greenblatt 1984) and is due to transposition during replication, possibly associated with high *Ac* transposition activity (Peterson and Yoder 1995). Later, proliferation of *Ac* was shown to occur also in heterologous hosts, like tomato (Yoder 1990) and rice (Greco et al. 2001b). Moreover, amplification of *Ds* copy number was reported after ratoon-culture in rice (Chin et al. 1999). Here, restriction of *Ds* proliferation to T_0 and T_1 actively transposing plants could be also related to high levels of transposition promoted by the CaMV 35S promoter driving the *Ac* transposase. The propagation of such lines with multiple independent *Ds* insertions will help in reducing the number of lines needed to saturate the genome.

Some of the enhancer trap lines developed (construct ET1) bear *lox* sequences for site-specific recombination (reviewed in Ow and Medberry 1995; Ow 1996) in the *Ds* transposon and the T-DNA. Combining insertional mutagenesis with site-specific recombination provides additional advantages for mutant identification and gene cloning (Van Haaren and Ow 1993). The general principle is that, once transposition has occurred, site-specific recombination can induce chromosomal rearrangements between transposed *Ds* and the T-DNA. Due to prevalent *Ac*/*Ds* transposition to linked loci, this will mainly result in the occurrence of intra-chromosomal deletions or inversions. Mapping the donor T-DNA in the lines used will then provide a way to induce rearrangements at specific locations. Such an approach has been applied in Arabidopsis, tobacco and tomato, using *Ac*/*Ds* and the Cre-*lox* (Medberry et al. 1995; Osborne et al. 1995; Stuurman et al. 1996) or the R-*RS* system (Machida et al. 1995). Using the latter system, Nakagawa et al. (2001) described the recovery of genomic deletions in rice. In case of early transposition with amplification of *Ds* copy number in a single cell, as was observed for *Ac* (Greco et al. 2001b), the multiple transposed *Ds* at cis-linked loci will provide a substrate for recombination with the possibility to create sequential deletions. At the same time, the presence of sequences for site-specific recombination could allow Cre recombinase-mediated delivery of foreign DNA in the rice genome, encompassing the limitations due to the transformation process (reviewed in Ow 2002).

Enhancer trapping efficiency

Gene detection systems in rice have been previously described, which made use of T-DNA (Jeon et al. 2000) or transposons. In the latter case, *Ds* elements modified to serve as gene or enhancer traps have been employed (Chin et al. 1999; Upadhyaya et al. 2002) and the reported frequencies of GUS staining patterns identified were comparable to Arabidopsis (Sundaresan et al. 1995). In our system, the frequency of lines showing GUS expression was surprisingly low. Perhaps too few independent insertions were represented in the subset of T_1 plants analysed, while the high rate of somatic transpositions could have hampered the detection, especially in the case of staining restricted to small sectors of the plant. All the three GUS-positive patterns identified failed to be inherited, strongly suggesting the occurrence of *Ds* insertions in sectors of the inflorescences used for screening, which were not transmitted to the next generation. Indeed, several flowers from the same plant were stained, but GUS expression was identified only in some of them. Alternatively, the presence of a *lox* site between the minimal promoter and the *gus* gene might also be reducing the expression of the GUS marker. Improved

protocols to increase the sensitivity of staining detection are currently being tested on T_2 stable lines.

Gene insertional specificity

A pilot sequencing experiment of Insertion Tagged Sites (ITSs) was performed on three subsets of T_1 lines, active or stabilised by genetic segregation of the transposase.

Plants with active transposition showed a higher number of *Ds* insertions in comparison to stabilised plants, in accordance with the high somatic activity revealed in the former by Southern analysis. As previously discussed, most of those inserts will likely not be transmitted to the next generation. Nevertheless, the different ITSs obtained clearly indicate that many independent insertions can be potentially recovered after stabilisation, even from plants originating from a single T_0 line, as in the case of line 13.

The percentage of insertions in transcribed regions of the rice genome is about 30% of all the ITSs, but rises to more than 50% on considering only the ITSs that give homology to rice sequences, indicating that more than half of the insertions are actually in genes. A compilation of the tagged genes shown in Table 4 points out that more inserts are in genes either upstream or downstream, demonstrating again the insertional specificity. This preference for *Ds* insertion in transcribed genes confirm previous reports about the behaviour of *Ac* (Enoki et al. 1999; Greco et al. 2001b), and implies that a lower number of lines will be needed to saturate the genome for insertions.

Furthermore, as the lines contain enhancer trap constructs, they are supposed to reveal also neighbouring genes that reside within 5 kb upstream or downstream of the insertion, the range at which strong enhancers in the genome can work. Examination of the genes flanking the insertion tagged sites (Table 4) reveal a number of interesting genes like transcription factors, whose expression patterns can be monitored using a reverse genetics approach.

Based on those results, extensive sequences of flanking sites is now being carried out on stabilized T_2 lines that underwent inactivation of transposition.

Selectable markers

The effectiveness of a high-throughput transposon tagging system for gene function identification relies on the choice of efficient selectable markers. A critical step is the selection of lines carrying independent stable insertions. Double assays based on the use of a counter-selectable marker linked to the transposase source and a positive selection marker inside the mobile transposon were devised and successfully employed in Arabidopsis, to select for independent unlinked stable insertions (Fedoroff and Smith 1993; Sundaresan et al. 1995). The use of *bar* and *su1* genes as positive and negative selection markers turned out to be particularly efficient for greenhouse-based screenings in Arabidopsis (Tissier et al. 1999; Marsch et al. 2002). To date, only the *bar* gene was proven to work efficiently in rice as a transposon marker (Chin et al. 1999; Greco et al. 2001b), while the effectiveness of *su1* is still controversial. The functionality of *su1* in monocot species was previously reported by Koprek et al. (1999), which demonstrated its potential applicability in barley for large-scale greenhouse screenings. In rice, Chin et al. (1999) described the use of this marker in their original T-DNA, but did not report about its efficiency. Preliminary tests performed on seedlings of some of our rice enhancer trap lines grown in the greenhouse and sprayed with the R7402 pro-herbicide, did not result in an effective selection. However, further testing using different experimental conditions and larger samples are required for a more proper evaluation.

On the other hand, *sgfp* seems to offer a promising alternative (Upadhyaya et al. 2002; our unpublished results). Although its suitability as an excision marker could be debatable (Greco et al. 2001b; this work), the possibility of easy detection at the seed level as a segregation marker could allow effective visual selection for independent insertions. Transposon constructs using the *sgfp* gene as a negative selection marker are currently being tested. Alternatively, hygromycin painting on greenhouse leaves could represent another effective way to screen for transposase presence and segregation (Cotsaftis et al. 2002). Effective selection for plants with stable *Ds* insertions could be achieved using a leaf painting-assay for Basta and hygromycin separately on greenhouse seedlings. The Basta resistant/hygromycin sensitive plants can then be selected as stable lines.

Conclusions

International public efforts to build a rice gene machine require a concerted effort from a number of different labs, providing genotypes and information available to the public. The use of a number of different systems like T-DNA, retrotransposons and cut-and-paste type of transposons, such as the *Ac*/*Ds* system, will ensure that biases due to insertional specificity will be avoided.

The important consistent observation derived from this study is that the *Ds* transposons, like the autonomous *Ac* (Enoki et al. 1999; Geco et al. 2001b), insert at a higher frequency in gene-rich regions and tag genes. This suggests that a much smaller population of inserts would be required to be able to tag most of the genes in the genome. In addition, our choice of using multiple transposon lines allows us to be able to generate a large population of transposon inserts in a smaller number of plants. Following the identification of an insert in a gene of interest, segregation analysis has normally to be carried out, and in this effort other inserts can be segregated out.

The results reported here help in the development of more efficient *Ac*/*Ds* transposon populations using dif-

ferent markers and insertion types. Parallel efforts are thus being made to produce a rice activation tagging population. In this case, the *Ds* mobile transposon is modified to carry strong enhancer sequences that could cause dominant mutant phenotypes by mis-expressing adjacent genes. This system has been successfully employed for gene function discovery in Arabidopsis using T-DNA (i.e., Weigel et al. 2000) and recently described in rice (Jeong et al. 2002). However, its effectiveness in terms of mutant phenotype recovery in Arabidopsis was demonstrated to be even higher when transposons were used as vectors (Wilson et al. 1996; Marsch et al. 2002), offering a powerful strategy in rice to unravel novel gene functions that would be otherwise not possible due to gene redundancy.

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