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Characterization of enhancer trap and gene trap harboring *Ac*/*Ds* transposon in transgenic rice*

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Abstract: Insertion mutagenesis has become one of the most popular methods for gene functions analysis. Here we report a two-element *Ac*/*Ds* transposon system containing enhancer trap and gene trap for gene tagging in rice. The excision of *Ds* element was examined by PCR amplification. The excision frequency of *Ds* element varied from 0% to 40% among 20 F_2 populations derived from 11 different *Ds* parents. Southern blot analysis revealed that more than 70% of excised *Ds* elements reinserted into rice genome and above 70% of the reinserted *Ds* elements were located at different positions of the chromosome in rice. The result of histochemical GUS analysis indicated that 28% of enhancer trap and 22% of gene trap tagging plants displayed GUS activity in leaves, roots, flowers or seeds. The GUS positive lines will be useful for identifying gene function in rice.

Key words: *Ac*/*Ds*, Enhancer trap, Excision, Gene trap, Reinsertion, Rice (*Oryza sativa* L.)
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INTRODUCTION

After the whole rice genome was sequenced successfully, more and more genes can be predicted with different softwares and annotation system. However, the functions of predicted genes have to be further identified by biological analysis. One of the most powerful methods assigning function to gene is through insertion mutagenesis with transposable element as DNA sequence tag. *Ac*/*Ds* transposon system has become a very popular tool for gene tagging and functional genomics in various plant species (Altmann *et al.*, 1995; Chin *et al.*, 1999; Enoki *et al.*, 1999; Greco *et al.*, 2001; Izawa *et al.*, 1997; Koprek *et al.*, 2000, Long *et al.*, 1993a).

Ac/*Ds* transposon system has been modified into one-element system or two-element system having the great advantage of controlling the copy number of *Ac* and *Ds*. *Ac* Transposase gene can be segregated away from non-autonomous element (*Ds*) in an F_2 population derived from the cross between the parents containing immobilized *Ac* transposase and non-autonomous element (*Ds*), respectively. Thereby stabilized *Ds* insertion mutants can be obtained. Furthermore, the revertants by *Ds* element excised from insertion site can be regenerated by induction of *Ac* element again. In rice, both one-element and two-element transposon system had been used for gene tagging (Enoki *et al.*,

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1999; Izawa *et al.*, 1997; Nakagawa *et al.*, 2000).

Enhancer trap and gene trap elements harboring a reporter gene can respond to cis-acting transcriptional signals at the insertion site. The GUS gene as a reporter gene had been extensively applied in gene expression patterns in many organisms because of its easy detection by histochemical staining and the tolerance of N-terminal translational fusion in its enzyme activity (Campisi *et al.*, 1999; Chin *et al.*, 1999; Jeon *et al.*, 2000; Sundaresan *et al.*, 1995). In addition, a mutation in a pleiotropic gene that is required at multiple stages of development is likely to be lethal. Gene trap mutation can reveal the gene expression pattern at multiple developmental stages in viable heterozygote (Springer *et al.*, 1995). Functionally redundant genes whose disruption does not produce a significant phenotype can also be identified by their expression patterns. This character favors its extensive application in functional genomics of model plants.

We constructed a two-element *Ac*/*Ds* transposon system harboring gene trap and enhancer trap for gene tagging in rice. For easy selection of lines containing transposon, a *bar* gene cassette for resistance to phosphinothricin (PPT) was inserted into *Ds* element. Analysis of transposition frequency and pattern were performed using a PCR-based strategy in 20 F_2 populations, GUS activity was examined in *Ds* reinsertion plants. Our results indicated that the *Ac*/*Ds* transposon would be a useful tool for gene tagging and that the *DsG* (gene trap) or *DsE* (enhancer trap) tagged lines will be useful for detection of gene function in rice.

MATERIALS AND METHODS

Construction of vectors

Standard cloning procedures were used throughout. The vectors used in this research were derived from the binary vector pCAMBIA 1300 (GenBank accession number AF234296). The procedure for constructing the plasmid of pDsE and pDsG (Fig.1a and 1b) is described below: pWS31 (Sundaresan *et al.*, 1995) was digested with *Hin*dIII and *Xba*I, and blunted with T4 DNA polymerase. The 2.5 kb fragment carrying a *bar* gene cassette was inserted into blunted *Hin*dIII/*Xba*I site to give pBAR31. pCAMBIA 1300 was digested with *Sac*I, treated with CIAP (Calf Itestinal Alkaline Phosphatase). The 6 kb *Ds* element fragment from pBAR31 was inserted into *Sac*I site of pCAMBIA 1300 to give pDsE. The same procedures were used for construction of pDsG. For pAc construction (Fig.1c), NOS termini digested from plasmid pAHC25 (Christensen and Quail, 1996) was inserted into *Eco*RI and

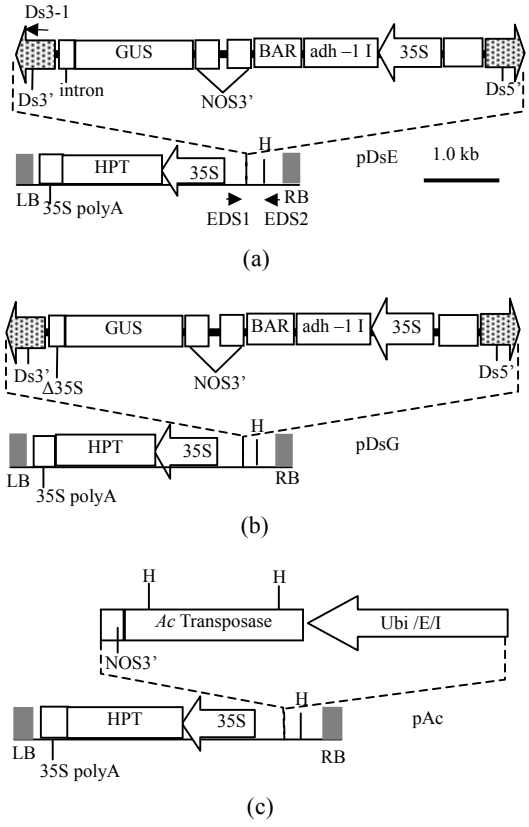

Fig.1 Schematic representation of the T-DNA containing the *Ds* element harboring gene trap (*DsG*) (a) and enhancer trap (*DsE*) (b), and *Ac*TPase (c), as well as selectable markers associated with each.

All three vectors were derived from pCAMBIA1300. Abbreviations: LB, T-DNA left border; RB, T-DNA right border; HPT, hygromycin phosphortransferase; 35S, CaMV35S promoter; NOS3', 3'-end signal of nopaline synthase; GUS, *β*-glucuronidase; 35S polyA, CaMV35S polyA signal; *Ds*5', 5' termini of dissociation from maize; *Ds*3', 3' termini of dissociation from maize; Δ35S, CaMV 35S mini promoter; intron, intron of gene for G-protein *α*-subunit from *Arabidopsis*; UBI/E/I, maize ubi-1 promoter/Exon/Intron; *Ac* transposase, transposase of activator from maize. EDS1, EDS2 and Ds3-1 are primers used in PCR analysis. One kb size bar is included

*Sac*I site of pCAMBIA 1300 to give plasmid p1300NOS. *Ac* transposase element was cloned between *Sac*I and *Bam*HI to give plasmid pNOSTS. pNOSTS was digested with *Bam*HI, blunted with T4 polymerase and treated with CIAP to remove phosphate from the 5' end. Then the blunted UBI pro- moter was cloned into upstream of *Ac* transposase gene to give the last plasmid pAc. The binary vec- tors were introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood *et al*., 1993) for rice transformation.

Rice transformation

Immature embryos from developing seeds of a *joponica* rice variety Zhonghua-11 were excised aseptically after sterilization with 70% ethanol for 2 minutes and 1.5% NaClO for 1.5 hours. They were cultured on 2N6 medium at 25 ºC for 4 days. Then the shoots were dissected and the calli were cultured on a new 2N6 medium for another 3 days for co-cultivation with *Agrobacterium* harboring binary vector of interest. The transformation and regeneration procedures were carried out as described previously (Hiei *et al.,* 1994).

Development of a tagging F_2 population

Transgenic rice plants harboring immobilized *Ac* element were crossed to plants carrying low copy *Ds* element (*DsE* and *DsG*) to get F_1. The immobilized *Ac* element carried a *promoter* UBI:: *Ac transposase* fusion for triggering *Ds* excision. The *Ac* element lacked 5' and 3' termini, and so cannot transpose but it provides transposase. The *Ds* element contained a *β*-glucuronidase (GUS) reporter gene and a *bar* gene for resistance to phosphinothricin (PPT). The expression of GUS reporter gene can be examined by histochemical staining. The enhancer trap element *DsE* had a CaMV35S minimal promoter upstream of GUS reporter gene, which can express when inserted near cis-acting chromosomal enhancer. Gene trap *DsG* had a multiple splice acceptors sequence fused to the promoterless but intact GUS gene, which would express when inserted into a transcribed region.

F_1 plants were selfed to get F_2. Two-week old seedlings of F_2 population were sprayed with 3 mg/L PPT solution. One week later, the normal growing plants were analysed for excision, reinsertion events and GUS activity.

Genomic DNA preparation

Genomic DNA was extracted from each of the PPT^R plants for PCR analysis. One leaf of each young seedling was collected into separate microfuge tube and ground into powder in liquid nitrogen with dispensable grinder. Then 500 μl extraction buffer (100 mmol/L Tris-HCl pH 8.0, 20 mmol/L EDTA, 500 mmol/L NaCl, 1.5% SDS) was added into each tube. The mixture was vortexed for 5 sec and then incubated in 65 ºC water bath for 20 min. Iso-volume 500 μl cholorform:ethanol:pantenol (20:4:1) was added into the mixture and vortexed for 10 sec, then spun 5 min at the 12 000 rpm. The 500 μl supernant was transferred to a new tube and nucleic acids were precipitated by adding 1 ml precooled ethanol. After spinning at 12 000 rpm for 5 min, pellets were dissolved in 100 μl H_2O containing 0.1 mg/ml RNaseA for PCR analysis.

PCR analysis

For detecting empty donor site (EDS), primers EDS1 (5'-atgcttccggctcgtatgttgtgt-3') next to 3' end of *Ds* element and EDS2 (5'-aagcgtaaggtatatttggaagta-3') adjacent to 5' end of *Ds* element were designed. It was expected to amplify approximately 520 bp EDS fragment in PCR reaction after the *Ds* element excised from the donor site (Fig.1). For full donor site (FDS) fragment amplification, primers EDS1 and Ds3-1 were used. The resulting fragment was approximately 290 bp. Three primers EDS1, EDS2 and Ds3-1 were put together in a reaction tube to perform PCR amplification. The reaction was performed in 30 μl reaction volume (ca. 50 ng genomic DNA, 200 μmol/L dNTP, 1 μmol/L EDS1, 0.5 μmol/L EDS2 and Ds3-1a, 1.5 mmol/L MCl_2, 50 mmol/L KCl, 0.1% Triton X-100, 10 mmol/L Tris-HCl, pH 9.0, 1 unit Taq DNA polymerase) at cycling condition: 94 ºC for 4 min; 94 ºC for 20 sec, 56 ºC 30 sec, 72 ºC for 1 min for 35 cycles; 72 ºC for 5 min.

DNA gel-blot analysis

Genomic DNA was isolated from mature

leaves. DNA (5 μg) was digested with *Hin*dIII, fractionated on a 0.7% agarose gel, blotted onto a nylon membrane (Hybond-N+, Amersham) and hybridized with a ^{32}P labeled probe.

To determine the copy number of *Ac*-T-DNA and *Ds*-T-DNA in parents, blotted DNA was probed by radiolabelled *hpt* fragment. Because there is no *Hin*dIII site in the sequence of *hpt* gene (Fig.1), the bands number probed with *hpt* fragment represents the copy number of *Ac*-T-DNA or *Ds*-T-DNA in transgenic rice.

hpt fragment was used as probe to detect EDS after *Ds* excision from donor site through Southern hybridization. As described above, there is only one *Hin*dIII site outside of *Ds* element. The full donor site indicated by *hpt* probe must be larger than 8.5 kb in *Hin*dIII digested blot. After the 6 kb *Ds* element excised from the donor site, the residual EDS must be 6 kb less than FDS. The distance between FDS and EDS was calculated with software Gel-Pro ANALYZER (version 4.0.00.001).

To detect transposition events in F_2 plants, the *Hin*dIII digested DNA was probed by *bar* fragment that was specific to *Ds* element. Excision and re-insertion of *Ds* element into a new location will create a different size *Hin*dIII fragment.

The *bar* and *hpt* probes used here were PCR products from amplification with primers described above. The PCR products were purified with glass milk. Labeling of probes was performed by standard procedure.

Gus staining analysis

The samples were collected in 1.5 ml Eppendorf tubes containing 1 ml GUS staining solution (100 mmol/L NaH_2PO_4 buffer pH 7.0, 0.5% Triton X-100, 0.5 mg/ml X-Gluc and 20% methanol). After a brief period of vacuum infiltration, the samples were incubated at 37 ºC up to 24 hours. After staining, tissues were fixed in FAA solution. The samples were examined under a dissection microscope.

RESULTS

Development of F_2 populations

A two-element transposon system for gene tagging in rice was developed in this study. Three constructions (pAc, pDsE and pDsG) were introduced into rice by *Agrobacterium*-mediated gene transfer. The transgenic rice plants harboring immobilized *Ac* element were crossed to plants carrying one of the two different *Ds* elements (*DsE* or *DsG*). Before crossing, Southern blot and genetic analysis of hygromcin resistance (for *Ac* plant) or PPT resistance (for *Ds* plant) were conducted to determinate the T-DNA insertion number and loci in *Ac* and *Ds* parent plants. The four transgenic plants carrying single copy *Ac* element were used as male parent for crossing. The *Ac*-transposase expression was identified with RT-PCR in all four lines (data not shown). The selfed progenies of 11 *Ds* parents appeared 3:1 segregation of PPT resistance, except CE14. However, multiple copies of *bar* gene or *hpt* gene were observed in CE70, CE72, CE78 and CE80 (Table 1). The regenerated T_0 plant or selfed T_1 plants were used for crossing experiment. F_2 seeds were collected independently from 20 F_1 plants carrying both *Ds* element and *Ac* transposase. These F_1 plants obtained from 11 combinations are shown in Table 2.

Table 1 Genetic analysis of *Ac/Ds* starter lines

Parents line	HYG^R plants	HYG^S plants	Segregation ratio	χ^2	Copy number[a]	
					HPT	BAR
Ac line						
CAc24	18	10	3:1	1.17	1	No
CAc46	31	10	3:1	0.13	1	No
CAc48	28	8	3:1	0.13	1	No
CAc52	25	9	3:1	0.13	1	No
Ds line						
CE14	37	3	15:1	0.11	3	4
CE37	34	10	3:1	0.13	1	1
CE59	19	7	3:1	0.04	1	1
CE68	26	7	3:1	0.26	1	1
CE70	30	7	3:1	0.71	1	3
CE72	22	9	3:1	0.26	2	2
CE78	33	10	3:1	0.07	2	2
CE80	32	15	3:1	1.25	1	2
CG38	31	6	3:1	1.46	1	1
CG32	36	17	3:1	1.78	-	-

[a]The copy number was determined in the T_0 generation by Southern blot analysis with *bar* or *hpt* gene fragment as probe

Table 2 Frequency of *Ds* excision and reinsertion in F_2 plants

F_1 plant	*Ds*×*Ac*	PPT^R F_2 plants	F_2 plants showing			Ac-	Excision (%)	Reinsertion (%)	Independent reinsertion (%)	Stable reinsertion (%)
			EDS	Reinsertion	Independent					
C03-02	CE70×CAc46	60	16	12	10	4	27	75	83	33
C03-13	CE70×CAc46	60	18	13	10	4	30	72	77	31
C03-14	CE70×CAc46	100	34	28	21	7	33	82	75	25
C05-05	CE76×CAc46	35	2	-	-	-	6	-	-	-
C05-06	CE76×CAc46	35	0	-	-	-	0	-	0	-
C06-14	CE37×CAc46	35	2	-	-	-	6	-	-	-
C06-22	CE37×CAc46	35	0	-	-	-	0	-	-	-
C11-02	CE14×CAc46	35	5	-	-	-	14	-	-	-
C11-09	CE14×CAc46	35	6	-	-	-	19	-	-	-
C19-01	CE68×CAc46	39	13	10	8	2	33	77	80	20
C13-04	CE78×CAc48	35	0	-	-	-	29	-	-	-
C13-09	CE78×CAc48	35	9	-	-	-	25	-	-	-
C15-01	CE80×CAc48	35	10	7	6	1	29	70	86	14
C15-43	CE80×CAc48	100	40	30	22	8	40	73	73	27
C14-03	CE42×CAc52	35	8	-	-	-	23	-	-	-
C18-05	CE72×CAc52	35	0	-	-	-	0	-	-	-
C18-06	CE72×CAc52	35	1	-	-	-	3	-	-	-
C20-03	CG38×CAc52	84	18	15	12	4	22	83	80	27
C20-05	CG38×CAc52	60	14	12	10	3	23	86	83	25
C26-01	CG32×CAc24	39	9	-	-	-	23	-	-	-

Ac-: *Ds* reinsertion plants lacking *Ac* element;
-: not determined;
Excision (%): the number of F_2 plants showing EDS/the number of F_2 PPT^r plants analysed;
Reinsertion (%): the number of F_2 plants showing reinsertion/the number of F_2 plants showing EDS;
Independent reinsertion (%): the number of F_2 plants showing independent reinsertion/the number of F_2 plants showing reinsertion;
Stable reinsertion (%): the number of reinsertion plants showing Ac-/the number of F_2 plants showing reinsertion

Excision events of *Ds* element in F_2 populations

PCR was carried out using genomic DNA taken from mature leaves for detecting empty donor site (EDS) in all 20 F_1 plants. As expected, no EDS fragment was obtained.

PCR analysis for *Ds* excision was carried out in F_2 plants to provide PPT resistance. Striking increase in *Ds* excision frequency was found. As designing, four kinds of amplification patterns (EDS^+FDS^-, EDS^+FDS^+, EDS^-FDS^+, EDS^-FDS^-) were observed in F_2 plants (Fig.2). The pattern EDS^-FDS^- (C19-01.002, C19-01.005 and C19-01.010) represented the plants containing reinserted *Ds* only (providing PPT resistance) but no EDS and FDS loci. EDS^+FDS^- (C19-01.001, C19-01.009 and C19-01.011) indicated plants carried an empty donor site and reinserted *Ds* element. EDS^-FDS^+ indicated the plants carried only full donor site or both full donor site and transposed *Ds* element. EDS^+FDS^+ (C19-01.004) indicated the existence of both empty donor site and full donor site in a plant at the same time. *Ds* reinsertion occurred in EDS^+FDS^- and EDS^-FDS^- plants.

To prove that the 520 bp EDS fragment obtained from PCR amplification presented the result of excision of the *Ds* element from the donor site, ten different EDS fragments from three independent *Ds* parents were recycled and sequenced. The results indicated the EDS bands were produced from the excision of *Ds* element in all ten cases. Base

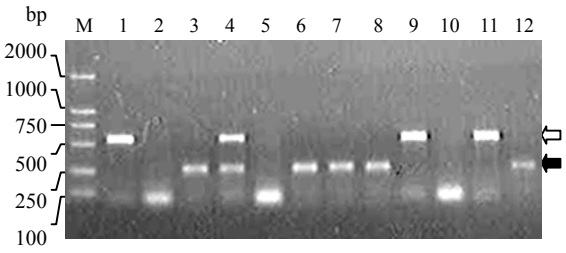

Fig.2 PCR analysis of EDS in F_2 PPT^R plants. (The number 1 to 12 represents C19-01.001 to C19-01.012 respectively. ⇨: EDS; ➡: FDS. No amplification band in plants C19-01.002, 005 and 010 means reinsertion *Ds* element unlinked to donor site. EDS only in C19-01.001, 009 and 011 indicate that plants contain a reinsertion *Ds* element providing PPT resistance. FDS and EDS existing in C19-01.004 and the plants containing FDS only are not the plants with high possibility containing a reinsertion *Ds* element)

substitutions and deletions appeared in EDS sequences (Fig.3) as reported previously in maize, *Arabidopsis*, tobacco and rice (Izawa *et al.*, 1997, Rinehart *et al.*, 1997, Suzuki *et al.*, 2001).

To confirm the EDS and FDS indicated by PCR amplification in F_2 plants, Southern hybridization was carried out. Consistent with PCR analysis results, plants C15-43.186, 182 and 062 with EDS only in PCR amplification (data not shown) showed EDS band only in Southern hybridization. Otherwise plant C15-43.172 with EDS and FDS in PCR amplification, gave two bands in 8800 bp and 2800 bp size (Fig.4), corresponding to FDS and EDS, respectively.

Because PCR amplification could reflect the *Ds* excision in F_2 generation, excision events (including somatic excision and germinal excision) of 20 independent F_2 populations derived from 11 different *Ds* parents were detected by PCR analysis. The results are listed in Table 2. The excision frequency varied from 0 to 40% among different crossing combinations. *Ds* excision frequency varied widely among cross combinations with different *Ds* parents. For example, *Ds* excision frequency of combination CE76×CAc46 (C05-05 and C05-06) was 6% and 0% respectively. In contrast, *Ds* excision frequency of combination CE80×CAc48 (C15-1 and C15-43) was as high as 29% and 40% respectively. Different F_2 populations from the same cross combination exhibited similar excision frequency. For example, the 3 different F_2 populations (C03-02, C03-13, C03-14) from the same cross combination CE70×CAc46 exhibited excision frequency of 27%, 30% and 33% respectively. However, in two F_2 populations with the same pollen origin (CAc46) and two different *Ds* parents (CE37 and CE76), smaller excision freque-

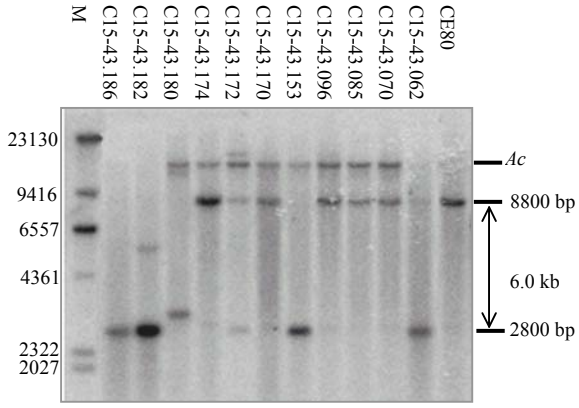

Fig.4 Southern blot analysis of *Ds* excision events in F_2 plants. (Genomic DNAs from C15-43 F_2 plants were digested by *Hin*dIII and probed with radiolabelled *hpt* fragment. M, lambda *Hin*dIII marker; *Ac*, *Ac* Transponsase element; signal at 8800 bp and 2800 bp indicate the presence of FDS and EDS respectively. EDS existed in plants C15-43.186, 182, 153 and 062. Both EDS and FDS band existed in C15-43.172. The band length of FDS to EDS calculated with software Gel-Pro ANALYZER (version 4.0.00.001))

Ds element

GGAATTCGAGCTC GGTCACGCAACGC donor site
GGAATTCGAGCTC **C**GTCACGCAACGC C03-14.272, C03-14.047, C03-14.003, C11-02.003
GGAATTCGAGCT**G A**GTCACGCAACGC C03-14.267, C15-43.153, C15-43.186
GGAATTCGAGCT— —GTCACGCAACGC C03-14.024, C03-14.058, C15-43.182, C19-01.001

Fig.3 Sequence analysis of empty donor sites (EDS). PCR products of EDSs amplified with EDS1 and EDS2 were sequenced. Base changes induced by *Ds* element excision are shown. Deletion bases are indicated in dash lines, and substitution bases in boldfaces

ncy was detected (0% and 6%). The results accorded with the earlier report in *Arabidopsis* (Smith *et al.*, 1996). The copy number of *Ds* element did not affect the transposition frequency (Table 2).

Based on the result of Southern blot analysis, high frequency of germinal excision was detected in the excision events. Germinal excision must leave EDS only in donor site after *Ds* excision. For example, among the 6 different plants with EDS band, only C15-43.172 displayed the FDS band at the same time (Fig.4). This means that germinal excision occurred in at least 5 out of 6 plants.

Reinsertion of *Ds* element in F_2 generation

To examine reinsertion of *Ds* elements into F_2 plants, Southern hybridization using radiolabeled *bar* gene fragment as probe was conducted. Eight F_2 populations originated from 4 *Ds* parents (CE70, CE68, CE80 and CG38) were investigated. Fig.5 shows the representative results, in which the *Ds* band patterns were different from those of *Ds* parents. Different band pattern indicated that *Ds* element was transposed to a novel locus in the rice chromosome.

By comparing the band pattern from Southern hybridization, independent reinsertion of *Ds* element in F_2 progenies was calculated. The result is listed in Table 2. In 8 examined F_2 populations, the independent frequency reinsertions were all above 70%. The result was not the same as previously report in *Arabidopsis* that most of the reinsertion transposants from the same F_2 population showed the same hybridization pattern (Sundaresan *et al.*, 1995).

The excision *Ds* element did not always reinsert into plant genome DNA. The PCR amplification of EDS fragment provided us the information on *Ds* excision events in F_2 progenies. Southern hybridization gave us the *Ds* reinsertion information. The frequency of *Ds* reinsertion was calculated as the percentage of the number of F_2 plants with reinsertion of excision *Ds* element out of the number of F_2 plants with *Ds* excision. The *Ds* reinsertion frequency is listed in Table 2. The reinsertion frequency was all above 70% in 8 examined independent F_2 populations. Most of the reinsertion plants contained *Ac* element, which may induce *Ds* retransposing in F_3 families. The stable reinsertion frequency (*Ac* lacking reinsertion plants) varied from 14% to 33% in the 8 examined F_2 populations (Table 2). This ratio accorded with the Mendelian rule for single *Ac* element segregation.

GUS expression mediated by enhancer trap and gene trap

The reporter gene GUS would express when gene trap and enhancer trap insert into gene region of rice chromosome. To evaluate the efficiency of gene trap and enhancer trap via GUS activity, roots, leaves flowers and immature seeds from *Ds* reinserted plants were collected for GUS staining analysis. Based on Southern blot analysis, F_2 plants with *Ds* reinsertion were selected for GUS staining

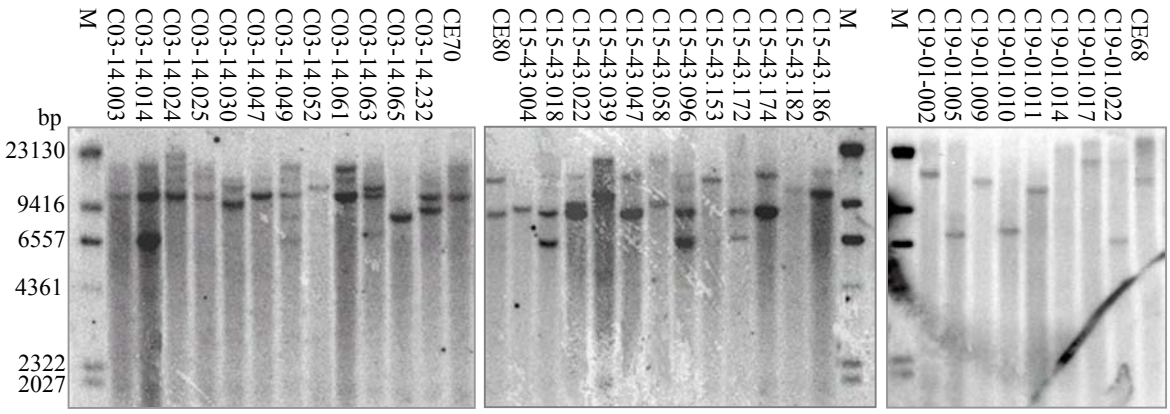

Fig.5 Southern blot analysis of reinsertion pattern in F_2 plants derived from C03-14, C15-43 and C19-01. DNA of F_2 plants was digested with *Hin*dIII and blots were probed with radiolabelled *bar* fragment. CE70, CE80 and CE68 are *Ds* parents. The hybridization bands exhibit different patterns in different lane among F_2 plants

analysis. Among 100 F_2 plants containing reinsertion *DsE* element from 6 different F_1 families (Table 2), 28 plants (28%) displayed GUS expression. Among 27 F_2 plants containing reinsertion *DsG* elements from 2 independent F_1 (C20-3 and C20-5) families, 6 (22%) displayed the GUS expression. Fig.6 shows the examples of organs or tissues expressing GUS activity of the F_2 plants containing *DsE* or *DsG* element. Different GUS staining patterns were obtained both in *DsE* or *DsG* lines. The mutant phenotypes in the organs where the GUS gene was activated will be studied in the F_3 generation.

DISCUSSION

In the present study, a two-element *Ac*/*Ds* transpo son system harboring gene trap and enhancer trap was constructed for creating *Ds* insertion mutants in rice. Because rice was insensitive to kanamycin, a *bar* gene cassette resistant to herbcide phosphinothricin was inserted into *Ds* element instead of *npt*II which was used effectively in arabidopsis (Sundaresan *et al*., 1995).

Transposition frequency of *Ds* element varied widely in different system and different organisms. It had been reported that the transposition frequency ranged from 50%–60% in *Arabidopsis* (Bancroft and Dean, 1993; Ito *et al*., 1999), 0%–90% in tobacco (Suzuki *et al*., 2001), and 0%–47% in barley (Koprek *et al*., 2000). Nakagawa *et al*.(2000) reported that the *Ds* transposition frequency ranged from 0% to 51% in rice. In our case, the transposition frequency of *Ds* element varied from 0% to 40% in different F_2 populations. Large difference in the transposition frequency of *Ds* element was found in the F_2 populations derived from different *Ds* parents crossed with the same *Ac* parent. This result indicated that genome sequence around *Ds* element would affect the transposition frequency in rice.

It had been reported that both the same reinsertion site and different reinsertion sites could be

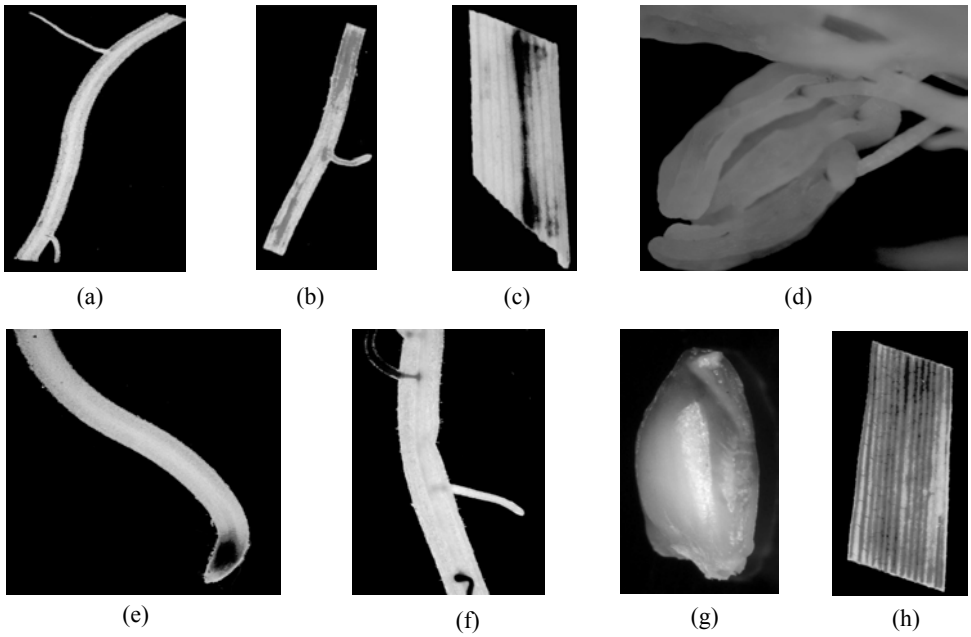

Fig.6 GUS activity analysis of F_2 plants containing *DsE* or *DsG* element. Gene trap plant: (a) line C20-05.090 exhibited GUS activity at the base region of lateral roots; (b) line C20-05.112 exhibited strong GUS activity in whole roots; (c) line C20-05.194 exhibited strong GUS activity in main vein of leaf; (d) line C20-05.311 exhibited GUS staining at junctions between filament and anther; Enhancer trap lines: (e) line C03-13.015 exhibited strong GUS activity in root tips; (f) line C03-14.174 exhibited strong GUS activity in lateral roots; (g) line C03-14.272 exhibited GUS activity in seed capsule; (h) line C15-43.058 exhibited strong GUS activity in leaf

observed in F_2 plants derived from the same F_1 line in *Arabidopsis*, barley and rice (Bancroft and Dean, 1993; Koprek *et al.*, 2000; Nakagawa *et al.*, 2000). High ratio of F_2 plants from the same F_1 family carrying the same reinsertion patterns was regarded as resulting from transposition occurring during the earlier development stage, which had been shown to be associated with high levels of transposase expression driven by relatively strong promoters, such as maize UBI and CaMV 35S (Balcells and Coupland, 1994; Long *et al.*, 1993b). However, in the present case, relatively high frequencies (above 70%) of *Ds* reinsertion in different sites were obtained in all 8 F_2 populations (Table 2) with *Ac* transposase driven by the relatively strong promoter maize UBI. In our experiment, no excision events were detected in all 20 F_1 plants based on PCR analysis with DNA template from leaf tissues before flowering. The results suggested that the *Ds* transposition events should happen at later development stage in F_1 plants, which might lead to higher frequency of *Ds* reinsertion at different sites in F_2 plants.

Gene trap and enhancer trap had been proposed to be powerful tools for gene identification, especially for functionally redundant genes and genes necessary for development in multiple stages (Springer *et al.*, 1995). GUS staining plants are very useful for cloning gene and analyzing expression pattern in growth and in different developmental stages. In our results, 22% gene trap and 28% enhancer trap plants showed different GUS expression patterns. A variety of GUS staining patterns were observed from the *DsE* and *DsG* tagged lines. The GUS staining result also indicated that *Ds* element reinserted in different position of chromosome in rice. *DsE* or *DsG* tagged lines that exhibited a particular GUS staining pattern should facilitate identification of genes that are regulated spatially and temporally for plant development. The rice genome sequence flanking *Ds* element is being isolated in GUS positive lines.

Based on information from this research, the strategy using *Ac/Ds* transposon system can be used for creating large number of independent *Ds* insertion mutants. If one F_1 seed produces 1000 F_2 seeds, the independent stable *Ds* insertion plants could be calculated as: 1000×1/4(theoretical ratio of plants without donor site in an F_2 population)×30%(transposition frequency)×70%(independent reinsertion frequency)×1/4(number of plants without *Ac* in one F_2 population) = 12. In order to get much more insertion F_2 plants without considering whether *Ds* linked to donor site and *Ac* element or not, EDS^-FDS^- or EDS^+FDS^- F_2 plants resistant to PPT identified by PCR amplification should be selected and preserved independently. Furthermore, through GUS activity analysis of *DsE* and *DsG* tagged plants, many genes can be identified by GUS expression patterns.

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