ORIGINAL PAPER

Y. Ito · M. Eiguchi · N. Kurata

Establishment of an enhancer trap system with *Ds* and GUS for functional genomics in rice

Abstract To develop an efficient means of enhancer trapping, a two-element system employing *Ds* and an *Ac* transposase (AcTPase) gene was tested in rice. We generated 263 transgenic rice plants, each of which harboured the maize transposable element *Ds* together with a GUS coding sequence under the control of a minimal promoter (*Ds-GUS*), and a gene that confers resistance to the herbicide chlorsulfuron. Among the 263 lines generated, 42 were shown to have a single copy of the *Ds-GUS* element. Four single-copy lines were crossed with each of six transgenic plants that carried the AcTPase gene. Excision of the *Ds-GUS* in leaves of F_1 plants was detected in eight combinations out of seventeen examined. The frequency of transposition of *Ds-GUS* in germ cells in the F_1 plants was examined using 10,524 F_2 plants, and 675 (6%) were judged to be transposants. Their frequencies differed among F_1 plants depending on the AcTPase x *Ds-GUS* cross considered, and also among panicles on the same F_1 plant. This suggests that *Ds-GUS* tends to transpose during panicle development. Southern analysis with a GUS probe showed different band patterns among transposants derived from different panicles. Therefore, the transposants derived from different panicles must have arisen independently. Transposants showing tissue-specific GUS activities were obtained, and enhancers thus trapped by the *Ds-GUS* element were identified. These results demonstrate that the system is suitable for the isolation of large numbers of independent *Ds-GUS* transposants, and for the identification of various tissue-specific enhancers. The *Ds-GUS* lines generated in this study offer a potentially powerful tool for studies on the functional genomics of rice.

Keywords *Ac* / *Ds* · Gene tagging · Enhancer trap · Chlorsulfuron resistance · Rice

Communicated by M.-A. Grandbastien

Y. Ito · M. Eiguchi · N. Kurata (✉)
Plant Genetics Laboratory,
National Institute of Genetics,
1111 Yata, Mishima, Shizuoka-ken
411-8540, Japan
E-mail: nkurata@lab.nig.ac.jp
Tel.: +81-55-9816808
Fax: +81-55-9816879

N. Kurata
Department of Life Science,
Graduate University for Advanced Studies,
1111 Yata, Mishima,
Shizuoka-ken 411-8540, Japan

Introduction

Rice is one of the most important suppliers of nutrients for humans, and is a model plant with several advantages for molecular genetic studies (Shimamoto 1995; Izawa and Shimamoto 1996). It has a relatively small genome (430 Mb), and there are a large number of established markers useful for mapping and subsequent positional cloning of genes of interest. In spite of these advantages, however, rice still lacks an efficient technique for cloning genes responsible for mutant phenotypes. Though the number is growing, relatively few genes have been disabled and cloned using the endogenous retrotransposon *Tos17* (Hirochika 2001) and by positional cloning approaches (Song et al. 1995; Ashikari et al. 1999; Takahashi et al. 2001; Yamanouchi et al. 2002; Li et al. 2003; Sasaki et al. 2003). The *Tos17* system is the most useful tagging system to date. However, the efficiency of tagging of newly emerging mutations has been estimated to be around only 10% (H. Hirochika, personal communication). In *Arabidopsis*, the T-DNA of *Agrobacterium* and heterologous transposable elements are often used as insertional mutagens, and target genes have been successfully cloned (Bhatt et al. 1996; Azpiroz-Leehan and Feldmann 1997). Therefore, it is obvious that efficient gene-tagging systems are necessary to facilitate the cloning of mutant genes, and for studies on the functional genomics of rice. In this species, about 18,000 insertional lines have so far

been generated using a gene trap system based on T-DNA; about 7,000 of these have been examined, and 1.6–2.1% of tested organs showed reporter gene activity (Jeon et al. 2000).

The maize transposable element *Ac / Ds* is known to be active in many heterologous plant species, including rice (Enoki et al. 1999). In rice, it was first reported that autonomous *Ac* had transposed during shoot regeneration and leaf development (Izawa et al. 1991), and this discovery was followed by the detection of transposition of non-autonomous *Ds* in the presence of the *Ac* transposase gene (Shimamoto et al. 1993). *Ds* has been successfully used as an insertional mutagenic fragment, as well as a vehicle for enhancer trap systems, in *Arabidopsis* (Ito et al. 2002). An enhancer trap system is a modified version of a gene-tagging system. The enhancer trap vector contains the coding sequence of a reporter gene, such as a *β*-glucuronidase (GUS) gene, with a minimal promoter, in addition to a selectable marker gene. Insertion of such a reporter gene in or near an enhancer element of a gene may cause activation of the reporter gene by the enhancer and/or disruption of the inserted gene. The genes can be identified if disruption of the gene causes a phenotypic change, and can be cloned using the vector as a tag. Even if no phenotypic changes are observed, the gene can be identified by screening for tissue-specific expression of the reporter. Thus, this system can be utilized to identify organ-, tissue- or cell-type-specific enhancers or genes, as well as for conventional gene tagging.

In rice only limited attempts have been made to use such a system as a tool for gene tagging. There have been only a few reports which have shown transposition of autonomous *Ac*, or several types of modified *Ds*, using a small number of primary transformants (Izawa et al. 1997; Chin et al. 1999; Solis et al. 1999; Nakagawa et al. 2000). Chin et al. (1999) demonstrated efficient transposition of *Ds* and obtained several lines that displayed tissue-specific reporter gene activity using a gene trap vector. Because *Ac/Ds* tends to transpose preferentially, but not always, to sites close to its original position on the same chromosome (Smith et al. 1996; Machida et al. 1997; Nakagawa et al. 2000; Ito et al. 2002), one needs to use multiple *Ds* transgenic plants with independent insertions in various chromosomal positions. To apply *Ac / Ds* as a tool for functional analysis of the rice genome, it is also necessary to select a large number of transposants by an efficient method. Recently, a number of *Ds* transposants were obtained from many independent transformants, but neither the original positions of the *Ds* nor the patterns of transposition in plants were analysed (Upadhyaya et al. 2002).

In this study we introduced the enhancer trap vector *Ds-GUS* (Fedoroff and Smith 1993) into rice, and analysed the frequency and timing of transposition of the *Ds-GUS* element after crossing insertion lines with a line expressing the Ac transposase (AcTPase). To establish a set of independent insertion lines carrying the *Ds-GUS* at different positions distributed over most or all rice chromosomes, we chose 42 original transgenic

lines, each of which harboured a single copy of the *Ds-GUS*. Mapping of the original *Ds-GUS* sites (T-DNA integration sites) showed that ten rice chromosomes out of twelve harboured one or more *Ds-GUS* insertions at various positions. Crossing of these *Ds-GUS* lines with the AcTPase lines induced excision and transposition of the *Ds-GUS* in leaves and in germ line cells at high frequency, resulting in the identification of many independent transposants in the F$_2$. Furthermore, we showed that simple and efficient selection methods based on herbicide and antibiotic resistance can be utilized to screen for stable transposants of the *Ds-GUS*. We further identified several transposants that showed tissue-specific GUS activity, and we present examples of the molecular identification of the trapped enhancers. These results demonstrate that we have succeeded in establishing an efficient experimental system capable of generating a large number of *Ds-GUS* transposant lines and various tissue-specific enhancers. These lines should serve as useful resources for functional genomics in rice.

Materials and methods

Enhancer trap vectors

Plasmids bearing *Ds-GUS* and 35S-AcTPase T-DNAs were obtained from Dr. N. V. Fedoroff (Fedoroff and Smith 1993). The 35S-AcTPase T-DNA plasmid was modified to make it suitable for rice transformation and selection. First, the *tms2* gene driven by the CaMV 35S promoter (35S-tms2) was replaced with a bialaphos resistance gene driven by the same promoter. To do so, a 3′ portion of the AcTPase gene was first amplified by PCR using the primer pair Ac1 (5′-TGAA GCTTTGATATGCACAA-3′) and Ac3 (5′-CCTCTA GAGGTACCCTGCAGGTCGGTAACGGTCGGTA AA-3′). The amplified PCR fragment was digested with *Xba* I, and then ligated to the 35S-AcTPase T-DNA plasmid digested with the same enzyme. The resultant plasmid was digested with *Kpn* I, and then blunt-ended with T4 DNA polymerase, followed by digestion with *Pst* I. This DNA was ligated to the *Eco* RI (filled in with T4 DNA polymerase)-*Pst* I fragment of pARK8, which contains the bialaphos resistance gene driven by the CaMV 35S promoter (35S-bar). The resultant plasmid, named pBAc1, contains the 35S-bar in place of the 35S-tms2 in the original plasmid. Then the CaMV 35S promoter of pBAc1 was replaced by an ubiquitin promoter. For this purpose, pBAc1 was digested with *Pst* I, and then blunt-ended with T4 DNA polymerase, followed by digestion with *Bam* HI. This DNA was ligated to the *Hind* III (filled in with T4 DNA polymerase)-*Bam* HI fragment of pUBA, which contains the ubiquitin promoter. The resulting plasmid, named pBAc3, contains the bialaphos resistance gene driven by the ubiquitin promoter in place of the 35S-tms2 in the original plasmid (Fig. 1b).

Transformation of rice

Oryza sativa cv. Nipponbare was transformed by the method described by Hiei et al. (1994). When pBAc3 was used for transformation, bialaphos (5 mg/l) was used instead of hygromycin for the selection of transformants.

Southern analysis

DNAs were isolated from rice leaves with a cetyltrimethylammonium bromide (CTAB)-based method (Murray and Thompson 1980). DNAs were digested with restriction enzymes, and blotted onto a nylon membrane. Hybridization and detection were carried out using ECL direct labelling and detection systems (Amersham).

Mapping of insertions

Sequences flanking *Ds-GUS* T-DNA insertions were amplified by TAIL-PCR (Liu et al. 1995) or adaptor-ligated PCR, cloned, and sequenced using an ABI377 autosequencer (ABI). Two sets of primers specific for *Ds-GUS* T-DNA were used: the first set comprised ALS2 (5′-GTTGATCTCTTCATCATTCAATGG-3′) and its nested primers ALS3 (5′-AGCTTAACTAGTAAACTAAAGTAGTC-3′) and ALS4 (5′-AATATTACATGAATAATCCAAAACGAC-3′), while CGN3 (5′-GCTGCATTAATGAATCGGCCAAC-3′) and its nested primers CGN2 (5′-GGAGAGGCGGTTTGCGTATTG-3′) and CGN1 (5′-GAGACCTCAATTGCGAGCTTTC-3′) made up the other set. For adaptor-ligated PCR, genomic DNAs were digested with *Bcl* I, *Nsi* I, *Pst* I, *Sal* I or *Xho* I, and ligated to an adaptor which was generated by annealing the complementary oligonucleotides 5′-GTACATATTGTCGTTAGAACGCGTAATACGACTCACTATAGGGAN-3′ and 5′-NTCTCCCTATAGTGAGTCGTATTACGCGTTCTAACGACAATATGTAC-3′ (N indicates sequences that are cohesive to a corresponding cleaved restriction site when annealed). After ligation, PCR was carried out with the *Ds-GUS* specific primer ALS2 and the adaptor-specific primer CPC1 (5′-GTACATATTGTCGTTAGAACGCGTAATACGACTCA-3′), and then with the nested primers ALS4 and CPC2 (5′-CGTTAGAACGCGTAATACGACTCACTATAGGGAGA-3′). PCR products were cloned into pBluescript (Stratagene) and sequenced with the ABI377 autosequencer. A database search was carried out at the DDBJ (http://spiral.genes.nig.ac.jp/homology/welcome-e.shtml) and at the RGP (http://rgp.dna.affrc.go.jp/).

Detection of somatic excision and heritable transposition

DNAs were isolated from a leaves of F_1 plants by a simple CTAB-based method. A young leaf (∼2 cm long) was homogenized with a motor-driven pestle in 100 µl of CTAB buffer (100 mM TRIS-HCl pH 8.0, 20 mM EDTA, 3% CTAB, 1.4 M NaCl, 1% polyvinylpyrrolidone K 30). The homogenate was incubated at 60°C for 30 min after the addition of another 400 µl of the CTAB buffer. After incubation, 500 µl of chloroform was added to the homogenate and gently mixed for 30 min, followed by centrifugation at 15,000 rpm for 10 min at room temperature. The supernatant was transferred to a new tube, and DNAs were precipitated with an equal volume of isopropanol. DNAs were dissolved in 20 µl of 10T-0.1E (10 mM TRIS-HCl pH 7.6, 0.1 mM EDTA) supplemented with a small amount of RNase A. PCR was carried out for 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by a final 5-min incubation at 72°C. The reaction mixture contained 1×ExTaq buffer, 2.5 mM MgCl₂, 0.25 mM dNTPs, each primer at 0.2 µM, and 0.5 U of ExTaq (Takara Shuzo) in a volume of 20 µl. The following primers were used: 35S-F2 (5′-GTGGATTGATGTGATATCTC-3′), ALS1 (5′-CTAAAGCTTCGACGAGGATA-3′), GUS-F (5′-ACGCTCACACCGATACCATC-3′), GUS-R (5′-AACGCTGATCAATTCCACA-3′), NOS-R2 (5′-ATCGCAAGACCGGCAACAGG-3′), H-1 (5′-AACCAGTTGTTGATCTGCTT-3′) and H24-1 (5′-GCTCTTCTGAGGAGGACCAA-3′ (Ito et al. 1999).

Selection for chlorsulfuron resistance

F_3 seeds were surface sterilized with 1% sodium hypochlorite and rinsed several times with sterilized water. The seeds were then placed on an MS plate (MS basal salts and vitamins, 3% sucrose, 0.8% agar, pH 5.8) containing various concentrations of chlorsulfuron and 50 mg/l hygromycin, and incubated at 28°C under continuous light for 2 weeks. To screen for transposants, F_2 seeds were cultured on an MS plate containing 1 mg/l chlorsulfuron and 50 mg/l hygromycin for 2 weeks. Resistant seedlings were then transferred to soil.

Enhancer trap screening

F_3 seeds were surface sterilized with 1% sodium hypochlorite and rinsed several times with sterilized water. The sterilized seeds were imbibed in water overnight at 28°C, or placed on MS plates containing 50 mg/l hygromycin, and incubated at 28°C for 2 weeks. Hygromycin-resistant plants were transferred to soil and grown on a cycle of 14 h at 28°C in the light and 10 h at 25°C in the dark.

Samples were incubated in X-gluc solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide and 0.5% Triton X-100 in 50 mM phosphate buffer (pH 7.0) at 37°C overnight, fixed (in 5% formaldehyde, 5% acetic acid, 20% ethanol), and cleared in ethanol.

Cloning of enhancers

TAIL-PCR and adaptor-ligated PCR were carried out as described above, except that the following *Ds-GUS* specific primers were used: Ds1 (5′-GGTGAAACGG TCGGGAAACTAG-3′), Ds2 (5′-CTACCGTTTCCG TTTCCGTTTAC-3′) and Ds3 (5′-GTTTTGTATATC CCGTTTCCGTTC-3′). The PCR products were directly sequenced on an ABI3100 autosequencer. A database search was carried out at the DDBJ (http:// spiral.genes.nig.ac.jp/homology/welcome-e.shtml) and the RGP (http://cdna01.dna.affrc.go.jp/cDNA/ and http://rgp.dna.affrc.go.jp/). Insertion of the *Ds-GUS* was confirmed by PCR using the primer combination P1772-F1 (5′-GTAGGTAGTATCTGTATGCA-3′) and Ds3 for P1772, and the primer pair P2503-F1 (5′-TTAATC GCACTCGTGCAAAG-3′) and Ds3 for P2503 (see below).

Young leaves were cut into pieces 1 cm in length and floated on water for the indicated periods at 28°C in light. RNA isolation and RT-PCR were carried out as described previously (Ito et al. 2001) using the primer pairs P1772a-F2 (5′-GGAAGCCTATCGGAGTCTTC-3′) and P1772a-R2 (5′-AAAGAGCCTTGCACAACATG-3′) for AK073621, P2503-F3 (5′-GCAGTACACC-AAGGACTCTG-3′) and P2503-R3 (5′-CACA CCCGTCAGAAATCCTC-3′) for *OsLEA3*, and P2503-F4 (5′-CAAAATGTCCTCAGTAGTTG-3′) and P2503-R4 (5′-GGCAAGCTGAGACAGCATAT-3′) for AK062488 (see below).

Results

Generation of *Ds-GUS* lines and 35S-AcTPase lines of rice

We utilized as enhancer-trap vectors two plasmids developed by Fedoroff and Smith (1993) with some modifications (Fig. 1a, b). One is a vector containing *Ds* between a cauliflower mosaic virus (CaMV) 35S promoter and the coding region of a chlorsulfuron resistance gene, inserted in a T-DNA region (Fig. 1a). The *Ds* contains a GUS coding sequence under the control of a minimal version of the CaMV 35S promoter, and a hygromycin resistance gene. This *Ds* is called *Ds-GUS*. The other vector carries an Ac transposase (AcTPase) gene driven by the CaMV 35S promoter (35S-AcTPase) (Fig. 1b). This vector was modified from the original AcTPase vector (Fedoroff and Smith 1993) as follows. The 35S-tms2 cassette in the original vector was replaced by a gene that confers resistance to the herbicide biala-phos, which is controlled by a maize ubiquitin promoter, so that the 35S-AcTPase gene and the bialaphos resistance gene are now located in the T-DNA region. Thus, the hygromycin resistance and the bialaphos resistance can be used to check for the presence of the *Ds-GUS* and the AcTPase gene, respectively. Furthermore, the
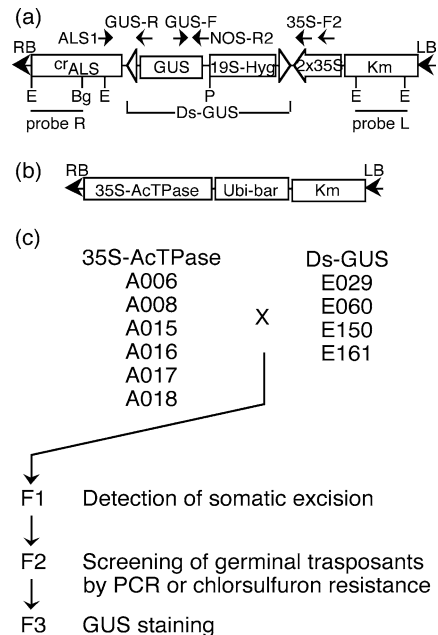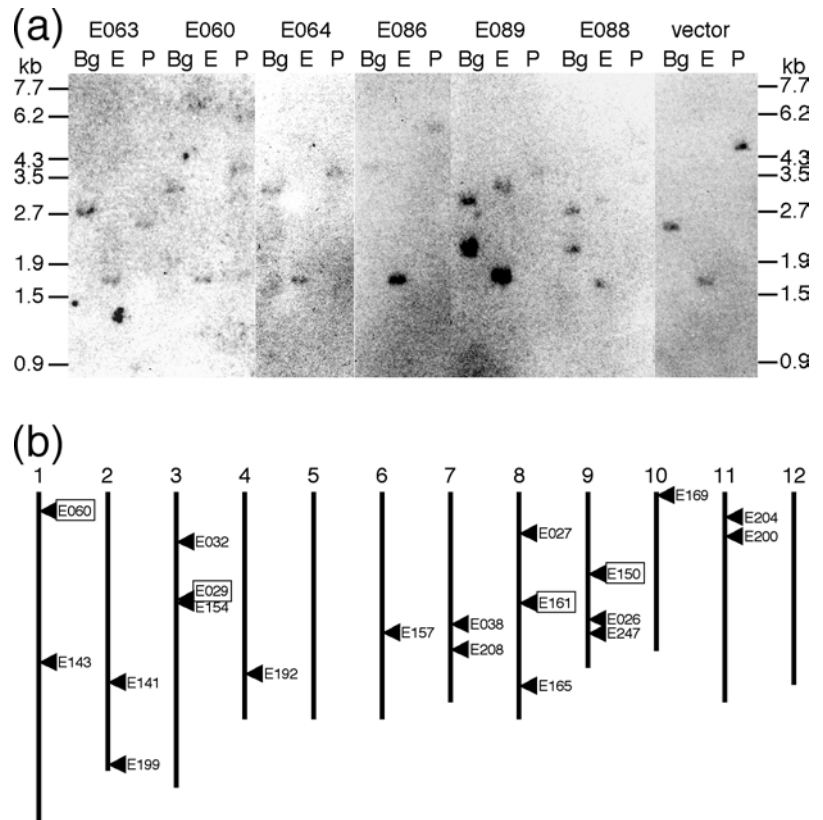

Fig. 1a–c Strategy used for enhancer trapping. **a** Structure of *Ds-GUS* T-DNA and positions of probes and primers. The *arrows* indicate positions and directions of primers. crALS, the coding sequence of the chlorsulfuron resistance gene; 19S-Hyg, the hygromycin resistance gene driven by the CaMV 19S promoter; 2×35S, two tandemly repeated copies of the CaMV 35S promoter; Km, the kanamycin resistance gene; RB, the right border of T-DNA; LB, the left border of T-DNA. Restriction enzyme sites: Bg, *Bgl* II; E, *Eco* RI; P, *Pst* I. **b** Structure of 35S-AcTPase T-DNA. 35S-AcTPase; the AcTPase gene driven by the CaMV 35S promoter; Ubi-bar, the bialaphos resistance gene driven by the ubiquitin promoter; Km, the kanamycin resistance gene; RB, the right border of T-DNA; LB, the left border of T-DNA. **c** Strategy used for enhancer trapping. Six of the 35S-AcTPase lines were crossed with each of the four *Ds-GUS* lines. F_1 plants were examined for somatic excision in leaves. F_2 seeds were subjected to screening for heritable transposition events by PCR, and for chlorsulfuron resistance to examine the frequency and timing of transposition in each F_1 plant. In the F_3 generation, transposants selected by PCR were subjected to screening for tissue-specific GUS expression to identify enhancer trapping events

chlorsulfuron resistance can serve to monitor excision of the *Ds-GUS* from its initial position in the vector.

The *Ds-GUS* T-DNA was introduced into rice by *Agrobacterium* -mediated transformation (Hiei et al. 1994). We obtained 263 transgenic rice plants, and the copy number of the integrated T-DNA was estimated by Southern analysis (Fig. 2a) using the probes shown in Fig. 1a. Since probes R and L detect a right end and a left end, respectively, of the T-DNA including the chlorsulfuron resistance gene, *Ds-GUS* lines that showed *Eco* RI fragments of the same sizes as those in the vector must contain the complete sequence of the *Ds-GUS* and the flanking chlorsulfuron resistance gene. The number of *Bgl* II or *Pst* I bands indicates the copy number of the *Ds-GUS* T-DNA. Forty-two lines were judged to contain a single copy of the *Ds-GUS* and the chlorsulfuron resistance gene necessary for its transposition and selection. Fifty lines were judged to contain two copies, at least one of which retained the complete region. The

other 171 lines contained more than three copies or no complete region.

We generated six lines of rice that contained 35S-AcTPase T-DNA. Southern analysis suggested that the numbers of integrated T-DNA copies ranged from one to six depending on the line considered (data not shown).

Both *Ds-GUS* lines and 35S-AcTPase lines were self-pollinated, and seeds were collected for further studies.

Mapping of the original *Ds-GUS* insertions on rice chromosomes

To determine map positions of the *Ds-GUS* inserts in the original lines, the sequences flanking the *Ds-GUS* T-DNA were amplified by TAIL-PCR or adaptor-ligated PCR, and sequenced. A database search was carried out using the amplified flanking sequences as queries. If the sequence matched a rice genome sequence that had already been mapped, this simultaneously localized the original *Ds-GUS* insertion. With this method we were able to map the *Ds-GUS* sequences in twenty lines. These sequences were distributed in various locations on ten of the twelve rice chromosomes (Fig. 2b).
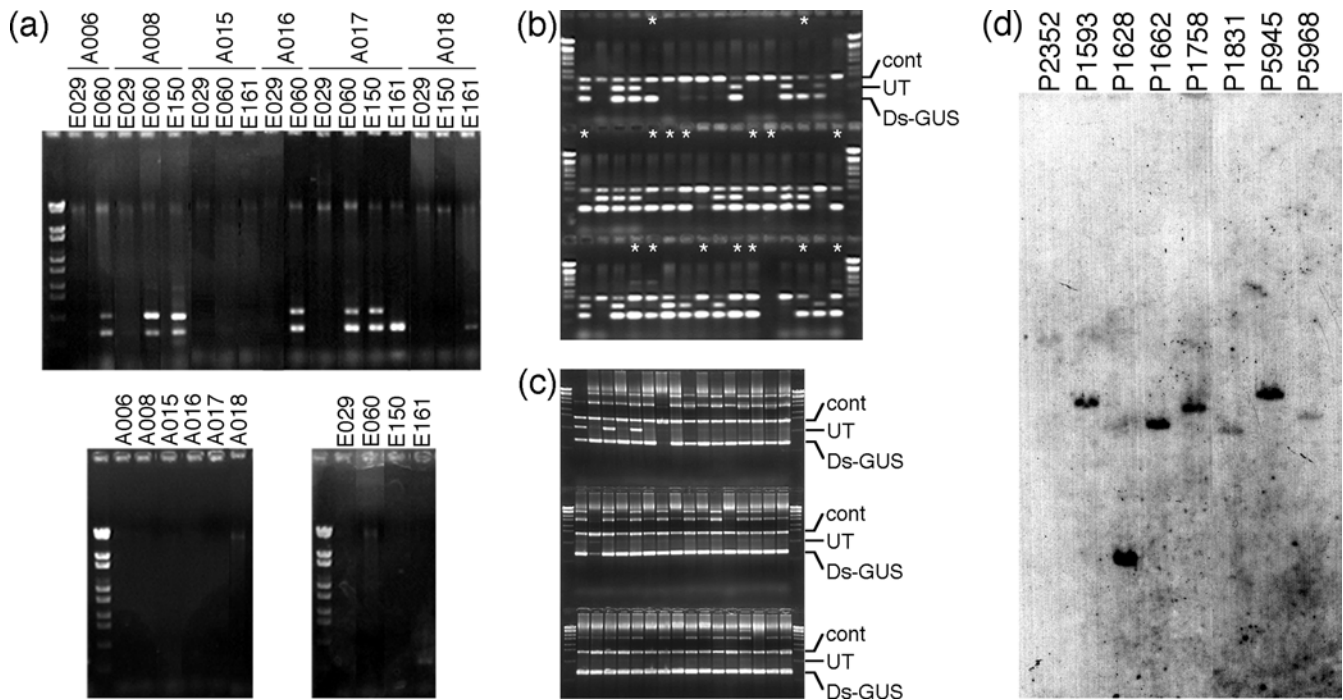
Somatic excision of the *Ds-GUS* in F$_1$ leaves

To examine how frequently the *Ds-GUS* element can transpose in rice, we chose four lines containing single copies of the *Ds-GUS* (E029, E060, E150 and E161) and crossed them with each of six 35S-AcTPase lines (A006, A008, A015, A016 A017 and A018) (Fig. 1c). F$_1$ seeds were obtained from 17 of these crosses, and the resulting plants were used for the detection of somatic excision by PCR. For this purpose, DNAs were isolated from a single leaf of each F$_1$ plant. Primers were designed to flank the original *Ds-GUS* insert (Fig. 1a); one (35S-F2) anneals in the CaMV 35S promoter and the other (ALS1) anneals in the coding sequence of the chlorsulfuron resistance gene. Because the CaMV 35S promoter is repeated in tandem, there are two annealing sites for the primer 35S-F2. If the *Ds-GUS* has been excised, 0.6-kb and 0.9-kb bands are amplified from an inner site and an outer site, respectively. If the *Ds-GUS* has remained in place the size of the amplified band is about 6 kb, or no band is amplified due to the fact that long-range PCR is inefficient. Out of the 17 combinations examined, eight showed the 0.6-kb and 0.9-kb bands, whereas none of the other nine combinations, nor any of the parental lines, showed such bands (Fig. 3a). The *Ds-GUS* line E029 showed no excision with any of the 35S-AcTPase lines, and the 35S-AcTPase line A015 failed to induce excision of the *Ds-GUS* in any of the combinations examined. Nevertheless, these results demonstrated that many combinations of the *Ds-GUS* and the 35S-AcTPase permit excision of the former in leaves.

Heritable transposition of the *Ds-GUS*

To examine the frequency and timing of germ-line transposition of the *Ds-GUS* in the F$_1$ plants, F$_2$ seeds

Fig. 3a–d Somatic excision and heritable transposition of the *Ds-GUS*. **a** Detection of excision of the *Ds-GUS* in leaves of F_1 plants by PCR. The *upper panel* shows somatic excision of the *Ds-GUS* in F_1 plants. In some F_1 plants 0.6-kb and 0.9-kb bands appear. The parental lines used for each cross are indicated *above* the lanes. The two *lower panels* show the results of PCR analysis of the parental lines. The *leftmost* lanes contain a size marker (lambda DNA digested with *Sty* I). **b** Screening for germline transposants by PCR. The *asterisks* indicate transposants that show a *Ds-GUS* band and a positive control band (cont) but no untransposed *Ds-GUS* band (UT). Lanes that show all three bands indicate that the plants have *Ds-GUS* at the original position, and these were classified as non-transposants. Presence of the control band only indicates a plant without the *Ds-GUS*. **c** Confirmation of transposants selected for chlorsulfuron resistance by subsequent PCR. Plants with a *Ds-GUS* band and a positive control band (cont) but no untransposed *Ds-GUS* band (UT) were confirmed to be transposants. Plants with three bands are either non-transposant or carry both a transposed *Ds-GUS* and an untransposed *Ds-GUS*. **d** Independent transposants derived from different panicles of the same F_1 plant. Genomic DNAs of transposants (P2352–P5968) obtained from different panicles of the same F_1 plant (A008×E060) were digested with *Sac* I, blotted onto nylon membrane and hybridized with a GUS probe

were collected separately from each panicle of the F_1 plants. Using leaf DNAs from each F_2 individual, transposants were selected by PCR with three combinations of primers (Fig. 1a). The first combination of primers (GUS-F and NOS-R2) amplifies an inner portion of the *Ds-GUS*, and tests for the presence of the *Ds-GUS*. The second combination (ALS1 and GUS-R) amplifies a junction region between the *Ds-GUS* and the coding sequence of the chlorsulfuron resistance gene, and detects the presence of untransposed *Ds-GUS*, but not transposed *Ds-GUS*. The third combination (H24-1 and H-1; Ito et al. 1999), which amplifies a part of *OSH1*, an endogenous rice gene (Matsuoka et al. 1993), was used as a positive control for the PCR. If *Ds-GUS* is detected but untransposed

Ds-GUS is not, the plant harbours only transposed *Ds-GUS* and it is scored as a transposant. A representative sample of the results of the PCR screen for transposants is shown in Fig. 3b and summarized in Table 1. Based on this screen, we identified 678 transposants (6%) among 12,123 F_2 plants derived from all 17 cross combinations. The 678 transposants were obtained from 143 panicles. The frequency of transposition differed greatly in the different F_1 plants, and differences in transposition frequency were even found among panicles from the same F_1 plant. Examples are shown in Table 2. Thus, in an F_1 plant derived from the cross between A008 and E060, 64% (16/25) of the seeds of panicle #19 were transposants, while no transposant was obtained from nine other panicles (Table 2). In an F_1 plant derived from the A017×E150 cross, the transposition frequency ranged from 0% in 13 panicles to 56% (5/9) in panicle #24 (Table 2). These results indicate that the *Ds-GUS* can transpose in the germ-line cells of many F_1 plants at a high frequency.

The differences in transposition frequency among panicles suggests that the *Ds-GUS* tends to transpose at certain stages during panicle development (see Discussion). This further suggests that transposants obtained from different panicles are independent. To check this, Southern analysis was carried out using DNAs isolated from the transposants derived from different panicles of the same F_1 plant. Most of the F_2 plants obtained from different F_1 panicles showed a unique band with a specific size when hybridized with a GUS probe (Fig. 3d), while most of the F_2 plants from the same panicle showed same sized bands (data not shown). These results indicated that transposants obtained from different panicles arise independently.

Table 1 Frequency of heritable transposition

Ds-GUS line	PCR screen				Chlorsulfuron and hygromycin screens			
	TP[a]	Panicle[b]	Total number examined[c]	%	TP[a]	Panicle[b]	Total number examined[c]	%
E029	2	2	1690	0.1	0	0	1406	0
E060	327	57	2831	12	447	145	1992	22
E150	296	59	2006	15	1755	213	5512	32
E161	56	25	5833	1	0	0	2116	0
total	681	143	12360	6	2202	358	11,026	20

[a]Number of transposants
[b]Total number of panicles from which transposants were obtained
[c]Total number of F2 plants derived from several different F1 plants generated by crossing the indicated *Ds-GUS* line with each of the 35S-AcTPase lines

Chlorsulfuron resistance and screening of the transposants

The *Ds-GUS* in the vector prevents expression of the chlorsulfuron resistance gene because it is inserted between the CaMV 35S promoter and the coding sequence of the resistance marker (Fig. 1a), and it is expected that the excision of the *Ds-GUS* from its original position should activate the chlorsulfuron resistance gene and confer the resistance phenotype upon transposants. To confirm this, F_3 seeds derived from self-pollinated transposants were germinated on an MS plate containing various concentrations of chlorsulfuron. As a positive control, we used transgenic rice plants which harbour the chlorsulfuron resistance gene driven by the CaMV 35S promoter (35S-ALS). As a negative control non-transformed wild-type plants were used.

Transposants derived from E060 and E150 showed strong resistance to chlorsulfuron (Fig. 4). They grew on plates containing 1 mg/l chlorsulfuron. However, transposants derived from E161 were as sensitive as the untransposed parental lines or non-transformed plants (Fig. 4). These results revealed that the transposants can show resistance to concentrations of chlorsulfuron that are more than 100 times that tolerated by the untrans-

posed parental lines, but the level of chlorsulfuron resistance observed in the transposants greatly depends on the parental line from which they are derived.

In order to screen many transposants for chlorsulfuron resistance, F_2 seeds were germinated and grown on an MS plate containing 1 mg/l chlorsulfuron and 50 mg/l hygromycin, and resistant seedlings were selected. Out of 11,026 F_2 seeds examined, 2202 (20%) showed resistance to both chlorsulfuron and hygromycin (Table 1). These resistant plants were obtained from 358 panicles. The frequency of transposants observed in the chlorsulfuron screen was higher than that found by PCR screening, because we preferentially used F_2 seeds derived from the F_1 plants which produced transposants at high frequency, as judged by PCR, such as A008×E060 and A008×E150. In this screen, the proportion of the transposants differed from plant to plant and from panicle to panicle, as in the PCR-based screen. In agreement with the above results, no transposants were obtained from E161 (Table 1). PCR analysis using primers ALS1 and 35S-F2, which amplify an empty donor site, showed that the resistant plants contained the chlorsulfuron resistance gene (data not shown), and PCR analysis with the three combinations of the primers used for PCR screening confirmed that the resistant

Table 2 Number of transposants identified in each panicle

Cross		Panicle number																									
35S-AcTPase line	Ds-GUS line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
A008	E060																										
Total[a]		35	21	25	20	29	18	10	34	22	23	45	28	11	18	38	23	12	25	25	12	17	37	15	14	20	21
TP[b]		0	11	0	10	0	5	2	18	2	0	0	0	6	7	0	10	5	15	16	2	7	23	0	1	1	0
%[c]		0	52	0	50	0	28	20	53	9	0	0	0	55	39	0	43	42	60	64	17	41	62	0	7	5	0
A017	E150																										
Total[a]		16	9	9	29	39	17	25	43	21	17	34	32	34	39	15	41	23	39	12	43	20	21	5	9	4	17
TP[b]		0	0	0	7	2	8	3	16	11	1	3	1	6	13	1	0	0	0	0	0	0	0	0	5	0	0
%[c]		0	0	0	24	5	47	12	37	52	6	9	3	18	33	7	0	0	0	0	0	0	0	0	56	0	0

[a]Number examined of F2 plants derived from each panicle of the F1 plant generated by the indicated cross
[b]Number of transposants in each panicle
[c]Percentage of total transposants in each panicle
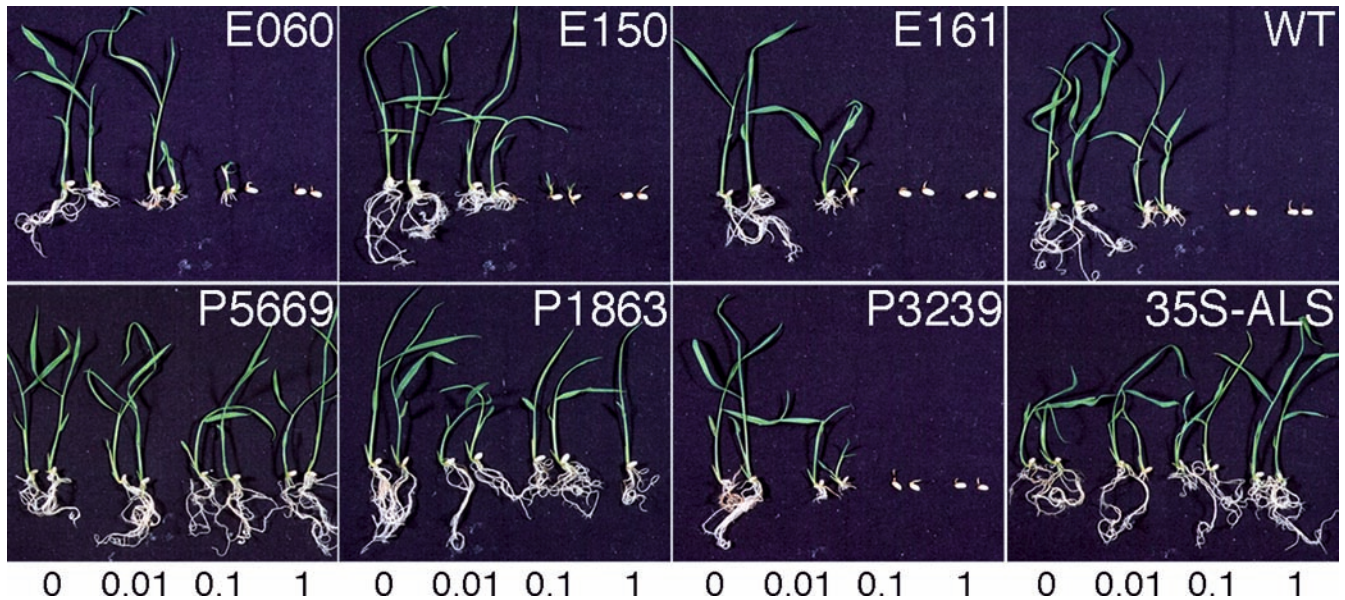
Fig. 4 Chlorsulfuron resistance of transposants. P5669, P1863 and P3239 are transposants derived from crosses between A017 and E060, E150 and E161, respectively. The *numbers* at the bottom indicate the concentration of chlorsulfuron (mg/l) used

plants were transposants in most cases (Fig. 3c). These results showed that the transposants could be effectively screened by testing for chlorsulfuron and hygromycin resistance.

Fig. 5 Tissue-specific GUS activities detected in the transposants in the F_3 generation. P2097, P4583, P2449, P1652 and P6129 show GUS staining in different organs of young flowers. P5097 shows staining in the young inflorescence, P5816 in a restricted region in the developing embryo. In P1772 and P2503, staining is observed at the cut edges of the leaf blade. In P4692 and P5664, GUS staining is found in the ligular region of the leaf. In P4555 the stem, and in P4581 and P4583 the roots, are stained in specific patterns
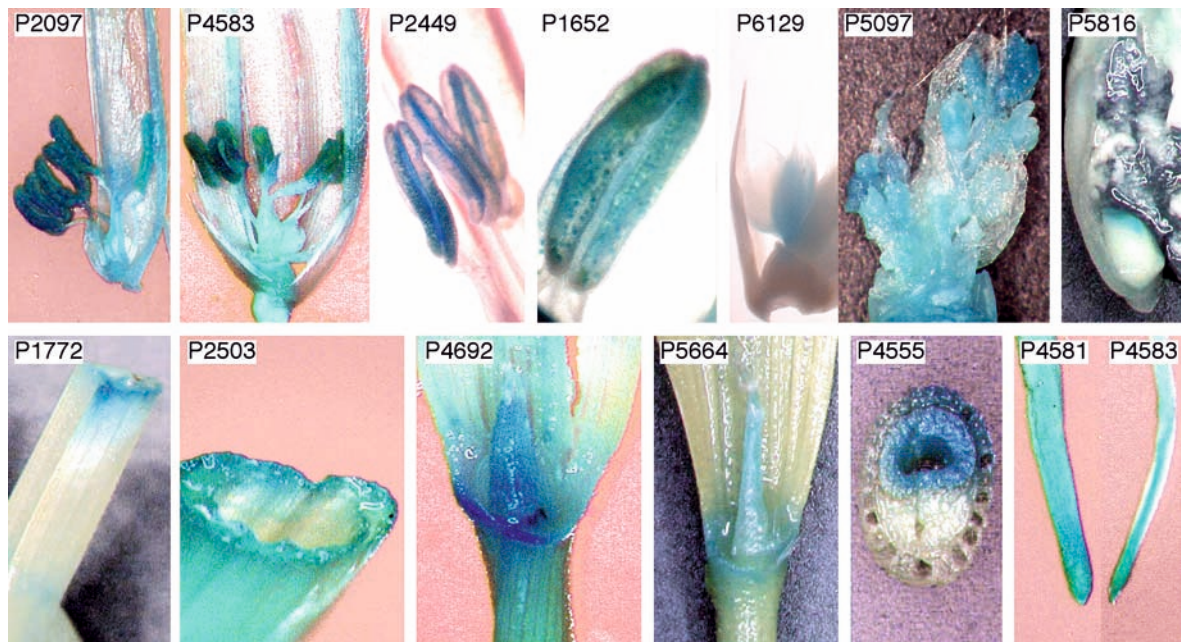
GUS activities in the transposants

To test whether transposed *Ds-GUS* elements can trap enhancers and induce GUS activity, the transposants selected by the PCR were stained with X-gluc. We examined F_3 seeds imbibed overnight, 2-week seedlings grown on MS plates containing hygromycin, and various organs at the flowering stage. Parental lines in which the *Ds-GUS* had not transposed were also stained to evaluate background GUS activity in every organ examined.

The parental *Ds-GUS* lines showed weak GUS activity in various organs which were examined after overnight incubation. The parental *Ds-GUS* lines also showed high GUS activity in some organs, such as nodes at all stages, and lodicules, styles, anthers and pollen

only after flowering. It would thus appear to be difficult to identify enhancers trapped in these organs at these stages. This pattern of background staining is due to the structure of the vector, and not to the position of T-DNA insertion, because all parental lines showed a similar staining pattern (data not shown).

Among 400 lines examined, we identified 44 lines that showed specific GUS activities in various organs. Examples are shown in Fig. 5. Transposant lines P2097, P4583, P2449, P1652 and P6129 showed GUS activities in developing floral organs. In P2097 and P4583, GUS staining was observed in entire anthers, while in P2449, the staining was restricted to the septa of the anthers (Fig. 5). In P1652, GUS staining was observed in the pollen, and in P6129, it was observed in the lodicules (Fig. 5). P5097 showed GUS staining in a young panicle with a length of about 2 mm (Fig. 5). P5816 showed GUS staining in the dorsal region of a developing embryo (Fig. 5). P1772, P2503, P4692 and P5664 showed GUS activity in leaves. In P1772 and P2503, the GUS staining was observed in the cut edge of a leaf (Fig. 5). Thus, wounding stress may have activated certain enhancers in these two lines. In P4652 and P5664, the GUS staining was observed in a ligular region including a ligule and auricles, but not in the leaf blade or leaf sheath (Fig. 5). P4555 showed GUS activity in a stem (Fig. 5). P4581 and P4583 showed GUS activity in roots with distinct regional specificities. In P4581, the staining was observed in an entire root (Fig. 5). In contrast, P4583 showed GUS staining specifically in the inner layers of a root (Fig. 5).

Although we detected various tissue-specific enhancers, the enhancer trapping efficiency of 11% (44/400) in the transposants may have been overestimated. Because the transposants used for staining were not selected for the absence of the 35S-AcTPase, some of the GUS activities observed may have been caused by a second transposition in the F₃ plants that retained the 35S-AcTPase. In such a case, the staining pattern may be shared with neither their siblings nor progenies. Confirmation of the staining with plants that are free from 35S-AcTPase is therefore necessary.

Identification of enhancers trapped by the *Ds-GUS*

To identify enhancers trapped by the *Ds-GUS* element, we chose as examples two lines (P1772 and P2503), both of which displayed GUS activity in surfaces exposed by wounding. P1772 was derived from the parental line E060, in which the *Ds-GUS* was located on chromosome 1. P2503 was derived from E150, in which the *Ds-GUS* was located on chromosome 9. Sequences flanking the transposed *Ds-GUS* were amplified by TAIL-PCR or adaptor-ligated PCR, sequenced, and a database search was carried out using the flanking sequences as queries. The results showed that in P1772 the *Ds-GUS* was located in the BAC clone OSJNBa0018L16, which covers the region between 64.4 and 65.3 cM on chromosome 12 (http://rgp.dna.affrc.go.jp/). We searched for cDNAs

located near the insertion site and identified a full-length cDNA, AK073621 (Fig. 6a). The *Ds-GUS* was located 1.4 kb downstream of the 3′ end of this cDNA. No cDNA has yet been identified or annotated within 10 kb on the opposite side of the insertion site. The expression pattern of this gene was examined by RT-PCR (Fig. 6b). RNAs were isolated from leaves at the indicated time after wounding. Expression was observed at 4 h and 24 h after cutting, while no signal was detected at 0 h (Fig. 6b). Because this expression pattern was consistent with the GUS activity observed in P1772 (Fig. 5), it was concluded that the *Ds-GUS* had trapped the enhancer of this gene in P1772.

In P2503, the *Ds-GUS* was located in the BAC clone OJ1362_G11, which has been mapped at 109 cM on chromosome 5 (http://rgp.dna.affrc.go.jp/). Two cDNAs were identified near the insertion site (Fig. 6c). These were the full-length cDNA AK062488 and *OsLEA3* cDNA (Moons et al. 1997). The *Ds-GUS* was located 3.2 kb upstream of the 5′ end of AK062488 and 1.1 kb upstream of the 5′ end of the *OsLEA3* cDNA (Fig. 6c). We examined the expression patterns of these genes and found that *OsLEA3* was expressed transiently after wounding (Fig. 6e), while AK062488 was expressed in the absence of wounding stress, and actually showed
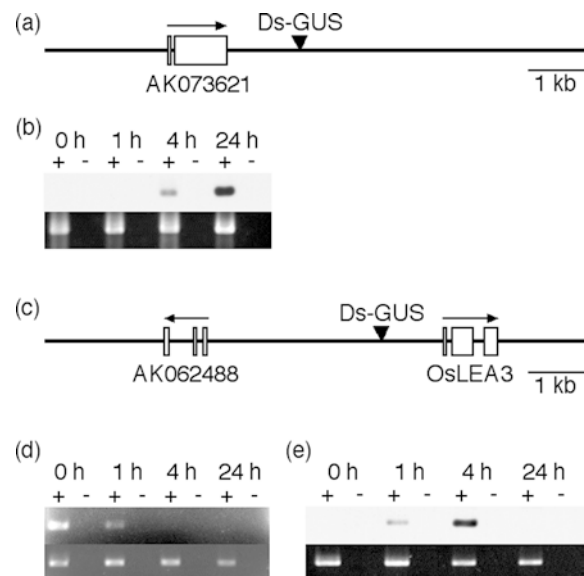
Fig. 6a–e Identification of enhancers trapped in P1772 and P2503. **a** Genome structure around the *Ds-GUS* insertion site in P1772. The *open boxes* indicate exons, the *arrows* mark the orientation of transcription. **b** RT-PCR analysis of AK073621. The *upper* (Southern blot) and *lower* (EtBr staining) *panels* show the expression of AK073621 and actin (internal control), respectively, at the indicated times (in h) after wounding; + and − indicate whether reverse transcriptase was added to the reaction mixture or not. **c** Genome structure around the *Ds-GUS* insertion site in P2503. The *open boxes* and the *arrows* indicate exons and the orientation of transcription, respectively. **d** RT-PCR analysis of AK062488. The *upper and lower panels* show the expression of AK062488 and actin, respectively, as indicated in **b**. **e** RT-PCR analysis of *OsLEA3*. The *upper* (Southern blot) and *lower* (EtBr staining) panels show the expression of *OsLEA3* and actin, respectively, as indicated in **b**

decreased expression after wounding (Fig. 6d). Because the expression pattern of *OsLEA3* was consistent with the GUS activity observed in P2503 while that of AK062488 differed from the profile of GUS activity (Fig. 5), it was concluded that the *Ds-GUS* element had trapped the enhancer of *OsLEA3* in P2503.

Discussion

In this study we examined the utility of an enhancer trap vector based on *Ac / Ds*, GUS and a chlorsulfuron resistance gene, and showed that this system serves as an efficient system for enhancer trapping in rice. (1) We have shown that the *Ds-GUS* element can transpose in developing panicles at an acceptable frequency in the presence of a 35S-AcTPase gene (Fig. 3b, Table 1). Comparison of frequencies of transposition among panicles, and subsequent Southern analysis, revealed that the transposants obtained from different panicles are the products of independent events (Fig. 3d, Table 2). This makes it easy to obtain a large number of independent transposants by simple repetitive ratoon culturing of the F_1 plants as described by Chin et al. (1999). (2) We confirmed that excision of the *Ds-GUS* permits the expression of the chlorsulfuron resistance gene (Fig. 4, Table 1). This also enabled us to obtain a large number of transposants simply by screening the F_2 seeds for chlorsulfuron and hygromycin resistance. (3) Tissue-specific enhancer activities were detected in a proportion of the transposant lines by GUS staining (Fig. 5). This finding indicates that transposed *Ds-GUS* can reveal the activity of various enhancers in a dominant manner. (4) We identified some of the enhancers trapped by the *Ds-GUS* element. This clearly demonstrates the usefulness of our system for the molecular identification of enhancers. (5) We obtained stable *Ds-GUS* lines which carry single copies of the element in ten of the 12 rice chromosomes. Each line can be used for the efficient isolation of region-specific transposants. Efficient isolation of transposants harbouring *Ds-GUS* dispersed at various positions throughout the rice genome should therefore be feasible. Thus, the results obtained here show that a reliable system which allows one to generate a number of independent transposants and to further select various enhancer-trapped lines has been successfully established in rice. Successful identification of the trapped enhancers at the molecular level also demonstrated that the enhancer trapping system described here represents a marked improvement over those previously described for rice.

Temporal pattern of transposition
of the *Ds-GUS* element

The differences in transposition frequency observed among panicles from the same plant suggests that the *Ds-GUS* tends to transpose at certain stages during panicle development. If the *Ds-GUS* transposes before differen-tiation of the primordial cells of the panicles, 75% of the F_2 plants should be transposants with the same insertion pattern in many panicles (25% will lose the *Ds-GUS* by segregation). For example, in an extreme case, if the *Ds-GUS* transposes in a fertilized ovule of an F_1 plant, all the panicles will show a transposition frequency of 75% and the transposant seeds set will be identical. In contrast, if the *Ds-GUS* transposes after differentiation of the primordial cells of the panicles, the frequency of transposition will depend on the stage at which the *Ds-GUS* transposes during panicle development. In this case, the proportions of the transposants in panicles may differ from panicle to panicle, as was found in our system. If the *Ds-GUS* transposes at an earlier step in panicle development, the proportion of transposants will be higher than when the *Ds-GUS* transposes later. This is indeed what we found (Table 2). The variation in the timing of transposition also indicates that the transposants obtained from different panicles contained *Ds-GUS* elements that had reinserted at different chromosomal positions. This is a great advantage, allowing one to obtain large numbers of independent transposants in rice, because rice produces many panicles in ratoon cultures (Chin et al. 1999).

Transposition frequency and chlorsulfuron resistance

We observed a correlation between the frequency of transposition and the level of chlorsulfuron resistance. Thus, the transposants derived from E060 and E150, which showed a higher frequency of heritable transposition (11% and 15%, respectively, by PCR screening), showed greater resistance to the herbicide, while the transposants derived from E161, which showed a lower frequency of transposition (1%), were as sensitive as the non-transformed control plants (Fig. 4). One possible explanation for this correlation is that both characteristics are determined by the chromosomal structure of the site at which the original *Ds-GUS* is located. For the transposition of the *Ds-GUS*, the AcTPase needs to access the site where the original *Ds-GUS* is located. Similarly, for the expression of the chlorsulfuron resistance gene, the transcription machinery needs to access the empty donor site where the *Ds-GUS* is located before transposition. The accessibility of the site to these proteins may determine the frequency of transposition, as well as the level of expression of the resistance gene. Such epigenetic regulation of both transcription and transposition has also been reported in *Arabidopsis* (Miura et al. 2001). The chromosomal structure may thus regulate both transposition and gene expression. The plants generated in this study may also be useful for studying the influence of chromatin structure on gene expression and transposition.

Strategy for distributing the *Ds-GUS* element
over whole rice chromosomes

To permit the isolation of many *Ds-GUS* insertional mutants and the trapping of various tissue-specific

enhancers, it is important that the *Ds-GUS* can transpose to sites on all twelve chromosomes of rice. It is well known that *Ac* / *Ds* tends to transpose to linked sites in both maize and heterologous plant species, such as *Arabidopsis* and rice (Smith et al. 1996; Machida et al. 1997; Nakagawa et al. 2000; Ito et al. 2002). It would be difficult to obtain numerous lines habouring single transposed *Ds-GUS* elements in various independent chromosomal positions if a limited number of original *Ds-GUS* lines is used. This difficulty can be overcome by using multiple original lines, in which the *Ds-GUS* is distributed evenly at different positions on each chromosome. These lines can produce a number of transposants with reinserted *Ds-GUS* in various positions on each of the twelve chromosomes. Given that the size of the rice genome is 1522 cM (Harushima et al. 1998), and a single *Ds-GUS* line can be used for saturated transposition of a 20-cM interval (10 cM each direction), a total of 76 original lines with *Ds-GUS* dispersed in various chromosomal positions would be necessary. We generated 263 transgenic rice plants harbouring *Ds-GUS*, and identified 42 single-copy lines and 50 two-copy lines. Single-copy lines can easily be obtained from the two-copy lines in the next generation after segregation of the two copies by self-pollination. If the *Ds-GUS* elements in the available 92 lines are distributed evenly in the rice genome, saturated transposition can be carried out with these lines. We have therefore begun to map the elements in the 42 single-copy lines taking advantage of the availability of the genome sequence of rice. We amplified and sequenced the flanking sequences of the *Ds-GUS* T-DNA, and carried out a homology search in the database. The position of the *Ds-GUS* can thus be mapped on chromosomes if it has inserted within a genome sequence that has already been mapped. With this method, we have mapped the insertion sites of the *Ds-GUS* in 20 of the original lines to ten different chromosomes out of the twelve (Fig. 2b). These lines could be used for targeted mutagenesis or enhancer trapping in a specific chromosomal region.

Enhancers trapped by *Ds-GUS* insertion

In this study, we identified enhancer trapped lines which show the tissue-specific GUS activities at a relatively high frequency (Fig. 5). Since these lines may have inherited the 35S-AcTPase, and the GUS activities may have resulted from a second transposition of the *Ds-GUS* in the F_3 plants, it is necessary to confirm the enhancer activities in their siblings or their progenies which are free from the 35S-AcTPase. Alternatively, it is necessary to clone the enhancers and compare their expression with the GUS staining patterns. Successful identification of the enhancers in two lines out of two examined suggested that the GUS activities shown in Fig. 5 may indeed be representative of each line in many cases, and are not due to a second transposition of the *Ds-GUS* in the given organs. Once the enhancer activity is confirmed, the temporal and spatial activities of the enhancers, as well as the environmental conditions under which the enhancers are activated, can be studied in detail. In addition, the enhancers or their genes can be cloned easily by using the *Ds-GUS* as a tag, as we demonstrated. Such enhancer-trapped lines and their genes can be used as tissue-specific marker lines and molecular markers, respectively, in a wide range of studies on rice. In addition to these characteristic benefits of enhancer trapping, insertional mutants can be obtained in the same way as with conventional transposon tagging. We have screened some of the *Ds-GUS* transposed lines, and identified a mutant line which produced a giant embryo. This clearly shows that the system can be utilized for mutant isolation and analysis. This enhancer trap system should therefore serve as a very useful tool for functional genomics in rice.

References

Ashikari M, Wu J, Yano M, Sasaki T, Yoshimura A (1999) Rice gibberellin-insensitive dwarf mutant gene *Dwarf1* encodes the α-subunit of GTP-binding protein. Proc Natl Acad Sci USA 96:10284–10289

Azpiroz-Leehan R, Feldmann KA (1997) T-DNA insertion mutagenesis in *Arabidopsis*: going back and forth. Trends Genet 13:152–156

Bhatt AM, Page T, Lawson EJR, Lister C, Dean C (1996) Use of *Ac* as an insertional mutagen in *Arabidopsis*. Plant J 9:935–945

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

Enoki H, Izawa T, Kawahara M, Komatsu M, Koh S, Kyozuka J, Shimamoto K (1999) *Ac* as a tool for the functional genomics of rice. Plant J 19:605–613

Fedoroff NV, Smith DV (1993) A versatile system for detecting transposition in *Arabidopsis*. Plant J 3:273–289

Harushima Y, et al (1998) A high-density rice genetic linkage map with 2275 markers using a single F2 population. Genetics 148:479–494

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

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

Ito T, Motohashi R, Kuromori T, Mizukado S, Sakurai T, Kanahara H, Seki M, Shinozaki K (2002) A new resource of locally transposed *Dissociation* elements for screening gene-knockout lines in silico on the *Arabidopsis* genome. Plant Physiol 129:1695–1699

Ito Y, Eiguchi M, Kurata N (1999) Expression of novel homeobox genes in early embryogenesis in rice. Biochim Biophys Acta 1444:445–450

Ito Y, Eiguchi M, Kurata N (2001) *KNOX* homeobox genes are sufficient in maintaining cultured cells in an undifferentiated state in rice. Genesis 30:231–238

Izawa T, Shimamoto K (1996) Becoming a model plant: the importance of rice to plant science. Trends Plant Sci 1:95–99

Izawa T, Miyazaki C, Yamamoto M, Terada R, Iida S, Shimamoto K (1991) Introduction and transposition of the maize transposable element *Ac* in rice (*Oryza sativa* L.). Mol Gen Genet 227:391–396

Izawa T, Ohnishi T, Nakano T, Ishida N, Enoki H, Hashimoto H, Itoh K, Terada R, Wu C, Miyazaki C, Endo T, Iida S, Shimamoto K (1997) Transposon tagging in rice. Plant Mol Biol 35:219–229

Jeon J-S, et al (2000) T-DNA insertional mutagenesis for functional genomics in rice. Plant J 22:561–570

Li X, Qian Q, Fu Z, Wang Y, Xlong G, Zeng D, Wang X, Liu X, Teng S, Fujimoto H, Yuan M, Luo D, Han B, Li J (2003) Control of tillering in rice. Nature 422:618–621

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

Machida C, Onouchi H, Koizumi J, Hamada S, Semiarti E, Torikai S, Machida Y (1997) Characterization of the transposition pattern of the *Ac* element in *Arabidopsis thaliana* using endonuclease I-*Sce*I. Proc Natl Acad Sci USA 94:8675–8680

Matsuoka M, Ichikawa H, Saito A, Tada Y, Fujimura T, Kano-Murakami Y (1993) Expression of a rice homeobox gene causes altered morphology of transgenic plants. Plant Cell 5:1039–1048.

Miura A, Yonebayashi S, Watanabe K, Toyama T, Shimada H, Kakutani T (2001) Mobilization of transposons by a mutation abolishing full DNA methylation in *Arabidopsis*. Nature 411:212–214

Moons A, De Keyser A, Van Montagu M (1997) A group 3 LEA cDNA of rice, responsive to abscisic acid, but not to jasmonic acid, shows variety-specific differences in salt stress response. Gene 191:197–204

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 19:4321–4325

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

Sasaki A, Itoh H, Gomi K, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Jeong D-H, An G, Kitano H, Ashikari M, Matsuoka M (2003) Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant. Science 299:1896–1898

Shimamoto K (1995) The molecular biology of rice. Science 270:1772–1773

Shimamoto K, Miyazaki C, Hashimoto H, Izawa T, Itoh K, Terada K, Inagaki Y, Iida S (1993) *Trans* -activation and stable integration of the maize transposable element *Ds* cotransfected with the *Ac* transposase gene in transgenic rice plants. Mol Gen Genet 239:354–360

Solis R, Takumi S, Mori N, Nakamura C (1999) *Ac* -mediated trans-activation of the *Ds* element in rice (*Oryza sativa* L.) cells as revealed by GUS assay. Hereditas 131:23–31

Smith D, Yanai Y, Liu Y-G, Ishiguro S, Okada K, Shibata D, Whittier RF, Fedoroff NV (1996) Characterization and mapping of *Ds* -GUS-T-DNA lines for targeted insertional mutagenesis. Plant J 10:721–732

Song W-Y, Wang G-L, Chen L-L, Kim H-S, Pi L-Y, Holsten T, Gardner J, Wang B, Zhai W-X, Zhu L-H, Fauquet C, Ronald P (1995) A receptor-like protein encoded by the rice disease resistance gene *Xa21*. Science 270:1804–1806

Takahashi Y, Shomura A, Sasaki T, Yano M (2001) *Hd6*, a rice quantitative trait locus involved in photoperiod sensitivity, encodes the α-subunit of protein kinase CK2. Proc Natl Acad Sci USA 98:7922–7927

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

Yamanouchi U, Yano M, Lin H, Ashikari M, Yamada K (2002) A rice spotted leaf gene *Spl7* encodes a heat stress transcription factor protein. Proc Natl Acad Sci USA 99:7530–7535.