Title: Construction and application of efficient Ac-Ds transposon tagging vectors in rice

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Abstract

Transposons are effective mutagens alternative to T-DNA for generation of insertional mutants in many plant species including those whose transformation is inefficient. The current strategies of transposon tagging are usually slow and labor intensive and yield low frequency of tagged lines. We have constructed a series of transposon tagging vectors based on three approaches: (i) AcTPase controlled by GVG chemical-inducible expression system, (ii) deletion of AcTPase via Cre-lox site-specific recombination that was initially triggered by Ds excision, (iii) suppression of early transposition events in transformed rice callus through a dual-functional hygromycin resistance gene in a novel Ds element (HPT-Ds). We tested these vectors in transgenic rice and characterized the transposition events. Our results showed these vectors are useful resources for functional genomics of rice and other crop plants. The vectors are freely available for the community.

Key words: Ac-Ds transposable element; GVG-inducible expression; Cre-lox site-specific recombination; rice.
**Introduction**

Insertional mutagenesis is a powerful strategy for gene identification and functional genomics in plants (Parinov and Sundaresan 2000). While the T-DNA approach is applicable to the model plants *Arabidopsis* and rice where effective transformation methods are available, it may not be feasible in many other plant species whose transformation is inefficient. Transposon can be alternatively used for insertional mutagenesis in those plants, since the generation of new insertions occurs through crossing or propagation rather than through transformation.

The maize *Ac-Ds* transposable element has been shown to be active in the plant kingdom widely (Ramachandran and Sundaresan 2001; Sundaresan 1996). A number of important plant genes have been cloned using the *Ac-Ds* element (Ramachandran and Sundaresan 2001). *Ds* insertion libraries have been generated in *Arabidopsis* (Parinov et al. 1999; Ito et al. 2002; Raina et al. 2002; Muskett et al. 2003; Kuromori et al. 2004; Ito et al. 2005) and rice (Greco et al. 2003; van Enckevort et al. 2004; Kolesnik et al. 2004; Park et al. 2007). However, the current strategies of transposon tagging are usually slow and labor intensive and have several drawbacks. For example, in presence of *Ac* transposase (*AcTPase*), transposed *Ds* elements may continue secondary transpositions. Unstable *Ds* insertions and serial transposition events may cause untagged mutations because imprecise excision or a transposition footprint can result in a mutation that is no longer associated with the transposon (Smith et al. 1996; Ramachandran and Sundaresan 2001). Another problem is that the *Ac-Ds* transposable elements are highly active in rice and can transpose early in newly transformed callus cells (Greco et al. 2001a), which results in many sibling plants carrying the same *Ds* insertions and consequently will decreases gene tagging efficiency. In this study, we constructed 12 *Ac-Ds* transposon tagging vectors based on three approaches: (i) *AcTPase* controlled by GVG chemical inducible expression system, (ii) deletion of *AcTPase* via Cre-**lox** site-specific recombination that was initially triggered by *Ds* excision, (iii) suppression of early transposition events in transformed rice callus through a dual-functional hygromycin resistance gene in a novel *Ds* element. We have tested these vectors in transgenic rice and characterized the transposition events. Our results showed these vectors are useful in functional genomics of rice and they will be useful for other crop plants as well.

**Results**

*Ac-Ds* transposon tagging vectors carrying GVG-inducible *AcTPase*

We constructed *Ac-Ds* transposon tagging vectors using a GVG-inducible expression system
(Aoyama and Chua 1997; Ouwerkerk et al. 2001). The vectors pJJ86 and pDs-Ac-GVG (Figure 1A and 1B) carry an in cis two-element system that consists of Ds, 35S:GVG that expresses the chimaeric GVG transcription activator, and AcTPase controlled by a GVG-inducible promoter. The inducible promoter is transactivated through interaction between GVG and the 4xGAL4-upstream activating sequence (4xUAS). The transactivating activity of GVG is regulated by treatment with the steroid chemical dexamethasone (DEX). The Ds element in pJJ86 contains the 4x CaMV 35S enhancer for activation tagging (Weigel et al. 2000), while the Ds in pDs-Ac-GVG does not. Excision of Ds from pJJ86 can be detected because in the resulting T-DNA fragment, the GUS gene is driven by a CaMV 35S promoter. We also constructed a two-vector tagging system in which GVG-inducible AcTPase and Ds are in separate vectors (pINDEX1-Ac and pJJ85, Figure 1C and 1D). The strategy of two-vector system is that transgenic plants carrying the GVG-inducible AcTPase and Ds are generated, respectively, and the AcTPase and Ds are combined in F1 by genetic crosses. In this case, Ds is mobilized in the presence of AcTPase in F1 plants, but stabilized after it is uncoupled from AcTPase in the subsequent generation.

To test whether the inducible Ac-Ds system is functional in rice, we transformed rice cultivar Nipponbare with pJJ86. Independently transformed rice calli were cultured 5 days on media with DEX to induce expression of AcTPase. Because Ds transposition can be detected by GUS activity, the DEX-treated calli and untreated controls were stained for GUS activity. DEX treatment of pJJ86-transformed calli exhibited stronger GUS staining than controls (Figure 2), indicating DEX-inducible system in this vector is functional in rice. At the same time, there was low background of GUS activity in the untreated rice calli (“-DEX” in Figure 2), suggesting that some background transposition occurred in the pJJ86 transformants.

Vectors carrying a novel Ds element for suppression of early transposition events

Because the Ac-Ds transposable elements are active in newly transformed callus cells (Greco et al. 2001a) and early transposition events lead to same Ds insertions in sibling plants, we constructed a novel Ds element, designated HPT-Ds, and used the hygromycin resistance gene (HPT) to suppress transposition. The pHPT-Ds1 vector carrying HPT-Ds and GVG-inducible AcTPase in cis is shown in Figure 1E. The HPT gene in HPT-Ds has the same intron and triple splice acceptors (3xSA, Figure 1E) as in the gene-trap Ds (Sundaresan et al. 1995). Because HPT-Ds is immediately downstream of maize ubiquitin 1 promoter (Ubi) in T-DNA, the Ubi:HPT-Ds fusion confers hygromycin resistance and transformed rice cells are thereby selected on hygromycin media. In case of transposition, HPT-Ds in the rice genome
may not have a promoter nearby for transcription and the rice cells lose hygromycin resistance and can be counter-selected by hygromycin.

To examine the efficacy of the HPT-Ds element, we made a test construct containing Ubi:HPT-Ds and confirmed the function of the Ubi-driving HPT gene in a rice transformation experiment. A total of 250 rice calli were transformed using a particle bombardment method and hygromycin-resistant cells were selected from 30 callus explants after 50 days of selection on hygromycin media. In constructing the pHPT-Ds1 vector, HPT-Ds was cloned between Ubi and GUS so that transposant cells can be detected by GUS assay. The pHPT-Ds2 vector (Figure 1F) is similar to pHPT-Ds1 except that pHPT-Ds2 carries a Bar gene and transposition can be selected by herbicide resistance (De Block et al. 1987).

pHPT-Ds1 was introduced into rice cultivar Nipponbare. A total of 26 stably transformed callus lines were obtained. In the condition without DEX treatment, five calli were randomly selected from each callus line and GUS-assayed for detection of transpoant cells. Transposant cells were detected in 84.6% (22 among 26) of callus lines but mosaic GUS patterns occurred at low frequency (Figure 3A) as compared to the GUS patterns of untreated pJJ86 calli (Figure 2). GUS assays were also performed on 14 of pHPT-Ds1 transformed plantlets; 57.1% (8 among 14 assayed) plantlets contained transposant cells that were rarely distributed in the tissue (Figure 3B). The results of pJJ86 transformants (Figure 2, “-DEX”) and pHPT-Ds1 transformants indicated that there was background transposition activity in the rice calli and plantlets selected from hygromycin media, and that the growth of rice cells containing HPT-Ds transposition events were partially suppressed by hygromycin counter selection.

To characterize the HPT-Ds excision events, rice genomic DNA of eight GUS-positive pHPT-Ds1 transformants was extracted and examined in nested PCR reactions using Ubi- and GUS-specific primers (Figure 3C). Reconstructed Ubi:GUS sequence containing the HPT-Ds empty donor site (EDS) was confirmed by sequencing the 657-bp PCR product (Figure 3C). These results suggested that HPT-Ds elements in the pHPT-Ds1 transformants excised from the T-DNA.

To get more information about the background transposition in the GVG-inducible AcPTase system, we constructed pHPT-Ds3 and pHPT-Ds4 (Figure 1G and 1H) by removing the 35S:GVG from pHPT-Ds1 and pHPT-Ds2, respectively. According to the GUS assay results of pHPT-Ds3 transformed callus lines, 57.1% (8 among 14) of the callus lines showed somatic transposition. The mosaic GUS patterns of pHPT-Ds3 transformants (Figure 3D and 3E) were similar to those of pHPT-Ds1 transformants and the transposition frequency (57.1%)
was a little lower than 84.6% of the pHPT-Ds1 calli. Our explanation for the results of pHPT-Ds1 and pHPT-Ds3 is that the background transposition in GVG-inducible Ac-Ds system was primarily due to a low-level leaky expression of 4xUAS:AcTPase (see Discussion).

Ac-Ds vectors whose AcTPase is controlled by Cre-lox site-specific recombination

The system of bacterial Cre-lox site-specific recombination was shown to be a useful tool for generation of chromosomal rearrangements in plants (Medberry et al. 1995; Osborne et al. 1995). To stabilize transposed HPT-Ds, we utilized Cre-lox system to delete AcTPase after HPT-Ds transposition. pHPT-Ds5 and pHPT-Ds6 (Figure 1I and 1J) carry AcTPase flanked by two lox sites and the Cre gene that is separated from the upstream Ubi by HPT-Ds. To control AcTPase, the two vectors were designed in such a way that (i) HPT-Ds can transpose in the rice genome and excision of HPT-Ds reconstructs Ubi:Cre (Figure 4B) and (ii) Cre recombinase mediates lox-lox recombination and thereby deletes AcTPase (Figure 4C). For examination of rice cells containing deletion events, GUS and Bar were utilized in pHPT-Ds5 and pHPT-Ds6, respectively.

We transformed pHPT-Ds5 into rice cultivars Taipei 309 and Nipponbare. Three of four Taipei 309 transformants and 23 of 30 (76.6%) Nipponbare transformants showed transposition as shown by mosaic GUS patterns (Figure 5A). Genomic DNA of the Taipei 309 transformants was examined in nested PCR reactions using Ubi- and Cre-specific primers. Reconstructed Ubi:Cre sequence containing the HPT-Ds EDS was confirmed by sequencing the 0.6-kb PCR product (Figure 5B and 5C). A 4.3-kb fragment containing the HPT-Ds FDS in T-DNA was also amplified in PCR of the transformants. Additionally, the pHPT-Ds6 vector was transformed into Nipponbare and the Ubi:Cre sequence was detected in genomic DNA of five of the eight pHPT-Ds6 transformants (Figure 5B). Using adaptor-ligation PCR (Alonso et al. 2003), we successfully cloned rice genomic sequences flanking the HPT-Ds terminus from one pHPT-Ds5-transformant and one pHPT-Ds6-transformants (Figure 5D). BLAST analysis showed that the flanking sequences were from the rice chromosomes 6 and 4, respectively, thereby confirming the reinsertion of excised HPT-Ds in rice genome. In analysis of T1 populations of pHPT-Ds5 plants, four of six T1 families showed transposition based on spotted GUS staining of the leaf tissues. These results indicated that the HPT-Ds element in pHPT-Ds5 and pHPT-Ds6 transformants transposed in rice and that the transposition restored Cre expression and induced deletion of AcTPase.
Ac-Ds transposon tagging vectors containing GVG-inducible AcTPase, HPT-Ds and Cre-lox recombination system

On the basis of the examination of the GVG-inducible AcTPase, HPT-Ds and the Cre-lox recombination system, we further constructed two vectors, pHPT-Ds7 and pHPT-Ds8, containing all the components in cis in the same T-DNA. pHPT-Ds7 and pHPT-Ds8 (Figure 1K and 1L) contain (i) HPT-Ds, (ii) Cre-lox system, (iii) GVG-inducible AcTPase and (iv) GUS (in pHPT-Ds7) or Bar (in pHPT-Ds8). Leaf tissues of eight pHPT-Ds7 transformants were GUS-assayed and all plants showed mosaic GUS patterns (data not shown), indicating that HPT-Ds in the plants was active. At T1 generation, transposition was detected in two (E4 and E5) families according to GUS assay results (Table 1). To test whether excised HPT-Ds reintegrated in the rice genome, we performed Southern hybridization of genomic DNA of pHPT-Ds7 and pHPT-Ds8 transformants (T0) using HPT as probe (Figure 6). A 5.4-kb hybridizing band derived from the HPT-Ds FDS in T-DNA were detected in most of the transformants. pHPT-Ds7 transformants E1 and E8 did not contain the 5.4-kb FDS fragment, suggesting HPT-Ds excision from FDS in the T-DNA. Because the HPT-Ds element in E1 and E8 transformants was active based on GUS assay results of their leaf tissues, the hybridizing bands larger than 5.4 kb (Figure 6, indicated with arrowheads) represented reintegrated HPT-Ds elements in the rice genome. In pHPT-Ds7 transformants E2 and E4 and pHPT-Ds8 transformants D4, D6, D7 and D8, the hybridizing bands larger or smaller than 5.4 kb might be either from transposition or from transgene rearrangement (see Discussion). Therefore, in the condition without DEX treatment, HPT-Ds in a part of pHPT-Ds7 transformants underwent transposition due to the background expression activity of AcTPase and HPT-Ds excision triggered Cre-lox recombination, which were similar to the results of the pHPT-Ds5 transformants.

Discussion

The HPT-Ds element described in this paper is a novel Ds whose HPT gene has a dual function. During plant transformation and selection, HPT expression relies on the upstream Ubi promoter to confer resistance to hygromycin in selection media. In case of transposition, the HPT gene may be inactive because the 5' flanking sequence of HPT-Ds at a new genomic site may not be able to provide promoter activity. It is conceivable that most of the transposant cells become sensitive to hygromycin. Therefore, the counter-selection nature of the HPT gene in HPT-Ds can be utilized to diminish transposant cells in newly transformed
rice calli on hygromycin media. In testing pHPT-Ds1 and pHPT-Ds3, it was observed that early transposition events in transformed calli and plantlets were suppressed by hygromycin. Few transposant cells in the calli and plantlets were able to grow under the hygromycin selection pressure, which might be due to escaping transposant cells or because of promoter activity of the 5' transposon flanking sequence.

Because transposition requires transposase, an important theme in transposon tagging research is how to efficiently control transposase activity. It was reported that AcTPase driven by strong promoters mediated high-frequency Ds excision in several dicot plants (Becker et al. 1992; Swinburne et al. 1992; Long et al. 1993). Strong double enhancers of CaMV 35S promoter adjacent to wildtype Ac element induced high-frequency Ac excision in rice transformation (Greco et al. 2001a). In this study, we have utilized the GVG-inducible promoter to control AcTPase expression and transposition was induced to high levels by DEX treatment of pJJ86 transformed callus. However, we also observed a leaky expression of AcTPase in GVG-inducible Ac system in the transformants of pJJ86, pHPT-Ds1 and pHPT-Ds7 based on GUS assay results. Our explanation is that the transposition background was primarily from a low level of leaky expression of 4xUAS:AcTPase. Consistently, in the pHPT-Ds3 and pHPT-Ds5 vectors that do not have 35S:GVG, 57.1% of the pHPT-Ds3 transformants and 76.6% of the pHPT-Ds5 transformants still showed transposition in somatic cells. In spite that the wildtype Ac element has a weak promoter that supports only 0.2% expression of the CaMV 35S promoter (Scortecci et al. 1999), the wildtype Ac itself can transpose in rice with a relatively low activity for three successive generations (Enoki et al. 1999). These indicate that a weak expression of AcTPase can cause transposition events.

In Southern blot analysis of genomic DNA of pHPT-Ds7 and pHPT-Ds8 transformants, the 5.4-kb hybridizing band represented the HPT-Ds at FDS in T-DNA. For the hybridizing bands larger or smaller than 5.4 kb, we explain that some of the bands might be from transposed HPT-Ds. The pHPT-Ds7 transformants showed transposition in somatic cells as suggested by GUS assay results. Because the rice genomic DNA for Southern hybridization was extracted from few leaves of a transformant, transposition in other leaves might not have been detected in the results. Also, since a rice transformant may have more than one T-DNA copy and may contain rearranged T-DNA, the hybridizing bands larger or smaller than 5.4 kb might possibly be from transgene rearrangement. Nevertheless, the efficacy of the HPT-Ds element when it was brought together with the GVG-inducible-AcTPase and the Cre-lox recombination system in pHPT-Ds7 and pHPT-Ds8 was confirmed by GUS assay and Southern blot analysis.
For the strategy of inducible Ac-Ds system, it was reported that in Arabidopsis AcTPase controlled by a heat shock promoter transactivated Ds upon heat shock treatment of flowering plants and the transposition was subsequently stabilized by release of the heat shock treatment (Nishal et al. 2005). The heat shock method used in Arabidopsis seems impractical for rice because of the difficulty of heat shock treatment of a large number of rice plants. But for GVG-inducible Ac-Ds system, the transgenic rice plants can be treated with DEX by hydroponics or by spray to induce transposition to higher frequency given that the treatment condition is optimized. Because the Cre-lox-based strategy will help delete AcTPase and thereby stabilize transposed HPT-Ds elements, we will be able to utilize GVG-inducible AcTPase to induce higher level of transposition while using Cre-lox system to stabilize transposition. The pHPT-Ds7 and pHPT-Ds8 vectors contain both GVG-inducible AcTPase and Cre-lox system and therefore provide a good solution to major drawbacks in the Ac-Ds system. Further work needs to be done with the pHPT-Ds8 vector as how to enhance transposition by DEX induction and how to use the Bar gene to select Basta-resistant transposant progeny.

In summary, we have constructed a series of Ac-Ds transposon tagging vectors and tested individual approaches to control AcTPase expression and transposition in transgenic rice. The pJJ86 and pDs-Ac-GVG vectors were made for testing GVG-inducible AcTPase; the pHPT-Ds1 vector was for testing both GVG-inducible AcTPase and HPT-Ds that contains a dual-functional HPT gene; the pHPT-Ds5 and pHPT-Ds6 vectors were for testing deletion of AcTPase via Cre-lox recombination. The pHPT-Ds7 and pHPT-Ds8 vectors contain all the features of GVG-inducible AcTPase, HPT-Ds and Cre-lox recombination and were tested for comprehensive control of AcTPase and HPT-Ds. The Ac-Ds transposon tagging vectors described in this paper are publicly available, and provide useful resources for the functional genomics of a wide range of plants and especially for that of monocot plants.

Materials and Methods

Construction of Ac-Ds transposon tagging vectors

Molecular manipulation of DNA constructs was carried out using standard methods of molecular cloning (Sambrook et al. 1989). The Ac-Ds transposon tagging vectors (Figure 1) were constructed by combining the components of HPT-Ds, GVG-inducible AcTPase, or/and Cre-lox system. pJJ86, pDs-Ac-GVG, pINDEX1-Ac and pJJ85 were constructed using the backbone of binary vector pC1300intC (Ouwerkerk et al. 2001; GenBank Accession AF294978). The backbone of pRTAC8 (Qu et al. 2003), a transformation-competent artificial
chromosome (TAC) vector, was used in pHPT-Ds1, pHPT-Ds2, pHPT-Ds3, pHPT-Ds4, pHPT-Ds5, pHPT-Ds6, pHPT-Ds7 and pHPT-Ds8. Construction of HPT-Ds is described in the Results section. For the components of HPT-Ds, the 1785-bp 5’Ds, 222-bp 3’Ds, and 138-bp splice acceptors (3xSA) were from the pWS32 vector (Sundaresan et al. 1995; GenBank # AF433043). The hygromycin resistance gene (or hygromycin phosphotransferase, HPT) was from pYLTAC7 (Liu et al. 1999; GenBank Accession AB020028); the 4x CaMV 35S enhancers (4xEn) was from the AcREH construct (Suzuki et al. 2001). The AcTPase fragment was nucleotides 939-4356 of the Ac element (Kunze et al. 1987; GenBank Accession X05424). Components of the GVG-inducible expression system including 35S:GVG and “4xUAS” inducible promoter were from pINDEX3 (Ouwerkerk et al. 2001; GenBank Accession AF294982). The maize ubiquitin 1 promoter (Ubi) (Christensen and Quail 1996) in vectors pHPT-Ds1 to pHPT-Ds8 (Figure 1E to 1L) was from pGA1611 (Kim et al. 2003; GenBank Accession AY373338). The β-glucuronidase (GUS) coding region in pJJ86, pDs-Ac-GVG, pJJ85, pHPT-Ds1, pHPT-Ds3, pHPT-Ds5 and pHPT-Ds7 was from pBI221 (Jefferson et al. 1987; GenBank Accession AF502128). The Bar gene coding region (De Block et al. 1987) in pHPT-Ds2, pHPT-Ds4, pHPT-Ds6 and pHPT-Ds8 was from pSKI1015 (Weigel et al. 2000; GenBank Accession AF187951). The wildtype Cre gene was from pMM23 (Qin et al. 1994). The intron-containing Cre recombinase gene of pHPT-Ds5, pHPT-Ds6, pHPT-Ds7 and pHPT-Ds8 was constructed by introducing the Arabidopsis KOR1 intron into Cre coding sequence according to published method (Zuo et al. 2001; GenBank Accession AF330636). More details of vector construction are available upon request. All the vectors listed in Figure 1 are in the public domain and will be made available by Guo-Liang Wang (Department of Plant Pathology, Ohio State University, Columbus, OH 43210, USA; email wang.620@osu.edu) upon request.

Rice transformation, GUS histochemical assay and dexamethasone treatment

All the T-DNA vectors containing Ac-Ds elements were transformed into japonica rice cultivar Nipponbare or Taipei 309 via Agrobacterium-mediated method (Yin and Wang 2000; Qu et al. 2003). Embryogenic calli derived from mature seeds were used as transformation explants. Rice transformation of the Ubi:HPT-Ds testing construct was performed via bombardment (Li et al. 1993). GUS activity in rice calli and plants was assayed using the histochemical method described by Jefferson (1987). For GVG-inducible expression, the steroid chemical dexamethasone (DEX; Sigma, USA) was prepared as 100 mM stock solution in dimethyl sulfoxide (DMSO). Transformed rice calli were treated in NB medium
(Li et al. 1993) containing 10 μM of DEX for 5 days.

**PCR of transposon EDS sequences and adaptor-ligation PCR of transposon flanking sequences**

For PCR of empty donor site (EDS) sequence of the *HPT-Ds* element in pHPT-Ds1 transformants, rice genomic DNA was amplified in nested PCR reactions using Ubi- and GUS-specific primers. Primers in first-round PCR were U1 (5'-CTAGATCGGAGTAGAATTCTGT-3') and G1 (5'-TCC TGA TTA TTG ACC CAC ACT TT-3'); primers in second-round PCR were U2 (5'-TCATACATCTTCTAGTTACGAGT-3') and G2 (5'-GTA ATG AGT GAC CGC ATC GAA A-3'). For PCR of EDS sequence in pHPT-Ds5 and pHPT-Ds6 transformants, rice genomic DNA was amplified in nested PCR reactions using Ubi and Cre-specific primers. Primers in first-round PCR were U1 and C1 (5'-GGTCGAAATCAGTGCGTTCG-3'); primers in second-round PCR were U2 and C2 (5'-CCAGGTATGCTCAGAAAACGC-3'). The first PCR reactions were performed with a 5 min denaturing at 94°C, 25 cycles of amplification (94°C for 30 sec, 61°C for 40 sec, 72°C for 1 min), and a final extension of 10 min at 72°C. Products of the first-round PCR were diluted 50 times and used as templates in the second PCR. The second PCR reactions were initiated at 94°C for 5 min, continued with 30 cycles of 94°C for 30 sec, 62°C for 40 sec and 72°C for 1 min, and the final extension was at 72°C for 10 min.

Sequences flanking *HPT-Ds* were amplified by adaptor-ligation PCR as described (Alonso et al. 2003). About 100 ng of rice genomic DNA was subject to digestion-ligation reactions using the *HindIII* and *EcoRI* adaptors and the enzymes of *HindIII*, *EcoRI* and T4 DNA ligase (Alonso et al. 2003). The reaction products were amplified using two rounds of nested PCR in the same conditions. The primers in the first PCR were Ds1 (5'-acggtcggaaactagctctacc-3') and AP1 (Alonso et al. 2003); primers in the second round PCR were Ds2 (5'-ctcgggttcgaaatcgatcgggat-3') and AP2-C (5'-tggtcagggccgggctgc-3'). The amplified fragments were purified by electrophoresis on agarose gel and cloned into the pGEM-T easy vector (Promega, USA) and sequenced.
Genomic DNA extraction and Southern hybridization

Rice genomic DNA was isolated using a CTAB-based method (Saghai-Maroof et al. 1984). About 3 µg of rice DNA was digested with EcoRI, fractionated by electrophoresis on 0.8% agarose gels and transferred by alkaline blotting onto Hybond-N+ membranes (Amersham). For hybridization, 835-bp HPT fragment was prepared by PCR using specific primers Hpt-F (5'-TACTTCTACACAGCCATC-3') and Hpt-R (5'-TATGTCCTGCGGGTAAAT-3') and labeled in the presence of $[^\alpha\text{32P}]$-dCTP. The DNA blot was hybridized to HPT probe in hybridization buffer containing 350 mM Na$_2$HPO$_4$, 150 mM NaH$_2$PO$_4$, 7% of SDS, 1 mM EDTA-Na$_2$, and 100 mg/L of heat-denatured salmon sperm DNA. Final washes of the membranes were performed in a solution containing 0.1x SSC and 0.1% SDS, 65°C for 30 min.

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References


Figure legends

Figure 1. Schematic representation of Ac-Ds transposon tagging vectors containing GVG-inducible AcTPase, HPT-Ds or/and Cre-lox recombination system. Abbreviations: RB and LB, right and left T-DNA borders; HPT, hygromycin resistance gene; 35S, CaMV 35S promoter; GVG, transcription activator (Aoyama and Chua 1997; Ouwerkerk et al. 2001); 4xUAS: 4x upstream activation sequences; AcTPase, Ac transposase; 5’Ds and 3’Ds: Ds 5’ and 3’ terminal sequences; 4X En, 4X CaMV 35S enhancers; GUS, β-glucuronidase coding sequence; Bar, the Bar gene conferring resistance to the herbicide Basta; Ubi, maize ubiquitin 1 promoter; lox, P1 phage 34 bp recombination site; 3xSA, 3x transcript splicing acceptors; Cre, Cre recombinase.

Figure 2. DEX treatment and GUS assay of rice callus lines independently transformed with vector pJJ86. Four stably transformed rice callus lines (No 1, 2, 3, and 4) were cultured 5 days on NB medium (Li et al. 1993) containing 10 μM of dexamethasone (DEX; Sigma, USA) (“+DEX”) or without DEX (“-DEX”). GUS activity in the rice calli was further assayed using the histochemical GUS staining method described by Jefferson (1987).

Figure 3. Characterization of HPT-Ds excision in pHPT-Ds1 and pHPT-Ds3 transformants. A and B, GUS assay of a callus and a plant transformed with pHPT-Ds3; C, PCR and sequencing of HPT-Ds empty donor site (EDS) in eight pHPT-Ds1 transformants (1 through 8). Nested PCR primers U1, U2, G1 and G2 were used in two rounds of PCR reactions. The U1 and U2 primers are Ubi specific and G1 and G2 are GUS specific. The sequences shown are HPT-Ds full donor site (FDS) in the pHPT-Ds1 T-DNA and EDS from a pHPT-Ds1 transformant. The nine italicized characters are the first three codons of the GUS gene. Compared to the HPT-Ds FDS sequence, altered nucleotides flanking the HPT-Ds EDS are indicated in bold characters. M, 1-kb ladders DNA marker; P, pHPT-Ds1 plasmid DNA; CK, untransformed wildtype rice. D and E, GUS assay of pHPT-Ds3 transformed calli and plantlets, respectively.

Figure 4. Schematic representation of genome structures including HPT-Ds excision, Cre-lox-based AcTPase deletion and HPT-Ds reintegration.

Figure 5. Characterization of HPT-Ds excision and reinsertion events in pHPT-Ds5 and pHPT-Ds6 transformants. A, GUS assay of HPT-Ds excision and AcTPase deletion in a
pHPT-Ds5 transformant. As shown in Figure 4, deletion of AcTPase via Cre-lox site-specific recombination was initially triggered by HPT-Ds excision. B, PCR of HPT-Ds FDS and EDS in three pHPT-Ds5 transformants and eight pHPT-Ds6 transformants. CK, untransformed wildtype rice; M, 1-kb ladders DNA marker; CK, untransformed wildtype rice. C, Diagram of PCR primers and amplified regions in pHPT-Ds5 and pHPT-Ds6 T-DNA and sequencing of HPT-Ds EDS and FDS cloned from the rice transformants. PCR primers U1 and U2 are specific to Ubi, and primers C1 and C2 are specific to Cre. The sequences below are (i) HPT-Ds FDS in pHPT-Ds5 and pHPT-Ds6 transformants; (ii) EDS in pHPT-Ds5 transformant 2 and pHPT-Ds6 transformants 1, 2, 6 and 7; (iii) EDS in pHPT-Ds6 transformant 8. In the FDS sequence, Ubi, lox and HPT-Ds positions are indicated, and the six italicized characters represent the first two codons of the Cre gene. Compared to HPT-Ds FDS sequence, altered nucleotide(s) flanking HPT-Ds EDS is indicated in bold characters. Asterisks(s) represent deleted nucleotide(s). D, (i) Sequence of HPT-Ds 5’ terminus is shown in bold characters; (ii) Downstream HPT-Ds 5’ terminus is the genomic sequence (GenBank FJ899735) of rice chromosome 6 in pHPT-Ds5 transformant 2, where a transposed HPT-Ds element was reinserted; (iii) Downstream HPT-Ds 5’ terminus is the genomic sequence (GenBank FJ899736) of rice chromosome 4 in pHPT-Ds6 transformant 2, where the HPT-Ds element was reinserted. The four asterisks represent a 4-bp deletion at the HPT-Ds 5’ terminus resulting from HPT-Ds transposition.

Figure 6. Southern analysis of transposition in pHPT-Ds7 and pHPT-Ds8 transformants. A, Genomic DNA of rice transformants was digested with BamHI and probed with an 835-bp HPT fragment of HPT-Ds (see 6B and 6C). D1 to D9, nine pHPT-Ds8 transformants; E1 to E8, eight pHPT-Ds7 transformants. B and C, T-DNA structures prior to or after transposition, respectively, in the genome of rice transformants.
Table 1. Transposition detected in the T1 families of pHPT-Ds7 transformed plants

<table>
<thead>
<tr>
<th>T1 families</th>
<th>GUS-positive plants</th>
<th>GUS-negative plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>E4</td>
<td>18</td>
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<td>17</td>
</tr>
<tr>
<td>E7</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>
**C**

- **Full donor site**: TAGAATTTCGATC: HPT-Ds:GGTCACGCAACGCGC
  CACGTACAGCCTCCCCCTCCGGCAGATCAATT
  CGATGGGTGGTCAGTCCCTTATGTTACG
  "657 bp"

- **Empty donor site**: TAGAATTCA:GTACGCAACGCGC
  CACGTACAGCCTCCCCCTCCGGCAGATCAATT
  CGATGGGTGGTCAGTCCCTTATGTTACG
  "657 bp"

**D** & **E**

- Images of plant tissue showing expression patterns.

**Note:**
- "3xSA" indicates a repeated sequence.
- "4xUbi" refers to a Ubiquitin promoter.
- "4xEnUbi" refers to an Enhanced Ubiquitin promoter.
- "U1-G1" and "U2-G2" suggest different splicing events.
- "pHPT-Ds1" denotes a construct involving the HPT-Ds1 gene.

**Legend:**
- RB: Repressor Binding Site
- Ubi: Ubiquitin
- GUS: Green Fluorescent Protein
- Ac: Acetoclastic
- pHPT-Ds: pHPT-Ds1
- GVG: GUS Gene
- 35S: 35S Promoter
A  T-DNA structure before HPT-Ds transposition

B  T-DNA structure after HPT-Ds transposition

C  T-DNA structure after lox-site recombination
(i) HPT-Ds 5′ terminal sequence

ACGTCGGTGACCGGATTTTACCCGTTTCTACTTCC

(ii) HPT-Ds reinsertion in the genome of a pHPT-Ds5 transformant (#2)

ACGTCGGTGACCGGATTTTACCCGTTTCTACTTCC

(iii) HPT-Ds reinsertion in the genome of a pHPT-Ds6 transformant (#2)

ACGTCGGTGACCGGATTTTACCCGTTTCTACTTCC

(i) Full donor site

P pHPT-Ds6

RB Ubi Cre AcTPease:4xUAS Bar LB

(iii) Empty donor site 2

TAGAATTCGAAT*GTCACGCAA...

(ii) Empty donor site 1

TAGAATTCGAAT*GTCACGCAA...

(i) Full donor site

P pHPT-Ds5

RB Ubi Cre AcTPease:4xUAS GUS LB