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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 the 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) *Ac*TPase controlled by glucocorticoid binding domain/VP16 acidic activation domain/Gal4 DNA-binding domain (GVG) chemical-inducible expression system; (ii) deletion of *Ac*TPase *via* Cre-*lox* site-specific recombination that was initially triggered by *Ds* excision; and (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 that these vectors are useful resources for functional genomics of rice and other crop plants. The vectors are freely available for the community.

Available online at www.jipb.net

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 (Sundaresan 1996; Ramachandran and Sundaresan 2001). 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; Kolesnik et al. 2004; van Enckevort et al. 2005; Park et al. 2007). However, the current strategies of transposon tagging are usually slow and labor intensive and have several drawbacks. For example, in the presence of *Ac* transposase (*Ac*TPase), 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,b), which results in many sibling plants carrying the same *Ds* insertions and consequently decreasing gene tagging efficiency. In the present study, we constructed 12 *Ac-Ds* transposon tagging vectors based on three approaches: (i) *Ac*TPase controlled by glucocorticoid binding domain/VP16 acidic activation domain/Gal4 DNA-binding domain (GVG) chemical inducible expression system; (ii) deletion of *Ac*TPase *via* Cre-*lox* site-specific recombination that was initially triggered by *Ds* excision; and (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 that 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 *Ac*TPase

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,B) carry an *in cis* two-element system that consists of *Ds*, 35S:GVG that expresses the chimeric GVG transcription activator, and *Ac*TPase 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 enhancers 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 β-glucuronidase (GUS) gene is driven by a CaMV 35S promoter. We also constructed a two-vector tagging system in which GVG-inducible *Ac*TPase and *Ds* are in separate vectors (pINDEX1-Ac and pJJ85, Figure 1C,D). The strategy of the two-vector system is that transgenic plants carrying the GVG-inducible *Ac*TPase and *Ds* are generated, respectively, and the *Ac*TPase and *Ds* are combined in F_1 by genetic crosses. In this case, *Ds* is mobilized in the presence of *Ac*TPase in F_1 plants, but stabilized after it is uncoupled from *Ac*TPase 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 for 5 d on media with DEX to induce expression of *Ac*TPase. 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 that the 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 the 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 *Ac*TPase *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 d of selection on hgromycin media. In constructing the pHPT-Ds1 vector, *HPT-Ds* was cloned between Ubi and GUS so that transposant cells can

Figure 1. Schematic representation of *Ac-Ds* transposon tagging vectors containing GVG-inducible *Ac*TPase, *HPT-Ds* or/and Cre-*lox* recombination system.

3xSA, 3x transcript splicing acceptors; 4X En, 4X CaMV 35S enhancers; 4xUAS, 4x upstream activation sequences; 5′*Ds* and 3′*Ds*, *Ds* 5′ and 3′ terminal sequences; 35S, CaMV 35S promoter; *Ac*TPase, *Ac* transposase; Bar, the Bar gene conferring resistance to the herbicide Basta; Cre, Cre recombinase; GUS, β-glucuronidase coding sequence; GVG, transcription activator (Aoyama and Chua 1997; Ouwerkerk et al. 2001); HPT, hygromycin resistance gene; RB and LB, right and left T-DNA borders; Ubi, maize ubiquitin 1 promoter; *lox*, P1 phage 34 bp recombination site.

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 transposant 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 with the GUS patterns of untreated pJJ86 calli (Figure 2). GUS assays were also carried out on 14 of pHPT-Ds1 transformed plantlets; 57.1% (eight 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 trans-formants indicated that there was background transposition activity in the rice calli and plantlets selected from hygromycin
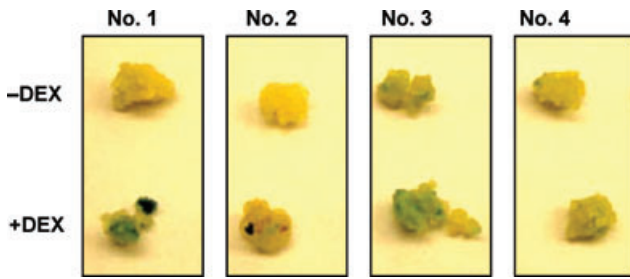
Figure 2. Dexamethasone (DEX) treatment and β-glucuronidase (GUS) assay of rice callus lines independently transformed with vector pJJ86.

Four stably transformed rice callus lines (No. 1, 2, 3, and 4) were cultured 5 d on NB medium (N6 minerals plus Gamborg's B5 vitamins) (Li et al. 1993) containing 10 μM of dexamethasone (DEX) ("+DEX") or without DEX ("–DEX"). GUS activity in the rice calli was further assayed using the histochemical GUS staining method described by Jefferson (1987).

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 polymerase chain reaction (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 *Ac*PTase system, we constructed pHPT-Ds3 and pHPT-Ds4 (Figure 1G and H) 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% (eight among 14) of the callus lines showed somatic transposition. The mosaic GUS patterns of pHPT-Ds3 transformants (Figure 3D,E) 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 the GVG-inducible *Ac-Ds* system was primarily due to a low-level leaky expression of 4xUAS:*Ac*TPase (see Discussion).

Ac-Ds vectors whose *Ac*TPase 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 the generation of chromosomal rearrangements in plants (Medberry et al. 1995; Osborne et al.

1995). To stabilize transposed *HPT-Ds*, we used Cre-*lox* system to delete *Ac*TPase after *HPT-Ds* transposition. pHPT-Ds5 and pHPT-Ds6 (Figure 1I,J) carry *Ac*TPase flanked by two *lox* sites and the Cre gene that is separated from the upstream Ubi by *HPT-Ds*. To control *Ac*TPase, 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 *Ac*TPase (Figure 4C). For examination of rice cells containing deletion events, GUS and Bar were used 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,C). A 4.3-kb fragment containing the *HPT-Ds* full donor site (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 the rice genomic sequences flanking the *HPT-Ds* terminus from one pHPT-Ds5-transformant and one pHPT-Ds6-transformant (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 the rice genome. In analysis of T_1 populations of pHPT-Ds5 plants, four of six T_1 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 *Ac*TPase.

Ac-Ds transposon tagging vectors containing GVG-inducible *Ac*TPase, *HPT-Ds* and Cre-*lox* recombination system

On the basis of the examination of the GVG-inducible *Ac*TPase, *HPT-Ds* and the Cre-*lox* recombination system, we further constructed two vectors, pHPT-Ds7 and pHPT-Ds8 containing all of the components *in cis* in the same T-DNA. pHPT-Ds7 and pHPT-Ds8 (Figure 1K,L) contain (i) *HPT-Ds*, (ii) Cre-*lox* system, (iii) GVG-inducible *Ac*TPase 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 T_1 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
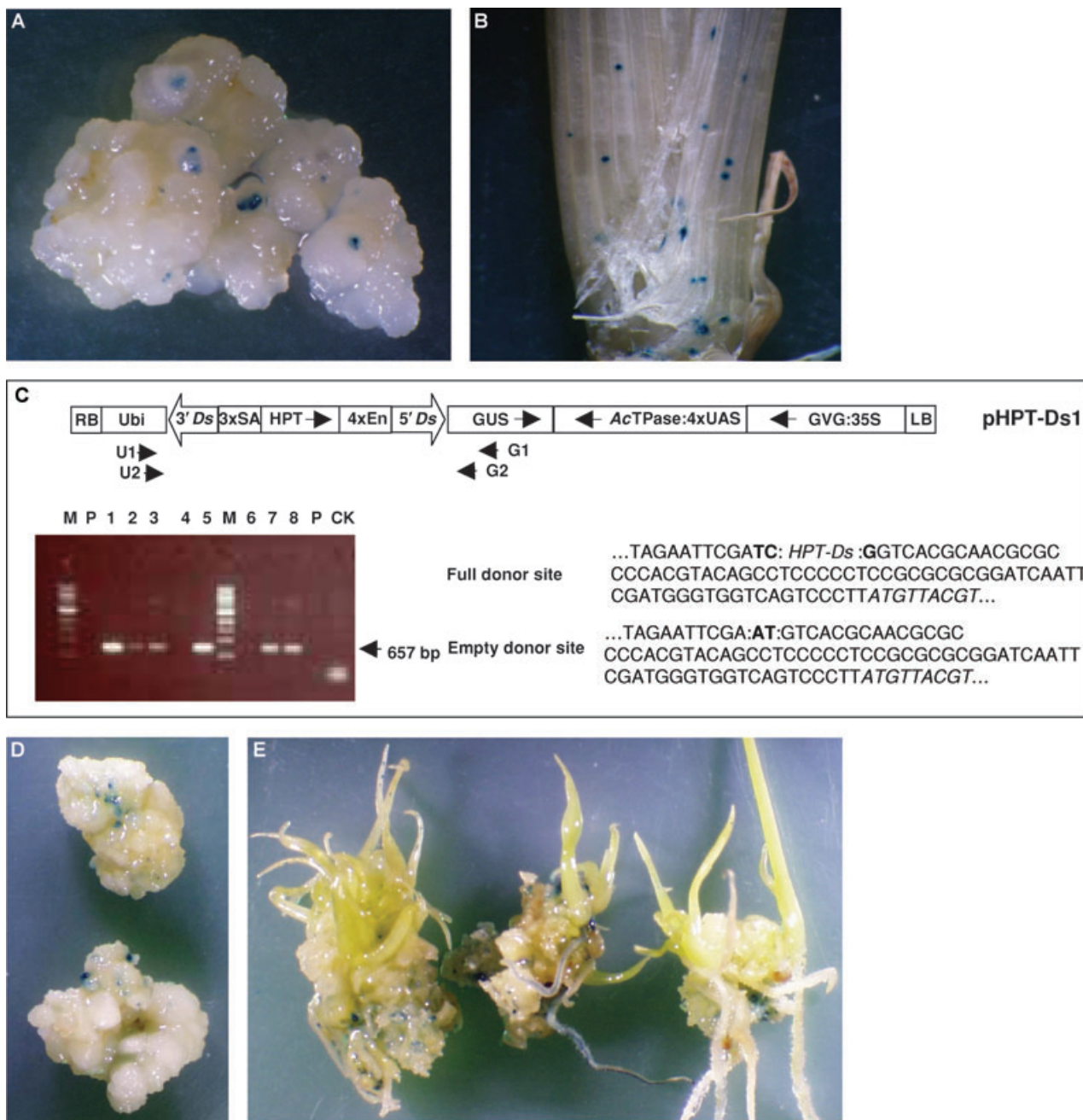
Figure 3. Characterization of *HPT-Ds* excision in pHPT-Ds1 and pHPT-Ds3 transformants.

(A,B) *β*-glucuronidase coding sequence (GUS) assay of a callus and a plant transformed with pHPT-Ds1.

(C) Polymerase chain reaction (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 with the *HPT-Ds* FDS sequence, altered nucleotides flanking the *HPT-Ds* EDS are indicated in bold characters. CK, untransformed wildtype rice; M, 1-kb ladders DNA marker; P, pHPT-Ds1 plasmid DNA.

(D,E) GUS assay of pHPT-Ds3 transformed calli and plantlets, respectively.

A   T-DNA structure before *HPT-Ds* transposition

B   T-DNA structure after *HPT-Ds* transposition

C   T-DNA structure after *lox*-site recombination
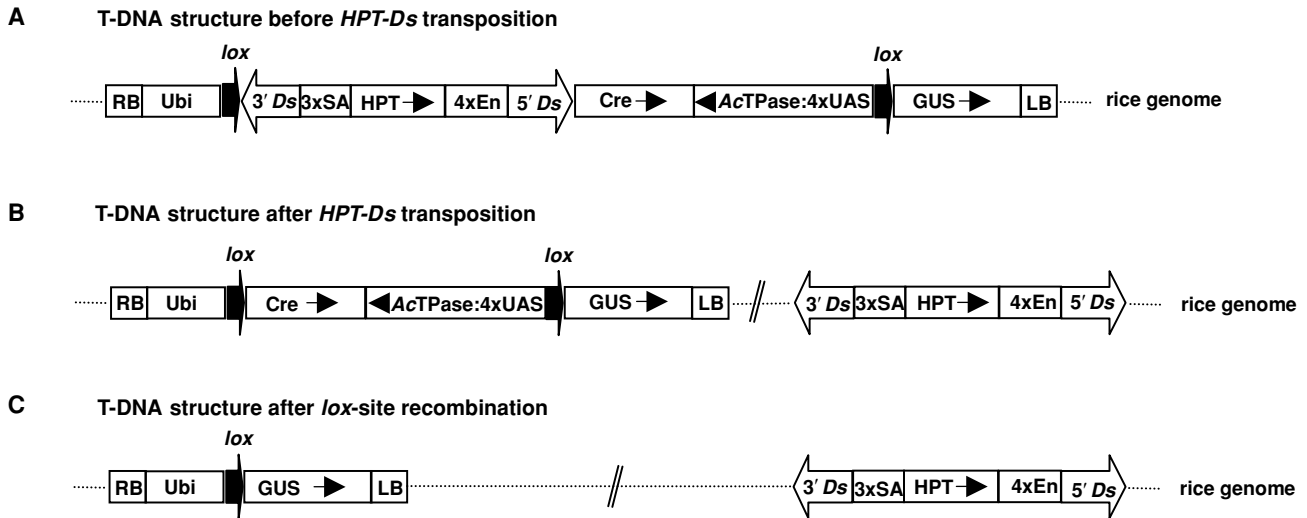

Figure 4. Schematic representation of genome structures including *HPT-Ds* excision, Cre-*lox*-based *Ac*TPase deletion and *HPT-Ds* reintegration.

the rice genome, we carried out Southern hybridization of genomic DNA of pHPT-Ds7 and pHPT-Ds8 transformants (T_0) using HPT as a 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 conditions without DEX treatment, *HPT-Ds* in a part of pHPT-Ds7 transformants underwent transposition due to the background expression activity of *Ac*TPase, and *HPT-Ds* excision triggered Cre-*lox* recombination; the results were similar to those of the pHPT-Ds5 transformants.

Discussion

The *HPT-Ds* element described in the present study 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 used 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 *Ac*TPase 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 the present study, we have used the GVG-inducible promoter to control *Ac*TPase expression and transposition was induced to high levels by DEX treatment of pJJ86 transformed callus. However, we also observed a leaky expression of *Ac*TPase in the 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:*Ac*TPase. 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 of the wildtype *Ac* element having a weak promoter that supports only 0.2% expression of the CaMV 35S promoter (Scortecci et al. 1999), the wildtype *Ac*
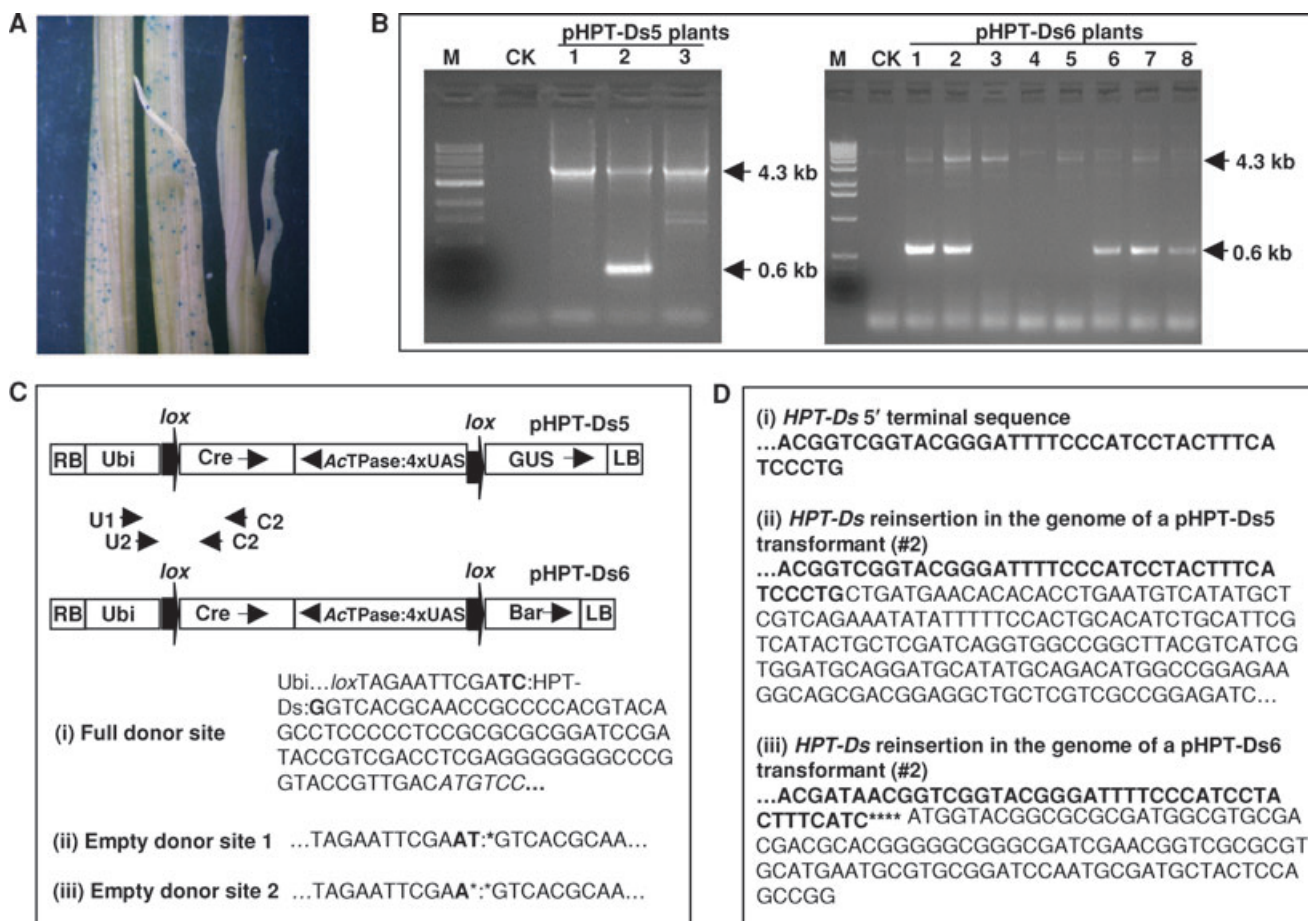
Figure 5. Characterization of *HPT-Ds* excision and reinsertion events in pHPT-Ds5 and pHPT-Ds6 transformants.

(A) *β*-glucuronidase (GUS) assay of *HPT-Ds* excision and *Ac*TPase deletion in a pHPT-Ds5 transformant. As shown in Figure 4, deletion of *Ac*TPase *via* Cre-*lox* site-specific recombination was initially triggered by *HPT-Ds* excision.

(B) Polymerase chain reaction (PCR) of *HPT-Ds* full donor site (FDS) and empty donor site (EDS) in three pHPT-Ds5 transformants and eight pHPT-Ds6 transformants. CK, untransformed wildtype rice; M, 1-kb ladders DNA marker.

(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 with the *HPT-Ds* FDS sequence, altered nucleotide(s) flanking *HPT-Ds* EDS is indicated in bold characters. Asterisks(s) represent deleted nucleotides(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.

itself can transpose in rice with a relatively low activity for three successive generations (Enoki et al. 1999). This indicates that a weak expression of *Ac*TPase 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

Table 1. Transposition detected in the T$_1$ families of pHPT-Ds7 transformed plants

T$_1$ families	GUS-positive plants	GUS-negative plants
E2	0	4
E4	18	11
E5	31	17
E7	0	20

GUS, β-glucuronidase.

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-*Ac*TPase and the Cre-lox recombination system in pHPT-Ds7 and pHPT-Ds8 was confirmed by GUS assay and Southern blot analysis.

For inducible *Ac-Ds* system, it was reported that in *Arabidopsis Ac*TPase 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
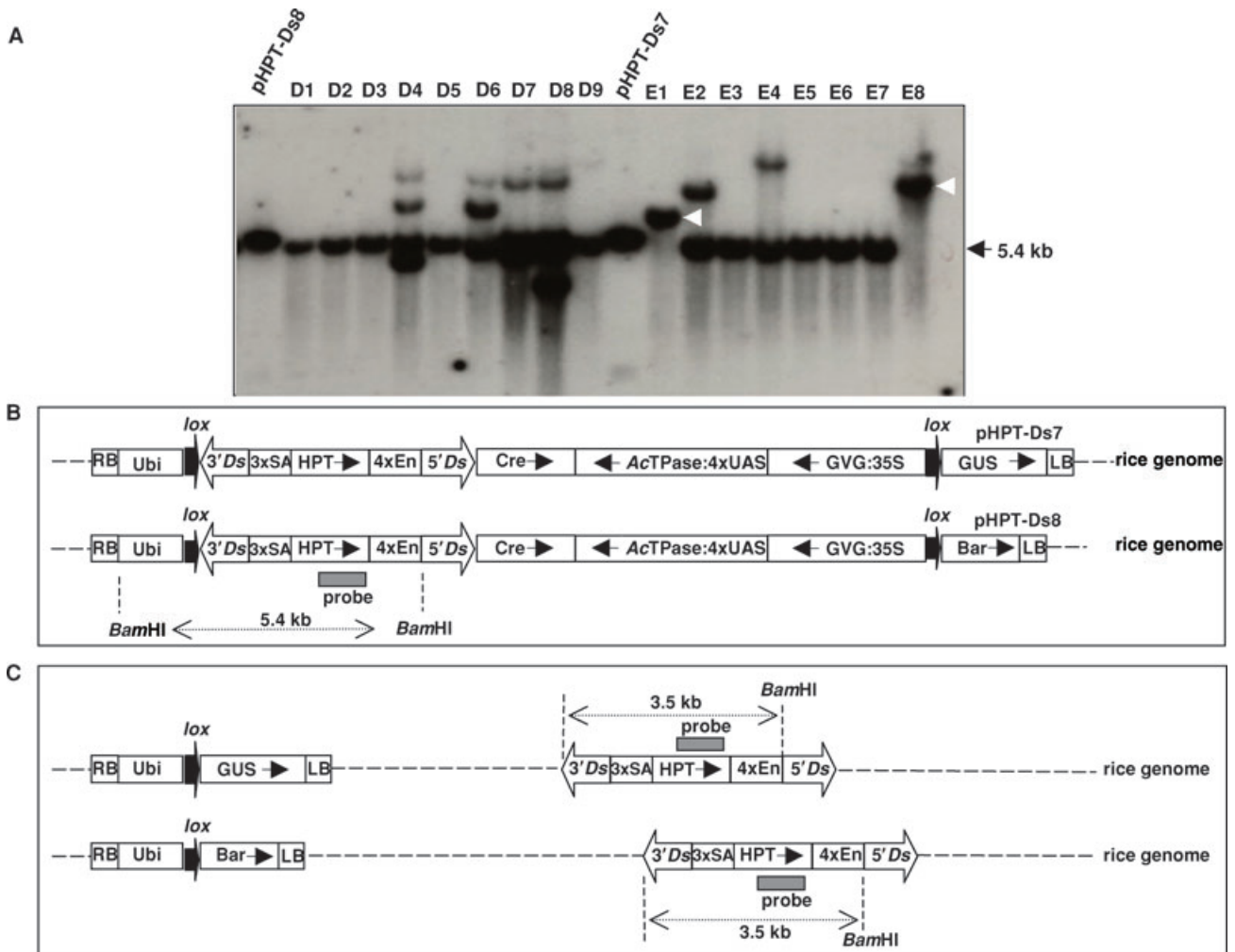

Figure 6. Southern analysis of transposition in pHPT-Ds7 and pHPT-Ds8 transformants.

(A) Genomic DNA of rice transformants was digested with *Bam*HI and probed with an 835-bp hygromycin resistance gene (HPT) fragment of *HPT-Ds* (see 6B and 6C). D1 to D9, nine pHPT-Ds8 transformants; E1 to E8, eight pHPT-Ds7 transformants.

(B,C) T-DNA structures prior to or after transposition, respectively, in the genome of rice transformants.

used in *Arabidopsis* seems impractical for rice because of the difficulty of heat shock treatment of a large number of rice plants. But for the 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 *Ac*TPase and thereby stabilize transposed *HPT-Ds* elements, we will be able to use GVG-inducible *Ac*TPase to induce higher levels of transposition while using the Cre-*lox* system to stabilize transposition. The pHPT-Ds7 and pHPT-Ds8 vectors contain both GVG-inducible *Ac*TPase and Cre-*lox* systems 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 to determine 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 *Ac*TPase expression and transposition in transgenic rice. The pJJ86 and pDs-Ac-GVG vectors were made for testing GVG-inducible *Ac*TPase; the pHPT-Ds1 vector was for testing both GVG-inducible *Ac*TPase and *HPT-Ds* that contains a dual-functional HPT gene; the pHPT-Ds5 and pHPT-Ds6 vectors were for testing the deletion of *Ac*TPase *via* Cre-*lox* recombination. The pHPT-Ds7 and pHPT-Ds8 vectors contain all the features of GVG-inducible *Ac*TPase, *HPT-Ds* and Cre-*lox* recombination and were tested for comprehensive control of *Ac*TPase and *HPT-Ds*. The *Ac-Ds* transposon tagging vectors described in the present 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 *Ac*TPase, 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 Accession 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) were from the AcREH construct (Suzuki et al. 2001). The *Ac*TPase 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–L) 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 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. 1995). 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 the Cre coding sequence according to the method of Zuo et al. (2001; GenBank Accession AF330636). More details of vector construction are available upon request. All of 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 of the T-DNA vectors containing *Ac-Ds* elements were transformed into japonica rice cultivar Nipponbare or Taipei 309 *via* the *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 carried out *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, St. Louis, MO, USA) was prepared as 100 mM stock solution in dimethylsulfoxide (DMSO). Transformed rice calli were treated in N6 minerals plus Gamborg's B5 vitamins (NB) medium (Li et al. 1993) containing 10 μM of DEX for 5 d.

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′-CTAGATCGGAGTAGAATTCTGTT-3′) and G1 (5′-TCCTGATTATTGACCCACACTTT-3′); primers in second-round PCR were U2 (5′-TCATACATCTTCATAGTTACGAGT-3′) and G2 (5′-GTAATGAGTGACCGCATCGAA A-3′). For PCR of the 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 carried out with a 5 min denaturing at 94 °C, 25 cycles of amplification (94 °C for 30 s, 61 °C for 40 s, 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 s, 62 °C for 40 s 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 *Hin*dIII and *Eco*RI adaptors and the enzymes of *Hin*dIII, *Eco*RI 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′-acggtcgggaaactagctctacc-3′) and AP1 (Alonso et al. 2003); primers in the second round PCR were Ds2 (5′-ctcgggttcgaaatcgatcgggat-3′) and AP2-C (5′-tggtcgacggcccgggctgc-3′). The amplified fragments were purified by electrophoresis on agarose gel and cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced.

Genomic DNA extraction and Southern hybridization

Rice genomic DNA was isolated using a cetyltrimethylammonium bromide (CTAB)-based method (Saghai-Maroof et al. 1984). About 3 μg of rice DNA was digested with *Eco*RI, fractionated by electrophoresis on 0.8% agarose gels and transferred by alkaline blotting onto Hybond-N$^+$ membranes (Amersham Pharmacia, Piscataway, NJ, USA). For hybridization, an 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 [α-^{32}P]-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 sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA)-Na$_2$, and 100 mg/L of heat-denatured salmon sperm DNA. Final washes of the membranes were carried out in a solution containing 0.1× standard saline citrate (SSC) and 0.1% SDS, 65 °C for 30 min.

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