Precise marker excision system using an animal-derived piggyBac transposon in plants

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SUMMARY

Accurate and effective positive marker excision is indispensable for the introduction of desired mutations into the plant genome via gene targeting (GT) using a positive/negative counter selection system. In mammals, the moth-derived piggyBac transposon system has been exploited successfully to eliminate a selectable marker from a GT locus without leaving a footprint. Here, we present evidence that the piggyBac transposon also functions in plant cells. To demonstrate the use of the piggyBac transposon for effective marker excision in plants, we designed a transposition assay system that allows the piggyBac transposition to be visualized as emerald luciferase (Eluc) luminescence in rice cells. The Eluc signal derived from piggyBac excision was observed in hyperactive piggyBac transposase-expressing rice calli. Polymerase chain reaction, Southern blot analyses and sequencing revealed the efficient and precise transposition of piggyBac in these calli. Furthermore, we have demonstrated the excision of a selection marker from a reporter locus in T0 plants without concomitant re-integration of the transposon and at a high frequency (44.0% of excision events), even in the absence of negative selection.

Keywords: Marker excision, piggyBac, rice, transposon, precision genome engineering, marker-free plant, technical advance.

INTRODUCTION

Gene targeting (GT) is a relatively new tool that is available for plant genome engineering (Paszkowski et al., 1988; Lee et al., 1990; Puchta, 2002; Terada et al., 2002; Endo et al., 2007; Yamauchi et al., 2009; Saika et al., 2011; Moritoh et al., 2012; Ono et al., 2012). We have succeeded in the introduction of targeted point mutations into the rice genome via GT (Endo et al., 2007; Saika et al., 2011); herbicide-tolerant and high-tryptophan rice plants were generated by changing the amino acids in acetolactate synthase (ALS) and the α-subunit of anthranilate synthetase (OASA2), respectively, via GT (Endo et al., 2007; Saika et al., 2011). GT rice plants that harbor these mutations acquired herbicide BS resistance and tolerance to the tryptophan analog BMT, and allowed easy selection of GT cells on medium that contained BS or 5MT, respectively. Conversely, positive/negative selection has been used in most cases to isolate rare clones that have undergone targeted gene replacement by GT (Yamauchi et al., 2009; Moritoh et al., 2012; Ono et al., 2012), as this system allows the modification of any gene of interest. However, the positive selectable marker gene should be excluded completely from the target locus to produce mutants in which only the desired mutations are introduced into the target gene.

To date, several methods, such as co-transformation, transposition, and site-specific recombination, have been employed successfully to remove selectable marker genes from transgenic plants (reviewed in Hohn et al., 2001; Darbani et al., 2007; Woo et al., 2011). Among several transposable elements, the maize Ac/Ds transposon system is used widely; this system is reported to be capable of producing maker-free transgenic rice, tomato, tobacco and aspen plants (Goldsbrough et al., 1993; Ebinuma et al., 1995).
1997; Cotsaftis et al., 2002). However, a major problem when using a transposable element system for the elimination of marker genes is the residual footprint at the excised site and the low transposition activity in heterologous hosts. Furthermore, the efficiency of marker-free transgenic plant generation using a transposable element system has been demonstrated to be low, because of the tendency for the transposon to reinsert elsewhere in the genome (Goldsborough et al., 1993; Ebinuma et al., 1997; Cotsaftis et al., 2002).

Marker excision systems using site-specific recombination that include the Cre/loxp system from bacteriophage P1 and the FLP/FRT system from Saccharomyces cerevisiae, have been well studied in plants (reviewed by Wang et al., 2011; Woo et al., 2011). A large number of studies has demonstrated that site-specific recombination methods allow the generation of marker-free plants at a high frequency in model plants and crop species (Woo et al., 2011). Furthermore, Terada et al. (2010) succeeded in Cre/loxp-mediated marker elimination from a GT locus. However, marker excision using site-specific recombination leaves dispensable sequences such as the recognition sequences of recombinase at the excised site. In mammalian cells, it has been reported that the recognition sequences of recombinase have the potential to affect adjacent gene expression (Meier et al., 2010). Therefore, the development of an efficient and accurate marker excision system is an indispensable part of the production of desired point mutation plants via GT.

The piggyBac transposon derived from the lepidopteran cabbage looper moth Trichoplusia ni (Cary et al., 1989) integrates into the host genome at TTAA elements and has been used for transgenesis and insertional mutagenesis in a variety of organisms that include Drosophila melanogaster (Horn et al., 2003), chicken (Lu et al., 2009), mouse (Ding et al., 2005), and human (Wilson et al., 2007). Additionally, the piggyBac transposon excises without leaving a footprint at the excised site TTAA elements (Cary et al., 1989). Recently, Yusa et al. (2011b) have shown that the piggyBac transposon system enables excision of a selectable marker from a GT locus in the host genome without residual ectopic sequences in mammalian cells.

We have developed a stable and efficient Agrobacterium-mediated transformation system for rice (Toki et al., 2006), and have recently established an efficient sequential monitoring system for stable transformation by visualization of cells using a non-destructive and highly sensitive visible marker [click beetle luciferase (Eluc)] in rice (Saika et al., 2012). Using a stable and efficient transformation system and an effective sequential monitoring system for stable transformation of rice, we present evidence that the animal-derived piggyBac transposon is capable of accurate and effective transposase-mediated transposition in plant cells, and suggest that a high-frequency marker excision system for plant genomes could be established using the piggyBac transposon system.

RESULTS AND DISCUSSION

High-frequency transposase-mediated transposition of the animal-derived piggyBac transposon in plant cells

To test the use of the piggyBac transposon for effective marker excision in plants, we designed two assay systems in rice cells (Figure 1). These systems allow the transposition of piggyBac transposon to be visualized as luminescence derived from reconstituted luciferase expression cassettes. The reporter constructs pBL1 and pBL2 (Figure 1) carry a rice elongation factor-1α promoter:emerald luciferase (Eluc) cassette that contains the piggyBac transposon that harbors a rice actin terminator and a mutant cytosine deaminase (codA) gene [D314A, mutcodA, (Mahan et al., 2004)] expression cassette as a negative selection marker in the Eluc gene or in the intron in the 5' untranslated region (5' UTR), respectively. A codA gene converts the non-toxic 5-fluorocytosine (5-FC) into the toxic 5-fluorouracil (5-FU) (Perera et al., 1993). Transgenic rice calli with pBL1 or pBL2 are Eluc negative and 5-FC sensitive. Using pBL1, if the piggyBac transposon is excised

![Figure 1. Schematic representation of excision assay to detect transposition of piggyBac transposon as luciferase luminescence in rice calli.](image)

(a) The pBL1 reporter constructs carry an Eluc expression cassette that contains a rice actin terminator and a mutcodA expression cassette as a negative selection marker in the Eluc gene. Transgenic rice calli with pBL1 are Eluc negative (−) and 5-FC sensitive (S). Upon precise transposon excision from the reporter locus, transgenic calli become Eluc positive (+) and 5-FC resistant (R). However, if the piggyBac excision leaves a footprint, the Eluc gene is inactivated and the calli remain Eluc negative.

(b) The pBL2 reporter constructs carry an Eluc expression cassette that contains a rice actin terminator and a mutcodA expression cassette as a negative selection marker in the 5' UTR intron. Transgenic rice calli with pBL2 are Eluc negative (−) and 5-FC sensitive (S). The piggyBac-excised calli become Eluc positive and 5-FC resistant, regardless of whether the piggyBac excision leaves a footprint or not. LB, left border; RB, right border.
precisely by the activation of transposase, the *Eluc* gene is restored and the calli become *Eluc* positive and 5-FC resistant. However, if the *piggyBac* excision leaves a footprint, the *Eluc* gene is inactivated and the calli remain *Eluc* negative (Figure 1a). Using pBL2, the *piggyBac*-excised calli become *Eluc* positive and 5-FC resistant regardless of whether the *piggyBac* excision leaves a footprint or not (Figure 1b).

Seven-day-old rice calli were infected with *Agrobacterium* that harbored pBL1 or pBL2 and selected against hygromycin sulfate for 4 weeks (Figure 2). To evaluate the frequency of *piggyBac* transposition and the effect of 5-FC negative selection, transgenic lines with a single copy of the reporter constructs (pBL1-5, pBL1-10, pBL2-3 or pBL2-9) were selected by Southern blot analysis using a mutcodA probe (Figures 3a,b, S1(A,B), S2(A,B) and S3(A,B)), and were transferred to N6D medium without the antibiotic meropenem, which kills *Agrobacterium*. If the transgenic lines carried the multi copy of reporter constructs, the transposon excision from one locus, but not other locus, did not confer 5-FC-resistance on the transgenic calli or plants. After 4 weeks of cultivation, transgenic lines pBL1-5, pBL1-10, pBL2-3 and pBL2-9 calli were transformed with *Agrobacterium* to introduce the control vectors (pBL1-5_c, pBL1-10_c, pBL2-3_c or pBL2-9_c) or an expression vector that encoded either insect *piggyBac* transposase (ePBase, [Tamura et al., 2000]) (pBL1-5_e, pBL1-10_e, pBL2-3_e and pBL2-9_e) or hyperactive *piggyBac* transposase, which carries seven amino acid substitutions (I30V, S103P, G165S, M282V, S509G, N570S, and N538K) (hyPBase, [Yusa et al., 2011a]) (pBL1-5_hy, pBL1-10_hy, pBL2-3_hy and pBL2-9_hy) under the control of the maize polyubiquitin 1 promoter, and selected with or without 5-FC (Figure 2). After 4 weeks of selection, *Eluc* luminescence was detected on hyPBase and ePBase transgenic rice calli but not on control calli. In addition, the *Eluc* signal observed on hyPBase rice calli was significantly higher than that on ePBase-expressing rice calli, which indicated that the frequency of hyPBase-inducible transposition was higher than that of ePBase-inducible transposition (Figures 3c, S1(C), S2(C) and S3(C)). However, no significant differences in *Eluc* luminescence were seen between transgenic rice calli grown on N6D with and without 5-FC (Figures 3c, S1(C), S2(C) and S3(C)). Furthermore, the *Eluc* luminescence detected in pBL1-5 and pBL1-10 transgenic rice calli was comparable with that of pBL2-3 and pBL2-9 transgenic rice calli (Figures 3c, S1(C), S2(C) and S3(C)), which suggested that the *piggyBac* transposon was excised precisely from the *Eluc* expression cassette via the activation of PBase. Thus, pBL1 transgenic rice calli were used for further analysis.

**Efficient and precise excision of *piggyBac* from the reporter locus**

To confirm the *Eluc* expression results, polymerase chain reaction (PCR) and Southern blot analysis were performed with genomic DNA extracted from three independent chimeric calli with *Eluc*-negative and *Eluc*-positive pBL1-5_e and pBL1-5_hy, with or without 5-FC treatment. Using PCR analysis, a 5.9-kb band that was specific to the original pBL1 vector was detected by the primers illustrated in Figure 4(a) in all transgenic lines (Figure 4b). A 1.65-kb band from the *piggyBac*-excised fragment was detected in pBL1-5_hy transgenic calli regardless of 5-FC treatment, but was observed only in pBL1-5_e transgenic calli that had been treated with 5-FC (Figure 4b). Southern blot analysis with a specific mutcodA probe revealed 8.2-kb fragments derived from the full-length reporter construct.

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**Figure 2.** Experimental scheme for *piggyBac* excision in rice. Transgenic lines with a single copy of reporter constructs were selected by Southern blot analysis, and were transformed with *Agrobacterium* to introduce the control vector or expression vector of ePBase or hyPBase under the control of the maize polyubiquitin 1 (Ubi-1) promoter. After a 4-week selection period, *Eluc* luminescence was detected on hyPBase- and ePBase-expressing rice calli. *Eluc*-positive calli derived from hyPBase-expressing calli were transferred to shoot regeneration medium with or without 5-FC. These plants were subjected to marker excision analysis.
in all transgenic lines (Figure 4c,d). In addition to 8.2-kb fragments, 3.9-kb fragments expected from the piggyBac-excised reporter cassette were detected using the 3′-flanking region of the Eluc gene probes in pBL1-5_hy transgenic calli treated with or without 5-FC (Figure 4c,e). Only faint 3.9-kb fragments were observed in pBL1-5_e transgenic calli even in the presence of 5-FC selection (Figure 4e), while no 3.9-kb fragments were detected from pBL1-5_c (Figure 4e). Similarly, the full-length reporter-specific fragments were recognized in pBL1-10 all transgenic lines, while, the piggyBac-excised reporter-specific fragments were detected in pBL1-10_hy transgenic calli but not pBL1-10_c and pBL1-10_e transgenic calli (Figure S4).

The hyperactive mutant of PBase that contained seven amino acid substitutions has been reported to show a 17-fold increase in excision activity, and increased protein expression compared with wild-type PBase in mouse embryonic stem (ES) cells (Yusa et al., 2011a). Therefore, our results also might be explained by high levels of hyPBase protein expression under the control of the maize polyubiquitin 1 promoter, although quantitative RT-PCR analysis revealed that transcript levels of the hyPBase and ePBase genes in pBL1-5_hy and pBL1-5_e transgenic rice calli were comparable (Figure 4f).

In mouse ES and in induced pluripotent stem (iPS) cells, piggyBac-excision-derived footprints were detected in 5% (Wang et al., 2008), 9% (Woltjen et al., 2009) or 0.8% (Yusa et al., 2011a) of PBase-mediated excision events. No difference in footprint frequency between hyPBase- and insect PBase-induced piggyBac transposition was seen in mouse ES cells (Yusa et al., 2011a). To determine if the piggyBac transposon leaves a footprint at the excised site in the rice genome, we analyzed the sequence of the 1.65-kb PCR products; 20 clones from each transgenic line of pBL1-5_e, pBL1-5_hy, pBL1-10_e and pBL1-10_hy were sequenced; a TTAA element was restored after transposon removal in all cases (Figure 4g). These results indicated that rice cells are just as conducive as animal cells to piggyBac transposition. In mammalian cells, the piggyBac transposon system allows efficient excision of a selectable marker from a GT locus in the host genome without changing any nucleotide sequence (Yusa et al., 2011b). Thus, the development of improved marker excision strategies using piggyBac to eliminate selectable marker genes from GT loci seems a promising strategy.

High-frequency marker excision via piggyBac transposition in T1 plants

Eluc-positive calli derived from pBL1-5_hy and pBL1-10_hy transgenic calli were transferred to shoot regeneration medium with or without 5-FC. Ten plants from pBL1-5_hy-3 line and 20–30 plants from pBL1-10_hy-2, -3 and -4 were regenerated under 5-FC selection, while 20–40 plants from pBL1-5_hy-1–7 and pBL1-10_hy-1–4 were regenerated without 5-FC selection, respectively. PCR analysis using genomic DNA extracted from leaves of regenerated plants showed that a 1.65-kb band from the piggyBac-excised fragment, but not the original pBL1 vector-specific 5.9-kb fragment, was detected in 100 and 75.0% of pBL1-5_hy and pBL1-10_hy 5-FC-resistant regenerated plants, respectively.
Figure 4. Molecular analysis of hyPBase- or ePBase-induced piggyBac excision in rice calli. 
(a, c) Structure of the original pBL1 vector and piggyBac-free reporter locus. Arrowheads represent PCR primers. Bars represent DNA probe fragments used for Southern blot analysis.
(b) PCR analysis of genomic DNA isolated from wild-type (Wt), control (pBL1-5_c), hyPBase (pBL1-5_hy) and ePBase (pBL1-5_e) transgenic calli treated with (+) or without (−) 5-FC. Bands of 1.65 kb (open arrowhead) that resulted from piggyBac excision are detected in addition to the original 5.9 kb pBL1 bands (filled arrowhead). Primer pairs are as shown in (a).
(d, e) Southern blot analysis using a specific codA (D) or 3′-flanking region of Eluc gene probe (e) in wild-type (Wt), control (pBL1-5_c), hyPBase (pBL1-5_hy) and ePBase (pBL1-5_e) transgenic calli treated with (+) or without (−) 5-FC. When digested with AscI/Paci, bands of 3.9 kb (open arrowhead) derived from the Eluc gene restored by piggyBac transposition were detected in addition to the original 8.2 kb pBL1 bands (filled arrowhead).
(f) Expression of hyPBase (left) and ePBase (right) in wild-type (Wt), control (C), hyPBase (pBL1-5_hy) and ePBase (pBL1-5_e) transgenic calli treated with (+) or without (−) 5-FC. Relative transcript levels were normalized to OsAct1 mRNA. Error bars represent ± standard deviation (SD) of three individual experiments.
(g) Nucleotide sequences of the original pBL1 (top) and piggyBac excision site in the Eluc reporter cassette (bottom) in hyPBase and ePBase transgenic rice calli. Open boxes highlight the duplicated TTAA sequence that flank the piggyBac transposon.

A higher proportion of T₀ plantlets (on average, 71.7 and 91.0%; range, 44.4–90.0 and 80.0–96.0% of pBL1-5_hy and pBL1-10_hy, respectively, Table 1 and SII, and Figure S5). A higher proportion of T₀ plantlets (on average, 71.7 and 91.0%; range, 44.4–90.0 and 80.0–96.0% of pBL1-5_hy and pBL1-10_hy, respectively, (Tables 1 and SII, and Figure S5)). A higher proportion of T₀ plantlets (on average, 71.7 and 91.0%; range, 44.4–90.0 and 80.0–96.0% of pBL1-5_hy and pBL1-10_hy, respectively, Table 2 and SII) contained the 1.65-kb band and lacked the 5.9-kb fragment without 5-FC selection at the time of regeneration, which indicated efficient marker excision in

Table 1 PCR analysis of piggyBac excision events with 5-FC treatment in pBL1-5_hy-expressing T₀ plants

| Line no. | No. of T₀ plants analyzed | piggyBac excision from reporter locus | Frequency of piggyBac excision |
|----------|---------------------------|--------------------------------------|-------------------------------|
|          |                           | Without CodA | With CodA | Total | Without re-integration | With re-integration | Total |
| 3        | 10                        | 6           | 4          | 10    | 60.0                  | 40.0                | 100   |
hyPBase transgenic plants at the callus stage. However, 40.0 and 20.6% of pBL1-5_hy and pBL1-10_hy 5-FC-resistant regenerated plants and 41.0 and 9.0% of pBL1-5_hy and pBL1-10_hy regenerated plants treated without 5-FC, respectively, were shown to harbor a re-integrated piggyBac transposon, as a mutcodA-specific fragment was detected by PCR analysis (Figure S5). Namely, the re-integration frequency of excised piggyBac was 40.0 and 25.4% in pBL1-5_hy and pBL1-10_hy regenerated plants treated with 5-FC and 56.0 and 9.9% in pBL1-5_hy and pBL1-10_hy regenerated plants without 5-FC, respectively. These results suggested that 5-FC negative selection neither enriched piggyBac-excised transgenic rice cells nor suppressed re-integration of the piggyBac. The negative selection by codA might be not stringent or not sufficient for facilitation of the growth of piggyBac-excised rice cells.

Furthermore, we performed Southern blot analysis with genomic DNA extracted from each three T0 plants of three independent lines with pBL1-5_hy (line nos. 2, 4 and 5), which contained the piggyBac-excised fragment and lacked the original pBL1 vector-specific fragment and the mutcodA-specific fragment as far as analyzed by PCR. In all analyzed T0 plants, a fragment derived from the piggyBac-excised reporter cassette was detected using the 3'-flanking region of the Eluc gene probes (Figure 5a,c), whilst the full-length reporter construct and the re-integrated piggyBac transposon were absent using the 3'-flanking region of the Eluc gene probes and a specific mutcodA probe, respectively (Figure 5a,b). However, the fragment derived from full-length reporter construct was only detected in the pBL1-5_c T0 plant. Self-pollinating T1 progeny of these pBL1-5_hy T0 plants were obtained for further analyses.

It has been reported that 60% of piggyBac excision events are not accompanied by re-integration of the transposon in mouse ES cells without negative selection (Wang et al., 2008). In addition, previous studies have demonstrated that piggyBac preferentially re-integrates close to the excision site (Wang et al., 2008; Li et al., 2013a), a finding that suggested that it might be difficult to segregate re-integrated piggyBac transposons from the donor site in T1 progeny. Recently, Li et al. (2013b) have exploited an excision-competent/integration-defective PBase. Thus, negative selection may not be absolutely required for the generation of marker-free rice plants using the piggyBac transposon system. Accordingly, our results suggested that the piggyBac transposon system could be used widely to eliminate the selectable marker in several plant species, regardless of whether the negative selection via 5-FC was effective or not.

Table 2  PCR analysis of piggyBac excision events without 5-FC treatment in pBL1-5_hy-expressing T0 plants

| Line no. | No. of T0 plants analyzed | piggyBac excision from reporter locus | Frequency of piggyBac excision |
|---------|-------------------------|--------------------------------------|-------------------------------|
|         |                         | Without CodA | With CodA | Total            | Without re-integration | With re-integration | Total |
| 1       | 36                      | 4            | 12         | 16               | 11.1                   | 33.3                | 44.4  |
| 2       | 40                      | 12           | 22         | 34               | 30.0                   | 55.0                | 85.0  |
| 3       | 20                      | 3            | 14         | 17               | 15.0                   | 70.0                | 85.0  |
| 4       | 30                      | 24           | 3          | 27               | 80.0                   | 10.0                | 90.0  |
| 5       | 21                      | 4            | 10         | 14               | 19.0                   | 47.6                | 66.7  |
| 6       | 20                      | 5            | 6          | 11               | 25.0                   | 30.0                | 55.0  |
| 7       | 29                      | 10           | 12         | 22               | 34.5                   | 41.4                | 75.9  |
| Average |                         | 30.7         | 41.0       | 71.7             |                        |                     |       |

Figure 5. Southern blot analysis of the reporter locus in pBL1-5_hy T0 plants. Southern blot analysis with genomic DNA extracted from pBL1-5 and each three T0 plants of three independent lines with pBL1-5_hy (line nos. 2, 4 and 5) was performed using a specific codA (a) or 3'-flanking region of the Eluc gene probe (b) shown in Figure 4(c). Filled and open arrowheads represent the original 5.9 kb pBL1 bands and 1.65 kb bands derived from the piggyBac-excised reporter cassette, respectively.
species. In addition, many transposons, including Drosophila P elements, are non-functional in heterologous species, a finding that suggests that host factors are needed for transposon activity (Handler et al., 1993). However, there have been multiple reports of successful transposition of the moth-derived piggyBac transposon in heterologous animal species (Horn et al., 2003; Ding et al., 2005; Wilson et al., 2007; Lu et al., 2009). Sarkar et al. (2003) reported the presence of many piggyBac-like sequences in the genomes of a phylogenetically diverse range of organisms that included fungi, plants, insects, crustaceans, urochordates, amphibians, fishes and mammals. In this report, we have shown that piggyBac transposons can also transpose effectively and accurately in rice cells, a finding that suggested that host factors or additional regulatory factors might be widely conserved in many organisms or, alternatively, are not required for piggyBac transposition in rice cells.

The production of marker-free transgenic plants in T1 progeny

PCR analysis was performed with genomic DNA extracted from 34 T1 progeny from pBL1-5_hy-4 to investigate further whether marker-free plants were obtained in T1 progeny. Twenty-one T1 progeny were each found to contain a 1.65-kb band from the piggyBac-excised fragment, but not from the original pBL1 vector-specific 5.9-kb fragment and a mutcodA-specific fragment. In addition, a hyPBase-specific fragment was absent from 11 T1 progeny, a finding that indicated that eight out of the 34 T1 progeny were marker-free transgenic plants that contained piggyBac-excised reporter cassettes and lacked hyPBase expression cassettes.

Furthermore, two (line nos. 1 and 2) and six plants (line nos. 3–8) of pBL1-5_hy-4 T1 progeny with or without the hyPBase expression construct, respectively, were selected and were subjected to Southern blot analysis. Southern blot analysis with the 3′-flanking region of the Eluc gene probes exhibited the presence of 3.9-kb piggyBac-excised reporter fragments in the pBL1-5_hy-4 T0 plant and in all pBL1-5_hy-4 T1 progeny, but not pBL1-5_c T0 plants, and showed the full-length reporter construct only in pBL1-5_c T0 plants (Figure 6a–c). Furthermore, no additional fragment derived from a re-integrated piggyBac transposon was observed in all transgenic plants (Figure 6b). In agreement with the results of the PCR, the lack of hyPBase expression constructs was seen in pBL1-5_hy-4 T1 progeny (line nos. 3–8, Figure 6e). These results suggested that marker-free transgenic progeny that lack the PBase expression cassette can be obtained via segregation by crossing.

Ebinuma et al. (1997) reported the production of marker-free transgenic tobacco and aspen plants using the multi-autotransformation (MAT) vector system, in which the selectable marker is composed of a chimeric isopentenyl transferase (ipt) gene inserted into the maize transposable element Ac, although the frequency of marker-free transgenic tobacco plants was about 100-fold lower compared with that of hyPBase-mediated marker excision in this study. About 10% of Ac elements excised from the transgenic tomato genome without re-integration, or re-integrated into a sister chromatid that was subsequently lost by somatic segregation (Belzile et al., 1989), whilst 60 or 44% of piggyBac were shown to be lost from the genome after excision in mouse ES cells and rice cells (this study),

![Figure 6](image_url)

**Figure 6.** Analysis of piggyBac excision and re-integration in T1 progeny.

(a) Structure of the original pBL1 vector and piggyBac-free reporter locus. Bars represent DNA probe fragments used for Southern blot analysis.

(b), (c) Southern blot analysis using a specific codA (b) or 3′-flanking region of Eluc gene probe (c) in wild-type (Wt), T0 regenerated plants that carry pBL1 (pBL1-5) or hyPBase-expressing vector (pBL1-5_hy-4); T1 progeny of pBL1-5_hy-4 (pBL1-5_hy-4 T1).

(d) Structure of hyPBase-expressing vector. Bars represent DNA probe fragments used for Southern blot analysis.

(e) Southern blot analysis using a specific hyPBase gene probe shown in (d) and 2 μg of genomic DNA extracted from wild-type (Wt), T0 regenerated plants that carry pBL1 (pBL1-5) or hyPBase-expressing vector (pBL1-5_hy-4), T1 progeny of pBL1-5_hy-4 (pBL1-5_hy-4 T1) and digested with SpeI.
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EXPERIMENTAL PROCEDURES

Vector construction

The pBL1 reporter vector (Figure 1a) was constructed as follows: (i) The Eluc cDNA was amplified by PCR from pEluc-test (TOYO-OBO) using primer sets Eluc-F/Eluc-R and cloned into the NcoI/blunt end site between the rice elongation factor-1α promoter (Pef) and the transcription terminator of the rice heat shock protein 16.9b gene (Thsp16.9b) in pENTR L1-L2 (Life Technologies), yielding pE(L1-L2)Pef:ELUC:Thsp16.9b. (ii) A 680-bp artificially synthesized fragment that contained the piggyBac inverted-repeat transposable element (IVR) and multi-cloning site (Hpal-IVR-Avrl-Aartl-Ivr-Hpal) was cloned into the Hpal site (GTGAAAC to GTTAAAC) introduced by site-directed mutagenesis with the Quick change mutagenesis kit (Agilent Technologies) using the primers 5′-gtcgcgccgtctggttcttcaggacctc-3′ and 5′-gtacagatgcctgaggatggac-3′. (iii) The 8.4-kb fragment that contained the rice actin terminator (Tact), cauliflower mosaic virus 35S (CaMV35S) promoter (P35S), a mutant codA gene and the Agrobacterium nopaline synthase (NOS) terminator (Tnos) was digested with Avrl/Aarfl and integrated into pE(L1-L2)Pef:ELUC:Thsp16.9b, yielding pE(L1-L2)Pef:ELUC:Thsp16.9b. (v) The 8.4-kb fragment that contained PefpCodAb:ELUC:Thsp16.9b was re-cloned into the pZD202Hyg vector using a Gateway LR cloning reaction (Life Technologies).

Expression vectors for hygBase and ePBase were constructed as follows: (i) A 4.7-kb fragment that contained the maize poly-ubiquitin1 promoter + hygBase or ePBase + the Arabidopsis ribulose-bisphosphate carboxylase small subunit gene terminator (TrbcS) was cloned into the HindIII/Pacl site in pZPP200MCS [a derivative of pZPP200 (Hajdukiewicz et al., 1994), with the addition of restriction sites (PacI-Ascl-SnaBI) to the multi-cloning site of pZPP200], yielding pPZP/Pubi:hygBase:TrbcS or pPZP/Pubi:ePBase:TrbcS. (ii) A kanamycin resistance (nptII) gene cassette that harbored the open reading frame (ORF) under the control of the rice actin promoter (Pact) and the Arabidopsis poly-A-binding protein terminator, was digested with Ascl/SnaBI and cloned into pPZP/Pubi:hygBase:TrbcS or pPZP/Pubi:ePBase:TrbcS, yielding pPZP/hyPBase and pPZP/ePBase, respectively. (iii) For construction of the control vector, a cassette of P35S:nptII was cloned into the pENTR L1-L4 (Life Technologies), Pact:GFP:Tact in pENTR R4-R3 (Life Technologies), P2x3SS:HT:Tnos in pENTR L3-L2 (Life Technologies) was combined and cloned into the pZD202 vector (Kwon et al., 2012) using Multisite-gateway Pro (Life Technologies) in accordance with the manufacturer’s protocol (Life Technologies).

Agrobacterium-mediated transformation

The binary vectors described above were transferred into Agrobacterium tumefaciens strain EHA105 (Hood et al., 1993) by electroporation. Agrobacterium-mediated transformation of rice (O. sativa L. cv. Nipponbare) was performed as described previously (Toki, 1997; Toki et al., 2006).

Genomic DNA extraction and Southern blot analysis

Genomic DNA was extracted from rice calli using the NucleoSpin Plant II kit (Macherey-Nagel) in accordance with the manufacturer’s protocol. Firstly, 2 μg of genomic DNA was digested with EcoRI or Ascl/Pacl or SphI and fractionated in a 1.0% agarose gel. Southern blot analysis was performed in accordance with a standard protocol. Specific DNA probes for the mutcodA gene and the piggyBac IVR fragment were generated by PCR amplification of genomic DNA, using the primers 5′-gtcgctgactccgtgactctgattc3′ and 5′-gtgaggctgagagaggctc-3′. PCR amplification

Genomic DNA was extracted from rice calli using the NucleoSpin Plant II kit (Macherey-Nagel) in accordance with the manufacturer’s protocol. PCR amplifications were performed with the PrimeSTAR GXL DNA polymerase (TaKaRa) using the primer sets as follows: (i) for analysis of piggyBac excision, PeF-F 417 (5′-aactatatagaccggtgcaaagtg-3′) and codA D314A-R 435 (5′-tctagaatggtgtcgaataacgc-3′); and (ii) for analysis of piggyBac re-integration, codA D314A-F Xbal (5′-ttactatatagaccggtgcaaagtg-3′) and codA D314A-R BamHI (5′-gctggtctgccgcttgattc3′).

Observation of LUC luminescence

Rice calli were treated with 0.2 mM Beetle luciferin potassium salt (Promega). LUC luminescence images were taken using a high resolution photon counting camera (C2400-700 VIM camera,
Hamamatsu Photonics, Japan) with a 10-min exposure time, and processed using Aquacosmos (Hamamatsu Photonics).

**RNA extraction and quantitative RT-PCR analysis**

Total RNA was extracted from calli and seedlings of rice calli using an Qiagen Plant Mini Kit (QIAGEN). Quantitative reverse transcription (qRT)-PCR was performed with a Power SYBR Green PCR Master Mix (Life Technologies) and an ABI7300 (Life Technologies) in accordance with the manufacturers’ protocols. Primer pairs for qRT-PCR were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and are as follows: OsaAct1 (5'-ctctggctcagatgagca-3' and 5'-gggccgaccacagtctt-3'); hyPBase (5'-gtgtgatctgctagcagaaaaa-3' and 5'-gcctacgtggctgctgtaa-3'); and ePBase (5'-tttttgagcagccccctaa-3' and 5'-tgggcttaccggtactttc-3').

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Analysis of hyPBase- or ePBase-induced piggyBac excision from pBL1-10 reporter locus in transgenic rice calli.

**Figure S2.** Analysis of hyPBase- or ePBase-induced piggyBac excision from pBL2-3 reporter locus in transgenic rice calli.

**Figure S3.** Analysis of hyPBase- or ePBase-induced piggyBac excision from pBL2-9 reporter locus in transgenic rice calli.

**Table S1.** PCR analysis of piggyBac excision events with 5-FC treatment in T2 plants.

**Table S2.** PCR analysis of piggyBac excision events without 5-FC treatment in pBL1-10 hy expressing T2 plants.

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