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Full Length Research Paper

The use of hygromycin phosphotransferase gene (*hpt)* with an artificial intron to obtain marker-off transgenic plants

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Since maize transposon *Ac* can move to a new location within genome, it has been used in removing selectable markers in transgenic plants. Previously, we have developed a marker-off system to truncate a selec marker in transgenic plants by locating one end of the transposon in the intron of the marker gene (glyphosate-tolerant *epsps* gene). In order to expand this technique to those marker genes without intron in this report, we created an artificial intron containing one end of the transposon to achieve marker-off. The hygromycin phosphotransferase gene (*hpt*) from *E. coli* which has been proved to be very effective marker for plant transformation was used in this study. The artificial intron which contains a 250 bp *Ac* 5' end and partial sequences of the first intron of rice *epsps* gene was introduced into the intronless *hpt* gene. This modified marker gene was flanked by *Ac* 3' end and transposase gene, which is under the control of the inducible promoter (PR-1a), yielding the new marker-off transposon, *KH*. The behavior of *KH* was analyzed in transgenic rice and tobacco. We determined the expression of the modified *hpt* gene and the transposition events in transgenic plants. The *KH* element thus exhibits more applications to create marker-off transgenic plants.

Key words: *Ac* transposase, inducible transposon, selectable marker, transgenic plants.

INTRODUCTION

Genetic modification of plants offers improvement in agricultural practices, food safety, and human health. In current plant transformation systems, a selectable marker gene is co-delivered with the gene of interest (GOI) to identify and separate rare transgenic cells from non-transgenic cells. However, marker genes usually are not needed once transgenic plants have been identified. The presence of marker genes, especially antibiotic markers and herbicide resistance markers in transgenic crop plants may elicit envi ronmental and consumer concerns. The development of marker-free transgenic plants is desirable in agricultural biotechnology. Many strategies to produce marker-free transgenic plants have been described, including co-transformation, cre/lox and transposon systems (Ebinuma et al., 2001; Hare and Chua, 2002; Hohn et al., 2001; Miki and McHugh, 2004). Among these, the transposon system (example *Ac*/*Ds*) offers the advantage that information about the new location of the removed marker's DNA for commercial regulatory approval.

The maize transposon *Ac* is an autonomous transposable element of 4565 bp and is active in a wide range of plant species. It codes for a single gene product, *Ac* transposase, which, together with the inverted repeats and about 250 bp of both ends (terminal regions) of the transposon and putative host factors, is the only prerequisite for transposition of the *Ac* element in plants (Haring et al., 1991). In the transformation vector, the marker gene is inserted into the *Ds* element. The expression of the *Ac* transposase excises both end of the transposon and usually re-integrate into other locations on

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Abbreviations: *hpt*, Hygromycin phosphotransferase gene; ***epsps*,** 5-enolpyruvylshikimate-3-phosphate synthase gene; **GOI,** gene of interest; ***TPase*,** transposase gene; **SA,** salicylic acid.

the chromosome. When the transposon transposes within the same chromosome (linked transposition), both insertion sites of the T-DNA (harboring the marker gene) and the transposon (harboring the GOI) need regulatory approval for commercialization. With unlinked transposition, the marker gene can be removed by out-crossing. Although the work is time consuming, all removed information remains clear for regulatory approval. Furthermore, with the transposon system, one successful transformation can create more independent transgenic lines because of the re-integrated loci. This feature is valuable to create transgenic plants in species with low transformation efficiency. However, out-crossing with this system cannot be used with vegetatively propagated plants and woody tree species. According to this, we have designed a system to truncate a marker gene after transposition, termed "marker-off," which respects the marker-free system and does not require segregating the marker away (Charng et al., 2008). The system involves introducing an intron-containing marker gene (glyphosate tolerant *epsps* gene), accompanied by a transposon whose one end is located in the intron of the marker gene, into a transformation vector. The new construct, termed as KCEH, contains the *Ac*-based inducible transposon *KCEH* and the glyphosate-tolerant marker gene containing one end of *KCEH* in its first intron. When KCEH was transformed into rice calli, the marker gene acted glyphosate resistant to yield transgenic rice. Subsequently, the *KCEH* in the transgenic rice was induced to transpose, which truncated the marker gene, and consequently the transgenic plants became marker-off. In this report, we expand this technique to those marker genes without intron. This was achieved by inserting an artificial intron containing one end of transposon into the *hpt* gene, which is the most frequently used marker gene in monocots and dicots. The new marker-off system, KH, was introduced into rice and tobacco plants. After obtaining successful transgenic plants (transgenesis), we studied the expression of the modified *hpt* gene in transgenic plants. Finally, we induced transposition events to terminate the hygromycin-tolerance ability in transgenic rice.

MATERIALS AND METHODS

DNA manipulation and plant transformation

The construction procedures for the KH system are depicted in supplementary material. All cloning and DNA manipulations followed standard procedures (Sambrook and Russel, 2001) with the use of chemicals from Roche (Basel, Switzerland). All transformations involved the use of rice (*Oryza sativa* L. cv TNG67) and tobacco (*Nicotiana tabacum* cv. W38) as described previously (Toki, 1997; Charng et al., 2004).

PCR analysis for successful transgenic plants and *hpt* gene expression

Existence of *KH* in transgenic plants was analyzed by PCR with oligonucleotide primers: Primer TF (5'-TATCATGGTGGAGGGGAAGG -3') and primer TR (5'-CTCATGGAGAGGAGCC -3'). RNA extracted from transgenic rice or tobacco was reverse transcribed with the use of SuperScript™ First Strand Synthesis System (Invitrogen). For RT-PCR analysis, primers specific to the *hpt* target sequences were used: HF (5'-GGCACTTTGCATCGGCCGCG -3') and HR3 (5'-CATCGACCCTGCGCCCAAGCTGC -3').

Induction of *KH* transposition

For experiments inducing *KH* in transgenic rice, the T1 rice seeds of each transformed line were incubated on callus induction medium containing hygromycin for 4 weeks to yield enough calli. Hyg^R calli were incubated with 1 mM salicylic acid (SA) for 7 days, then transferred to callus induction medium without SA for 4 to 8 weeks before PCR analysis. To induce the KH transposition in transgenic tobacco, Hyg^R calli of an average diameter of 1 cm were incubated on callus regeneration medium containing 1 mM SA for 3 days and then transferred to callus regeneration medium without SA for 4 weeks before transposition analysis.

Isolation of genomic DNA and southern blot analysis

Genomic DNA was isolated from transformed plants by use of a kit (Genemark, Tainan, Taiwan). In brief, fresh leaves (2 g) or callus tissue (0.1 g) was frozen in liquid nitrogen and ground with the use of a mortar and pestle. Nuclei were isolated and lysed by protease treatment, and genomic DNA was precipitated with ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). About 10 μg of each rice DNA (or 30 μg for tobacco) was digested with the appropriate restriction enzyme under the conditions specified by the suppliers and fractionated on 0.8% agarose gels (in 1×TAE) overnight at 1 V/cm.

PCR analysis of KH excision events and determination of insertion sites in transgenic rice

Transposition of *KH* in transgenic plants was analyzed by PCR with oligonucleotide primers: Primer JuF (5'-ATTCCTAGGCCACCATGTTG-3') and primer HR3 (5'-CATCGACCCTGCGCCCAAGCTGC -3').

The flanking sequences of the T-DNA or *KH* integration sites in transgenic rice plants were determined by the use of arbitrary degenerate (AD) primers and TAIL-PCR as described previously (Liu et al., 1995; Sha et al., 2004), with modification: The primary TAIL-PCR involved approximately 150 ng of rice genomic DNA. The flanking sequences were amplified with the following oligonucleotide primers: TLn4 (5'- GGCCCAACATGGTGGCCTAG -3'), TLn3 (5'-GAATTAATTCGGCGTTAATTCAG -3') and TLn2 (5'-CGCAATGTGTTATTAAGTTG -3') for T-DNA and 3-1 (5'-GTGTGCTCCAGATTTATATGG-3'), 3-2 (5'-GATTTCGACTTTAACCCGACCGGA-3') and 3-3 (5'-CGTTTTCGTTACCGGTATATCCCG-3') for the 3' end of *KH*.

Assay of T1 progeny resistant to hygromycin

Successful transgenic rice containing *KH* (transposed or non-transposed) were self-pollinated to obtain T1 seeds. The seeds were imbibed in water for two days. The rice seedlings were incubated with water containing hygromycin for 50 μg/ml. The effect could be observed after one week.

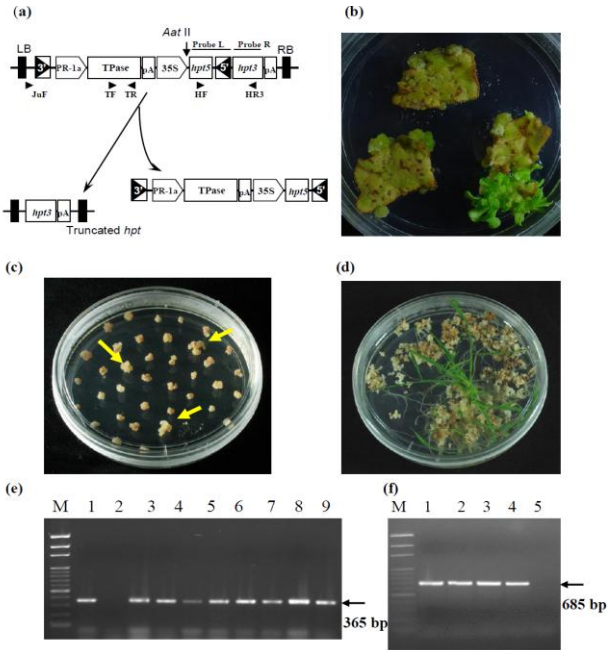

Figure 1. (a) Schematic diagram of the KH system and location of primers (shown as solid triangle). LB, Left border; RB, right border; 5' and 3', *Ac* left and right terminal-inverted repeat; PR-1a, PR-1a inducible promoter; HPT, hygromycin phosphotransferase gene; pA, poly(A) fragment; 35S, CaMV 35S RNA promoter. (b) Proliferation of tobacco calli and shoots in medium with hygromycin. (c) Proliferation of rice calli in growth medium with hygromycin over 5 weeks (yellow arrows). (d) Differentiation of proliferating calli into shoots and roots in shooting medium over 5 weeks. (e) PCR analysis of successful transgenic plants. PCR was performed with a positive controlled plasmid pKH (Lane 1) and genomic DNA from wild type rice TNG67 (Lane 2) and the transformants (Lanes 3 to 9). (f) RT-PCR analysis of modified *hpt* expression in transgenic rice lines (Lanes 1 to 4) and TNG67 (Lane 5) M, 100 bp marker.

RESULTS

Construction of the KH marker-off system

The construction of the new "marker-off" system is described in Figure 1A and supplementary data. First, the cloning vector pBC KS was digested with *Hinc* II then self-ligated to yield the plasmid pBCH, which was used as the vector to clone the *hpt* gene from pCambia1300. The resulting plasmid, pHYGP, was treated with *Hinc* II then ligated with the PCR amplified fragment containing the intron 1 of plasmid pKCEH (Charng et al., 2008). This resulted in the plasmid pHYG5P, of which the *hpt* gene was inserted with an artificial intron containing the 5' end of the *Ac* element. To complete the inducible transposon (termed as *KH*), the plasmid p3PTAS (Charng et al., 2008) which harbors the 3' end of *Ac* accompanied with the PR-1a: TPase and a promoter to trigger *hpt* was used as the vector to clone the *hpt* gene containing the artificial intron, yielding the plasmid p3PTASH5. The binary vector pCAMBIA 0380 was inserted with a synthesis DNA fragment containing *Sac* I and *Kpn* I restriction sites. Using these two sites, the resulting plasmid, p0380M, serves as the vector to clone the transposon system from p3PTASH5, yielding plasmid pKH. This new marker-off system, KH, composed with a PR-1a: *TPase*-based inducible transposon and the HPT marker gene was inserted with an artificial intron containing the 5' end of transposon. The KH system was then introduced into rice and tobacco plants by use of transfection with *Agrobacterium tumefaciens* strain LBA4404.

Normal transcripts yielded by the *hpt* marker gene in the KH construct

In construct KH, the 3' end of *Ac*, which accompanied with the first intron of *epsps* gene, was inserted in the *hpt* gene (Figure 1A). We determined whether the *hpt* gene was expressed normally in the transgenic plants. To this, the

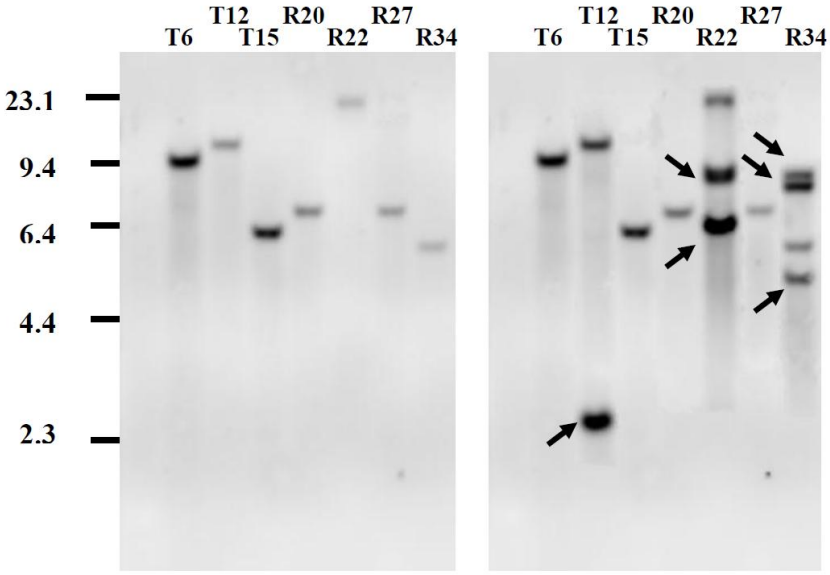

Figure 2. Southern blot hybridization of *Aat* II digested genomic DNAs isolated from different transgenic lines with the probe R or probe L, (construction is shown in Figure 1). The probe R (*left*) revealed the T-DNA copy and together with the probe L (*right*) revealed the un-transposed or transposed *INAc* (indicated by the *arrow*).

first evidence is revealed by obtaining the successful transgenic calli and subsequent regenerating shoots (Figure 1B to D), since the *hpt* gene is the only selectable marker for transformation. In order to determine the existence of *KH* in hygromycin resistant transgenic plants, we analyzed genomic DNA from primary rice and tobacco calli by PCR analysis (Figure 1E). All transgenic lines yielded single 365-bp DNA fragment with primers TF and TR. To ensure the normal expression of *hpt* transcripts, the total RNA of each transgenic line was extracted for RT-PCR. By using primers HF and HR3, PCR reactions of transgenic rice plants yielded single 685-bp DNA fragment (Figure 1F), indicating the normal expression of *hpt* gene. Sequencing analysis confirmed the normal transcripts of the *hpt* gene in *KH* (data not shown). All these results indicate that the *hpt* marker gene containing an artificial intron expressed normally in the transgenic plants.

Transposition events of *KH* and termination of the *hpt* in transgenic plants

Previously, we have constructed several inducible transposons based on PR-1a promoter and introduced these transposons into rice and tobacco plants (Charng et al., 2004, 2007). Transposition events are successfully triggered by applying the inducer, salicylic acid (SA). Thus, to remove the functional hygromycin-tolerant marker, we applied the same method to trigger the *KH* transposon in tobacco and rice plants. The excision events were determined by Southern blot and PCR analysis. Genomic DNAs extracted from 15 transformed rice lines and 20 tobacco lines were subjected to Southern blot analyses. As probes, a sub-cloned pBC based plasmid containing the DNA fragment specific to the *hpt*3 (probe R, Figure 1A), and the PCR amplified fragment (probe L, comprising the 5' end and *hpt*5 of the *hpt* gene) were used. The DNA samples were digested with *Aat* II and hybridized with probe L. Figure 2 shows that the transformed lines T-6, T-12, T-15, R-20, R-22, R-27 and R-34 (T designated for tobacco and R for rice) yielded single hybridizing fragments demonstrating that these transformed line harboring a single copy of T-DNA. After removal of the probe R, the same filter was hybridized with the probe L. The transformed lines T-6, T-15, R-20, and R-27 yielded the same hybridizing patterns, indicating the primary donor sites of the un-transposed *KH* element. For transformed line T-12, R-22 and R-34, in addition to the same hybridizing patterns with probe R (un-transposed *KH*), several new bands were yielded indicating the transposition of *KH* (Figure 2). By Southern blot analysis, 8 out of 15 transformed rice lines and 15 out of 20 tobacco lines showed single copy of T-DNA integration (with probe R). Among these, 2 transgenic rice lines and 1 tobacco lines yielded new bands with probe L. The experiments were expanded by PCR analysis, in order to determine the transposition event which yielded weak signal by Southern blot analysis. The same DNA samples were used for PCR analysis with the primers JuF and HR3. To determine the empty donor site, a 290-bp DNA fragment was expected (Figure 3A). Of 8 single-copy rice lines, 3

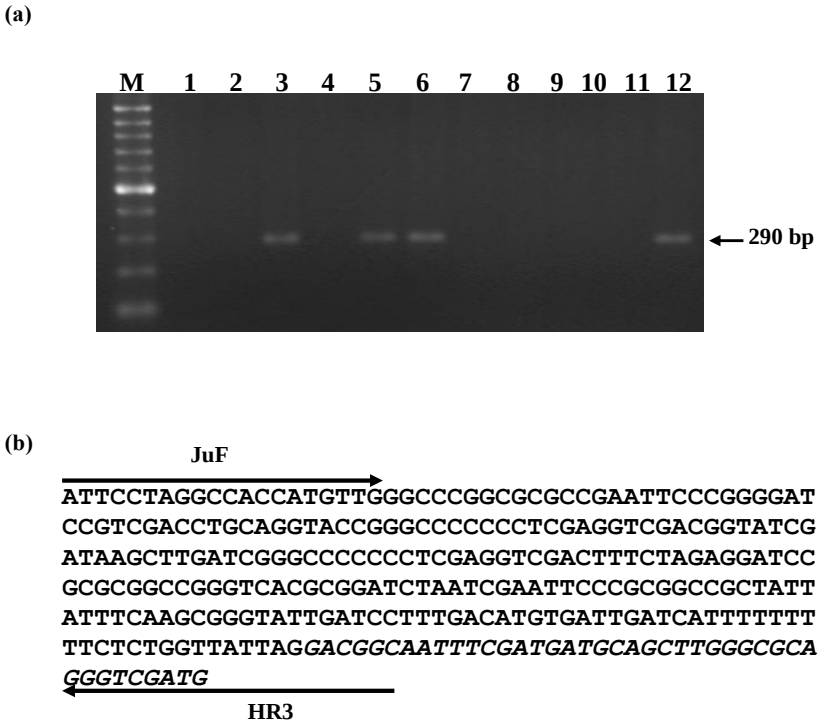

Figure 3. Analysis of transposition events of SA-induced transgenic plants harboring KH system. (A) PCR analysis of *KH* transposition with the primers JuF and HR3 and the expected fragments. (B) Sequence of the empty donor site of the transposition events, leaving the sequences from the truncated *hpt*, in which *hpt*3 are shown in italics. The primers JuF and HR3 are indicated as arrows. M,100 bp marker.

transgenic lines yielded the expected 290-bp DNA products, including those lines determined by Southern analysis. For transgenic tobacco, 10 out of 20 lines yielded the 290-bp DNA products. Sequencing analysis confirmed the residual DNA after the excision of the transposon (Figure 3B). These results indicate somatic transposition efficiency of *KH* is about 20% for transgenic rice and 50% for transgenic tobacco. Thus, the new transposon *KH* is as efficient as other PR-1a: *TPase*-based inducible transposons described previously (Charng et al., 2004, 2007).

The results described previously indicated that *Ac*-based inducible transposons are very active in induced rice and tobacco calli. Yet, our previous studies indicated that in transgenic rice, sometimes only a portion of cells contain the empty donor site (partial transposition pattern; Charng et al., 2007). We therefore determined whether the transposition events passed through the germ line and were inherited in the progeny (germinal transposition) or not (somatic transposition). The remaining calli of the transposed lines, as well as non-SA treated calli (controls), were cultured to set shoots then transplanted to soil for self-pollination. The seedlings of the progeny underwent PCR to determine the inheritance of the transposition events. Of 3 transgenic rice lines showing transposition, all showed the transposition events inherited in the progeny, which yielded 290-bp product with JuF and HR3 primers (data not shown). For transgenic tobacco, 4 out of 10 lines showed the transposition events inherited in the progeny. Furthermore, genomic DNA of the transgenic rice lines was subsequently used to amplify the flanking sequences of the transposed *KH* elements, in order to analyze the transposition patterns of *KH*. These were performed with transgenic rice plants, since the whole genome sequences database is available. The flanking sequences were determined by TAIL PCR (Liu et al., 1995). A summary of the significant homologies obtained after comparison of the flanking sequences from a public database is in Table 1. Of 3 independent transposition events, 1 showed linkages and 2 no linkages to the T-DNA locations.

Since the transposition of *KH* resulted in the termination of the marker gene, the progeny of transposed transgenic rice lines were determined for hygromycin-tolerance. The seedlings of the progeny were treated with hygromycin B. All seedlings were hygromycin sensitive, which indicates the loss of the hygromycin-tolerant function (Figure 4A). As a control, the same transgenic line which had not been induced with SA for transposition showed hygromycin-tolerance as a single Mendelian locus pattern (Figure 4B). Similar results were observed with the

Table 1. Genomic sequences flanking *KH* insertions in transgenic rice plants.

Line	Chromosome	BACs/PACs	Insertion position (bp)	GenBank accession no.	Identity
R-22	(T-DNA) 2	B1370C05	85146	AP005873	105/105 (100%)
22-1	10	OJ1014H12	42549	AC098694	204/206 (99%)
R-27	(T-DNA) 6	P0613F06	34266	AP003545	160/160 (100%)
27-1	1	OSJNBb0049O23	48912	AP003342	175/175 (100%)
R-34	(T-DNA) 9	OSJNBa0042H24	20645	AP005689	145/151 (98%)
34-1	9	OSJNBa0026C08	9435	AP006169	116/116 (100%)

The T-DNA integration site of each line is indicated after its designation.

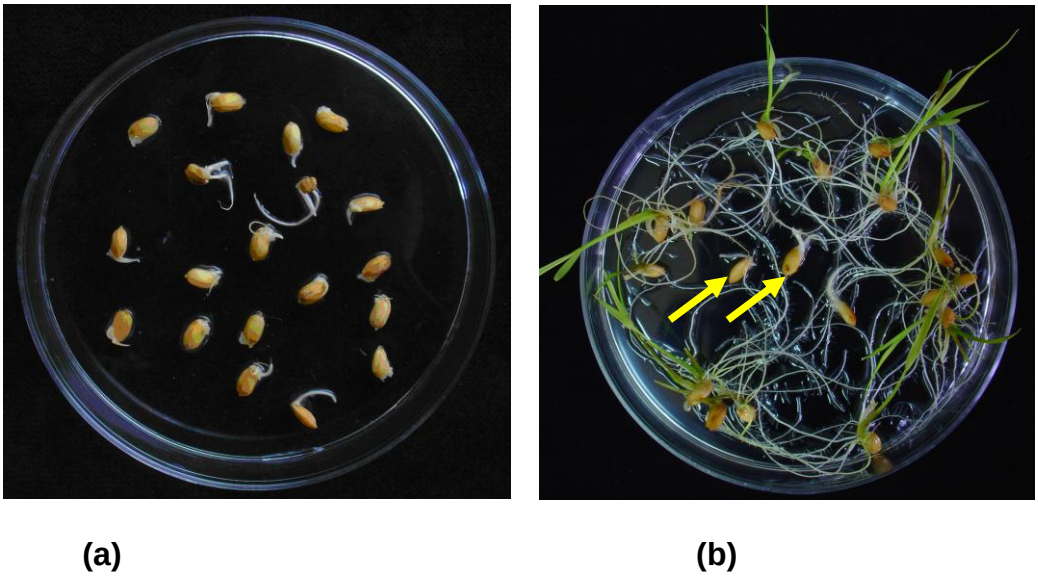

Figure 4. Hygromycin-tolerance analysis of the self-pollinated progeny of *KH* transposed (a) and untransposed line (b). Arrows indicate the null *KH* progeny, which are not resistant to hygromycin.

progeny of transposed transgenic tobacco lines (data not shown). Taken together, the results indicate that the KH system offers a desirable selectable marker for plant transformation and the ability to remove the marker thereafter.

DISCUSSION

Many marker-free systems have been estimated in transgenic plants including co-transformed genes, site-specific recombination system and transposon mediated system. Among these, transposon mediated system offers the possibility that the GOI and the marker integrate into different loci in the plant genome. Unlinked marker genes can then be segregated away from the GOI and allow the production of marker-free transgenic plants (Ebinuma et al., 2001). This technology is not useful for woody plants or plants that reproduce vegetatively. We therefore have applied an inducible transposon technology to develop a marker-off system without the need for out-crossing (Charng et al., 2008). The system, KCEH, involves introducing an intron-containing marker gene (rice *epsps* gene), accompanied by a transposon whose one end is located in the intron of the marker gene. After obtaining stable transgenic plants and inducing transposition, the transposon excised, the marker gene became truncated, and its expression was terminated. This strategy was demonstrated successfully in rice and tobacco plants. However, a conceptual problem limits the application of this system to the most frequently used marker genes, example, *nptII* and *hpt* genes, which have no introns. Therefore, in this report, we created an artificial intron to the marker gene, *hpt*. An artificial intron, which contains the 5' end of transposon was amplified from the KCEH construct and inserted into the *hpt* gene, yielding the new KH system for stable transgenic plants lacking marker function. The inserted intron, which

originated from the first inton of *epsps* gene, retained the semiconserved consensuses for splice donor and receptor sites, which were represented as NN/GTGAGA and YAG/N, respectively (bold designates exonic nucleotides; slashes designate splice sites; underlines designates highly conserved nucleotides; Schuler, 2008). Thus, to study the KH system, we first determined whether the modified *hpt*, which is splitted into 2 portions (*hpt*5 and *hpt*3; Figure 1A), was a functional marker. Since the modified *hpt* gene is the only selectable marker for transformation, the existence of successful transgenic calli suggested it is functional (Figure 1B to D). RT-PCR and sequence analysis indicated that the splice junctions of the artificial intron are identical to the junctions of the native *epsps* (Figure 1F). However, compared with other PR-1a: *TPase*-based inducible transposons, the KH construct yielded slightly lower transformation efficiency for either transgenic rice or tobacco (data not shown). This observation can be explained by the splicing efficiency of the modified *hpt* been lower than other genes with regular introns. As a result of this, some successful transgenic plants failed to act the tolerant activity against the regular concentration of hygromycin in the regeneration medium. Or alternatively, it is possible that the PR-1a: *TPase* was induced by endogenous stimuli and subsequent transposition truncated the *hpt* marker gene during the regeneration of the transgenic calli. Our previous studies indicated that the spontaneous transposition events did occur in transgenic plants containing the PR-1a: *TPase*-based inducible transposons (Charng et al., 2004, 2007). After obtaining the successful transgenic plants, we induced the transposase gene to determine the transposition. Our results show that *KH* harbors similar transposition efficiency as previous PR-1a: *TPase*-based inducible transposon in rice (Charng et al., 2007). Taken our previous and current works together, we located the end of the inducible transposon in an intron of a target gene for subsequently removing its function in transgenic plants (Charng et al., 2008; Li et al., 2008; Tai et al., 2011). All these works are based on the fact that before transposition, the insertion of the end of transposon in an intron did not obviously affect the normal splicing process of the target gene. Furthermore, the target genes are not restricted to those without native introns. All these features indicate the application of an inducible transposon for plant biotechnology.

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