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A one-time inducible transposon for creating knockout mutants

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Abstract The maize transposon *Ac* can move to a new location within the genome to create knockout mutants in transgenic plants. In rice, *Ac* transposon is very active but sometimes undergoes further transposition and leaves an empty mutated gene. Therefore, we developed a one-time transposon system by locating one end of the transposon in the intron of the *Ac* transposase gene, which is under the control of the inducible promoter (PR-1a). Treatment with salicylic acid induced transposition of this transposon, *COYA*, leading to transposase gene breakage in exons. The progeny plants inheriting the transposition events become stable knockout mutants, because no functional transposase could be yielded. The behavior of *COYA* was analyzed in single-copy transgenic rice plants. We determined the expression of the modified transposase gene and its ability to trigger transposition events in transgenic rice plants. The *COYA* element thus exhibits potential for development of an inducible transposon system suitable for gene isolation in heterologous plant species.

Keywords *Ac* transposase · Inducible transposon · Knockout mutant · Transposon tagging

Abbreviations

HPT	Hygromycin phosphotransferase
TPase	Transposase
SA	Salicylic acid

Introduction

The maize transposon *Activator* (*Ac*) is an autonomous transposable element of 4,565 bp 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). Transposition is a powerful genetic tool to yield knockout/activation mutants for finding new genes in plants and animals (Walbot 2000; Keng et al. 2005). Many researchers have modified native transposons to create more convenient tools for gene tagging, for example, for trapping and activation tagging (Greco et al. 2001; Marsch-Martinez et al. 2002).

Previously, the PR-1a promoter from tobacco was fused with the *Ac* transposase gene to develop an

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inducible transposon system (Charng et al. 2000). The transposon sometimes undergoes further transposition without induction, possibly because of endogenous stimuli. Schmitz and Theres (1994) constructed a self-stabilizing *Ac* derivative whose 3′ end located between the transposase gene and its promoter. The construct was improved for obtaining gain-of-function mutations (Suzuki et al. 2001). However, the transposon or T-DNA might locate downstream of a strong promoter or nearby enhancers for autonomous transposition. In this report, a new inducible transposon was constructed by locating one of its ends in the third intron of the transposase gene and introduced into rice. With application of the inducer, the transposase gene could be precisely expressed. Transposition events were triggered, and the transposase gene was then truncated via a break in exons. Somatic transpositions were determined in transgenic rice calli by LUC reporter and PCR analysis. Germinal transposition events were analyzed by Southern blot with progeny of SA-induced transgenic lines containing a single copy of the *COYA* transposon. We discuss the advantages of this novel transposon system for creating stable knockout mutants.

Materials and methods

DNA manipulation and plant transformation

The construction procedures for the *COYA* system are depicted in the supplementary material. All cloning and DNA manipulations followed standard procedures (Sambrook and Russel 2001) with use of chemicals from Roche (Basel, Switzerland). All transformations involved use of rice (*Oryza sativa* L. cv TNG67) as described (Toki 1997).

Induction of *COYA* transposition

For experiments inducing *COYA*, 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 5 mM salicylic acid (SA) for 7 days, then transferred to callus induction medium without SA for 4–8 weeks before LUC or PCR analysis. To induce rice plants, transgenic rice at 3-leaf age were cultured in pods for a month, then flooded in 5 mM SA solution for 2 days/week until heading.

RT-PCR analysis for transposase gene expression

RNA extracted from T_0 transgenic rice calli was reverse transcribed with use of the SuperScript™ First Strand Synthesis System (Invitrogen). Primers specific to each of the two target sequences were used in subsequent PCR amplification:

ER (5′-ACAGGGCCCTCATGGAGAGGAGCC-3′) and CHC2 (5′-ATACAAGTCAACTGTTGCTTC-3′) for *Ac* transposase.

In vivo and in vitro assays for luciferase gene activity

In vitro activity of luciferase enzyme was determined as described (Howell et al. 1989) by use of a Lumat LB 9501 luminometer (Berthold, München, Germany). For in vivo assay, 0.15 mg/l of luciferin aqueous solution was applied to rice calli, which were then placed in a dark room, then analyzed by use of a luminometer with an intensified CCD camera (Hamamatsu, Japan) and a Nikon 35-mm lens connected to a computer. The plant material and luminescent images were taken separately, the luminescent images revealing calli with luciferase activity.

PCR analysis of *COYA* excision events

Transposition of *COYA* from the *COYA*::LUC construct in transgenic plants was analyzed by PCR with oligonucleotide primers: primer 35S (5′-TCCTTCGC AAGACCCTTCCT-3′); and primer LUC2 (5′-CGGG CGCAACTGCAACTCC-3′).

The flanking sequences of the T-DNA or *COYA* integration sites in transgenic plants were determined by 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: CA1 (5′-TAGGGTTTCGCTCATGTGTT-3′), CA2

(5′-GTGTTGAGCATATAAGAAAC CCT-3′) and CA3 (5′-TTCGGCGTTAATTCAGTACA-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.

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 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 DNA 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. Southern blot analysis was performed as described (Charng and Pfitzner 1994).

Results

Construction of the one-time transposon system *COYA*

The construction of the one-time transposon system *COYA* is described in Fig. 1a. First, the 5′ end of the *Ac* element, together with a 35S promoter, was inserted into the third intron of the transposase gene, driven by a PR-1a promoter from tobacco. Then, the 3′ end of the *Ac* element flanking the hygromycin phosphotransferase (HPT) gene was constructed downstream of the transposase gene. Hence, the transposon contained the fourth and fifth exons of the transposase gene and HPT. Furthermore, the *COYA* element was constructed between the 35S promoter and the LUC gene. In principle, after transposition, LUC could be reactivated and the transposase gene would be truncated. The *COYA* system was inserted into the binary vector pCAMBIA 2200, yielding the plasmid pCOYA, which was then introduced into rice plants by use of transfection with *Agrobacterium tumefaciens* strain LBA4404. We used 21 single-copy T-DNA integration transgenic plants for the induction and detection of transposition events.

Normal expression of transposase gene with *COYA*

Previously, we created a construct by inserting the *Ac* 5′ end into the first intron of a glyphosate-tolerate rice EPSPS gene to create a "marker-off" transgenic system (Charng et al. 2008). The expression of this modified EPSPS gene showed normal and additional transcripts. Besides the first intron of the modified EPSPS gene, transcription proceeded to the partial 5′ end of the transposon, which resulted in a large RNA fragment (Charng et al., unpublished results). To understand whether the *Ac* transposase gene *COYA* was expressed successfully in transgenic rice, single-copy transgenic rice calli generated from T1 seeds were incubated with callus induction medium containing 5 mM SA for 7 days, then the total RNA of each transgenic rice line was extracted for RT-PCR. Two specific primers, CHC2 and ER (Fig. 1a), were used to determine functional splicing. As predicted, a 301-bp DNA fragment was obtained (Fig. 1b). Sequencing analysis suggested a normal expression of transposase gene with *COYA* (data not shown).

Transposition events of *COYA* in transgenic rice

Since the transposase gene could be expressed normally after 7 days' induction, SA-induced calli were then cultured on normal medium for further regeneration to determine whether the *COYA* transposition events could also be induced in rice calli. Transposition events were detected by in vivo LUC assay and PCR, then sequencing analysis. First, induced calli incubated with luciferin underwent direct LUC image capture (Fig. 2a and b). Then, two primers for PCR reaction were designed to confirm the excision of *COYA*. To determine the empty donor sites after excision, we used the primers 35S and LUC2, which are specific to DNA sequences flanking each end of *COYA*. After PCR amplification, a 588-bp DNA fragment was obtained (Fig. 3a) and sequenced to confirm the empty donor site (Fig. 3b).

Next, we studied the transposition efficiency of *COYA*. Although we had previously shown a high

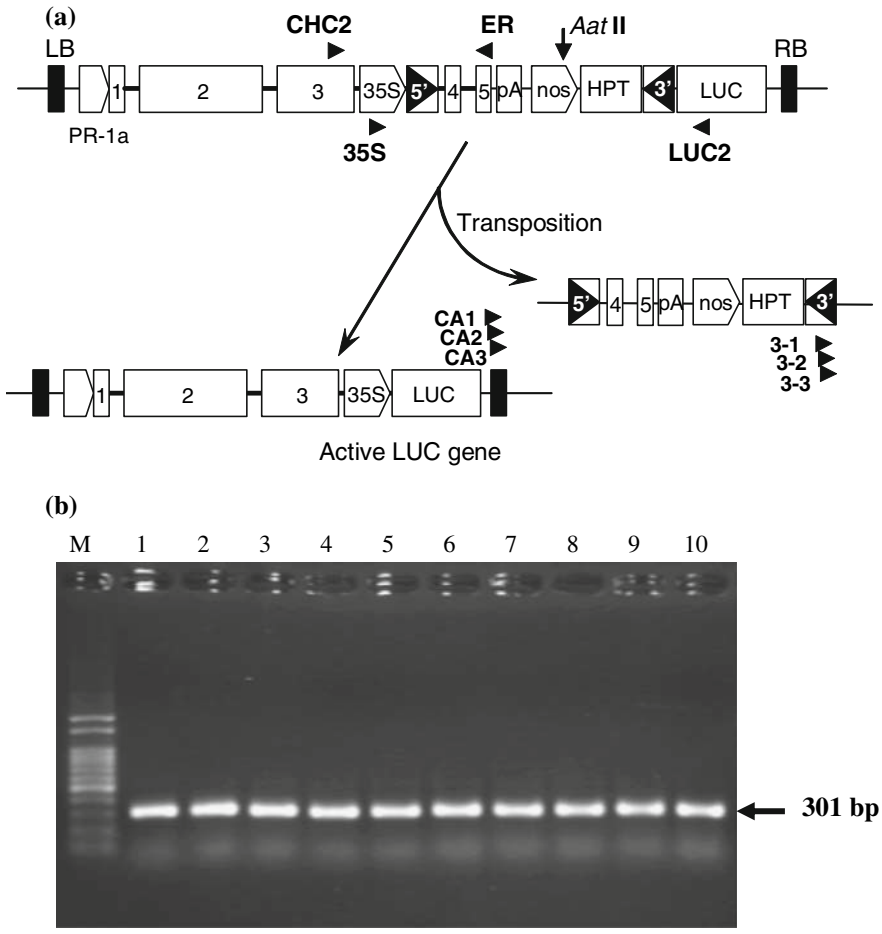

Fig. 1 (**a**) Schematic diagram of the one-time *COYA* transposon system and location of primers (shown as *solid triangle*). *LB* Left border; *RB* right border; *5′ and 3′ Ac* left and right terminal-inverted repeat; *PR-1a* PR-1a inducible promoter; *HPT* hygromycin phosphotransferase gene; *pA* poly(A) fragment; *NOS* nopaline synthase promoter; *LUC* luciferase gene; *1–5* transposase gene exon 1–exon 5. (**b**) RT-PCR analysis of induced transposase gene expression in transgenic rice lines (1–10) with the primers CHC2 and ER. M, 100 bp marker

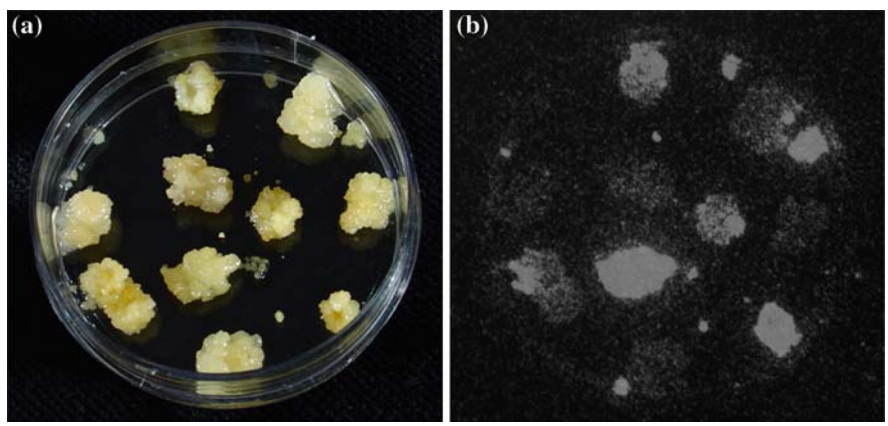

Fig. 2 Analysis of transposition events of SA-induced transgenic rice harboring *COYA* system. (**a**) Reflected-light image of induced transgenic rice calli harboring *COYA* system and (**b**) its luminescence image

induction efficiency of *INAc* transposon, which contains a PR-1a promoter fused with the cDNA of *Ac* TPase (Charng et al. 2000), the transposition efficiency of *COYA* could be affected because of the 5′ end inserted in the third intron of *Ac*. Transposition events were determined by in vitro LUC assay and PCR analysis described above. In Hyg^R rice calli regenerated from T1 seeds of each transgenic line, treatment with SA induced transposition of *COYA*, as analysed by in vitro LUC assay and PCR, and the transposition efficiency was dose dependent (Table 1). We discovered approximately 9% of

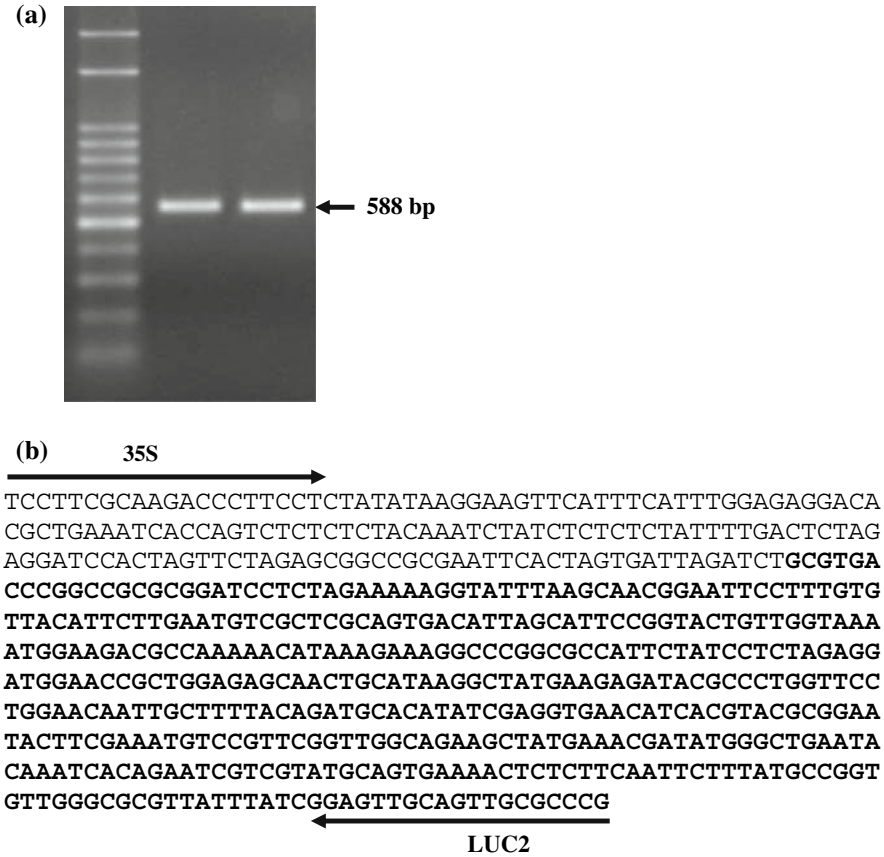

Fig. 3 Analysis of spontaneous transposition events of transgenic rice harboring COYA system. (**a**) PCR analysis of *COYA* transposition using primers 35S and LUC2 and the expected fragments. (**b**) Sequence of the empty donor site of the transposition events. The sequences flanking the *COYA* 5′ end are shown in *bold*

COYA transposition events to be spontaneous. Treatment with SA resulted in a high level of transposition efficiency; 52% for 5 mM SA and 76% for 10 mM SA (Table 1). Thus, with insertion of the third intron of the transposase gene by a DNA fragment containing a 35S promoter and the 3′ end of *Ac*, the transposase gene could express and target normally both ends of *Ac* with high efficiency.

Germinal transposition of *COYA* in transgenic rice

An important feature of an inducible transposable element is its ability to induce transposition in germinal tissue or in somatic tissues, which allows the transposition event to pass through the germ line and be inherited in the progeny. Such ability markedly increases the success of transposon tagging in isolating important plant genes. To determine the effectiveness of *COYA* for gene tagging in rice, we induced transposition in adult rice plants of eight transformed lines, which revealed no spontaneous transposition but, rather, SA-induced transposition (Table 1). We previously performed the germinal induction experiments by directly spraying SA on the floral tissue of tobacco (Charng 2000). Since rice floral tissues embed in the flag leaves when (or before) meiosis occurs, we flooded transgenic rice with 5 mM SA solution 2 days/week until seed set. The germinal transposition events/efficiencies were determined by the following steps: (1) LUC assay (in vitro) and PCR analysis to determine the presence of empty donor sites in progeny, which reveals the germinal transposition efficiency; (2) DNA blot analysis to identify each independent transposition events and thus determine the germinal transposition efficiency; and (3) flanking sequence analysis of each re-integrated *COYA* to reveal the transposition pattern of this one-time inducible transposon.

Of 1,065 seedlings, 273 (25.6%) exhibited luciferase activity and yielded PCR-specific products (Table 2). Among transformed plants without SA treatment, 376 seedlings yielded neither luciferase

Table 1 Transposition efficiencies of calli derived from progeny of 21 single-copy transgenic lines harboring the *COYA* transposon

Plant	None	Treatment	
		5 mM SA (5 days)	10 mM SA (3 days)
Y-2	3/30	21/30	28/30
Y-3	1/30	3/30	5/30
Y-7	4/30	9/30	22/30
Y-10	2/30	10/30	16/30
Y-11[a]	1/30	19/30	28/30
Y-12[a]	0/30	10/30	29/30
Y-15	6/30	8/30	27/30
Y-20[a]	1/30	17/30	25/30
Y-23[a]	0/30	16/30	30/30
Y-26[a]	0/30	26/30	29/30
Y-27	8/30	20/30	18/30
Y-32[a]	0/30	13/30	25/30
Y-38	9/30	28/30	30/30
Y-40[a]	0/30	16/30	24/30
Y-43	2/30	12/30	13/30
Y-46	0/30	2/30	14/30
Y-47	8/30	20/30	22/30
Y-48	5/30	25/30	26/30
Y-52[a]	0/30	16/30	26/30
Y-62	4/30	17/30	21/30
Y-65	3/30	15/30	19/30
Mean (%)	9	52	76

The regeneration calli derived from seeds of each line were divided into three portions for each treatment then incubated for 4 weeks before determination

All treatments were tested with a randomized collection of 30 samples for LUC and PCR analysis

Transposition events were recorded by the existence of LUC activities and PCR products described in "Results"

[a]These lines were selected for induction of germinal transposition experiments

activity nor PCR-specific products for the empty donor site.

LUC assay revealed the efficiency of *COYA* excision events that transmitted to progeny. Still, a single transposition event could happen early during rice growth and be inherited in many progeny, with siblings consequently containing the same *COYA* integration sites. This feature would lead to overestimation of the transposition efficiency. To rule out this possibility, we attempted to identify independent events. Since the results shown in Table 2 indicated that lines Y-12 and Y-52 yielded relatively low transposition frequencies, we used the six other lines. First, *COYA* (transposed or untransposed) in progeny determined by hygromycin resistance assay (data not shown) revealed out of 859 transgenic rice plants, 635 Hyg^R, which were selected for further analysis. The frequency and pattern of *COYA* transposition in transgenic rice were detected by Southern blot analysis, with the HPT gene used as a probe. Aat II digestion of the genomic DNA resulted in a unique hybridizing band, depending on the line (e.g., 4.2 kb for line Y-20 Starter line), which corresponds to the presence of an un-transposed *COYA* element (Fig. 4). A novel band of distinct size in progeny plants was considered an independent transposed *COYA* element. As an example, shown in Fig. 4, 21 progeny yielded 27 hybridizing signals composed of seven independent transposition events. Of 859 progeny assayed, 80 yielded *COYA*-reintegrated bands, with 27 independent integration signals (Table 2). Southern blot analysis revealed that all transformed lines used for germinal transposition experiments were heterozygous for the *COYA* T-DNA insertion site (Fig. 4; and data not shown). Since *COYA* was constructed for one-time transposition, the stability of the transposed *COYA* was determined with progeny of plants harboring single transposed *COYA* (e.g., lines 29 and 31; Fig. 4). Again, Southern blot analysis to detect the unique hybridizing band of *COYA* revealed all assayed progeny with the same hybridization patterns as their control samples (data not shown). Therefore, we concluded that the *COYA* element is self-stabilizing after transposition. The experiments were expanded to amplify the flanking sequences of the transposed *COYA* elements. We collected genomic DNAs containing the independent transposed *COYA*, as revealed by Southern blot patterns. The flanking sequences were isolated by TAIL PCR (Liu et al. 1995, see "Materials and methods"). A summary of the significant homologies obtained after comparison of the flanking sequences in a public database is shown in Table 3. For 27 independent transpositions, 16 were linked to the T-DNA locations, and 8 were un-linked. For three transposition events, the insertion locations were unclear, since the flanking sequences were matched to the repeat sequences in the database. Taken together, these results indicate that the transposase

Table 2 Number of plants with *COYA* excision, transposition in the progeny of transgenic lines treated with SA

Plant	Non-SA (control)[a]	SA-induced			
		Tested	Excision[b]	Transposition	Independent transposition
Y-11	0/48	127	34	18	5
Y-12	0/50	110	9	nd	nd
Y-20	0/50	160	56	21	7
Y-23	0/50	232	38	15	5
Y-26	0/50	212	42	13	6
Y-32	0/46	138	68	10	3
Y-40	0/50	140	22	2	1
Y-52	0/32	96	4	nd	nd
Total	(0%)	1,065	273 (25.6%)	80 (7.5%)	27 (2.5%)

[a] Number of LUC^+ progeny of transgenic rice without SA treatment as control (number of LUC^+/number tested)

[b] LUC assay was preliminary performed to reveal the excision efficiency. The results were used to exclude line Y-12 and 52 for Southern blot analysis

nd Southern analysis was not carried out

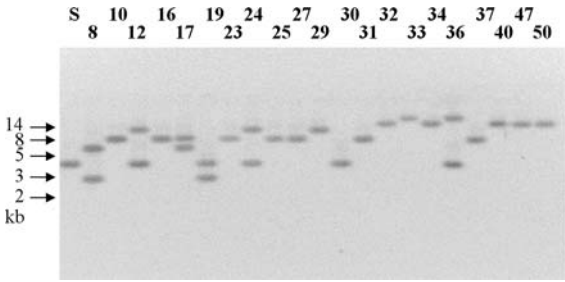

Fig. 4 Southern blot analysis of hygromycin-resistant *COYA* plants from a single starter line (Y-20) using the HPT gene as a probe. *S* Starter line; *numbers* represent selected plants from 21 independent Y-20 families after primary Southern blot analysis. Size markers after ethidium bromide staining are indicated on the left

gene could be expressed functionally, then could *cis*-activate the *COYA* transposon for one time.

Discussion

Transposon tagging has become a powerful tool to create mutants for isolating new genes. The genes mutated by the insertion of transposons and their sequences can be obtained by inverse or TAIL PCR, which uses transposon-based primers. However, the transposon should not undergo further transposition to leave an empty mutated gene (Knapp et al. 1994). Previously, a high frequency of further transposition of the native *Ac* transposon was reported in rice and tomato (Greco et al. 2001; Yoder 1990). For rice plants containing *Ac* elements, the frequency can be up to 10% (Izawa et al. 1997). Transcriptional control by fusing an inducible promoter with the TPase has been assessed (Charng et al. 2000, 2008). In rice, the inducible transposon undergoes further transposition was also observed (Charng et al., unpublished results). Therefore, in this study, we constructed a one-time inducible transposable element, *COYA*, for creating stable knockout mutants. We inserted the 5′ end of the *Ac* element in the third intron of the transposase gene, which was triggered by the SA-inducible PR-1a promoter. To complete the new transposon, the *Ac* 3′ end flanked with the HPT gene was constructed downstream of the transposase gene. The transposase gene could be induced to trigger the transposition. Then, after transposition, the transposase gene is truncated to terminate functional expression of the gene.

Our inducible one-time transposon, *COYA*, can be useful in creating stable knockout mutants in rice. Since *COYA* differs from previous inducible transposons because the 5′ end is inserted in the third intron of the transposase gene, we first confirmed the normal expression of the transposase gene, with no alternative transcript, by RT-PCR and subsequent sequence analysis (Fig. 2). With the expression of the transposase gene not affected, we determined the

Table 3 Genomic sequences flanking *COYA* insertions in transgenic rice plants

Line	Chromosome	BACs/PACs	Insertion position (bp)	GenBank accession no.	Identities
Y-11	(T-DNA) 2	OJ1191_G08	5415	AP004047	68/68 (100%)
11-11	1[a]	P0443D08	20572	AP003250	280/280 (100%)
11-12	2	OJ1479_B12	82024	AP004165	183/183 (100%)
11-19	10	OSJNBa0065C1	85441	AC074354	333/333(100%)
11-22	2	P0663F07	151215	AP005823	48/48(100%)
11-29	2[a]	P0487D09	153603	AP004880	56/56 (100%)
Y-20	(T-DNA) 12	OSJNBa0005P03	110785	AL935066	34/34 (100%)
20-8	6	P0417E03	48819	AP006054	198/198 (100%)
20-8	12	OSJNBa0073H17	28133	AL713909	127/127 (100%)
20-10	10	OSJNBa0096E22	38484	AC099400	138/138 (100%)
20-12	12	OSJNBa0090O14	7648	AL731763	280/280 (100%)
20-17	12	OSJNBb0085B24	40635	AL954871	83/83 (100%)
20-32	12	OSJNBa0011B18	104124	AL713908	400/400 (100%)
20-33	4	OSIGBa0125J07	59068	CR855046	75/75(100%)
Y-23	(T-DNA) 3	OSJNBa0093I13	119497	AC097279	184/184 (100%)
23-2	3	OSJNBb0081I10	52069	AC134240	147/147 (100%)
23-18	5	OSJNBb0088F07	47841	AC119292	124/124 (100%)
23-22	3	OSJNBa0076E06	17317	AC132215	66/66 (100%)
23-29	3	OJ1125B03	98463	AC134885	108/108 (100%)
23-36	3	OJ1125B03	93833	AC134885	87/87 (100%)
Y-26	(T-DNA) 9	P0635G10	42087	AP005396	334/334 (100%)
26-5	8	OJ1113_A10	78270	AP004643	94/94 (100%)
26-7	9[a]	OSJNBa0035B22	108545	AC137592	89/89 (100%)
26-18	9	OSJNBa0035B22	101135	AC137592	47/47 (100%)
26-21	9	OSJNBa0042B15	47964	AP006170	50/50 (100%)
26-22	12	OJ1005_B07	34060	AL713946	127/127 (100%)
26-38	9	P0489D11	7621	AP005742	240/240 (100%)
Y-32	(T-DNA) 10	OSJNBa0040D23	133921	AC074196	82/84 (98%)
32-2	10	OSJNBa0025B05	9103	AC096782	124/124 (100%)
32-8	5[a]	OSJNBa0032D15	45261	AC120989	155/155 (100%)
32-19	10	OSJNBa0031A07	38533	AC084884	200/200 (100%)
Y-40	(T-DNA) 7	OJ1119_B04	4292	AP003943	186/186 (100%)
40-11	7	OJ1119_B04	87349	AP003943	40/40 (100%)

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

[a] Indicates the flanking sequences matched to the repeat sequences in the database, and one record was singled out to present in this table

excision of the *COYA* element by LUC assay and PCR analysis and the precise empty donor site by sequencing analysis (Figs. 2 and 3). Finally, we used Southern blot and sequence analyses to demonstrate re-integration of the excised *COYA* and confirm the inheritance and stability of the transposed *COYA* element in progeny plants (Fig. 4 and Table 3).

Previously, we studied the behavior of our first inducible transposon, *INAc*. *INAc* contains the PR-1a promoter fused with the *Ac* transposase full-length cDNA fragment. We found that transposition efficiency may be overestimated for high copies of *Ac*, as well as partial transposition (some cells containing the empty donor site). In this report, we describe our

determination of somatic transposition efficiency by SA-induced transgenic calli containing one copy of *COYA*. The transposition efficiencies were 52 and 76% with 5- and 10-mM SA treatment, respectively, which indicated that the transposition efficiency was dose dependent. Yet, 9% of the rice calli yielded LUC activities and specific PCR products in the absence of SA. This result is likely attributable to activation of the PR-1a promoter by endogenous stimuli. Although the associated level of transposase expression may be low, it is likely sufficient to drive *Ac*-based transposons (Fusswinkel et al. 1991). This fact encouraged us to construct new inducible transposon bases in animal- or microbe-inducible systems, to rule out the induction of endogenous stimuli. However, our preliminary results indicated spontaneous transposition in rice plants containing the transposon with the Tet-ON inducible system, possibly activated by the nearby enhancer elements in the plant genome. All these findings confirm the necessity of constructing a one-time transposon. Nevertheless, since *COYA* differs from *INAc* in self-stabilizing ability, the resulting knockout mutants are still acceptable for tagging experiments.

After induction of *COYA* with SA, germinal transposition events were determined by LUC assay and Southern blot analysis in the rice progeny, which confirmed the existence of the *COYA* element by hygromycin resistance. The results in Table 3 indicated that this *Ac*-based inducible transposon harbors the tendency for linked transposition. LUC assay revealed 25.6% excision efficiency and Southern blot analysis revealed 27 of 859 progeny with *COYA* reintegrated bands of independent transposition events. In Fig. 4, six progeny yielded two hybridizing signals, with four of these—lines 12, 19, 24 and 36—showing the 4.2-kb band corresponding to the untransposed starter *COYA* element. Therefore, in these plants, transposition events could have occurred during embryogenesis and resulted in partially transposed chimeric plants, with two hybridization signals. However, this possibility is unlikely because all novel bands of these plants were consistent with bands in other progeny of the same size; for example, the 2.9-kb signal of progeny 19 was also observed in progeny 8. Further flanking sequence analysis indicated that these bands originated from the same transposition events. These results suggest that many knockout mutants resulted from an early transposition event, so the insertion locus was transmitted to several progeny; for example, an 8.9-kb band exists in progenies 10, 16, 17, 23, 25, 27, 31 and 37. In summary, 7.5% (80/1065) of the selfed progeny carried transposed *Ds* (*COYA*). Among these elements, 33% (27/80) were derived from independent transposition events. The rest of the elements were from the same transposition events. Interestingly, in contrast to the 9% spontaneous transposition efficiency observed in non-SA-induced calli tissue, no restored LUC activity was detected in the progeny of transgenic rice without SA treatment. This observation could be explained by spontaneous transposition of *COYA* being rare during the growth of rice, or the spontaneous transposition events occurred but were not transmitted to progeny. Optimization of induction conditions for germinal transposition of *COYA* requires further experimentation. Still, since rice is an ideal model plant because of the availability of its complete DNA sequence and its high regeneration efficiency from calli, even heterozygous mutants created by *COYA* are valuable for reverse genetics. Furthermore, a new strategy of inducing transposition by rice anther culture may allow us to obtain homozygous mutants and improve transposition efficiency.

In conclusion, we provide a new strategy for terminating transgene expression via inducible transposon-mediated break in exons. Our efforts expand the use of the one-time inducible transposon and indicate that this concept could have wide application.

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