Botanical Studies (2007) 48: 1-11.

The inducible transposon system for rice functional genomics

Yuh-Chyang CHARNG[1,*], Gideon WU[1], Chia-Shan HSIEH[1], Han-Ning CHUAN[1], Ji-Ying HUANG[1], Ling-Chun YEH[1], Ya-Hsiung SHIEH[1], and Jenn TU[2]

[1]*Department of Agronomy, National Taiwan University, No.1 Sec. 4 Roosevelt Rd., Taipei, Taiwan, Republic of China*

[2]*Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan, Republic of China*

(Received January 17, 2006; Accepted May 11, 2006)

ABSTRACT. The one-component inducible transposon system for rice functional genomic studies was assessed. In contrast to the native *Ac* transposon, *INAc* contains the transposase gene under the control of the inducible promoter (PR-1a) from tobacco. To examine whether *INAc* can be used in cereals, the behavior of *INAc* was analyzed with transgenic rice plants containing a single copy of the *INAc* element. Treatment of rice calli with salicylic acid induced transposition of *INAc* in somatic tissue, and the transposition efficiency of *INAc* was dose dependent. Furthermore, a high throughput method for detection of new transposed *INAc* was developed. Analyzing the flanking sequences of the transposed *INAc* indicated the independent insertions. Given the fact that a number of different types of *Ac*/*Ds* vectors have been already examined in rice, the importance of a "controlled" transposon system to yield knockout mutants or new transgenic plants was discussed.

Keywords: *Ac* transposase; Inducible promoter; Salicylic acid; Transposon tagging.

INTRODUCTION

Rice has become a model for the study of monocot plants because of the accumulating molecular information for this species (Harushima et al., 1998; Sakata et al., 2000; Temnykh et al., 2000; Yuan et al., 2000; for review see Jeon and An, 2001). Rice also has a high transformation efficiency (Tyagi and Mohanty, 2000), a small (430 Mb) genome (Arumuganathan and Earle, 1991), and is economically important (David, 1991). The complete DNA sequence for the rice genome is now known. Questions are now being asked about the function of the genes within it, and techniques are in development to address these questions on a genome-wide, cross-species scale. Because of the high degree of conservation among the gene sequences and orders among cereals, the structural and functional analyses of rice should have broad practical implications for developing products and technologies in both rice and other economically important cereals. Mutants represent one of the most effective ways to acquire information on a gene's function. Various mutants, such as gene knockouts or null mutations, are invaluable for understanding biological variability when assigning functions to such a large quantity of sequence information. To make full use of the information mutants provide, developing methods that efficiently utilize vast quantities of information regarding function are critical.

Transposon tagging has become a powerful tool to create mutants for isolating new genes. Several experimental approaches have been undertaken to develop rice lines in which genes are randomly tagged by insertion elements (Greco et al., 2001b; Hirochika, 2001, 2004; Izawa et al., 1997; Jeon and An, 2001). Since the first introduction of maize *Ac*/*Ds* into rice (Izawa et al., 1991; Shimamoto et al., 1993), a variety of modified constructs have been introduced into rice (Chin et al., 1999; Greco et al., 2001a; Nakagawa et al., 2000). Some important features of the *Ac* element have been characterized in transgenic rice plants: for example (1) The *Ac* element transposes in 18.9% of transformed rice; (2) The transposed *Ac* element continues to transpose and is transmitted to subsequent generations; (3) *Ac* transactivates transposition of the non-autonomous *Ds* element; (4) The germinal excision frequency of *Ac* could be as high as 40%; and (5) *Ac* transposes preferentially into protein-coding regions (Enoki et al., 1999). These observations indicate that *Ac*/*Ds* gene tagging systems are valuable for rice functional genomics. Although another transposon system, *En*/*Spm*, has been introduced into rice plants, its transposition efficiency seems quite low (Greco et al., 2004). Later, Kumar et al. (2005) showed that the *En/Spm* system works very well in rice. For the *Ac*/*Ds* system, several successful transposon tagging experiments

*Corresponding author: E-mail: bocharng@ccms.ntu.edu.tw; Tel: +886-2-33664774; Fax: +886-2-23620879.

in rice plants indicate that it is a valuable tool for rice functional genomic studies (Zhu et al., 2003; Komatsu et al., 2003; Zhu et al., 2004). However, one major concern when using a native *Ac* element is that it could undergo frequent excision from the target gene, resulting in variegation. This disadvantage can be eliminated with the use of a two-component system or a self-stabilizing *Ac* derivative (Schmitz et al., 1994). However, the integration of a stable non-autonomous element in the target gene requires segregation of the two components, which is very inconvenient. With this mind, we previously designed an inducible one-component transposon, *INAc*, and demonstrated that it could be induced to transpose in dicotyledon plants (Charng et al., 2000 and 2004). Then, in a review of strategies for producing rice gene tags, Jeon and An (2001) suggested that the development of an inducible transposable element system would be valuable. This encouraged us in the present study to introduce the *INAc* element into rice plants. We observed that the *INAc* transposition events increase after applying the inducer, salicylic acid (SA), and that this higher transposition efficiency of *INAc* is dose dependent. In this paper, we conclude with a discussion of the promotion of the inducible transposon to perform rice functional genomic studies.

MATERIALS AND METHODS

Plants and constructs

Construction of the plasmid pINAc and its introduction into the *Agrobacterium tumefaciens* strain LBA4404 has been reported previously (Charng et al., 2000). The rice variety used in this study was *Oryza sativa* L. Japonica cv. TNG67. Calli induced from immature rice seeds were co-cultured with *Agrobacterium* using the methods described by Hiei et al. (1994) and Toki (1997). Putative transformed calli were selected with hygromycin B.

For induction experiments, the T1 rice seeds of each transformed line were incubated on callus induction medium (CIM) containing hygromycin for 4 weeks in order to yield enough calli for induction experiments. The effect of SA on *INAc* transposition was determined by incubating Hyg^R calli for various times on CIM containing 0 mM, 5 mM, or 10 mM SA. The calli from the same seed were evenly divided (about 25 calli per plate) and incubated on CIM containing 0 mM, 5 mM, or 10 mM SA with respect to the induction time of 7, 5, and 3 days. The calli were then transferred to CIM without SA for about 2 weeks before sampling for PCR analysis.

Isolation of genomic DNA and Southern hybridization analysis

Genomic DNA was isolated from transformed plants with the use of a kit (BIO 101, Vista, CA). In brief, fresh leaves (2 g) or callus tissue (0.1 g) was frozen in liquid nitrogen in a mortar and ground with a 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 analysis was performed as described by Charng and Pfitzner (1994).

PCR analysis of *INAc* excision events

Transposition of *INAc* from the *INAc*::LUC construct in transgenic plants was analyzed by polymerase chain reaction (PCR) with three oligonucleotide primers: primer 1P (identical to the T-DNA promoter sequence from position 285 to 303 as numbered by Velten et al. (1984), 5′-GGTTGCCATGTCCTACACG-3′); primer LUC2 (complementary to the luciferase coding sequence from position 367 to 347 as numbered by De Wet et al. (1987), 5′-GCGGGCGCAACTGCAACTCCG-3′); and primer AC1 (complementary to the *Ac* sequence from position 155 to 137 as numbered by Müller-Neumann et al. (1984), 5′-ACCCGACCGGATCGTATCG-3′). Each reaction mixture contained ~0.1 μg of template DNA, 0.25 μg of each primer, 0.2 mM deoxynucleoside triphosphates, 1 U of Taq DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% (w/v) gelatin. The amplification protocol comprised 30 cycles of 1 min at 94 °C, 2 min at 55°C, and 2 min at 72°C and was performed in a T-gradient Thermocycler (Biometra, Göttingen, Germany).

The re-intergrated *INAc* after excision in transgenic rice was analyzed by single-primer PCR with AC1 as the primer, and the amplification protocol comprised 45 cycles of 1 min at 94°C, 1 min at 32°C, and 3 min at 72°C.

The flanking sequences of the *INAc* element in transgenic plants were amplified by TAIL-PCR analysis with the following oligonucleotide primers: primer AC8 (CCCGTTTCCGTTCCGTTTTC), primer AC2 (CTCGGGTTCGAAATCGATC), and primer AC10 (CG GTTATACGATAACGGTCGGTAC), which are identical to the *Ac* sequences from position 4446 to 4465, 4475 to 4493, and 4513 to 4536 as numbered by Müller-Neumann et al. (1984), respectively. Three arbitrary degenerate (AD) primers and the TAIL-PCR procedure were according to a previous report (Liu et al., 1995), except in the present study the primary TAIL-PCR contained about 150 ng of rice genomic DNA.

RESULTS

INAc construct and transgenic rice plants

The construction of the plasmid pINAc has been reported previously (Charng et al., 2000). This construct was transformed into rice varieties *Oryza sativa* L. Japonica cv. TNG67. 45 independent transformed lines were collected for primary analysis. The results of induced transposition of *INAc* were obtained from transformed rice plants containing a single copy of *INAc*.

Spontaneous transposition of INAc in rice

INAc transposes spontaneously in primary transformed shoots of tomato harboring the *INAc* element. The first step to assess the facilitation of *INAc* is to examine the spontaneous transposition events in transgenic rice. We analyzed genomic DNA from primary rice calli by PCR analysis (Figure 1) and Southern blot analysis (Figure 2). Two sets of primers were used to verify transposed vs. un-transposed *INAc* elements. With the primers AC1 and LUC2, a 580-bp PCR product was obtained with DNA from Hyg^R rice plants, which harbor the un-transposed *INAc* element (Figure 1A). With the primers 1P and LUC2, a 670-bp PCR product was obtained with DNA from transformed rice plants, indicating the excision of the *INAc* element. As shown in Figure 1, the transformed lines R7, R51 and R20 yielded a 580-bp fragment with primers AC1 and LUC2 (Figure 1B) but yielded no product with primers 1P and LUC2. These findings indicate that these

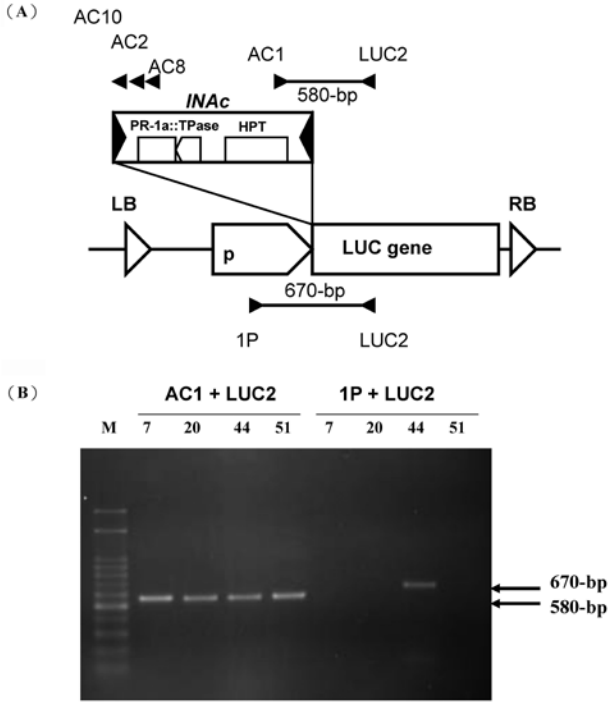

Figure 1. Construction of the *INAc* element and PCR analysis of its excision. (A) Structure of the *INAc* element and the location of primers (shown as solid triangles) used for PCR analysis. The *INAc* element contains a PR-1a::TPase fusion and a hygromycin resistant gene (HPT). The sizes of expected PCR products (580- and 670-bp before and after excision of *INAc*, respectively) are indicated. The sequential primers for TAIL-PCR, *Eco* RV site and probes for DNA blot analysis were also indicated. (B) Ethidium bromide-stained agarose gel on which PCR products were separated. PCR was performed with genomic DNA from the indicated transformants and with the indicated primers. The hatched boxes indicate the DNA fragments used as probes for Southern blot analysis. Lane M, 100-bp DNA ladder. Abbreviations: LB and RB, left and right borders of T-DNA; p, 1′ promoter; HPT, hygromycin phosphotransferase gene; LUC, luciferase gene.

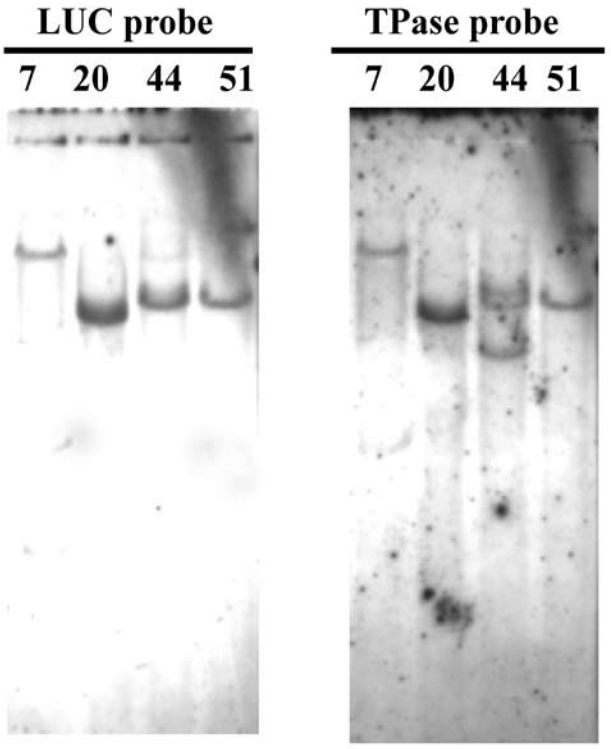

Figure 2. Southern blot hybridization of *Eco* RV digested genomic DNAs isolated from different transgenic rice lines with the LUC probe or with the TPase probe (for construction see Figure 1). The LUC probe (*left*) revealed the T-DNA copy and together with the TPase probe (*right*) revealed the un-transposed (indicated by the *arrow*) or transposed *INAc* (*star*).

Hyg^R rice plants represented successfully transformed lines and that the *INAc* elements were stable without induction treatment. In contrast, transformed line R44 yielded both a 580-bp fragment and a 670-bp fragment with these two sets of primers, indicating that this rice plant contained cells in which *INAc* had undergone spontaneous transposition and cells in which it had not. By using these two sets of primers, 8 out of 45 transgenic rice showed spontaneous transposition (data not shown). For the transformed lines yielding no 670-bp fragment, the spontaneous transposition event was monitored during the development of the T0 generation. PCR analysis indicated that the un-induced *INAc* element remained stable and transmitted to subsequent generations (data not shown). Also, genomic DNA from these transformed lines was subjected to Southern blot analyses. As probes, the 1.4-kb *Bam* HI/*Eco* RV fragment comprising the LUC reporter gene, and the 2.56-kb *Bam* HI fragment comprising the transposase gene were used. The DNA samples were digested with *Eco* RV and hybridized with LUC probe. Figure 2 shows that the transformed lines R7, R20, R44 and R51 yielded hybridizing fragments of 23-kb, 10-kb, 13-kb and 13-kb, respectively. These results demonstrate each transformed line harboring a single copy of T-DNA. After removal of the LUC probe, the same filter was hybridized with the TPase probe. In

agreement with the PCR experiment, the transformed lines R7, R20 and R51 yielded the same hybridizing patterns, indicating the primary donor sites of the un-transposed *INAc* element. For the transformed line R44, in addition to the hybridizing fragment of 13-kb (un-transposed *INAc*), bands of 14-kb and 7-kb indicated the transposition of *INAc* (Figure 2).

Induction of INAc transposition

The fact that *INAc* transposition occurred spontaneously in transformed line R44 indicates *INAc* is active in rice plants. To determine whether the *INAc* element could be induced in rice plants, we studied the induction of *INAc* transposition using salicylic acid (SA) as the inducer in transgenic rice calli and plants. The behavior of *INAc* in rice plants was studied with T1 seed-derived calli from four transformed rice lines, R7, R20, R44 and R51. Lines R7, R20 and R51 contain a single copy of the *INAc* element. The T1 rice seeds of each transformed line were incubated on CIM containing hygromycin to ensure the presence of the *INAc* element (transposed or un-transposed). Each progeny line was established by incubating the seed on CIM without SA, in order to yield enough calli for induction experiments. The effect of SA on *INAc* transposition was determined by incubating calli for various times on CIM containing 0, 5 mM or 10 mM SA. Since abundant calli could be regenerated from the rice seeds on the CIM, the calli regenerated from each seed (determined as an independent progeny line) were then divided into three portions and incubated on CIM with or without SA. Five Hyg^R seeds each from lines R7, R20, and R51 and 30 Hyg^R seeds from line R44 were used for induction experiments.

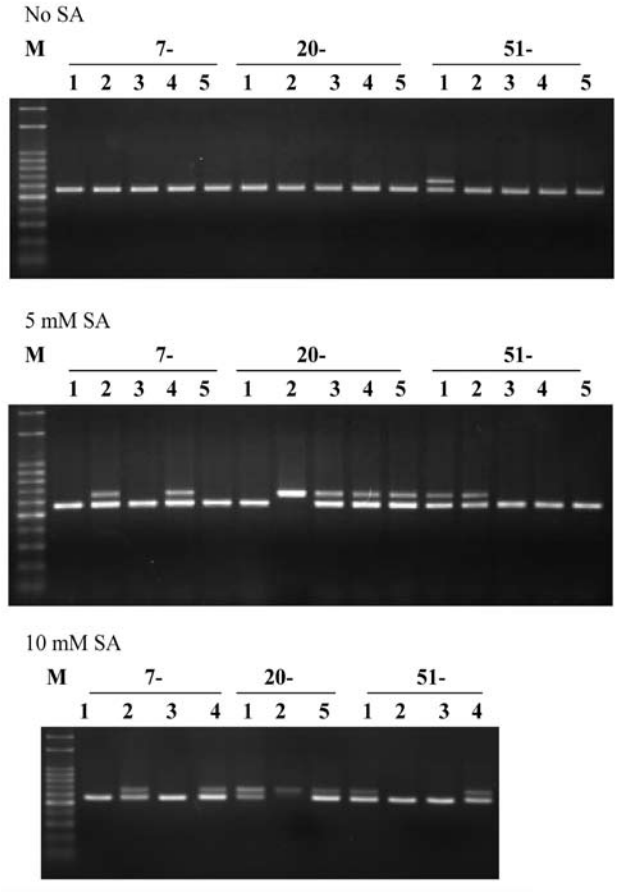

Figure 3. PCR analysis for detection transposition events for progeny lines of R7, R20 and R51. Each PCR reaction was performed with 1P, AC1 and LUC primers. The reaction yielded only 580-bp indicated un-transposed *INAc*. The reaction yielded only 670-bp, indicating completely-transposed *INAc*. The reaction yielded both 670 and 580-bp, indicating partially-transposed *INAc*. Lane M, 100-bp DNA ladder.

After the induction process, the regeneration calli of each line were collected for DNA extraction. The transposition events were determined by multiplex PCR analysis using three primers: AC1, LUC2 and 1P. The induced transposition efficiency of each transformed line was determined by the presence of the 580-bp fragment and/or the 670-bp fragment as shown in Figure 3 for progeny lines from R7, R20 and R51. For progeny lines of R44, the presence of the PCR products were expressed as "X" or "O" as described in Table 1. It is notable that exposure to 10 mM SA resulted in a marked decrease in the efficiency of callus regeneration. For some progeny lines treated with 10 mM SA, new calli formation was not observed, even after four weeks of propagation. For these progeny lines, DNAs could not be extracted for PCR analysis and are expressed as "—" (Table 1).

For transformed lines R7, R20 and R51, in which no spontaneous transposition event was detected, all un-induced progeny lines yielded a 580-bp fragment and one (line R50-1) out of 15 progeny lines also yielded a 670-bp fragment. These findings suggest that a spontaneous transposition event occurred in these transformed lines but with low frequency. However, when these progeny lines were treated with 5 mM SA, 8 out of 15 calli exhibited transposition events; when these same line were treated with 10 mM, 7 out of 11 calli exhibited transposition events. In addition to the spontaneous transposition events observed in un-induced progeny line R51-1, a 670-bp product was also detected in SA-induced progeny lines R7-2 (at both 5 mM SA and 10 mM SA), R7-4 (at both 5 mM SA and 10 mM SA), R20-1 (at 10 mM SA), R20-2 (at both 5 mM and 10 mM SA), R20-3 (at 5 mM SA), R20-4 (at 5 mM SA), R20-5 (at both 5 mM and 10 mM SA), R51-2 (at 5 mM SA), and R51-4 (at 10 mM SA). In summary, these findings demonstrate that transposition events were triggered under un-induced conditions with 0.6% efficiency while 5 mM- and 10 mM-SA induction produced a 53% and 64% efficiency, respectively. Taken together, these results show that the transposition efficiency of *INAc* was induced by SA in a dose-dependent manner. On the other hand, the transposition efficiency in line R7, R20, and R51 was 40%, 80% and 20%, respectively. These findings suggest that the location of the *INAc* element in the plant genome also contributes to the efficiency of transposition.

Table 1. PCR analysis for detecting transposition events for progeny lines of R-44.

Induction	no SA			5 mm SA (5 days)			10 mm SA (3 days)		
PCR products Progeny lines	580-bp	670-bp	Single primer	580-bp	670-bp	Single primer	580-bp	670-bp	Single primer
1	O	X		O	X		O	X	
2	O	O		O	X		O	O	
3	O	O		O	O		—	—	—
4	O	X		O	O		—	—	—
5	O	O		O	O		—	—	—
6	O	O		O	O		—	—	—
7	O	X		O	O		—	—	—
8	O	O		O	O		O	O	
9	O	X		X	O	O	O	O	
10	O	X		O	X		—	—	—
11	O	O	O	O	O		O	O	
12	O	X		O	O		O	O	O
13	O	O		O	O		O	O	
14	O	O		O	O	O	O	O	
15	O	X		O	O		O	O	O
16	O	X		O	O		—	—	—
17	O	O		O	O		O	O	
18	O	X		O	O		O	O	
19	O	O		O	O		O	O	O
20	O	O		O	O		O	O	
21	O	X		O	X		X	O	
22	O	O		O	O		O	O	
23	O	X		O	O		X	O	
24	O	X		O	X		—	—	—
25	O	O		O	O		—	—	—
26	O	X		O	O		X	O	
27	O	O		O	O		O	X	
28	O	O	O	O	O	O	O	O	
29	O	X		O	X		O	O	
30	O	X		O	O		O	O	

"O": symbol denotes the existence of PCR products (580-bp products for primers AC1 and LUC2; 670-bp products for primers 1P and LUC2; different size patterns for single primer (AC1) PCR; "X": no PCR product; "—": DNAs were not extracted for PCR analysis.

Interestingly, among the progeny lines of the transformed line R-44 that demonstrated spontaneous transposition, 15 out of 30 un-induced progeny lines yielded a 670-bp fragment when using primers 1P and LUC2 (Table 1). These findings indicated that new spontaneous transposition events occurred in the un-induced progeny of transformed line R-44. When the same 30 progeny lines were treated with 5 mM SA, 29 lines yielded a 580-bp fragment, and 24 lines yielded a 670-bp fragment. Exposure to 10 mM SA resulted in a marked decrease in the efficiency of callus regeneration, and hence 21 progeny lines were obtained for analysis. Among these 21 lines, 18 lines yielded a 580-bp fragment when using primers AC1 and LUC2, and 19 lines yielded a 670-bp fragment when using primers 1P and LUC2. Thus, the transposition efficiencies of 0 mM SA- (spontaneous), 5 mM SA-, and 10 mM SA-induced progeny lines were 50%, 80% and 90%, respectively. These results suggest that SA increases the frequency of production of the 670-bp product in a dose-dependent manner although, as mentioned above, treatment of plants with 10 mM SA resulted in a marked decrease in the efficiency of callus regeneration.

It is important to understand why some SA-induced progeny lines yielded both a 580 bp and a 670 bp product while others yielded only one of these products. Typical examples of this phenomenon were in progeny lines R20-2 (Figure 3, 10 mM SA induction), and R44-21, -23, and -26 (Table 1, 10 mM SA induction). When these progeny lines were incubated on CIM without SA, only a 580-bp fragment could be detected. When these same progeny lines were induced with 10 mM SA, only a 670-bp fragment could be detected. When progeny line R44-23 and R44-26 were induced with 5 mM SA, both a 580-bp fragment and a 670-bp product could be detected. It is likely that in these SA-treated progeny lines, *INAc* was transposed in most cells after 10 mM SA treatment. As a result, no 580-bp fragment was yielded when using AC1 and LUC2 primers for PCR analysis. We found that this phenomenon could also serve as an additional method to determine the effect of SA concentration. In Table 1, the transposition events of each progeny line with various treatments were interpreted as an un-transposed line, a partially-transposed line, or a completely-transposed line. The un-transposed lines were determined as those lines which yielded only a 580-bp fragment but no 670-bp fragment, e.g., progeny line R44-1 and R44-24. The partially-transposed lines were determined as those lines which yielded both a 580-bp fragment and a 670-bp fragment, e.g., progeny lines R44-3 to R44-8 (Table 1, 5 mM SA induction) and R7-2 (Figure 3, 5 and 10 mM SA induction). The completely-transposed lines were determined as those lines which yielded only a 670-bp fragment but no 580-bp fragment, e.g., progeny lines R20-2 (Figure 3, 5 mM and 10 mM SA induction), and R44-21, -23 and -26 (Table 1, 10 mM SA induction). The SA-induced transposition efficiency was then measured by the percentage of un-transposed, partially-transposed, and completly-transposed progeny lines of an individual transformed line. Using this method, we observed that partially-transposed efficiency reached 50%, 77% and 76% in un-treated, 5 mM SA- and 10 mM SA-treated progeny lines, respectively. Furthermore, completedly-transposed efficiency reached 0%, 3% and 14% in un-treated, 5 mM SA- and 10 mM SA-treated progeny lines, respectively. Once again, these results demonstrated that SA can induce the expression of PR-1a::TPase and transposition events in transgenic rice plants in a dose-dependent manner.

Detection of new transposition rice lines and the flanking genomic DNA

An important feature of an inducible transposable element for functional genomic studies is its tendency to create new transposition lines after induction. Thus, a high throughput method for screening the independent transposants (transposition lines) markedly increases the probability of success of transposon tagging when attempting to isolate important plant genes. Toward this end, in the present study we developed an identification system for new transposants in rice plants based on the single-primer PCR concept described by Karlyshev et al. (2000). The procedure involves just one transposon-specific primer and a single PCR reaction involving a low annealing temperature (32°C) for amplification of the region adjacent to the transposon insertion site. Amplification of this region is possible because at low annealing temperatures, the primer will bind to sites of limited sequence complementarity that are on the opposite strand from the specific primer binding site and close enough to the transposon insertion site for PCR to work. Subsequently, when the single primer PCR results indicate the existence of new independent transposants, the flanking sequence of the re-integrated *INAc* can be obtained by the TAIL-PCR technique.

As indicated in Table 1, the DNA extracts prepared from the progeny lines of R44 were analyzed by single-primer PCR. Principally, a single-primer PCR reaction produces several bands composed of specific and non-specific products. In this experiment, one end of the specific products was primed by AC1 containing a portion of DNA (155-bp) identical to the end of *INAc* while the non-specific products were amplified from the unknown sequence dispersed on the chromosome. All un-transposed progeny lines of R44 principally yielded the same DNA pattern, composed of non-specific products and/or a specific product. However, when the *INAc* element was transposed from the original site and integrated into another site on the chromosome, observing different DNA patterns by a single-primer PCR analysis became possible. Figure 4 presents a typical example of this analysis. For most of the progeny lines, single-primer PCR produced three major bands of 600-bp, 800-bp and 1.3-kb in size. However, for progeny line R44-14 (with 5 mM SA treatment), a 450-bp band was detected instead of a 800-bp band. These results indicate that for progeny lines harboring the un-transposed *INAc*, the 800-bp band was the specific product primed by the AC1 primer. Additional bands of various sizes but larger than 155-bp indicate new and different transposition sites of *INAc*. For example, the appearance of the 450-bp band in progeny line R44-14 strongly suggested a new transposition site of *INAc*. According to this, we detected 2 out of 30 progeny lines (no SA), 3 out of 30 progeny lines (5 mM SA-treated) and 3 out of 21 progeny lines (10 mM SA-treated) of R44 that yielded new PCR product patterns (Table 1).

The experiments were expanded to amplify the flanking sequences of the T-DNA and the transposed *INAc* elements. Genomic sequences flanking the *INAc* transposons were isolated from transgenic lines of R-44 and R-20 with induced somatic transposition, due to observation of the new single-primer PCR product patterns. The flanking sequences were isolated using TAIL PCR (Liu et al., 1995, see Materials and Methods). Three specific primers AC8, AC2, and AC10 were used for the primary, secondary, and tertiary PCR reactions, respectively (Figure 1). A summary of the significant homologies obtained after comparison of the flanking sequences obtained in public database is shown in Table

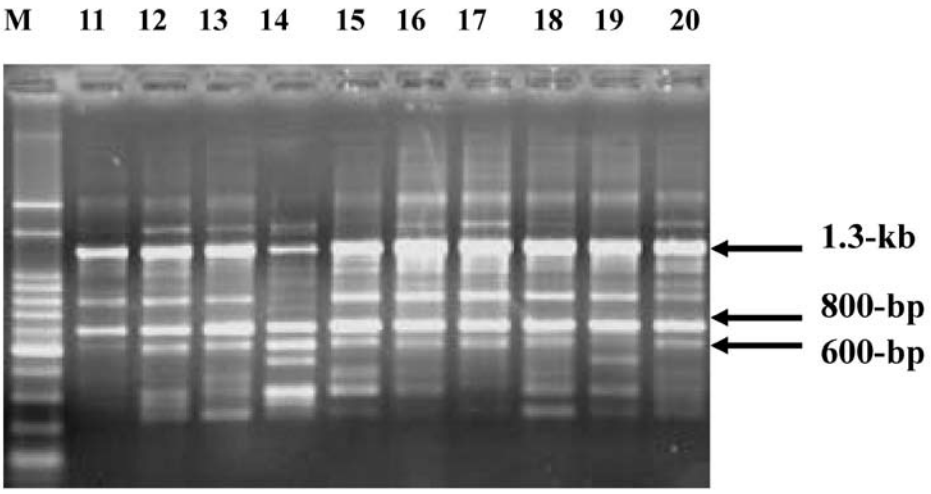

Figure 4. Single-primer PCR analysis of the new transposition events of *INAc*. Genomic DNA from transformed rice progeny lines were set for PCR reactions, which contained only AC1 primer, and the annealing temperature was set as 32°C.

2. For the transformed line R-20, only 1 out of 3 calli showed an un-linked transposition event of *INAc*, yet for the transformed line R-44, 4 out of 6 calli harbored the transposed *INAc* un-linked to the T-DNA. These results indicate that the independent transposition events of *INAc* could be induced efficiently by SA.

DISCUSSION

Rice, with its small genome size and well-characterized molecular information, is an ideal model plant for cereal genomics research. The entire sequence of the rice (*Oryza sativa*) genome has been determined by the International Rice Genome Sequencing Project (IRGSP). Still, based on current technologies, a large population of mutant plants will likely be required to adequately assign function to the abundance of sequence information.

Jeon and An have summarized various strategies for producing gene tags that may be invaluable for understanding the functional genomics of rice (2001). Both T-DNA and *Ac* element have been used as the insertional mutagen for rice gene tagging. Previously, we reported an inducible transposable element for higher plants (Charng et al., 2000). In the present study, we studied the activity of *INAc* in order to further develop rice gene tagging systems.

For the *INAc* construct, the native PR-1a promoter was fused with the transposase gene of the *Ac* element, and this PR-1a::TPase construct was inserted together with the HPT gene directly into a small *Ds* element (Figure 1A). The resulting *INAc* element was placed between the 1′ promoter and the LUC gene. In principal, LUC expression should be triggered after excision of the transposable element. However, when the *INAc* construct

Table 2. Genomic sequences flanking *INAc* insertions in transgenic rice plants. The T-DNA integration site of each line is indicated after its designation.

Line	Chromosome	BACs/PACs	Insertion position (bp)	GenBank accession no.	Identities
R-44	(T-DNA) 3	OJ1124_H03	135923	AC087852	102/102 (100%)
0-11	10	OSJNBa0041L14	76009	AC099042	340/352 (96%)
0-28	1	P0443D08	120609	AP003250	467/478 (97%)
5-9	nd	nd	nd	nd	nd
5-14	3	OSJNBb0081B07	85729	AC093018	67/67 (100%)
5-28	9	P0528B09	88111	AP004703	89/89 (100%)
10-12	10	*Pseudo10p0.0-10p4.4	768203	AC145127	(100%)
10-15	nd	nd	nd	nd	nd
10-19	3	OSJNBa0002I03	72340	AC091246	40/40 (100%)
R-20	(T-DNA) 12				
5-2	9	OSJNBa0047P18	154242	AP005864	47/47 (100%)
	12	OSJNBa0014C10	45261	AL731787	27/27 (100%)
10-5	12	OSJNBa0052H10	29041	BX000494	104/104 (100%)

"nd": indicates no detectable product is obtained after TAIL PCR amplification.

was introduced into rice plants, our preliminary analyses indicated that although the transposition events could be detected by PCR analysis, no luciferase activities were detected (data not shown). This result is likely because the 1′ promoter is less functional in rice plants. Consequently, we set out to determine the transposition events using PCR analysis. 8 out of 45 primary transformed rice lines yielded a 670-bp band with primers 1P and LUC2 in the absence of the inducer SA. This result is likely attributable to activation of the PR-1a promoter by nearby enhancer elements in the plant genome (Beilmann et al., 1992).

In the transformed lines that exhibited no spontaneous transposition events, the *INAc* element remained stable during the development of the plant, and it was transmitted to the next generation (data not shown). The induction experiments were performed with the T1 progeny lines, which harbored the *INAc* element. We used PCR to determine transposition events by observing the 670-bp fragment with primers 1P and LUC2. The results shown in Figure 3 and Table 1 demonstrate that transposition events could be induced by SA treatment (5 mM or/and 10 mM) calli with various efficiencies. Generally, we incubated rice seeds on CIM for 4 weeks to produce enough calli for induction experiments. Spontaneous transposition events may have occurred during this calli regeneration process. Since the calli produced for induction experiments were divided into three equal portions prior to any artificial stimulation for transposition, it would seem that a spontaneous transposition event would happen equally in all three portions of the calli (control, 5 mM or 10 mM SA-treated). However, the fact that only one transposition event (1 out of 15) was detected in un-treated calli of progeny lines from R20, R7 and R50 strongly suggests that the transposition events detected in SA-treated calli mainly resulted in response to the induction treatment.

For those calli regenerated from the transformed line R44, in which spontaneous transposition events were detected in the T0 generation, it was necessary to consider that the 670-bp products detected in the SA-treated calli may have been spontaneous transposition events before or during SA treatment. To rule out this possibility, we determined the induced transposition events and efficiencies by comparing the presence of both a 580-bp (un-transposed) and a 670-bp (transposed) product. Any 670-bp product with primers 1P and LUC2 was recorded as a transposition event (spontaneous or non-spontaneous). The transposition efficiencies were based upon the presence of a 670-bp product in the progeny lines of transformed line R44. We found that, the transposition efficiency of un-induced, 5 mM SA- and 10 mM SA-treated R44 progeny lines were 50%, 80% and 90%, respectively. Alternatively, for progeny lines R44-24, R44-34 and R44-55, 10 mM SA-treated calli yielded only a 670-bp fragment but no 580-bp product. This was likely because transposition events were triggered in most cells of these calli, and hence the un-transposed *INAc* could not be detected. We considered these calli as completely-transposed after treatment. The presence of completely-transposed calli from each progeny line served as a means to determine the effect of SA concentration. On this basis, completedly-transposed efficiency reached 0%, 3% and 14% by the un-treated, 5 mM SA- and 10 mM SA-treated progeny lines, respectively. Taken together, these results strongly suggest that the transposition efficiency of *INAc* can be induced by SA in a dose-dependent manner.

An important feature of an inducible transposable element is its ability to induce transposition in germinal tissue. Induction of the germinal transposition in rice is supposedly more complicated than in tobacco, since the rice floral tissues embed themselves in the flag leaves when (or before) meiosis occurs. For our primary induction experiments, the highest germinal transposition efficiency was detected by flooding the transgenic rice with 5 mM SA solution; two out of 40 R20 progeny lines showed transposition events under those condition, indicating a 5% germinal transposition efficiency (data not shown). Compared with the germinal transposition of the native *Ac* element in rice (up to 40%; Nakagawa et al., 2000), optimization of induction conditions for germinal transposition of *INAc* requires further experimentation. Still, since rice is an ideal model plant due to the availability of its complete DNA sequence and its high regeneration efficiency from calli, even heterozygous mutants created by *INAc* are also valuable for reverse genetics. We believe that the induced *INAc*-mutants regenerated from rice calli are also valuable.

Our experiences in the present study lead us to suggest a procedure for inducible transposon tagging in rice. First, the *INAc* was induced to create abundant mutant lines. Then, as a high throughput tool, the single-primer PCR method was used to identify the new transposants. Finally, these transposants were collected for TAIL-PCR to identify the flanking sequence of the *INAc* element. By aligning the DNA sequences obtained from the TAIL-PCR technique to ensure the existence of the region from the specific primer for tertiary TAIL-PCR (primer AC10 in this paper) to the end of *INAc* (5-end in this paper), the new transposants could be recorded for insertion-tagged sequence libraries for rice.

All the features described above indicate that the inducible transposon system would be a valuable tool for Agro-biotechnology, e.g. either to create knockout mutants or to generate selectable marker-free transgenic plants. Nevertheless, several improvements must be considered in order to design a new inducible transposon for plant gene tagging systems. First, the fact that *INAc* can transpose spontaneously in tomato, tobacco, and rice indicates that the PR-1a inducible promoter can be activated by endogenous stimuli in these plants. Although the expression level in this condition might be low, the resulting abundance of the transposase is likely sufficient to drive transposition events. Previous studies indicated that *Ac*/*Ds* transposons are easily triggered by fewer transposases (Fusswinkel et al., 1991). Thus, it seems that spontaneous transposition events of

INAc may occur generally in heterologous plants, with various efficiencies. This fact encourages us to consider the use of an animal inducible system for constructing new inducible transposons. Recently, several inducible systems from animals were used in plants (Padidam et al., 2003; Ouwerkerk et al., 2001; Zuo et al., 2000; Böhner et al., 1999). All of these systems should be assessed for the creation of new inducible transposon. On the other hand, since a self-stabilizing *Ac* concept has been reported (Schmitz and Theres, 1994), a one-step inducible transposon that undergoes inducible transposition but is stable after integration should also be valuable. Alternatively, many activation-tagging systems have been developed in *Arabidopsis* for cloning genes (Weigel et al., 2000; Marsch-Martinez et al., 2002). An entrapment tagging system allows for monitoring gene activity by creating fusions between tagged genes and a reporter gene (Meissner et al., 2000). According to these, several new inducible transposons based on *INAc* have been demonstrated to be functional and as efficient as *INAc* in rice (Charng et al., unpublished data). All of these strategies, combined with the one-step inducible transposon concept, will allow us to develop more efficient transposon systems for future rice functional genomic studies.

Acknowledgments. This project was supported by the National Science Council (Grant No. NSC93-2317-B-002-007-) of Taiwan.

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可誘導轉位子應用於水稻功能基因體學

常玉強[1] 吳紀德[1] 謝嘉珊[1] 莊函寧[1]
黃紀穎[1] 葉凌君[1] 謝亞雄[1] 杜 鎮[2]

[1]國立台灣大學農藝系
[2]中央研究院植物暨微生物研究所

Ac/Ds 轉位子為研究植物功能基因體學之重要工具。本研究以 *Ac* 轉位子為基礎建構「可誘導轉位子」系統，並應用於水稻功能基因體學。可誘導轉位子 *INAc* 以 *PR-1a* 啟動子驅動 *Ac* 轉位酶，在水稻癒合組織中可受水楊酸誘導而轉位，且水楊酸劑量影響轉位效率；分析跳出之轉位子側翼序列顯示轉位子跳入染色體新位置後可遺傳至下一代，證明「可誘導轉位子」系統可應用於水稻功能基因體學研究。

關鍵詞：*Ac* 轉位酶；可誘導啟動子；水楊酸；基因釣取。