Transposition of the maize *activator* element in transgenic rice plants

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ABSTRACT

Transposition of the maize *Activator* (*Ac*) element was observed in transgenic rice. After protoplast transformation, *Ac* excision from an interrupted hygromycin phosphotransferase gene was monitored by appearance of the hygromycin-resistant colonies. The frequency of *Ac* excision, based on the biological assay was up to 19%. Southern hybridization analysis indicated that at least one copy per genome of the hygromycin-resistance gene was reconstituted after *Ac* excision and that the transposed *Ac* element was reintegrated into the rice genome. Analysis of DNA sequences at 14 empty donor sites indicated that the *Ac* element was excised in rice in a similar manner as maize. The excision of an *Ac* mutant in which a 1.3 kbp *Tn903* fragment was inserted at a unique *Bam*HI site so as to disrupt binding of the putative transposase was not detected by DNA analysis. These results demonstrated that the maize *Ac* element might be used as an effective heterologous transposon for mutagenesis and gene tagging in rice, an important food crops.

INTRODUCTION

The autonomous controlling element *Activator* (*Ac*) and non-autonomous *Dissociation* (*Ds*) of maize were the first transposon family identified by McClintock (1). The structure and function of *Ac/Ds* elements have been among the best characterized in higher plants (2). The *Ac* element is 4563 bp in length and contains one long open reading frame encoding a 3.5 kb transcript for the putative transposase. The *Ac* element has an 11 bp terminal inverted repeat and generates an 8 bp duplication at the site of insertion.

Transposon gene tagging in maize has facilitated the isolation of the *a1* (3), *bz* (4), *bz2* (5), *c1* (6,7), *c2* (8) and *R-nj* genes (9) in the anthocyanin biosynthetic pathway. The *opaque-2* gene which regulates the expression of the maize storage protein zein was also isolated using this approach (10). A principal difficulty in these tagging experiments arose in identifying the active transposon sequence at the genetic locus of interest among a

background of multiple copies of defective *Ac* (*Ds*) present in the maize genome. In this sense, introduction of an *Ac* element to a heterologous genetic background provides an unique advantage since there will be no endogenous element that would interfere in a search for the *Ac* insertion site.

The *Ac* element has been introduced into five dicotyledonous plant species, tobacco (11), tomato (12), potato (13), carrot and *Arabidopsis thaliana* (14). In all cases the *Ac* element transposed at a high frequency with a non-reproductive mechanism as maize. In addition, a second autonomous transposable element *Enhancer/Suppressor* has been shown to transpose in transgenic tobacco plants (15,16). We are interested in studying transposition of the *Ac* element in rice with a long term goal of developing a method for transposon mutagenesis and gene tagging. Rice is one of the most important food crops in the world. There exists a refined genetic linkage map with morphological and isozyme markers as well as a restriction fragment length polymorphism map of rice chromosomes (17). Currently, rice is so far one of the major cereal crops for which an efficient regeneration/transformation system is available. However, further advances in molecular analysis of the rice genome are hindered by the absence of a well-characterized endogenous transposable element as a mutagen and gene tag. Thus, introduction of the maize *Ac* element might substitute the need for an endogenous transposon for this purpose. Additionally, because rice is a member of the same taxonomic family as maize, rice might provide a genetic milieu more similar than tobacco in functional analysis of *Ac* transposition (18, 19).

We have previously reported an efficient protoplast regeneration and transformation system for production of fertile transgenic rice plants (20, 21). Here, we provide evidence for transposition of the maize *Ac* element in transgenic rice plants based on a hygromycin-resistance assay, southern blot hybridization and DNA sequence analyses.

MATERIALS AND METHODS

Construction of Transformation Vectors

Rice transformation vectors were constructed in such a way to allow a biological assay for excision of *Ac* in protoplast colonies.

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The *Ac* element isolated from an unstable waxy mutant, *wx-m7*, was kindly provided in the plasmid pAc7B by Dr. Peter Starlinger, University of Koln, FRG (22). A 4563 bp *Ac* sequence flanked by 18 and 35 bp of the maize *wx* sequence (23) was reisolated as a 4.6 kbp BssHII fragment from pAc7B. An aminoglycoside 3'-phosphotransferase gene of *Tn903* that confers resistance to kanamycin was isolated as a 1.3 kbp BamHI fragment from KSAC (Pharmacia).

The coding sequence of hygromycin phosphotransferase gene is under the control of Cauliflower Mosaic Virus 35S promoter and *Agrobacterium tumefaciens tml* polyadenylation site in pTRA132 (Figure 1). The maize *Ac* element with the 53 bp flanking *wx* sequences was inserted into a BssHII site of the 5'-untranslated leader region of the hygromycin resistance gene in pTRA137 and pTRA137R. The direction of transcription of the putative transposase gene is the same as the hygromycin-resistance gene in pTRA137, and the reverse orientation in pTRA137R. The *Tn903* neomycin phosphotransferase gene was inserted at the unique BamHI site of the *Ac* element, resulting in pTRA139 and pTRA139R. The *Ac* insertion site was located 60 bp downstream and 20 bp upstream, respectively, of the transcription initiation site of CaMV 35S promoter and the translation initiation codon of the *hph* gene.

Production of Transgenic Rice Plants

Protoplasts were isolated from suspension callus culture of rice (*Oryza sativa* L.) cultivars *Nipponbare* and *Taipei 309* as described (20). Protoplasts were transformed with plasmid DNA after treatment with 25%(w/v) polyethylene glycol as described (21).

Southern Hybridization Analysis

Rice DNA isolation and southern blot hybridization analysis were performed as described previously (21). Total DNA was isolated from young leaf tissue of transgenic plants during the booting stage prior to flowering. For southern blot hybridization analysis, 10 µg of DNA were digested with HindIII/EcoRI or BamHI, and digests were extracted with phenol/chloroform prior to electrophoretic separation in 0.8%(w/v) agarose gels. Three probe DNA fragments were used to analyze the structure of transferred DNA as shown in Figure 2. A 0.3 kbp HindIII/XbaI fragment corresponds to the CaMV 35S promoter. A 1.2 kbp EcoRI/PvuII fragment and a 1.6 kbp HindIII fragment represent the internal *Ac* sequences from nucleotide position 1172 to 2777, and from position 722 to 2075, respectively.

PCR amplification and DNA sequence analysis

DNA sequences surrounding empty donor sites in genomic DNA of transgenic rice plants were amplified by polymerase chain reaction (PCR) (24) and sequences were determined by the dideoxy chain termination method (25). One hundred ng of undigested genomic DNA from transgenic rice plants were subjected to 40 cycles of PCR using the GeneAmp kit (Perkin-Elmer/Cetus). The first cycle of the reaction was carried out at 93°C for 3 min, at 55°C for 1 min and at 72°C for 1 min. The following 2nd to 39th cycles were conducted at 93, 55 and 72°C for 1 min each. The 40th cycle was done at 72°C for 5 min and at 4°C overnight. The first oligonucleotide primer for the PCR reaction was 5'-GCA AGA AGC TTC CTC TAT ATA AGG AAG TTC-3'. It was complementary to the CaMV 35S promoter sequences from nucleotide position -46 to position -17 from the transcription initiation site. The 30-base primer contained two-base mismatches that would create a new HindIII site. The second

primer was 5'-CGC GGT GAA TTC AGG CTT TTT CAT ATC TCA-3'. It was complementary to the coding sequence of the hygromycin resistance gene from nucleotide position -6 to position 24 from the first base of the initiation codon (26). The 30-base primer contained one-base mismatch that would create a new EcoRI site. If *Ac* excision was precise as in the case of maize, the PCR reaction with these primers would generate a 218 bp fragment. The amplified fragments were cloned into a Bluescript plasmid (Stratagene) and the sizes of cloned fragments were determined by agarose gel electrophoresis. The 218 bp fragments were sequenced using the Sequenase kit (United States Biochemical), as recommended by the supplier.

RESULTS

A biological assay was developed to monitor excision of the maize *Activator (Ac)* element in rice. The *Ac* element was originally cloned from the *wx-m7* locus and was isolated as a 4.6 kbp BssHII fragment with 18 and 35 bp of the flanking maize *waxy (wx)* sequences containing no translation start signal (22, 23). The *Ac* fragment was inserted into the 5'-untranslated leader sequence of the hygromycin-resistance (*hph*) gene in order to interrupt proper gene expression and render the resistance gene non-functional. The direction of transcription of the putative transposase gene is the same as the hygromycin-resistance gene in the resulting plasmid pTRA137/139, and is the reverse orientation in pTRA137R/139R as shown in Figure 1. After transfer to rice protoplasts, the *hph* gene was expected to restore gene function to confer resistance to hygromycin if *Ac* were excised and if the promoter and hygromycin coding sequences were religated, including the 53 bp *wx* sequence, in the untranslated leader region of the gene.

As a negative control we constructed a mutant *Ac* element, in which a *Tn903* kanamycin resistance gene was inserted to the

a. pTRA132 :

b. pTRA137 and 137R:

c. pTRA139:

Figure 1. Physical map of rice transformation vectors pTRA132 and *Ac* element-insertion derivatives pTRA137, 137R, 139 and 139R. The coding sequence of hygromycin phosphotransferase gene is under the control of Cauliflower Mosaic Virus 35S promoter and *Agrobacterium tumefaciens tml* polyadenylation site in pTRA132. The maize *Ac* element with the 53 bp flanking *wx* sequences (solid box) was inserted into a BssHII site of the 5'-untranslated leader region of the hygromycin-resistance gene in pTRA137 and pTRA137R. The direction of transcription of the putative transposase gene is the same as the hygromycin-resistance gene in pTRA137, and the reverse orientation in pTRA137R. The *Tn903* neomycin phosphotransferase gene was inserted at the unique BamHI site of the *Ac* element, resulting in pTRA139 and pTRA139R. Abbreviations for restriction enzymes are Bh for BamHI, Bs for BssHII, Ec for EcoRI, Hd for HindIII, Pv for PvuII and Xb for XbaI.

unique BamHI site of *Ac*, forming pTRA139 and pTRA139R as shown in Figure 1. The BamHI site was chosen for insertion mutagenesis because of ease in construction. The BamHI site is located 149 bp upstream of the major transcription initiation site of the putative transposase gene in *Ac* (27). The BamHI site is also positioned at the middle of the 5'-end binding sites for the putative transposase protein (28). Thus, the insertion mutagenesis of the *Ac* element was expected to interfere with either the transcription initiation of the putative transposase gene, or with the binding of transposase at the 5'-terminus of *Ac*, thus providing a negative control for the biological function of *Ac* excision.

Hygromycin-resistance assay monitored *Ac* excision in protoplast colonies

The intact *Ac* element appeared to excise from the *hph* gene at a high frequency in protoplast colonies independently transformed with either pTRA137 or pTRA137R. We assayed for the phenotypic change of colonies from hygromycin-sensitivity to resistance as an indicator of *Ac* excision. We counted hygromycin-resistant colonies per million protoplasts and compared to the pTRA132 positive control as summarized in Table I. The frequency of phenotypic change was 12% in *Nipponbare* protoplasts, and 12 to 19% in *Taipei 309* protoplasts when regenerated protoplasts were selected with 95 μ M hygromycin. Results from *Nipponbare* and *Taipei 309* protoplasts represent an average of two and one independent experiments, respectively. These data suggest that up to 19% of all transformed cells generated colonies in which *Ac* excision occurred in at least one of the several *Ac*-containing *hph* genes present, leading to reactivation of the *hph* gene. The orientation of the *Ac* element in pTRA137/137R with respect to the *hph* gene promoter did not influence significantly these frequencies in the case of the *Nipponbare* transformation experiments (Table I). As expected, the higher selection concentration of hygromycin (190 μ M) reduced the number of resistant colonies in both cultivars to some extent, but did not alter significantly the conclusions of the results obtained at the 95 μ M concentration of hygromycin.

The insertion of the *Tn903* fragment at the BamHI site of the *Ac* element in pTRA139/139R essentially eliminated the excision function and reduced the transformation frequency by 10-fold to the background level (Table I). The background frequencies obtained with pTRA139 and 139R appeared to be due to the leaky

selection and did not represent true transformants. Only one out of seven pTRA139/139R colonies examined had transferred DNA and no empty donor site was detected in the transformants by southern and polymerase chain reaction analyses (Data not shown).

After *Ac* excision the hygromycin-resistance gene was reconstituted

Physical evidence for *Ac* excision was obtained by southern blot hybridization analysis of transferred DNA from transgenic plants. After hygromycin selection and colony propagation, *Nipponbare* plants were regenerated from resistant calli and grown to maturity in the greenhouse. Total DNA was isolated from leaf tissues during the booting (preflowering) stage of six transgenic plants independently-transformed with either pTRA137 or pTRA137R. Major agronomic traits of transgenic plants containing pTRA137 and 137R will be reported in the subsequent publication.

If the *Ac* element were excised from the integrated plasmid DNA and if the excised site were religated, the CaMV 35S promoter and hygromycin coding sequences would be joined through the 53 bp *wx* sequence to reconstitute a functional hygromycin-resistance gene. When HindIII/EcoRI digests of genomic DNA were probed with a 0.3 kbp HindIII/XbaI fragment representing the CaMV 35S promoter (Probe 1), 0.6 kbp bands were expected in the reconstituted resistance gene of both N137

Table I. Biological assay for *Ac* excision in rice protoplast colonies. Results from *Nipponbare* protoplasts represent an average of two independent experiments. Values for *Taipei 309* represent results from a single experiment. The percentage resistant colonies in parentheses are relative to the number of resistant colonies after transformation with pTRA132.

Plasmid DNA	Number of Resistant Colonies Per Million Protoplasts			
	Hygromycin 95mM		190 mM	
a. <i>Nipponbare</i>				
pTRA132	287.8	(100.0%)	176.0	(100.0%)
pTRA137	35.7	(12.4%)	9.0	(5.1%)
pTRA137R	36.2	(12.5%)	6.0	(3.4%)
pTRA139	4.9	(1.7%)	2.0	(1.1%)
pTRA139R	4.6	(1.6%)	1.0	(0.6%)
No DNA	0	(0%)	0	(0%)
b. <i>Taipei 309</i>				
pTRA132	471.4	(100.0%)	527.1	(100.0%)
pTRA137	60.0	(12.7%)	25.0	(4.7%)
pTRA137R	92.5	(19.6%)	78.8	(14.9%)
pTRA139R	8.5	(1.8%)	5.7	(1.1%)
No DNA	0	(0%)	0	(0%)

Figure 2. Expected size fragments in southern hybridization analysis of genomic DNA when hybridized with three separate probes. (a) (Upper) Structure of the hygromycin resistance gene and the *Ac* element inserted into the untranslated leader region of the resistance gene in pTRA137. (Lower left) Expected structure of the reconstituted resistance gene if the *Ac* element has been excised. (Lower right) Expected structure of a reintegrated copy of the *Ac* element in the rice genome. (b) Structure of the hygromycin-resistance gene and the *Ac* element inserted into the untranslated region of the resistance gene in pTRA137R. Three probe DNA fragments were a 0.3 kbp HindIII/XbaI fragment corresponding to the CaMV 35S promoter (P1), a 1.2 kbp EcoRI/PvuII fragment (P2) and a 1.6 kbp HindIII fragment (P3) representing the internal *Ac* sequences.

Figure 3. Southern blot hybridization analysis of genomic DNA isolated from leaves of transgenic rice plants after transformation with pTRA137 (Panels a–c) and pTRA137R (Panels d–f). (Panels a–c) Results of the hybridization analyses of genomic DNA from pTRA137-transformed plants. The same genomic blot of HindIII/EcoRI-digested DNAs was successively hybridized to probes 1 (Panel a) and 2 (Panel b). The genomic blot of BamHI-digested DNA was hybridized to probe 3 (Panel c). Lane 1, transformant N137-1 DNA; lane 2, N137-2 DNA; lane 3, N137-3 DNA. DNA fragments of 0.6 kbp generated from the reconstituted hygromycin-resistance gene are marked by open circles. Fragments of 0.9, 1.4 and 1.5 kbp generated from the intact *Ac* element are marked by asterisks (*). Fragments greater than 4.4 kbp DNA hybridizing to probe 3 are indicated by arrowheads. (Panels d–f) Results of the hybridization analyses of genomic DNA from pTRA137R-transformed plants. The same genomic blot of HindIII/EcoRI-digested DNAs were successively hybridized to probes 1 (Panel d) and 2 (Panel e). The BamHI-digested DNA blot was successively hybridized to probes 3 (Panel f) and 1 (data not shown). Fragments hybridizing to probe 3, but not probe 1 are indicated by arrowheads. Lane 1, transformant N137R-1 DNA; lane 2, N137R-2 DNA; lane 3, N137R-8 DNA. Each blot included lanes for non-transformed control DNA and one and five copy per diploid rice genome of reconstructed samples (Data not shown).

and N137R plants (*Nipponbare* transgenic plants containing pTRA137 and 137R, respectively) as illustrated in Figure 2-panels a and -b. As shown in Figure 3-panels a and d, the reconstituted *hph* gene fragments (marked by a circle) were indeed detected in all 6 lanes and were observed at least one copy per rice genome in DNA from all 6 transformants examined. One and five copy number controls of reconstructed samples were present for each blot but were not shown in Figure 3.

Further evidence for *Ac* excision was provided when HindIII/EcoRI digest blots were rehybridized with a second probe (Probe 2), a 1.2 kbp EcoRI/PvuII fragment corresponding to the internal region of the *Ac* element. As shown in Figure 2, HindIII/EcoRI digests of the transformation vectors pTRA137 and pTRA137R generated an internal fragment of 0.9 kbp and a border fragment of 1.4 and 1.5 kbp, respectively. If *Ac* remain without excision at its original donor site of the integrated plasmid

Figure 4. Agarose gel electrophoresis analysis of PCR-amplification products. Lane 1, positive control plasmid pTRA132; lane 2, H₂O control; lane 3, untransformed *Nipponbare* DNA; lane 4, transformant N137-2 DNA; lane 5, N137-3 DNA; lane 6, N137R-2 DNA; lane 7, N137R-8 DNA.

DNA, the relative intensity of the internal and border bands is expected to be roughly equivalent in DNA of N137 and N137R transformants. If *Ac* were excised, the relative intensity of the internal 0.9 to border 1.4 and 1.5 kbp bands would be greater. Southern hybridization results indicated that these pairs of bands (marked by an asterisk *) were detected in DNA of N137-1 and -3 (Figure 3-panel b, lanes 1 and 3), and N137R-1 and -2 (Figure 3-panel e, lanes 1 and 2). The internal 0.9 kbp band was more abundant than the border 1.4 and 1.5 kbp bands, based on the densitometric comparison of these two bands. This result provides additional evidence that *Ac* excision occurred in DNA of these four plants.

In addition to the *Ac* element(s) that had excised, southern hybridization analysis provided evidence that other *Ac* elements remained without excision at their original donor sites of the integrated plasmid DNA in the rice genome. If this is the case, 2.1 and 1.5 kbp fragments should be observed in N137 and N137R transformants, respectively, when HindIII/EcoRI digests of genomic DNA were probed with the CaMV 35S promoter (Probe 1) (Figure 2-panels a and -b). The expected bands were detected in DNA of four out of six transformants as shown in Figure 3-panels a and d. No corresponding signal was evident in digested DNA of N137-2 (Figure 3-panel a, lane 2) and N137R-8 plants (Figure 3-panel d, lane 3). This suggests either that all the *Ac* elements were excised from the integrated plasmid DNA in these plants, or that the sequence in question might have been rearranged during or after the integration. The detection of many unexpected bands which hybridized with probes 1 and 2 indicates that the integrated copies of the *Ac*-hygromycin vector were rearranged at a high frequency.

Excised *Ac* was reintegrated into the rice genome

The maize *Ac* element, once excised from the integrated plasmid DNA, appears to re-integrate into the rice genome. If the excised *Ac* element were reintegrated into the rice genome, bands larger than 4.4 kbp would be detected when BamHI digests of N137 DNA were hybridized with Probe 3, a 1.6 kbp HindIII fragment corresponding to the internal *Ac* sequence (Figure 2-panel a). As indicated in Figure 3-panel c, lane 1, two bands of 6 and 15 kbp (marked by an arrow) were detected in N137-1 DNA. However, the intensity of these two fragments was much less than that of the single copy of the *Ac* element per rice genome.

Table II. Structure of *Ac* Excision Sites in Transgenic Rice Plants.

Plant Sources	# of Clones	DNA Sequences
Original <i>wx-m7</i>	—	CCG <u>GGTCACGC</u> - <i>Ac</i> - <u>GGTCACGC</u> AACGCGCC
N137-3	1	CCG <u>GGTCACG</u> * <u>*GTCACGC</u> AACGCGCC
N137-3	1	CCG <u>GGTCACG</u> * <u>*****CGC</u> AACGCGCC
N137R-2	3	CCG <u>GGTCACG</u> * <u>*GTCACGC</u> AACGCGCC
N137R-2	6	CCG <u>GGTCACG</u> * <u>**TCACGC</u> AACGCGCC
N137R-8	2	CCG <u>GGTCACG</u> * <i>acc</i> <u>*GTCACGCAACGCGCC</u>
N137R-8	1	CCG <u>GGT<u>a</u>ACG</u> * <i>acc</i> <u>*GTCACGC</u> AACGCGCC

This suggests that only a fraction of leaf cells contained the reinserted *Ac* element when *Ac* excision occurred at a later stage of leaf development in this transgenic plant. Evidence for re-integration of the *Ac* element into the rice genome was also provided by BamHI digests of N137R plant DNA in Figure 3-panel f. If the *Ac* element were re-integrated into the rice genome, putative bands should be larger than 4.4 kbp (Figure 2-panel a). Furthermore, putative bands should hybridize with the *Ac* internal probe but not with the 35S promoter probe. A 6.5 kbp band in N137R-1 (Figure 3-panel f, lane 1), 6.6 and 9 kbp bands in N137R-2 (lane 2), and 20 and 30 kbp bands in N137R-8 (lane 3) essentially meet the above criteria for candidates as re-integrated *Ac* elements.

Most of the detectable bands larger than 4.4 kbp in N137R-1, an 11 kbp band in N137R-2 and a 5 kbp band in N137R-8 hybridized to both the *Ac* and 35S probes (Figure 3-panel f). This indicated that all these fragments arose from the integrated plasmid DNA in which *Ac* sequences remained at their donor sites without excision. Prominent bands smaller than 4.4 kbp were found in both N137-1 and N137-3 (Figure 3-panel c, lanes 1 and 3). These bands might originate from the initial integration event in which transferred plasmid DNA was inserted into the rice genome at a location near the 3'-end of the *Ac* element. Alternatively, they might arise from the rearrangement of the *Ac*-containing fragment after integration.

Structures of excision sites were similar in rice and maize

Direct evidence that the *Ac* element transposes in rice was obtained by analyzing the DNA sequences surrounding the empty donor sites after amplification by the polymerase chain reaction (PCR). The expected empty donor site sequences were amplified from undigested genomic DNA by PCR, using two 30 base primers which were designed to hybridize to sequences flanking the *Ac* insertion site. If *Ac* were excised from the donor site precisely, the PCR reaction would amplify a 218 bp fragment. The results from agarose gel separation of the PCR products indicated that the expected size fragment of 218 bp was detected in all N137 and N137R DNA (Figure 4).

DNA sequences of the PCR-amplified 218-bp fragments were determined after cloning into the pBluescript vector. The sequences of the 2 *Ac* excision sites from N137-3, 9 sites from N137R-2 and 3 sites from N137R-8 DNA were determined and summarized in Table II. In each transgenic plant examined, we obtained two distinguishable sequences of empty donor sites. This might suggest the occurrence of two independent excision events taking place in each plant. All of the 14 empty donor sites lost the eighth base of the left *wx* sequence repeat. In the right flanking sequence, 10 donor sites had a one base-pair deletion, 6 had a two base-pair deletion and one had a five base-pair deletion starting from the *Ac* excision site. In addition, 2 empty donor sites from N137R-8 had a three base-pair insertion at the junction

of right and left *wx* sequence repeat. In one case in N137R-8, we observed C to A change at the fourth base of the left flanking sequence. The DNA sequence analysis indicated that the *Ac* element was excised in rice in a similar manner as in maize.

DISCUSSION

Proper gene function was expressed after direct DNA transfer

This report represents the first demonstration in which an intact genetic element from a higher plant expressed a proper biological function after integration into the heterologous rice genome. All previous work on rice transformation reports the transfer of bacterial and viral genes. In fact, this may be the first report of functional expression of a plant gene after direct DNA transfer to the plant genome.

The *Ac* element was transferred to the rice genome as a circular plasmid DNA, in contrast to previous reports in which linear T-DNAs generated by *Agrobacterium tumefaciens* and *A. rhizogenes* were transferred to the plant genome (11, 14). Our previous analysis of transferred DNA in the rice genome indicated that circular plasmid DNA might form concatemers by intermolecular recombination prior to integration (21). After the insertion into the rice genome, concatemerization would result in a minimum of one copy of non-disrupted plasmid DNA, with a second copy of plasmid DNA through which integration of concatemers occurred.

The Maize *Ac* element transposed in transgenic rice

We demonstrated here that the maize transposable element *Ac* transposes in rice. Appearance of hygromycin-resistant colonies can be correlated with the presence of the empty donor sites in the reconstituted hygromycin-resistance gene in transformants. Based on the hygromycin-resistance assay, the excision frequency of *Ac* in rice appeared to be somehow lower than the average 25% frequency in the more evolutionary-distant tobacco (29). It would be difficult to compare strictly these excision frequencies since the selection conditions of the two experiments were different. However, the difference might reflect the variation of genetic background between rice and tobacco.

The biological assay system thus developed in rice can be applied to assess the functional significance of *cis*-acting sequences within the *Ac* element as have been reported with the tobacco system (18, 19). The demonstration that the insertion mutagenesis of *Ac* at the unique BamHI site reduced the excision frequency by 10-fold is a good beginning for this direction. In addition, the use of the rice system may offer a considerable advantage over tobacco in analyzing the expression of the putative transposase gene of *Ac* since rice is a member of the same monocot family as maize. It has been noted that transcription initiation and intron processing of monocot genes were inefficient in heterologous tobacco plants (19).

The *Ac* excision in rice was as precise as in maize

This is the first demonstration that *Ac* excision from the single *wx-m7* locus generated at least two distinguishable sites in each of three transgenic plants and thus in total five different excision sites. Sequence analysis of 14 empty donor sites was facilitated by PCR amplification of genomic DNA. Earlier studies with maize *wx-m9* locus reported 3 distinguishable *Ac* excision sites. In all cases one or two-base pair deletions were detected starting from the junction of *wx* eight-base sequence repeats. The excision site of a maize *Wx* revertant lost the first and second base of the left flanking sequence (11). There were 2 empty donor sites isolated from the introduced *wx-m9* (*Ac*) locus in tobacco (11). One of the tobacco excision sites had the same structure as the maize revertant. Three base-pairs insertion was detected at the junction of *wx* sequence repeats in a second tobacco and *Arabidopsis thaliana* empty donor sites (11, 14). Thus, the DNA sequence variations observed at the *wx-m7* excision sites in transgenic rice were essentially similar to the structure of the *wx-m9* excision sites reported previously. The sequence surrounding the *Ac* integration site in the rice genome is being isolated using the inverse PCR procedure in our laboratory (30).

CONCLUSION

We demonstrated here the utility of the hygromycin-resistance assay to monitor the excision of the maize *Ac* element in rice, a member of the same taxonomical family as maize. The introduced *Ac* element was excised from the donor site at a high frequency. The rice hygromycin-resistance assay system may be of considerable value in functional analysis of *Ac* transposition. Southern and DNA sequence analyses indicated that the *Ac* element was excised in rice in a similar manner as in maize and that the transposed *Ac* element was reintegrated into the rice genome. To further apply the *Ac* element for transposon mutagenesis and gene tagging, it is necessary to obtain evidence for germinal transposition of the *Ac* element in rice. Experiments in this direction are in progress in our laboratory.

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