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Transposon tagging in rice

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Abstract

To develop an efficient gene isolation method for rice we introduced the maize *Ac/Ds* system into rice. Extensive analysis of their behavior in rice for several generations indicated that *Ac* and Ds in the presence of *Ac* transposase gene actively transpose in rice. A wide spectrum of mutations affecting growth, morphogenesis, flowering time and disease resistance have been obtained in the population carrying *Ac/Ds* and some of them were genetically analyzed. Main efforts are currently being made to isolate genes responsible these mutations. In addition, a number of *Ac/Ds* were mapped on chromosomes and mapped elements will be used in the future for directed tagging of genes with known chromosomal positions.

Introduction

The controlling element firstly recognized by Barbara McClintock [15, 17] has become a powerful tool for gene isolation, since the element can be employed to correlate biological phenomena with molecular interactions [37, 39]. Many plant genes have been isolated using transposons as molecular tags without information on biochemical properties of their gene products and their expression patterns. The first cloning of a plant gene by transposon tagging was performed in 1984 and it was the maize *bronze* gene which encodes UDP-glucose: flavonoid 3-*O*-glucosyltransferase, a key enzyme of anthocyanin synthesis [16]. It was a victory of the classical maize genetics since the accumulated information of the maize genetics contributed tremendously for its successful isolation. Afterwards, a large number of genes were isolated by endogenous transposons as tags [39]. The use of transposon as a cloning tool, however, had been confined to plant species with active, well-characterized transposon systems, such as maize and snapdragon. Therefore, it was an epoch-making discovery that B. Baker and her colleagues [3, 4] demonstrated active transposition of the maize *Ac*/*Ds* elements in transgenic tobacco. Since then active transposition of maize elements has been demonstrated in other species including *Arabidopsis*, tomato, petunia, flax, carrot, potato, soybean and rice [18, 33, 37].

Seven years after the B. Baker's report, a flower color gene was successfully cloned in 1993 from petunia using *Ac* [12]. This is the first example for a gene to be cloned in heterologous host using an exogenous transposon. Concurrently, genes for leaf morphology and albino were isolated from *Arabidopsis* by the *Ac*/*Ds* system [7, 27] and an *Arabidopsis*

gene involved in male fertility was cloned by the use of another maize transposon system *En*/*Spm* [1]. Herein, the two element system was successfully employed to isolate the albino gene, in which non-autonomous element such as *Ds* is transposed by the *Ac* transposase (*Ac*TPase) gene driven by a strong promoter such as CaMV 35S promoter to increase the frequency of transposition. In the two-element system, one may avoid problems associated with the use of the autonomous element such as *Ac* since it is possible to genetically remove the TPase gene thus generating stable mutants. Autonomous transposons can transpose by themselves and leave behind non-functional genes carrying footprints (sequence alterations at the excision sites), and the generation of mutations due to footprints in mutant lines would make linkage studies of particular transposons with the phenotypes complicated. Furthermore, somatic excision that restores gene function would be another problem with the autonomous elements since such somatic reversion could make it difficult to identify insertional mutations.

In addition to these random tagging approaches, directed tagging of specific target genes has been performed. Three disease-resistant genes, the tomato *Cf-9* gene [21] for resistance to *Cladosporium fulvum*, the tobacco *N* gene [40] for resistance to tobacco mosaic virus and the flax rust-resistant gene, L^6 [24], and the *FAE1* gene [20] involved in fatty acid synthesis from *Arabidopsis* were cloned by directed transposon tagging. It is noteworthy that one *Ds* element located at 3 cM from the *Cf-9* gene was activated to tag the gene by the *Ac*TPase gene because *Ac*/*Ds* are known to transpose to closely linked loci even in heterologous hosts [5, 11, 14, 22]. Among approximately 160 000 progeny screened, at least 63 independent *Cf-9* mutants were obtained, of which 37 independent *Ds* insertions were detected. This indicates effectiveness of transposon tagging from a linked *Ds* to a target locus. However, this powerful method of gene isolation has not been applied yet to major cereals which do not carry active transposable elements.

Rice with a small genome size (ca. 430 Mb) is genetically well-characterized and suitable for molecular biological analysis [19, 34]. Recent advances in research on rice include efficient transformation, creation of a highly saturated molecular map, and the large-scale analysis of expressed sequence tags. Therefore, rice can be considered a model plant for monocotyledon. Nevertheless, to become a 'real' model plant, it is essential to develop effective methods for isolation of novel genes to be available to all researchers. Map-based cloning has already been applied in rice and the first example of genes isolated by this method was recently reported [36]. However, this approach is confined to relatively well-characterized mutants since target genes have to be genetically mapped beforehand. Transposon tagging must be another cloning system in rice and might become more accessible to many researchers. Indeed, we have already isolated mutants generated from rice lines carrying the *Ac*/*Ds* system. In this paper, we will review our approach and current status of transposon tagging in rice.

Transposition of Ac/Ds in rice

As a first step to develop a transposon tagging system in rice (*Oryza sativa* L.), we examined transposition of the maize transposon *Ac* in transgenic rice [18]. The 4.6 kb *Ac* element from the *wx-m7* allele of maize [8] that was inserted between the CaMV 35S promoter and the hygromycin-resistant (Hm^r) gene (Fig. 1) was introduced into rice protoplasts by electroporation [32]. When *Ac* is excised, the Hm^r gene is restored and rice cells acquire resistance to hygromycin. In our experiments, Hm^r transformed calli appeared with frequencies of ca. 10^{-3} per transformed callus. Southern blot analysis demonstrated that *Ac* actively transposes in rice cells and is inserted in different sites of the rice genome. Regenerated plants from *Ac*-containing calli showed distinct pattern of *Ac* insertions among different leaves of a plant indicating that *Ac* transposes in leaves during development. Sequence of the footprints left between the 35S promoter and the Hm^r gene were similar to those that were detected in maize, transgenic tobacco and transgenic *Arabidopsis* (Table 1).

We next introduced a non-autonomous *Ds* element carrying a 1.6 kb internal deletion in the *Ac* sequence (Fig. 1) [33]. Hm^r calli were obtained when co-transformed with the *Ac*TPase gene driven by the CaMV35S promoter (35S-*Ac*TPase, see Fig. 1), indicating that there is no endogenous *Ac* activity in rice and that *Ds* can excise only in the presence of the introduced *Ac*TPase gene. Footprints were similar to those found in the case of *Ac* in rice (Table 1). Since eight bp of target site duplications at the *Ds* integration sites were also confirmed by sequencing of inverse PCR products and genomic clones, it was concluded that *Ds* can be integrated into rice chromosomes by the mechanism similar to that in maize (Table 1).

Table 1. Target site duplication and footprints of *Ac/Ds* in rice. Bold letters represent mutated nucleotides in footprints. Asterisks represent deletions in footprints.

	Ac
Original site of *Ac* (*wx*-m7)[18]	GGTCACGC GGTCACGC
Y4,Y5,Y6,Y16	GGTCACG**G** **C**GTCACGC
Y19(A)	GGTCAC*****C ******TCACGC
Y19(B),Y20	GGTC******** *****GTCACGC
	Ds
Original site of *Ds*[33]	GGTCACGC GGTCACGC
162	GGTCACGC **C**GTCACGC
17,63(A),161	GGTCACG**G** **C**GTCACGC
63(B)	GGTCACG**G** *******CACGC
63(C)	GGTCA******* *****GTCACGC
14	GGTCA******* *******CACGC
	Ds 241
Integration site of *Ds*241	CTCCCTCC CTCCCTCC
C-2-3-213-8	CTCCCTC***** *****TCCCTCC
	Ds 80
Integration site of *Ds*80	CACACATC CACACATC
B-10-1-51-1	CACACAT**G** *****ACACATC
B-10-1-51-6	CACACAT**G** *****ACACATC
	Ds -b
Integration site of *Ds*-b[33]	GTCATGGA GTCATGGA
	Ds -c
Integration site of *Ds*-c[33]	TATGTCGC TATGTCGC

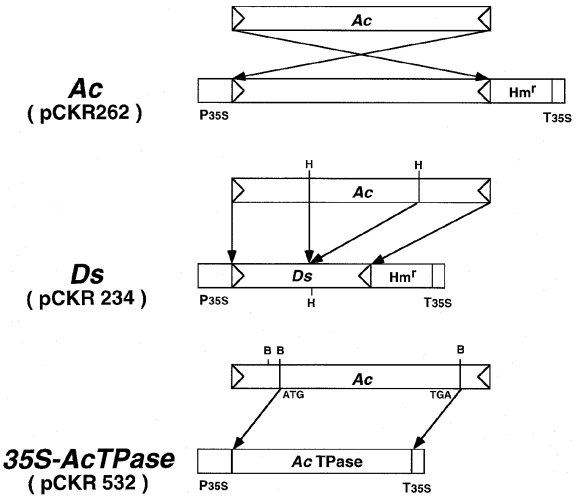

Figure 1. Schematic diagram of plasmids pCKR262, pCKR234 and pCKR532 used in this study. In *Ac* construct, the *Ac* element was inserted in the reverse orientation. Triangles at ends of *Ac* represent the inverted repeats. P_{35S}, CaMV 35S promoter; Hm^r, hygromycin phosphotransferase; T_{35S}, CaMV 35S terminator; H, *Hin*dIII; B, *Ban*II.

Interestingly, a callus line, named no. 17, did not have the *Ac*TPase gene and still had several transposed the *Ds* elements, suggesting that *Ds* elements on the introduced plasmid were excised by the action of the transiently expressed *Ac*TPase gene and that *Ds* was integrated into the rice chromosomes during early stages of protoplast culture. A regenerated plant derived from this callus line, plant 17-1, carried four copies of stable *Ds* elements which were transmitted to the progeny. We designated these *Ds* elements as *Ds*-a, *Ds*-b, *Ds*-c and *Ds*-d. Cloning of these *Ds* elements revealed that *Ds*-b and *Ds*-c are structurally intact while *Ds*-a has a 200 bp deletion and *Ds*-d, contains complicated DNA rearrangements including a 2.7 kb insertion (our unpublished results). Since all the four *Ds* elements have the intact 5′ and 3′ region, they are potentially capable of transposition when *Ac*TPase is provided. Segregation analysis of 21 progeny from the plant 17-1 demonstrated that the four *Ds* elements were segregated in the next generation in a Mendelian fashion. *Ds*-a and *Ds*-d were linked and co-segregated with the restored Hm^r gene. *Ds*-b and *Ds*-c segregat-

ed independently and did not co-segregated with the Hm^r gene. From these results, we concluded that plant 17-1 carry four functional *Ds* elements at three different chromosomes and does not have the *Ac*TPase gene. Therefore, we thought that this plant is suitable as a parent plant for transposon-induced mutagenesis. In addition, we have also constructed a transgenic line carrying a single 35S-*Ac*TPase gene (Fig. 1).

To induce transposition of inserted *Ds* elements, the plant 17-1 was crossed with the transgenic plant carrying a single active 35S-*Ac*TPase gene and 69 F1 plants were obtained carrying both the *Ds* elements and the 35S-*Ac*TPase gene (Fig. 2). Southern blot analyses of F2 progeny derived from these F1 plants revealed that germinal transposition of *Ds* in rice occurred with high frequency (data not shown). We could not accurately estimate the frequency of transposition because we did not use marker genes to monitor *Ds* excision. However, we could speculate on the excision frequency based on the results obtained by Southern blot analysis. A large number of F2 plants were subjected to Southern analysis and most of them showed newly transposed *Ds* bands. While some of the bands were sharp and unique, due to late germinal transposition, others were sharp and common to siblings, due to early germinal transposition. We have also observed unique and faint bands probably due to somatic transposition.

It has been reported that the frequency of *Ds* transposition varies from species to species. Elevated level of *Ac*TPase activity was shown to confer high frequencies of *Ds* excision in *Arabidopsis* [6, 38] and tobacco [31]. When the 35S promoter was used to drive the *Ac*TPase gene, the frequency of germinal transposition was 6.8% in tobacco and 10–74% in *Arabidopsis*. A previous report indicated that ca. 20% of *Arabidopsis* F2 plants carrying the excision maker inherited a transposed *Ds* after activation by the 35S-*Ac*TPase [38]. Taken together, germinal reinsertion frequencies of *Ds* in *Arabidopsis* in the range of 2–15%. Another study demonstrated that only a low portion (33%) of plants inheriting the marker gene used to monitor excision contained a transposed *Ds* when plants carrying *Ds* were crossed to those containing the *Ac*TPase gene driven by its own *Ac* promoter [5]. In this experiment, germinal reinsertion frequencies of *Ds* were generally less than 1%. Therefore, if F2 plants were subjected to Southern blot analysis without any selection, at most ten plants among 1000 plants would have contained a germinally transposed *Ds*. This number is much lower than that obtained in our study, even considering the fact that we used the 35S promoter to drive the *Ac*TPase gene. Therefore, we consider that *Ds* transposition in F1 plants of rice was much higher than that in *Arabidopsis*. In our experiment, however, one to four copies of the *Ds* elements were transmitted to F1 plants. Therefore, multiple active *Ds* elements may contribute high frequency of *Ds* transposition. Southern blot analysis revealed that frequency of transmission of original *Ds* elements to the next generation varied, suggesting that one or two *Ds* elements among the four elements were highly active in our case.

Similar results were obtained with the progeny of rice plants carrying the intact *Ac*. For example, eight transgenic rice plants of R5 generation obtained by selfing a R4 plant carrying two *Ac* elements were subjected to Southern blot analysis and one R5 plant was found to carry a newly transposed *Ac* (data not shown). Then, three of the eight R5 plants were selected at random and each 10 progeny was subjected to Southern blot analysis. The result indicated that four of 10 R6 plants from one selected R5 plant carry newly transposed *Ac* elements. Two of them seems to have a common band, suggesting a germinal *Ac* transposition. No new transposition was detected in the other two lines (data not shown). It has been reported that excision of *Ac* occurs in *Arabidopsis* with low frequency (0.2–0.5%) [13, 30]. With a derivative of *Ac*, the frequency can be elevated to 2–3% in *Arabidopsis* [26]. Therefore, *Ac* had very low germinal transposition frequencies in *Arabidopsis*. On the contrary, in rice it is not difficult to find newly transposed *Ac* that has been germinally transmitted to progeny.

Inactivation of Ac/Ds in rice

Despite of the highly active transposition of *Ds* in the F1 plants, we were unable to detect frequent transposition of *Ds* in later generations even in the presence of the 35S-*Ac*TPase gene. Only a few lines still show active transposition of *Ds* in F5 generation. For example, in the presence of the *Ac*TPase gene, only one line carries actively transposing *Ds* elements among 13 F4 lines originated from a F2 plant, B10-1 (data not shown). This indicates that *Ds* frequently becomes inactive in rice during successive generations after activation by the 35S-*Ac*TPase in F1 plants.

To overcome the inactivation of *Ds* transposition in rice, we have tried to reactivate the inactivated elements. Since it has been known that the tissue culture process activates silent transposable elements in maize [10, 25, 26], we applied plant regeneration via

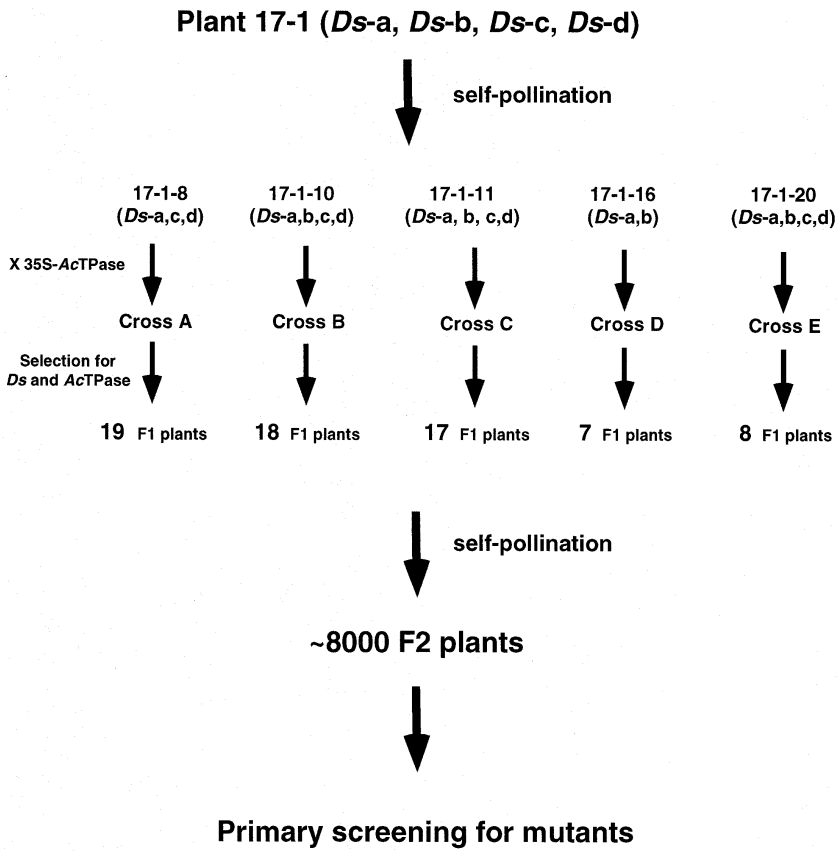

Figure 2. A strategy for transposon tagging in rice. Five progeny plants of a primary transgenic plant 17-1 were crossed with plants carrying the 35S-*Ac*TPase gene. Total of 69 F1 plants carrying *Ds* elements and the 35S-*Ac*TPase were selected by Southern analysis. About 8000 F2 plants from these F1 plants were subjected to mutant screening.

Table 2. Visible mutants found in F2 seedlings of Cross B. Eighteen F1 plants were obtained from the cross B between a *Ds*-containing plant, 17-1-10 and a plant carrying the 35S-*Ac*TPase gene. The 17-1-10 had four functional *Ds* elements, *Ds*-a, *Ds*-b, *Ds*-c and *Ds*-d. [a] A column represents F2 seedlings from a F1 plant. [b] The number of plants showing the phenotype found in F2 seedlings derived from a F1 plant.

	F1 plant																	
Phenotype	B2[a]	B7	B8	B10	B12	B13	B15	B16	B18	B19	B21	B25	B27	B28	B29	B32	B33	B40
stripe		2												1				
albino								1										
chlorina		4		6	8	14		1	1		1							
dwarf	7[b]	6						7	4				1	3	4	1		
twin		1		2	2	1		2			2			1				2
variegation																1		
number of F2 plants tested	97	88	109	110	106	108	110	109	109	110	109	106	109	107	108	110	107	109

protoplasts derived from four lines carrying the *Ds* elements and the *Ac*TPase gene to see whether the culture process activates transposition of the inactive *Ds* elements. Southern blot analysis was carried out with protoplast-derived plants and their progeny plants. The results indicated that *Ds* was highly reactivated in two lines (Fig. 3), while reactivation of *Ds* was observed only in some progeny in another line. Since no *Ds* transposition was found in a line in which the *Ac*TPase gene was absent, the observed reactivation of *Ds* via protoplast culture was likely to require the function of the *Ac*TPase. These results clearly demonstrated that

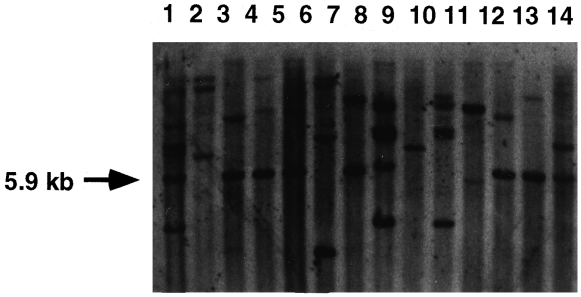

Figure 3. Southern blot analysis showing reactivation of *Ds*. One μg genomic DNA of 14 progeny plants of one regenerated plant from protolasts carrying inactive *Ds* and the *Ac*TPase gene was digested with *Bgl*II and subjected to Southern blot analysis. Digoxigenin-labelled 1.0 kb fragment derived from 5′ region of *Ds* was used as a probe. Only the 5.9 kb band segregated in a Mendelian fashion, indicating stable *Ds*. Other bands are likely to be due to new *Ds* transposition. Faint bands may be derived from somatic transpositions of *Ds*.

inactive *Ds* can be reactivated by the protoplast culture procedure in the presence of the *Ac*TPase gene.

To see whether a short culture procedure is sufficient to reactivate the inactive *Ds*, we examined ten independent calli induced for two weeks on a callus-inducing medium from rice seeds which carry both inactive *Ds* and the *Ac*TPase gene. Excisions of *Ds* were detected by PCR in all calli tested whereas no excision was observed from a control line, which carries the same *Ds* without the *Ac*TPase gene (data not shown). This indicates that inactive *Ds* elements can be also reactivated by a short culture without protoplast process. These results indicate that it is possible to increase the frequency of reversion events from mutant lines with inactive *Ds* elements by reactivating silent *Ds* through tissue culture.

Ac remains active in certain lines even in R6 generations as described above. However, with the results of Southern blot analyses, once it becomes inactive it seems to remain inactive in the subsequent generations. For example, 11 R4 plants obtained by selfing one R3 plant were subjected to Southern blot analysis and no transposition was detected. In the progeny of three of these R4 plants, no transposition was detected in 30 R5 plants (10 progeny each). In *Ac*-containing rice plants, some lines show methylation of *Msp*I/*Hpa*II and *Pvu*II sites within *Ac* in R2 through R4 generations (data not shown). Therefore, it is conceivable that methylation of *Ac* is involved in inactivation of *Ac* transposition although the more exact relationship between methylation and inactivation of *Ac* remains to be established.

Rice mutants generated by Ac/Ds

Sixty-nine F1 plants obtained from the cross were categorized into five groups (A, B, C, D and E) (Fig. 2). Each group consisted of 7 to 19 F1 plants carrying both the *Ds* elements and the 35S-*Ac*TPase gene. We screened various mutants using F2 lines originated from these five groups.

First, visible mutants were found, including stripe, albino, virescent, chlorina, dwarf, twin and chlorosis (Fig. 4). Generally, the number of plants displaying mutant phenotypes was not in good agreement with the Mendelian law. For example, seven of 97 F2 plants (7.2%) from one F1 plant, B2, exhibited a dwarf phenotype (Table 2). We assumed that most mutations found in this experiment were not caused by somaclonal mutation generated during culture process since a recessive mutation caused by somaclonal mutation present in F1 plants should give rise to 25% of the F2 plants displaying mutant phenotypes. Alternatively, fluctuations of segregation can be also possible.

Eight F2 families from the cross B (18 F2 families) produced dwarf seedlings with the frequencies of 1.0–7.2%. Similar frequencies in other mutant phenotypes were obtained (Table 3). Because no genetic analysis with these mutants was performed, we could not conclude that they were recessive mutants. If they were recessive, the same mutations should be carried in both egg cells and pollens of a few flowers of F1 plants to confer mutant phenotypes in a small portion of F2 plants. If this is the case, one can further speculate that *Ds* transposition must be not too early, and not too late events during development of F1 plants that results in homozygous mutations in F2 generation. It is noteworthy that multiple mutant phenotypes were often observed in F2 seedlings and frequencies of visible mutations in F2 generation varied among F1 plants (0–32%). This suggests that transposition frequency differs among F1 plants originated from the same cross. Similar non-uniformity of transposition frequencies has been reported in other systems such as *Arabidopsis* [38].

It has been reported that 559 families of *Arabidopsis* were subjected to a broad screening for mutants exhibiting visible phenotypes, each family representing at least one germinal *Ds* excision event [2]. Furthermore, 43 families representing at least one germinal *Ac* excision were also subjected to mutant screening in this experiment. Plants with abnormal phenotypes were observed in the progeny of 218 of these 602 families. One hundred and thirty four families of these

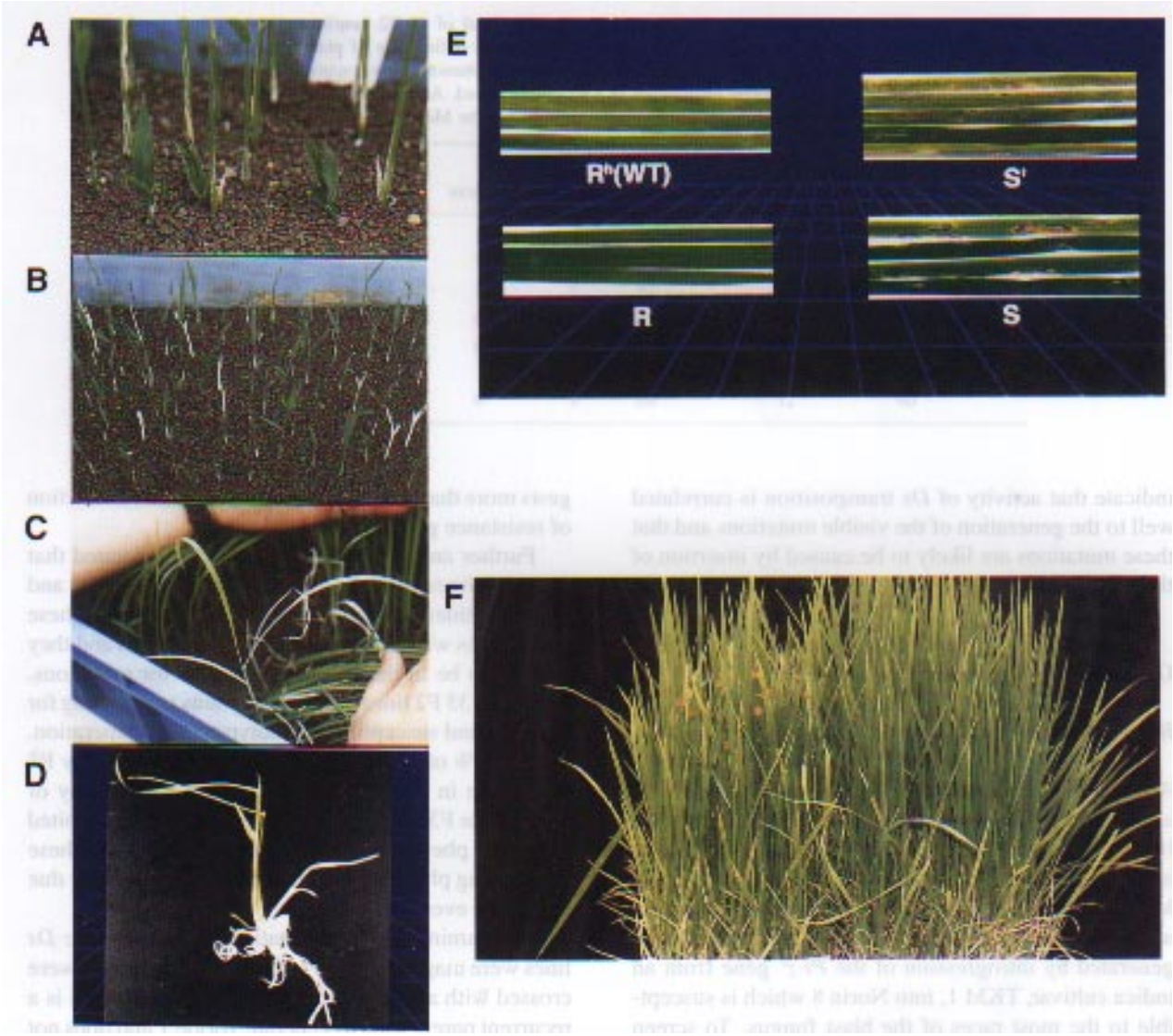

Figure 4. Phenotypes of mutants found in primary screening. A, dwarf; B, albino; C, virescent; D, twin; E, blast-sensitive; F, early-flowering. In E, S′ phenotype is similar to S phenotype. Therefore, S′ is treated as the same group with S in the text. In F, mutant plants from an early-flowering line are grown in the left part. Flowered plants are indicated with the red markers.

primary mutants were subjected to a second screening, 48 families turned out to contain plants with heritable defects. Similarly, 1000 families of *Arabidopsis* carrying ca. 500 independent germinal transposition events of *Ac* were screened for mutants showing visible phenotypes. Fifty different families segregated mutations in their progeny [9]. In our experiment, 48 of 69 families originated from different F1 plants exhibited visible mutant phenotypes (Table 3). Since the frequencies of visible mutants were much higher than those found in *Arabidopsis*, the frequency of germinal *Ds* transposition in rice is likely to be higher than that of *Arabidopsis*.

In F3 and F4 generations, new visible mutations were rarely found. This is consistent with the observation that *Ds* become silent in F3 and F4 generations. Visible mutants were often found in progeny of regenerated plants from protoplasts of four inactive lines carrying both *Ds* and the *Ac*TPase gene. Whereas none was found among around 500 progeny of regenerated plants from a line carrying only *Ds* (data not shown). Southern blot analysis confirmed reactivation of *Ds*

Table 3. Summary of primary screening for visible mutants. Total of 69 F2 families were screened. Each family consisted of approximately 100 F2 plants derived from selfing of a F1 plant. Number of F2 families which contained at least one F2 plant exhibiting the mutant phenotypes was scored. Number of F2 individuals showing the same mutant phenotypes in a F2 family varied. As shown in Table 2, frequencies of mutants appeared in a F2 family were not generally according to the Mendelian law.

	Number of F2 families	no phenotype	dwarf	chlorina	twin	stripe	chlorosis	albino
Cross A	19	12	4	0	1	0	1	0
Cross B	18	5	9	7	7	2	0	1
Cross C	17	1	15	2	1	1	1	0
Cross D	7	3	4	0	0	0	0	1
Cross E	8	0	8	0	0	0	1	0
	69	21	40	9	9	3	3	2

transposition in the four lines (Fig. 3). These results indicate that activity of *Ds* transposition is correlated well to the generation of the visible mutations and that these mutations are likely to be caused by insertion of the *Ds* elements.

Gene isolation by transposon tagging

Blast resistance genes

A *japonica* rice cultivar, Toride 1, which carries *Pi-z*^{t}, a gene conferring resistance to blast disease, was used for introduction of the *Ac* and *Ds* elements in this experiment [32]. The *Pi-z*^{t} locus is one of the 14 resistance loci identified in a differential set of Japanese cultivars and is located on the chromosome 6 [23]. Toride 1 was generated by introgression of the *Pi-z*^{t} gene from an indica cultivar, TKM 1, into Norin 8 which is susceptible to the most races of the blast fungus. To screen mutations of the blast resistance genes caused by *Ds* insertions about 8000 F2 seedlings were examined and 97 susceptible plants from 29 F1 plants were selected [35]. Toride 1 is highly resistant to the infection and very few necrotic lesions were observed when infected with the fungus. Plants showing altered resistance to the fungus are of two types; those exhibiting small lesions and those showing large lesions. We designated wild type as R^h for highly resistant, plants showing small lesions as R for resistant and those with large lesions as S for sensitive. These different phenotypes can be also microscopically distinguishable in young leaf sheaths infected with the fungus (data not shown). Therefore, the macroscopic differences reflect differences in early events during blast infection. In addition, observation of several distinct mutant phenotypes suggests more than one different alterations in the function of resistance genes.

Further analysis of F3 progeny demonstrated that eight F2 lines exhibited the stable S phenotype and other two lines exhibited the stable R phenotype. These phenotypes were apparently due to mutations and they seemed to be in the homozygous state for mutations. The other 35 F2 lines gave rise to plants segregating for resistant and susceptible phenotypes in F3 generation. About 50% of susceptible F2 lines exhibited only R^h phenotype in F3 generation. Because F4 progeny of susceptible F3 plants in the segregating lines exhibited the same phenotypes with their parent plants, these segregating phenotypes in F3 generation are likely due to genetic events (Table 4).

To examine whether mutations found in these *Ds* lines were mapped at the *Pi-z*^{t} locus, mutant lines were crossed with a *japonica* cultivar, Norin 8. Norin 8 is a recurrent parent used to generate Toride 1 and does not carry the resistance gene for blast disease. Therefore, if a mutation is recessive and F1 plants between a mutant and Norin 8 are susceptible to the blast fungus, the mutation is likely to be at the *Pi-z*^{t} locus. Six mutant lines exhibiting stable phenotypes in F3 plants crossed with Norin 8 and F1 plants were all susceptible (Table 4). Two of them were crossed with Toride 1 and all F1 plants were resistant, indicating that these two mutations are recessive. Therefore, we concluded that these mutations are at the *Pi-z*^{t} locus. Similarly, six F3 plants from segregating lines were also analyzed and identified as *Pi-z*^{t} mutants (Table 4). Together at least 7 mutant lines examined were all shown to have mutations at the *Pi-z*^{t} locus.

Table 4. Analysis of blast susceptible mutants. S, sensitive; R, resistant; R^h, highly resistant. Number in parenthesis is the number of plants showing the phenotypes. One F3 parent plant was selected from F3 plants of each line for this analysis.

Line	F3 phenotype	F3 parent	F4	X Norin8	X Toride1 (*Pi-z*^{t})
Segregating lines					
B10-2	S/R/R^h	S	S(8)	S(8)	R^h(3)
C9-1	S/R/R^h	S	S(24)	S(6)	R^h(5)
C7-6	S/R/R^h	S	S(18)	S(12)	
C10-6	S/R^h	S	S(24)	S(10)	R^h(7)
C12-25	S/R^h	S	S(13)	S(5)	R^h(14)
C18-4	S/R^h	S	S(7)	S(7)	R^h(5)
Stable lines					
C12-16	R	R	R(12)R^h(3)	R(15)	
C12-13	S	S	S(12)	S(3)	R^h(4)
C14-1	S	S	S(21)	S(6)	R^h(4)
A5-3	S	S	S(5)	S(5)	
A15-1	S	S	S(22)	S(9)	
D12-1	S	S	S(21)	S(15)	

To examine if these mutations were linked with specific *Ds* insertions, genomic DNAs of several F3 plants were isolated and subjected to Southern blot analysis. Results of the analysis revealed that four of the ten stable mutant lines did not carry any newly transposed *Ds*. Therefore, these four lines were not tagged by the *Ds* elements. We next examined the other six lines and compared the patterns of transposed *Ds* in mutant plants with those in F2 progeny showing resistance to the fungus infection. If the same *Ds* found in mutants were found in homozygous condition in disease-resistant relatives, it would mean that the *Ds* is not tagged. This comparison revealed that four of the other six mutants were not tagged. The other two lines, C6-2 and C14-1, carry *Ds* elements unique to susceptible plants. Further linkage analysis using F2 progeny between the mutant and the wild type and cloning of the region carrying *Ds* in these two lines are in progress.

A possible interpretation of these results is that *Ds* elements are frequently excised from the *Pi-z*^{t} locus giving rise to new mutations due to footprints and whether this interpretation is correct will be found out when the *Pi-z*^{t} gene is finally cloned. Similar phenomena were often observed in both *Ac*-induced *N* mutants of tobacco and L^6 mutants of flax [24, 40]. In addition, deletions of the L^6 locus detected among the flax plants carrying *Ac* might be induced due to chromosome breakages by *Ac* [25]. Therefore, it would be interesting to examine the chromosomal structures at the *Pi-z*^{t} locus of our mutants.

Flowering-time genes

Toride 1 is also known to carry several loci which affect flowering-time such as *Se-1*^{n}, *E1*, *Ef-1* [41, 42]. To isolate such genes which are involved in determination of flowering-time in rice, 21 lines originated from 10 F1 plants showing early-flowering plants were selected out of 80 F2 lines. Examination of flowering-time of F4 plants selected revealed that these lines confer either stable early-flowering phenotype or segregating phenotype. Under natural conditions (sowing at the beginning of May), they started flowering one to three weeks earlier than Toride 1. Among these early flowering lines, six stable lines were examined for flowering-time under conditions of long-day (14L 10D) and short-day (10L 14D) (Table 5). The results revealed two types of early-flowering mutants; constitutive early flowering and altered photoperiod sensitivity (Table 5).

One mutant termed C2-3 flowered 3 weeks earlier than the wild type under the long-day condition while this mutant flowered 2 weeks earlier under the short-day condition. This result indicates that the gene causing the C2-3 phenotype functions as a constitutive repressor. Similar results were obtained with the mutant lines C12-24, C16-2, C18-5 and C19-4. On

Table 5. Flowering-time of early-flowering mutants. Ten to twenty mutant plants were grown under long-day (14D10L) and short-day (10L14D) conditions. Flowering date (heading date) of each plant were scored and averages of days it tools from sowing to heading are presented with mean error. In parethesis, flowering-time of the wild-type plants in the same container is presented. The mutant phenotypes are evaluated in comparison with those of wild-type plants.

Mutant lines	Flowering time under SD (days)	Difference under SD (days)	Flowering time under LD (days)	Difference under LD (days)
B10-1-80	79.1 ± 2.5 (81.1 ± 1.6)	2.0	104.4 ± 2.1 (115.8 ± 0.8)	11.4
C2-3-241	69.3 ± 0.7 (75.2 ± 0.4)	5.9	107.7 ± 1.1 (121.3 ± 0.6)	13.6
C12-24-288	71.9 ± 1.1 (78.3 ± 1.1)	6.4	111.1 ± 1.1 (127.2 ± 1.0)	16.1
C16-2-308	65.6 ± 0.8 (78.3 ± 1.1)	12.7	112.4 ± 1.1 (127.2 ± 1.0)	14.8
C18-5-381	73.0 ± 0.5 (77.2 ± 0.9)	4.2	118.5 ± 1.0 (126.9 ± 1.0)	8.4
C19-4-424	71.4 ± 0.6 (86.4 ± 1.1)	15	106.4 ± 1.1 (119.5 ± 1.4)	13.1

the other hand, B10-1 flowered 2 weeks earlier than the wild type under the long-day condition but it did not differ from the wild type under the short-day condition, indicating that the mutation in B10-1 functions only under the long-day condition and may be involved in photoperiod sensitivity.

Southern blot analysis revealed that all lines but C18-5 carry multiple transposed *Ds* bands whereas C18-5 does not carries any *Ds* elements indicating that the C18-5 mutation is not tagged by *Ds*. To examine whether the other early-flowering mutants are linked with specific *Ds* elements, F2 analysis is in progress.

Conclusions

To make rice an attractive model system for molecular, genetic, and developmental studies of plants, development of gene tagging system is prerequisite. Therefore, we are trying to develop transposon tagging system in rice. Here, we demonstrated that the maize *Ac*/*Ds* system is an effective mutagen for rice. Mutagenesis of a disease resistant gene, *Pi-z*,^{t} was successful. However, no linkage of *Ds* elements with the mutant phenotypes indicates that integration and excision of *Ds* in F1 plants might be too frequent to identify a linked *Ds* using the segregating populations originated from F2 lines. To distinguish mutations caused by footprints due to multiple transposition of *Ds* from *Ds* insertion mutants, F2 analysis of the mutants remains to be done. Southern blot analysis using *Ds* as a probe revealed that inactivation of *Ds* transposition was often observed in F3 and F4 generations. To overcome this potential obstacle, we demonstrated that these inactive *Ds* can be reactivated through tissue culture. Use of progeny of tissue culture-derived plants would make it possible to screen revertants from mutant lines carrying inactive *Ds*. For further directed mutagenesis and gene isolation by the *Ac*/*Ds* system, mapping of transposed *Ds* elements is in progress. In the future, *Ds* elements closely linked to target genes will be used to isolate mutants and genes.

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References

1. Aarts MGM, Dirkse WG, Stiekema WJ, Pereira A: Transposon tagging of a male sterility gene in *Arabidopsis*. Nature 363: 715–717 (1993).
2. Altmann T, Felix G, Jessop A, Kauschmann A, Uwer U, Pena-Cortes H, Wilmitzer L: *Ac*/*Ds* transposon mutagenesis in *Arabidopsis thaliana*: mutant spectrum and frequency of *Ds* insertion mutants. Mol Gen Genet 247: 646–652 (1995).
3. Baker B, Coupland G, Fedoroff NV, Starlinger P, Schell J: Phenotypic assay for excision of the maize controlling element *Ac* in tobacco. EMBO J 6: 1547–1554 (1987).
4. Baker B, Schell J, Lörz H, Fedoroff NV: Transposition of the maize controlling element *Activator* in tobacco. Proc Natl Acad Sci USA 83: 4844–4848 (1986).
5. Bancroft I, Dean C: Transposition pattern of the maize element *Ds* in *Arabidopsis thaliana*. Genetics 134: 1221–1229 (1993).
6. Bancroft I, Dean C: Factors affecting the excision frequency of the maize transposable element *Ds* in *Arabidopsis thaliana*. Mol Gen Genet 240: 65–72 (1993).
7. Bancroft I, Jones JDG, Dean C: Heterologous transposon tagging of the *DRL* locus in *Arabidopsis*. Plant Cell 5: 631–638 (1993).
8. Behrens U, Fedoroff N, Laird A, Muller-Neumann M, Starlinger P, Yoder J: Cloning of *Zea mays* controlling element *Ac* from the *wx-m7* allele. Mol Gen Genet 194: 346–347 (1984).

9. Bhatt AM, Page T, Lawson EJR, Lister C, Dean C: Use of *Ac* as an insertional mutagen in *Arabidopsis*. Plant J 9: 935–945 (1996).
10. Brettell RIS, Dennis ES: Reactivation of a silent *Ac* following tissue culture is associated with heritable alterations in its methylation. Mol Gen Genet 229: 365–372 (1991).
11. Carrol BJ, Klimyuk VI, Thomas CM, Bishop GJ, Harrison K, Scofield SR, Jones JDG: Germinal transposition of the maize element *Dissociation* from T-DNA loci in tomato. Genetics 139: 407–420 (1995).
12. Chuck G, Robbins T, Nijjar C, Ralston E, Courtney-Gutterson N, Dooner HK: Tagging and cloning of a petunia flower color gene with the maize transposable element *Activator*. Plant Cell 5: 371–378 (1993).
13. Dean C, Sjodin C, Page T, Jones J, Lister C: Behavior of the maize transposable element *Ac* in *Arabidopsis thaliana*. Plant J 2: 69–81 (1992).
14. Dooner HK, Keller J, Haper E, Ralston E: Variable patterns of transposition of the maize element *Activator* in tobacco. Plant Cell 3: 473–482 (1991).
15. Döring HP, Starlinger P: Barbara McClintock's controlling elements: now at the DNA level. Cell 39: 253–259 (1984).
16. Fedoroff NV, Furtek DB, Nelson OE: Cloning of the *bronze* locus in maize by a simple and generalizable procedure using the transposable element *Activator* (*Ac*). Proc Natl Acad Sci USA 81: 3825–3829 (1984).
17. Fedoroff NV, Wessler S, Shure M: Isolation of the transposable maize controlling elements *Ac* and *Ds*. Cell 35: 235–242 (1983).
18. Izawa T, Miyazaki C, Yamamoto M, Terada R, Iida S, Shimamoto K: Introduction and transposition of the maize transposable element *Ac* in rice (*Oryza sativa* L.). Mol Gen Genet 227: 391–396 (1991).
19. Izawa T, Shimamoto K: Becoming a model plant: the importance of rice to plant science. Trends Plant Sci 1: 95–99 (1996).
20. James Jr DW, Lim E, Keller J, Plooy I, Ralston E, Dooner HK: Directed tagging of the *Arabidopsis FATTY ACID ELONGATION 1* (*FAE1*) gene with the maize transposon *Activator*. Plant Cell 7: 309–319 (1995).
21. Jones DA, Thomas CM, Hammond-Kosack KE, Balint-Kurti PJ, Jones JDG: Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266: 789–793 (1994).
22. Jones JDG, Carland F, Lim E, Ralston E, Dooner HK: Preferential transposition of the maize element *Activator* to linked chromosomal locations in tobacco. Plant Cell 2: 701–707 (1991).
23. Kiyosawa S: Establishment of differential varieties for pathogenicity test of rice blast fungus. Rice Genet Newsl 1: 95–97 (1984).
24. Lawrence GJ, Finnegan EJ, Ayliffe MA, Ellis JG: The L^6 gene for flax rust resistance is related to the *Arabidopsis* bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N*. Plant Cell 7: 1195–1206 (1995).
25. Lawrence G, Finnegan J, Ellis J: Instability of the L^6 gene for rust resistance in flax is correlated with the presence of a linked *Ac* element. Plant J 4: 659–669 (1993).
26. Lawson EJR, Scofield SR, Sjodin C, Jones JDG, Dean C: Modification of the 5′ untranslated leader region of the maize *Activator* element leads to increased activity in *Arabidopsis*. Mol Gen Genet 245: 608–615 (1994).
27. Long D, Martin M, Sundberg E, Swinburne J, Puangsomlee P, Coupland G: The maize transposable element system *Ac*/*Ds* as a mutagen in *Arabidopsis*: Identification of an *albino* mutation induced by *Ds* insertion. Proc Natl Acad Sci USA 90: 10370–10374 (1993).
28. Peschke VM, Philips RL, Gengenbach BG: Discovery of transposable element activity among progeny of tissue culture-derived maize plants. Science 238: 804–807 (1987).
29. Peschke VM, Phillips RL: Activation of the maize transposable element Suppressor-mutator (*Spm*) in tissue culture. Theor Appl Genet 81: 90–9 (1987).
30. Schmidt R, Willmitzer L: The maize autonomous element *Activator* (*Ac*) shows a minimal germinal excision frequency of 0.2%–0.5% in transgenic *Arabidopsis thaliana* plants. Mol Gen Genet 220: 17–24 (1989).
31. Scofield SR, Harrison K, Nurrish SJ, Jones JDG: Promoter fusions to the *Activator* transosase gene cause distinct patterns of *Dissociation* excision in tobacco cotyledons. Plant Cell 4: 573–582 (1992).
32. Shimamoto K, Terada R, Izawa T, Fujimoto H: Fertile transgenic rice plant regenerated from transformed protoplasts. Nature 338: 274–276 (1989).
33. Shimamoto K, Miyazaki C, Hashimoto H, Izawa T, Itoh K, Terada R, Inagaki Y, Iida S: *Trans*-activation and stable integration of the maize transposable element *Ds* cotransfected with the *Ac* transposase gene in transgenic rice plants. Mol Gen Genet 239: 354–360 (1993).
34. Shimamoto K: The molecular biology of rice. Science 270: 1772–1773 (1995).
35. Shimamoto K, Ohnishi T, Kume Y, Hashimoto H, Wu C, Izawa T: Toward molecular isolation of the blast resistance gene in rice by the maize *Ac/Ds* elements. In: Mills D, Kunoh H, Keen N, Mayama S (eds) Molecular Aspects of Pathogenicity and Resistance: Requirement for Signal Transduction, pp. 177–183. APS Press, St. Paul, MN (1996).
36. Song W-Y, Wang G-L, Chen L-L, Kim H-S, Pi L-Y, Holsten T, Gardner-J, Wang B, Zhai W-X, Zhu L-H, Fauquet C, Ronald P: A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science 270: 1804–1806 (1995).
37. Sundaresan V: Horizontal spread of transposon mutagenesis: new uses for old elements. Trends Plant Sci 1: 184–190 (1996).
38. Swinburne J, Balcells L, Scofield SR, Jones JDG, Coupland G: Elevated levels of *Activator* transposase mRNA are associated with high frequencies of *Dissociation* excision in *Arabidopsis*. Plant Cell 4: 583–595 (1992).
39. Walbot V: Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. Annu Rev Plant Physiol Plant Mol Biol 43: 49–82 (1992).
40. Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B: The product of the tobacco mosaic virus resistance gene *N*: similarity to *Toee* and the interleukin-1 receptor. Cell 78: 1–20 (1994).
41. Yamagata H: Heading-time genes of rice, *E1*, *E2* and *E3*. Rice Genet Newsl 1: 100–101 (1984).
42. Yokoo M, Kikuchi F: Multiple allelism of the locus controlling heading time of rice, detected using the close linkage with blast-resistance. Jpn J Breed 27: 123–130 (1977).