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Cre/*lox* site-specific recombination controls the excision of a transgene from the rice genome

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Abstract The Cre/*lox* site-specific recombination controls the excision of a target DNA segment by recombination between two *lox* sites flanking it, mediated by the Cre recombinase. We have studied the functional expression of the Cre/*lox* system to excise a transgene from the rice genome. We developed transgenic plants carrying the target gene, hygromycin phosphotransferase (*hpt*) flanked by two *lox* sites and transgenic plants harboring the Cre gene. Each *lox* plant was crossed with each Cre plant reciprocally. In the Cre/*lox* hybrid plants, the Cre recombinase mediates recombination between two lox sites, resulting in excision of the *hpt* gene. The recombination event could be detected because it places the CaMV 35S promoter of the *hpt* gene adjacent to a promoterless *gusA* gene; as a result the *gusA* gene is activated and its expression could be visualized. In 73 Cre/*lox* hybrid plants from various crosses of T0 transgenic plants, 19 expressed GUS, and in 132 Cre/*lox* hybrid plants from crosses of T2 transgenic plants, 77 showed GUS expression. Molecular data proved the excision event occurred in all the GUS^+ plants. Recombination occurred with high efficiency at the early germinal stage, or randomly during somatic development stages.

Keywords Rice · *Oryza sativa* · Site-specific recombination · Cre/*lox* recombination system

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Introduction

Site-specific recombination is a process involving reciprocal exchange between specific DNA target sites catalyzed by specialized proteins, the site-specific recombinases. As such, these recombinases can alter genomic DNA sequences in a highly specific manner providing powerful tools for the development of a new generation of molecular technology for crop improvement. The Cre/*lox* site-specific recombination system from *Escherichia coli* phage P1 is mediated by the Cre recombinase, which recognizes and interacts with the two 34-bp *lox* sites to direct the excision, integration or inversion of the intervening DNA sequences depending on the orientation of *lox* sites towards each other (Abremski et al. 1983; Hoess et al. 1986). Intramolecular recombination between the two *lox* sites in a direct orientation results in excision of the intervening DNA sequences, whereas an inverted orientation results in inversion of the intervening DNA segment.

The function of the Cre/*lox* system to excise DNA sequences from the yeast and mammalian genomes was reported by Sauer (1987). In plants, DNA rearrangements induced by Cre/*lox*-mediated recombination were recently well-documented. Such rearrangements included the deletion of a selectable marker from the host genomes (Dale and Ow 1991; Russell et al. 1992; Gleave et al. 1999), site-specific integration of the transgenes (Albert et al. 1995; Vergunst and Hooykaas 1998; Vergunst et al. 1998), creation of a hybrid chromosome (Koshinsky et al. 2000), and chromosome translocation or deletion (Vergunst et al. 2000).

These functions of the Cre/*lox* system have been mainly studied in model plants such as *Arabidopsis* and tobacco. Recently, Srivastava et al. (1999) used the Cre/*lox* system to reduce the copy number of DNA inserts in the wheat genome after bombardment. In this paper, we report the functions of this system in rice. Since rice is one of the most-important food crops in the world, the applications of this site-specific recombination system would be of a significant interest towards

the improvement of this crop. Our results showed that the function of the Cre/*lox* system controlling gene excision worked effectively in rice, resulting in a high recombination frequency. This suggests that the applications of the Cre/*lox* system for rice improvement could be feasible.

Materials and methods

Agrobacterium tumefaciens strain and plasmid constructions

The plasmids pED32A containing *lox* sites, and pMM23 containing the Cre gene, were graciously provided by Dr. D.W. Ow, Plant Gene Expression Center, Albany, Calif. The plasmid pAHC27 was obtained from Dr. Peter H. Quail, University of California, Berkeley. The plasmids pPUR was provided by Dr. Brian Larkins, University of Arizona. The plamids pSB11 and the *A. tumefaciens* strain LBA 4404 were provided by the Japan Tobacco Company.

In this study, three plasmids were constructed as follows. To obtain the first plasmid, pSB11-Lox-Hyg-Lox-GUS, the plasmid pSB11-GUS was constructed by isolating the 2.0-kb *Bam*HI-*Eco*RI *gusA* fragment from pPUR and cloning into the *Bam*HI-*Eco*RI sites of pSB11. The 53-bp *Xba*I *lox* oligonucleotide isolated from pED32 A was cloned into the *Bam*HI site of pHyg to obtain pHyg-lox. Another *lox* oligonucleotide from pED32A was cloned into the *Xba*I site of pSB11-GUS to yield pSB11-GUS-lox. The orientation of the two *lox* sites was checked. Lastly, a 2.152-kb *Hin*dIII 35S-Hyg-Lox fragment from pHyg-Lox was inserted into *Hin*dIII sites of pSB11-lox-GUS, previously equipped with a *lox* oligonucleotide in the *Xba*I site, yielding the designed construct, pSB11-Lox-Hyg-Lox-GUS (Fig. 1A).

To obtain the second plasmid pSB11Hyg-UbiCre, a 2.169-kb *Hin*dIII-*Eco*RI Enh 35S Hyg fragment from pFI19H was cloned into *Hin*dIII-*Eco*RI sites of pSB11 to yield pSB11-Hyg. Then, the 1.058-kb *Xba*I-*Eco*RI Cre gene from pMM23 was isolated and cloned into *Xba*I-*Eco*RI sites of pAHC27 to obtain pAHC27-UbiCre. The 3.859-kb *Hin*dIII fragment from pAHC27-UbiCre was cloned into *Hin*dIII sites of pSB11-Hyg, and the resulting plasmid was designated as pSB11Hyg-UbiCre (Fig. 1B). The third plasmid, pSB11bar-UbiCre, was constructed in the same way as pSB11Hyg-UbiCre but the Bar fragment was replaced for the Hyg fragment (Fig. 1C). These designed plasmids were introduced separately into *A. tumefaciens* by triparental mating.

Transformation of rice plants

The japonica rice cultivar (*Oryza sativa* L.) Taipei 309 was used for all tissue-culture and transformation experiments. Embryogenic calli derived from mature seeds were used for *Agrobacterium*-mediated transformation. The transformation procedures of Aldemita and Hodges (1996), which had been developed for immature embryos, were basically followed.

DNA isolation and Southern hybridization

Genomic DNA was isolated from leaf tissue of T0, T1, T2 transgenic plants and hybrid plants following the method of McCouch et al. (1988). Southern blot analyses were performed according to Sambrook et al. (1989) with 10 µg of genomic DNA after restriction with *Xba*I and *Eco*RI, or *Eco*RI or *Xho*I alone. The restricted DNA was fractionated through a 0.8% agarose-gel by electrophoresis at 50 V for 12 h, prior to capillary transfer to, and immobilization on, nylon membranes (Hybond-N$^+$, Amersham).

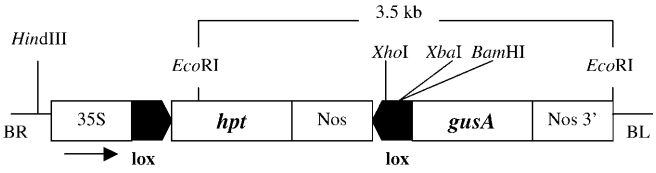

Fig. 1A

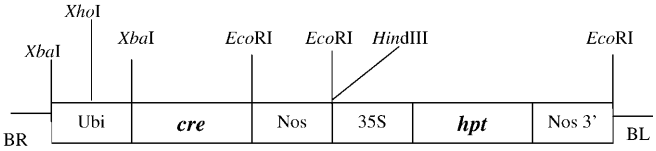

Fig. 1B

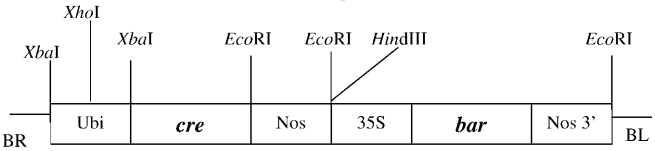

Fig. 1C

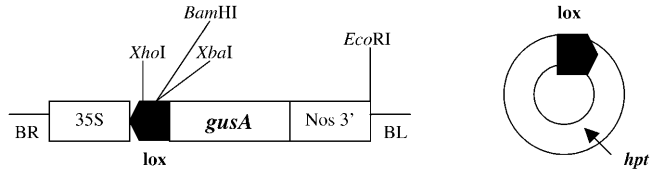

Fig. 1D

Fig. 1A–D A schematic diagram of the constructs to study the Cre/*lox* system controlling gene excision in rice. (**A**) pSB11-Lox-Hyg-Lox-GUS contains two *lox* sites flanking the *hpt* gene driven by the CaMV 35S gene promoter plus the *gusA* gene placed distally without a promoter. (**B**) pSB11Hyg-UbiCre contains the Cre gene driven by the Ubiquitin promoter plus the *hpt* gene driven by the CaMV 35S gene promoter. (**C**) pSB11bar-UbiCre contains the Cre gene driven by the Ubiquitin promoter plus the *bar* gene driven by the CaMV 35S gene promoter. (**D**) Cre-mediated recombination between two *lox* sites controlling excision of the *hpt* gene; consequently, the *gusA* gene fuses with the CaMV 35S gene promoter and is activated and can be detected by GUS$^+$ expression

Crossing of transgenic plants

Plants confirmed as transgenic by molecular analyses were assayed for transient expression of the Cre/*lox* system. The leaves of plants haboring the Cre gene were bombarded with the *lox*-containing plasmid, and those with *lox* were bombarded with the Cre-containing plasmid following the method of Christou (1992). The plants exhibiting a high level of Cre expression, based on the density of the blue spots in GUS staining, were identified (Fig. 2). Plants showing a simple integration of the transgenes and high transient GUS expression due to Cre-mediated activation were selected for crossing. Each *lox* plant was crossed with each Cre plant reciprocally. Ten independent crosses of T0×T0 transgenic plants and 12 crosses of T2×T2 transgenic plants were developed.

Assay of the GUS activity of the Cre/*lox* hybrids

GUS expression in the parental control, the hybrids from [T0×T0] and [T2×T2] crosses, and progeny F_1, F_2 of the hybrids from T0×T0 crosses was performed according to Jefferson (1987). GUS histochemical assays were evaluated at different developmental stages of the plant: the young seedling, the leaf, pollen, the ovary

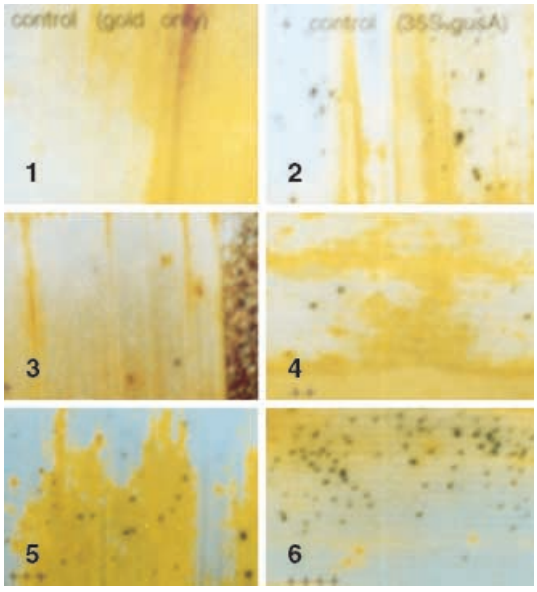

Fig. 2 GUS expression in rice leaves of transgenic plants containing the Cre gene after bomdardment with the *lox*-containing plasmid (pSB11-Lox-Hyg-Lox-GUS). *1* control (bombarded with gold only); *2* postive control (35S-*gusA*); *3–6* blue spots with varied density

and the mature seed. Plant tissues were scored as GUS-positive (GUS^+) when blue-stained tissues were detectable.

Molecular analysis of GUS^+ plants

Southern blotting was performed for all GUS^+ hybrid plants. DNA of these plants was digested with *Eco*RI and probed with a *gusA* fragment. The presence or absence of the 3.5-kb fragment was recorded. In addition, to test the presence of the Cre gene in the hybrids, these DNAs were digested with *Eco*RI and *Xba*I and probed with a Cre gene fragment. DNA of the *lox* parental plants was digested with *Eco*RI and probed with a *gusA* gene fragment to check the presence of the 3.5-kb fragment.

Results

Experimental design

The experimental strategy used in this study is described in Fig. 1. Three separate contructs were designed as follows. The first construct, pSB11-Lox-Hyg-lox-GUS, contained the 35S promoter of cauliflower mosaic virus (CaMV) driving the *hpt* gene for hygromycin resistance; the *hpt* gene was flanked by two *lox* sites, the *gus*A gene without a promoter was placed distally. The second construct, pSB11Hyg-UbiCre, contained the ubiquitin promoter driving the Cre gene followed by the *hpt* gene driven by the CaMV 35S promoter. The third construct, pSB11barB-UbiCre, also contained the Cre gene controlled by the CaMV 35S promoter and the *bar* gene driven by the CaMV 35S promoter. The hybrid plants which were developed by crossing *lox* plants with Cre plants contained both *lox/hpt/lox* and Cre. The Cre recombinase

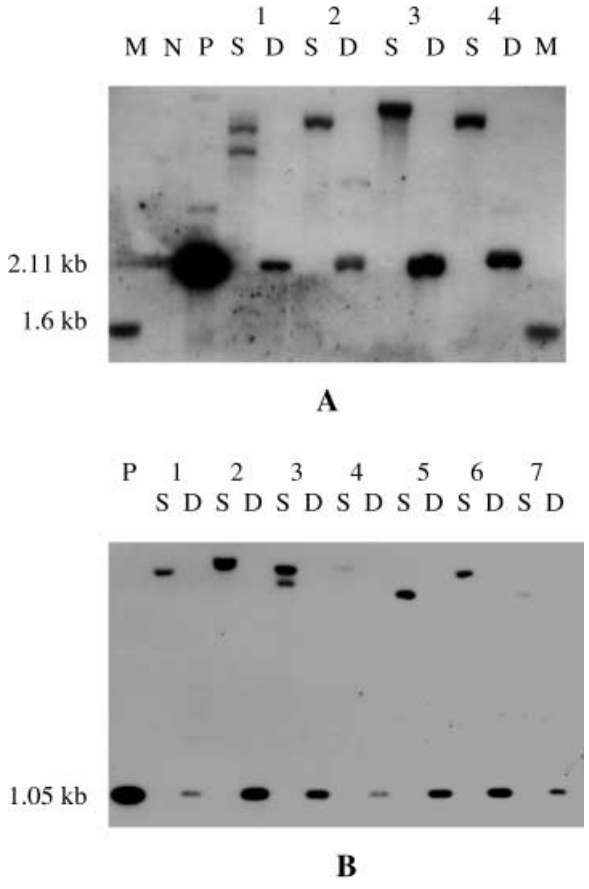

Fig. 3A, B Southern analysis of *lox* and Cre transgenic T0 plants. DNA of these plants was digested with *Xho*I (single cut: *S*) and with *Eco*RI and *Xba*I (double cut: *D*). **A** *M* marker, *N* non-transformed, *P lox* plasmid, *1–4* transgenic *lox* plants showing expected band of 2. 11 kb in double-cut (*D*) with the *gusA* gene fragment as a probe. **B** *P* Cre plasmid, *1–7* transgenic Cre plants showing expected band of 1.05 kb in double-cut (*D*) with the Cre gene fragment as a proble. The bands in single-cut (*S*) in **A** and **B** showed the simple integration of the transgenes

will catalyze recombination between two *lox* sites of the target resulting in the excision of the *hpt* gene. Such a deletion places the promoterless *gusA* gene adjacent to the CaMV 35S promoter; consequently, the g*usA* gene is activated and its expression could be visualized by histochemical staining. Two *Eco*RI sites restricted a fragment of 3.5 kb in the first contruct. If the *hpt* gene is excised, one *Eco*RI site will be removed and the diagnostic 3.5-kb fragment will be absent in the GUS^+ hybrids.

Development of parental plants

Applying *Agrobacterium*-mediated transformation, we obtained more than 300 T0 putative transgenic plants resistant to hygromycin (hyg) or phosphinothricin (ppt) (data not shown). Southern blotting was performed to ensure the presence of the *hpt* gene flanked by *lox* sites (Fig. 3A) or the presence of the Cre gene (Fig. 3B) in the primary transformants. In most of the cases, the Southern blots showed simple integration of the transgenes into

Table 1 Expression of GUS$^+$ in the hybrid of Cre/*lox* plants (from T0×T0 crosses) as a result of excision of the *hpt* gene from the rice genome

Cross no.	Parent 1 (female) T0 plant ID	Parent 2 (male) T0 plant ID	Hybrid plant ID	GUS$^+$plant no./ no. tested
1	Cre/*bar*108	*lox*/*hpt*90	6	1/2
2	Cre/*bar*132	*lox*/*hpt*7	17	1/2
3	Cre/*bar*226	*lox*/*hpt*511	131, 134	2/5
Sub-total				4/9
4	Cre/*hpt*405	*lox*/*hpt*27	51, 53	3/5
5	Cre/*hpt*406	*lox*/*hpt*9	54, 58,62	3/11
6	Cre/*hpt*406	*lox*/*hpt*3	93	1/12
Sub-total				7/28
7	*lox*/*hpt*28	Cre/*hpt*405	65, 68,69,79	4/16
Sub-total				4/16
8	*lox*/*hpt*29	Cre/*bar*132	27, 111	2/8
9	*lox*/*hpt*512	Cre/*bar*227	128	1/10
10	*lox*/*hpt*76	Cre/*bar*221	135	1/2
Sub-total				4/20
Total				19/73

Table 2 Expression of GUS$^+$ in the hybrid of Cre/*lox* plants (from T2×T2 crosses) as a result of excision of the *hpt* gene from the rice genome

Cross no.	Parent 1 (female) T2 plant ID	Parent 2 (male) T2 plant ID	Hybrid plant ID	GUS$^+$plant/ no. tested
1	*lox*/*hpt*26/4–2	Cre/*hpt*406/2–3	1–17	10/17
2	*lox*/*hpt*90/5–2	Cre/*hpt*405/5–2	18–32	15/15
3	*lox*/*hpt*26/9–4	Cre/*hpt*405/5–1	38–42	3/5
4	*lox*/*hpt*90/5–1	Cre/*hpt*405/3–4	43–48	6/6
5	*lox*/*hpt*26/4–1	Cre/*hpt*405/3–4	49–57	5/9
6	*lox*/*hpt*9/4–4	Cre/*hpt*405/2–2	101–133	14/33
7	*lox*/*hpt*26/9–1	Cre/*hpt*405/2–1	162–166	2/5
8	*lox*/*hpt*26/4–1	Cre/*hpt*405/3–4	167–170	1/4
9	*lox*/*hpt*26/4–4	Cre/*hpt*405/3–1	171–181	5/11
10	*lox*/*hpt*26/9–3	Cre/*hpt*405/3–1	204–206	3/3
Sub-total				64/108
11	Cre/*hpt*406/3–4	*lox*/*hpt*90/5–3	59–72	7/14
12	Cre/*hpt*405/3–2	*lox*/*hpt*9/5–4	194–203	6/10
Sub-total				13/24
Total				77/132

the genome. This was an advantage of the transformation method using *A. tumefaciens*. Transient assay of GUS expression through bombardment of a *lox* parent with a Cre construct, or vice versa, was performed. Out of 97 plants bombarded, 22 showed GUS expression and the density of blue spots varied among treatments. The negative control did not show any GUS expression (Fig. 2). Based on molecular data and transient GUS expression, ten independent T0 plants containing *lox/hpt/lox/gusA*, and seven containing Cre/*hpt* or Cre/*bar,* were selected for crossing. The T2 progeny of these primary transformants were also selected and crossed.

GUS expression in the Cre/*lox* hybrids

The expression of GUS in the Cre/*lox* hybrids from T0×T0 crosses is presented in Table 1. Ten crosses were made including three crosses of [Cre/*bar*]×[*lox/hpt/lox*], three crosses of [Cre/*hpt*]×[*lox/hpt/lox*], one cross of [*lox/hpt/lox*]×[Cre/*hpt*] and three crosses of [*lox/hpt/lox*]×[Cre/*bar*]. The number of individual hybrid plants from each cross varied from 5 to 24. In total we studied 73 hybrid plants. The GUS$^+$ expression was observed in 19 plants or 26.02%. The GUS$^+$ expression in individual crosses varied from 1/10 (10%) to 1/2 (50%) (Table 1).

Table 2 shows GUS expression in the Cre/*lox* hybrids from the T2×T2 crosses. Twelve crosses including ten crosses of [*lox/hpt/lox*]×[Cre/*hpt*] and two crosses of [Cre/*hpt*]×[*lox/hpt/lox*] produced 132 hybrids for study. The number of plants expressing GUS$^+$ was 77/132 or 58.33%. The percentage of GUS$^+$ plants varied from 1/4 (25%) to 15/15 (100%) among crosses. (Table 2).

GUS expression in the whole hybrid seedlings was visualized (Fig. 4B) and in some cases the staining intensity varied in different tissues of the hybrid plants (Fig. 4C). The F_2 progeny derived from GUS$^+$ hybrid plants (T0×T0) showed segregation for the *gusA* gene, and GUS$^+$ expression could be visualized in grains, seedlings, florets, anthers and pollens (Fig. 5).

Molecular analyses of gene excision

To obtain molecular evidence of the Cre-mediated excision event, Southern blotting was performed for the parental plants and all the GUS$^+$ hybrid plants. DNA from these

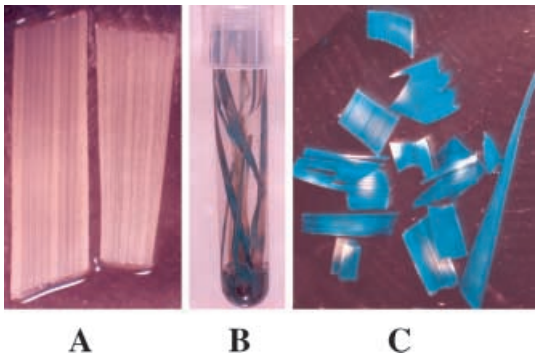

Fig. 4A–C Variation of GUS expression in the Cre/*lox* hybrid plants. **A** lox parental plant (control, no GUS expression), **B** blue staining of the whole hybrid seedling, **C** variation in blue-staining density in the leaves of one hybrid plant

Fig. 5 F_2 progeny derived from GUS$^+$ hybrid plants (T0×T0) showed that segregation for the *gusA* gene and GUS$^+$ expression could be visualized in grains, seedlings, florets, anthers and pollens

plants was digested with *Eco*RI and probed with a *gusA* fragment (Fig 6). In *lox* parental plants, the 3.5-kb fragment characteristic of the intact, non-recombined substrate DNA was present. In the hybrids, the 3.5 kb band was absent and only one band representing the recombined product was detected. This was observed for all the hybrids except ID. 111, which showed two bands as recombined products. It was noted that the *lox* parental plant of ID. 111 had two integration sites of the transgene (Fig. 6, lane 4) when digested with *Xho*I (single cut). Furthermore, the F_2 plants of this hybrid also showed a similar two bands (Fig. 7).

The hybrids plants (*Eco*RI digestion, Fig. 6, lanes 3, 6, 9 and 12) did not show this band but showed another band of recombined products. The hybrid plant ID. 111 (lane 6) was found to have two bands of recombined products and their F2 progeny also had two bands (Fig. 7). It was noted that, to confirm the presence of the Cre gene which mediates gene excision in the hybrid plants, DNA of the hybrid plants was digested with *Eco*RI and *Xba*I and probed with the Cre gene fragment.

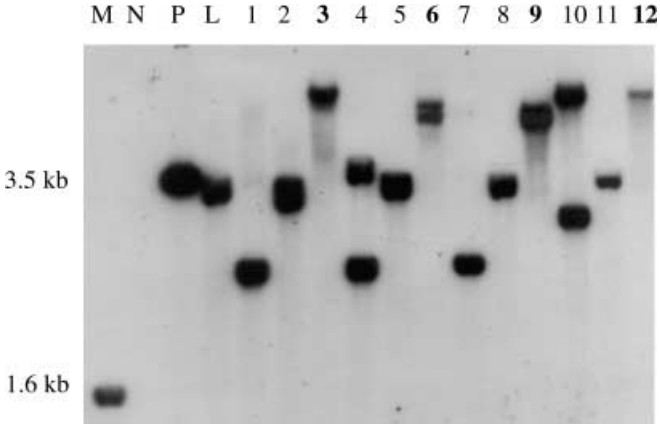

Fig. 6 Southern blotting proved the excision of the *hpt* gene. DNA was probed with a *gusA* gene fragment. *M* marker, *N* non-transformed, *P lox* plasmid and *L lox* plant showing 3.5 kb. *Lanes 1–12 lox* plants and corresponding hybrids. *Lanes 1 and 2* as parent *lox/hpt90* with a single cut (*Xho*I) and a double cut (*Eco*RI) respectively, *lane 3* as the hybrid ID. 6 from this parent with *Eco*RI. Similarly, *lanes 4 and 5* as parent *lox/hpt*29, *lane 6* as the hybrid ID. 111, *lanes 7 and 8* as parent *lox/hpt3*, lane 9 as the hybrid ID. 93, *lanes 10 and 11* as parent *lox/hpt*511, *lane 12* as the hybrid ID. 131. In the hybrids, the 3.5-kb band was absent (ID. of parental and hybrid plants, see Table 1)

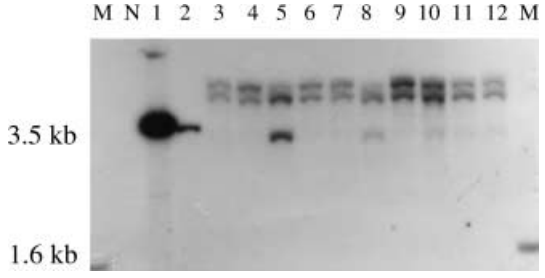

Fig. 7 F_1 and F_2 Progeny of the GUS$^+$ hybrid plant ID. 111 (*lox/hpt*29 as female×Cre/*bar*132 as male). DNAs were digested with *Eco*RI and probed with the *gusA* gene fragment. *M* marker. *N* non-transformed plant, *lane 1 lox* plasmid DNA, *lane 2 lox* parental plant (*lox/hpt*29), *lane 3* F_1 plant of the GUS$^+$ hybrid (plant ID. 111, Table 1), *lanes 4–12* F_2 progeny of the same GUS$^+$ hybrid 111

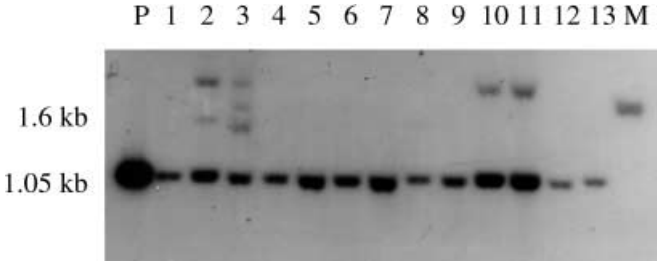

Fig. 8 Southern blotting showed the presence of the Cre gene in GUS$^+$ hybrid plants. DNA was digested with *Eco*RI and *Xba*I (double cut) and probed with the Cre gene fragment. *P* Cre plasmid, *M* marker, *lanes 1–13* GUS$^+$ hybrid plants showing an expected band of 1.05 kb

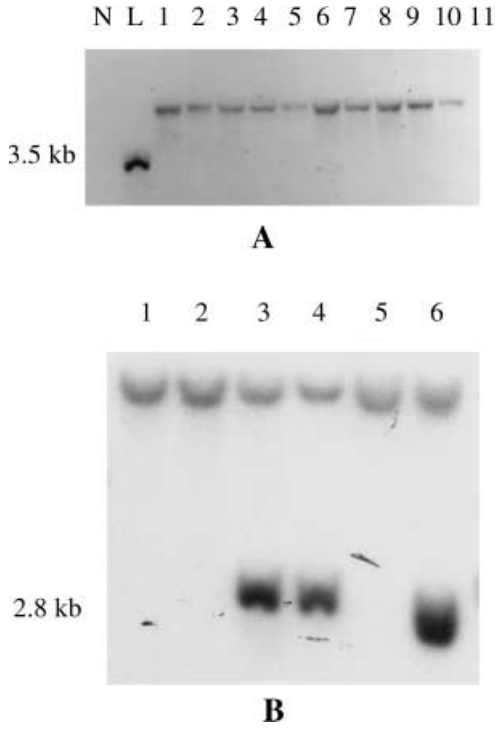

Fig. 9A, B Southern blotting proved the excision of the *hpt* gene in the hybrid plants of T2×T2 crosses. DNA was digested with *Eco*RI and probed with the *gusA* gene fragment. **A** *lox/hpt* 90/5–2 (female)×Cre/*hpt* 405/5–2 (male), *N* non-transformed plant, *L lox* parent (*lox/hpt* 90/5-2), *lanes 1–10* GUS$^+$ hybrid plants showing absence of the 3.5-kb band, *lane 11* Cre parent (Cre/*hpt* 405/5-2). **B** GUS$^+$ hybrid plants of the cross Cre/*hpt* 405/3-2 (female)× *lox/hpt* 9/5-4 (male), *lanes 1, 2 and 5* one band as recombined product; *lanes 3, 4 and 6* two bands as recombined product with an additional band at 2.8 kb, but in all the cases, the 3.5-kb band of the *lox* parent was absent

As shown in Fig. 8, the hybrids exhibited the expected band of 1.05 kb of the Cre gene fragment.

Apart from the Cre/*lox* hybrid plants from the crosses of T0×T0 transgenic plants, 77 Cre/*lox* hybrid plants derived from 12 crosses of T2×T2 transgenic plants were subjected to Southern-blot analysis. We found that all these plants did not have the 3.5-kb band (Fig. 9A) but there were two classes based on band pattern. One class consisting of 32 plants had one band of a recombined product, and another class consisting of 45 plants had more than one band of recombined products (Fig. 9B). The number of plants showing one band or two bands of recombined products varied among crosses. As shown in Fig. 9B, from the same cross some plants had one band of a recombined product while other plants had two bands of recombined products. The second bands of all four progeny had the same size of 2.8 kb (cross 12 in Table 2, Fig. 9B).

Discussion

The function of the Cre/*lox* site-specific recombination system in plant species has been reported. Such plants include tobacco (Dale and Ow 1991; Bayley et al. 1992; Medberry et al. 1995), tomato (Stuurman et al. 1996), Arabidopsis (Russell et al. 1992; Vergunst et al. 2000) and wheat (Srivastava et al. 1999). The application of the Cre/*lox* site-specific recombination system in plants has been discussed by Odell and Russell (1994). The functional expression of this recombination system has not yet been reported in rice. This study was therefore conducted to test the function of the Cre/*lox* site-specific recombination system to excise a transgene (selectable marker) from the rice genome with the following strategy. The Cre-mediated excision of the target *hpt* gene, flanked by two directly repeated *lox* sites, consequently leads to the fusion of the GUS gene to the CaMV 35S promoter. This event activates the expression of the GUS gene, resulting in its visible phenotype upon histochemical staining.

We were able to create a large number of transgenic plants harboring either *lox/hpt/lox/gusA* or Cre*/hpt* or Cre*/bar*. Southern blotting was conducted to help in selecting independent transformants showing simple integration of the transgenes. Further, these primary transformants carrying the *lox* sites or the Cre gene were evaluated for transient GUS expression by bombarding the Cre construct into the leaves of the *lox* plants and vice versa. The plants showing strong GUS expression were selected for crossing. Ten crosses were made from each *lox* plant with each Cre plant yielding 73 hybrids for GUS analyses. The efficiency of recombination, expressed as the percentage of GUS$^+$ plants, was 26.02%, being approximately the estimated recombination frequency of 1/4 for a hemizygous Cre/*lox* hybrid genotype. It is important to note that molecular data from Southern blotting proved the event of gene excision and the presence the Cre gene in all the GUS$^+$ hybrids. In the *lox* parental plants and non-transformed plants the Cre gene was absent indicating no endogenous Cre activity in the rice plants. However, upon activation the GUS expression was not uniform, some plants stained entirely blue while, in other plants, some leaf pieces uniformly stained blue but other leaf pieces showed higher staining intensity only at the cut ends (Fig. 4). The recombination efficiency also varied among crosses. For example, the cross Cre/*hpt*406×*lox/hpt* showed a recombination frequency of 1/12, whereas the frequency in the cross the *lox/hpt*28×Cre/*hpt*405 was 4/16 (Table 1). There was no obvious difference in recombination efficiency when the Cre plants were used as female or male parents.

The fact that GUS expression and recombination efficiency varied among and within crosses suggests that recombination is induced at the early germinal stage, or is random during the somatic development stages. Stuurman et al. (1996) have obtained efficient somatic and germinal recombination with the Cre/*lox* controlling inversion in tomato. Bar et al. (1996) in their study on the FLP/*FRT* system in tobacco, and Onouchi et al. (1995) on the R/RS-*gusA* system in *Arabidopsis*, observed similar variation for GUS expression and recombination efficiency among and within crosses. Chromosomal posi-

tions of the transgenic loci, or the difference in Cre recombinase activity in alternative parents, were likely to cause such variation. Bayley et al. (1992) proposed the selection of parents with strong expression of Cre for recombination achieved via sexual hybridization.

The efficiency of site-specific recombination in this study was comparable to that reported in the hemizygous FLP/*FRT* hybrids of tobacco (Bar et al. 1996). Earlier, Dale and Ow (1991) and Russell et al. (1992) found that the excision was more efficient when the Cre gene was introduced by re-transformation rather than by cross-fertilization. As compared to the FLP/FRT system controlling gene deletion with a recombination efficiency of 17.5% in tobacco (Lloyd and Davis 1994), the Cre/*lox* system demonstrated in this study was more efficient. In this study, we obtained a high efficiency of 58.3% in the Cre/*lox* hybrids from crosses of T2×T2 transgenic plants. In certain crosses (cross No. 2, cross No. 4) the efficiency was 100% (Table 2). Some T2 plants homozygous for the transgenes (data not shown) resulted in high recombination efficiency.

The progeny of the GUS^+ Cre/*lox* hybrids (F_2 plants) showed segregation for GUS expression, indicating that the control factors of gene excision were transmitted to subsequent generations. The Cre-mediated excision of the *hpt* gene was obviously proved by the absence of the 3.5-kb band in all the GUS^+ hybrid plants from T0×T0 crosses or T2×T2 crosses. Most of the hybrid plants showed one band representing the recombined product, as expected, but in some hybrid plants two bands were detected. The reason that the hybrids showed an unexpected hybridization pattern could be attributed to their *lox* parents, in which the transgene was integrated at two or more sites or the integration of T-DNA was incomplete. In the case of the hybrid ID.111 showing two bands, its *lox* parent exhibited two integration sites of the transgene (Fig. 6).

Our present study showed that the function of the Cre/*lox* site-specific recombination system worked effectively in the rice genome. As rice is the one of the most-important food crops in the world, this system could be applied in various ways for genetic studies or for practical rice breeding. For example, an alternative method of hybrid rice production could be developed to replace the conventional method using the cytoplasmic male-sterile system. The female parent harboring a male sterility gene flanked by two *lox* sites will be crossed with the male parent containing the Cre gene. In the hybrid, the male sterility gene is excised leading to the restoration of seed fertility. Luo et al. (2000) have used the FLP/*FRT* site-specific recombination system for the production of hybrid seeds in *Arabidopsis.* Earlier, the Cre/*lox* system was used to excise a selectable marker gene from the tobacco genome (Dale and Ow 1991) or from the tomato genome (Russell et al. 1992). Transgenic plants without the incorporation of marker genes for antibiotic or herbicide resistance would be more acceptable to the public. Our study revealed the feasibility of using the Cre/*lox* site-specific recombination system for rice breeding.

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