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Cre-*loxP* mediated marker elimination and gene reactivation at the *waxy* locus created in rice genome based on strong positive–negative selection

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Abstract The combination of homologous and site-specific recombination, if possible, could create mutants, such as tissue- or growth phase- specific gene knockout. In higher plants, there is no information on the site-specific recombination of the endogenous gene locus modified by gene targeting (GT) because of still limited success of reproducible GT for the endogenous gene. Our method of rice GT mediated by a strong positive–negative selection is appropriate for combined use with site-specific recombination because, in our method, it is possible to manipulate genome sequence variously without any unintentional random transformation, including ectopic targeting. We had previously created a targeted-*waxy* mutant by inserting the *hygromycin phospho transferase* (*hpt*) with *ΔEn* (a functional transcriptional stop sequence from *En*/*Spm* transposon), into the *Waxy* intron 1 region upstream of the translational initiation site. Since this inserted sequence was sandwiched by a pair of *loxP* in the same orientation, here, we tested Cre-*loxP* mediated elimination of this targeted insertion. The *Cre* was transiently expressed by a highly active promoter of CaMV 35S with the castor bean catalase intron 1 in the calli of the targeted-*waxy* homozygote. Cells derived from the treated calli were grown independently and applied to PCR screening as a bulk of calli clumps. Although the efficiency was quite low, Cre-*loxP* mediated *hpt-ΔEn* elimination and *Waxy* reactivation in the pollen of regenerated plants were detected as expected in some cells.

Key words: Cre-*loxP*, gene targeting, site-specific recombination, rice, *waxy*.

Gene targeting (GT) creates various mutants only in the target gene locus by homologous recombination (HR) between the endogenous gene and targeting vector. Site-specific recombination in the targeted gene locus creates more rigorous mutants, such as tissue- or growth phase-specific gene knockout, which is designated as conditional GT (Brian 1998; Lyznik et al. 2003; Branda and Dymecki 2004). In mouse, conditional GT of the *DNA polymeraseβ* in a tissue-specific manner was achieved (Gu et al. 1994). In this experiment, recognition sites for site-specific recombination were inserted in the first step of GT. Subsequent tissue-specific recombination generated T cell-specific gene knockout of the *DNA polymeraseβ*, which successfully overcame the lethal effect of the mutation in the whole body. In higher plants, however, there is no established protocol for site-specific recombination at the endogenous gene locus modified by GT, because of still limited success of reproducible GT.

Site-specific recombination has been used for the maker elimination (Keenan and Stemmer 2002) in random transgenic plants. The same marker-free at the targeted gene locus is further desirable especially for the improvement of molecular breeding. Inactivation and reactivation of targeted gene mediated by site-specific recombination is desirable for study of gene function. Since we have developed highly effective rice gene targeting system, application of site-specific recombination at targeted gene locus is expected to create further various mutants. To develop such effective gene manipulation system based on GT combined with site-specific recombination, we have firstly applied Cre-*loxP* (causes recombination/*locus of crossing over in P1*) to induce marker-free and simultaneous gene reactivation in the targeted-*waxy* locus, which had been created in previous experiments (Terada et al. 2002).

The earlier GT works with higher plants the endogenous genes of *AGL5*, *Protoporphyrinogen oxidase* (*PPO*) and

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Abbreviations: Cre, Causes recombination; GT, Gene Targeting; HR, Homologous Recombination; JS, Joint Sequence; *loxP*, Locus of crossing over in P1; NCre, Nuclear localization signal (NLS) tagged Cre recombinase
This article can be found at http://www.jspcmb.jp/

Cruciferin in Arabidopsis (Kempin et al. 1997; Hanin et al. 2001; Shaked et al. 2005) and of *Waxy*, *Alchol dehydrogerase* 2 (*Adh2*), *OsMet1a*, and *Acetolactate synthase* (*ALS*) in rice (Terada et al. 2002; 2007; Yamauchi et al. 2009; Endo et al. 2007), have been manipulated by different approaches for the selection of HR mediated GT events, which take place in extremely low frequency. The *PPO* (Hanin et al. 2001) and *ALS* (Endo et al. 2007) genes were modified into herbicide-resistant forms by GT-mediated sequence alterations, and GT events were selected by each herbicide. This approach, designated as gene-specific selection, is not applicable to the modification of general genes having no such selectable system. Another approach is visual selection of GT events after the HR-mediated promoter gain of the *GFP* coding sequence (Shaked et al. 2005). A promoter-less *GFP* was inserted just downstream of *Cruciferin* promoter by HR. Then GT event at the *Cruciferin* locus was searched by GFP expression. Although the application of this selection could be limited in highly expressed genes, site-specific recombination can be applicable to this selection method.

Our strong positive-negative selection for GT (Terada et al. 2002; 2007) seems to be the most adequate approach for combined use of site-specific recombination to create various mutants, for such as marker-free, facile switch on-off of the gene function, and conditional gene knockout. In our GT case, negative selection markers were placed at both ends of the DNA fragment to be transformed and can be removed later only through HR in GT, while all of the border-associated random integration events are eliminated by the toxic effect of the negative marker (Terada et al. 2007). This procedure is applicable to GT of any genes, indeed, we have succeeded in the modification of *Adh2*, and *OsMet1a* next to *Waxy* (Terada et al. 2002, 2007; Yamauchi et al. 2009). Moreover these obtained GT mutant plants have shown neither additional random integration nor ectopic targeting events (Terada et al. 2002, 2007; Yamauchi et al. 2009). Such undesirable recombination events were reported in the gene-specific selection procedures described above (Hanin et al. 2001; Endo et al. 2007). Therefore, the combined use of site-specific recombination with our strong positive-negative selection is expected to create various gene modifications at the targeted gene locus as in mouse conditional GT (Brian 1998; Gu et al. 1994; Lyznik et al. 2003).

As the first stage of gene manipulation at the targeted endogenous gene locus in rice (*Oryza sativa* L.), we applied Cre-*loxP* recombination of bacteriophage P1 (Abremski et al. 1983; Kilby et al. 1993) for the elimination of targeted insertion of positive selection marker of *hpt* (*hygromycin phosphotransferase*) with *ΔEn* (transcriptional stop from *En*/*Spm*). We tried to induce the functional recovery of *Waxy* gene in the targeted-*waxy* mutant line, obtained in the preceding GT (Terada et al. 2002). Since a pair of *loxP* in the same orientation was placed at both ends of the *hpt-ΔEn* in the middle of *Waxy* intron 1 (Figure 1A, B, C) (Terada et al. 2002), Cre can eliminate the insertion by reciprocal exchange of the DNA strands between their recognition sites (Abremski et al. 1983; Guo et al. 1997; Van Duyne 2001). Although a single *loxP* remains in the middle of intron 1, *Waxy* can be reactivated through the splicing (Isshiki et al. 1998).

Cre has been introduced by sexual cross (Odell et al. 1990) and re-transformation (Gleave et al. 1999) in higher plant. And it is recognized that continuous exposition of the genome DNA to Cre induces a toxic effect on the genome (Coppoolse et al. 2003; Hare and Chua 2001). To suppress Cre expression till the required time period for recombination, chemically inducible Cre was developed (Zuo et al. 2001; Hare and Chua 2001; Zhang et al. 2003; Sreekala et al. 2005). But the transient expression of Cre still has some advantages over the inducible Cre system; the one is that random integration of *Cre* is mostly evitable and the other is that the secondary selection marker is unnecessary for the *Cre* transformation to the targeted mutant, which already harbors the *hpt* gene.

In this report, N*Cre* gene under the control of a highly active promoter was delivered into targeted-*waxy* mutant cells by particle bombardment without any selection marker. The N*Cre* employed here has a nuclear localization signal (NLS) (Kanegae et al. 1995). Then the expected Cre-mediated recombination was directly selected by PCR screening. Although the efficiency of Cre-*loxP* mediated recombinants was quite low, the *hpt-ΔEn* elimination with expected *Waxy* reactivation occurred.

Materials and methods

Vector construction

The N*Cre* gene carrying a nuclear localization signal (NSL) of SimianVirus 40 in pSRNCre (Kanegae et al. 1995) was cut out by *Pst*I and *Eco*RI and connected between the CaMV 35S promoter-the castor bean catalase intron 1 and the *Nos* terminator of pIG221 (Terada et al. 2002). The construct was designated as pCKF1 (Figure 1D).

Callus induction from the targeted-waxy seeds

In earlier *Waxy* GT (Terada et al. 2002), we had obtained 6 independent targeted-*waxy* lines, named as lines A, B, C, D, E, and F (Terada et al. 2002). Among them the line E showed the best fertility. Therefore we have chosen line E as the first seed material for NCre-*loxP* recombination in this report. The calli were induced from about 30 of mature seeds from targeted-*waxy* homozygous plant(s) in the line E on 2N6 medium (Figure 2) (Terada et al. 2002) containing hygromycin B (30 mg l^{-1}).

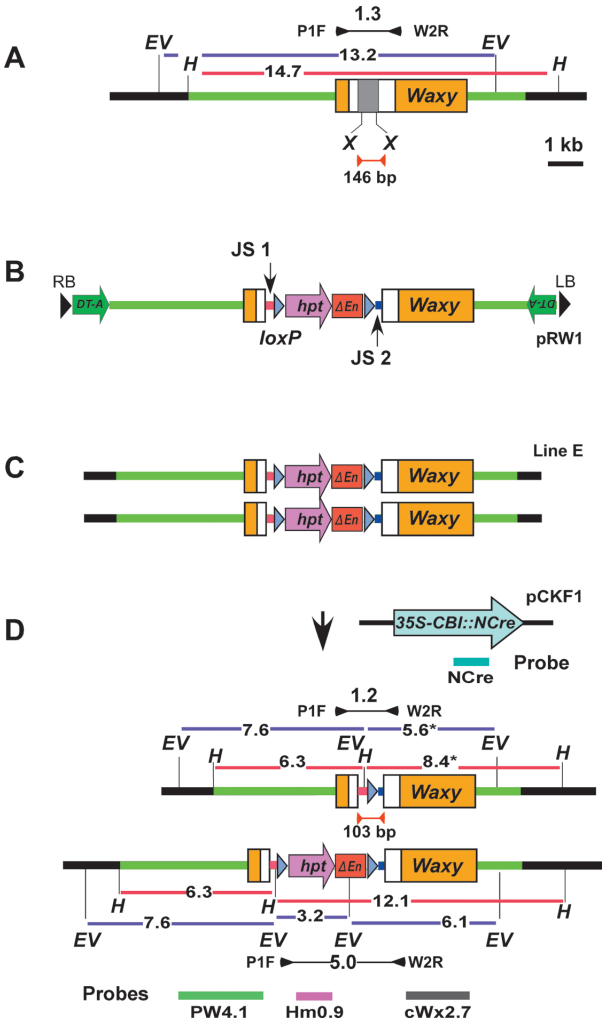

Figure 1. Structures of the endogenous *Waxy* locus and its modifications by positive-negative selection-mediated gene targeting and NCre-mediated recombination. (A) Genomic structure of the endogenous *Waxy* region. The white and gray rectangles with the two *X* (*Xba*I) sites in the orange *Waxy* box indicate the *Waxy* intron 1 and the replaced sequence of the 146 bp by the *Waxy* gene targeting and subsequent NCre-*loxP* recombination, respectively. The green bars represent the *Waxy* flanking sequences carried by pRW1 in (B). The primers, P1F and W2R are shown as black arrowheads. (B) Detailed structures between the left and right borders (LB and RB) of the gene targeting vector, pRW1. The blue arrowheads indicate *loxP*. The pink and blue bars outside of the *loxP* are JSs 1 and 2, including restriction enzyme sites of 50 bp and 19 bp, respectively, *hpt-ΔEn* is as described in Terada et al. (2002). (C) Detailed genomic structure of the targeted-*waxy* homozygote, line E. (D) Structures of pCKF1 and the NCre-*loxP* recombined-*Waxy*/*waxy* heterozygote. The red arrowheads in (A) and (D) in indicate endogenous and altered sequence of 146 bp and 103 bp, respectively. The bars with blue (NCre), green (PW4.1), pink (Hm0.9), and black stripes (cWx2.7) under the pCKF1 and the *Waxy* region indicate each probe for Southern blot (Terada et al. 2002). The numbers on each of the bars reveal the length of the fragment in kb. 35S-CBI in pCKF1 is the CaMV 35S promoter connected with the castor bean catalase intron 1. The restriction sites are EV, *Eco*RV, H, *Hin*dIII, X, *Xba*I.

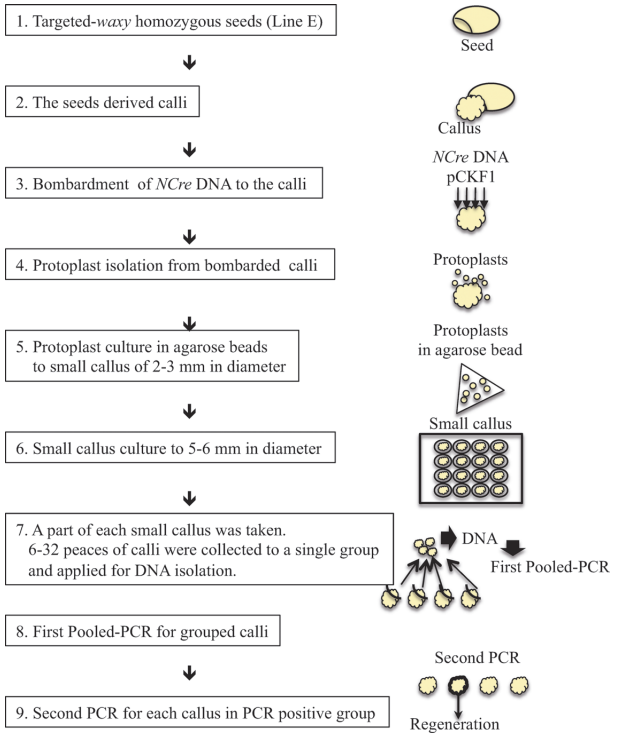

Figure 2. Schematic diagram of the procedure for NCre-*loxP* mediated recombination in the targeted-*waxy* homozygote. Each step was described in left side and the corresponding tissues of rice seed, callus, protoplasts, small calli, and DNA isolation from grouped calli was illustrated in right side.

Particle bombardment and protoplast isolation

Most of the procedures were according to Li et al. (2004). Briefly, calli derived from line E seeds were spread on 2N6 solid medium (Figure 2) (Terada et al. 2004) supplemented with 3% sucrose and 5% mannitol for a high-osmotic treatment. The pCKF1 for bombardment was purified using the QIA quick PCR Purification Kit (Qiagen Co.) and bound to gold particles. The particles were delivered into the rice calli through use of a helium-driven PDS-1000/He system (BioRad Co.) according to the manufacturer's instructions. After 12 h, the treated calli were transferred onto 2N6 medium and cultured for 2 weeks. Protoplasts were isolated according to Shimamoto et al. (1989) in the enzyme solution (4% Cellulaze RS, 1% Macellozyme R10, Yakulto Co., 0.1% $CaCl_2 \cdot 2H_20$, 0.4 M mannitol, pH 5.6).

Protoplast culture and plant regeneration

Each isolated single protoplast (Figure 3A) from bombarded calli was immobilized and grown separately in agarose bead (Figures 2, 3B) (Shimamoto et al. 1989) to a small cell cluster of 2–3 mm in diameter for 10 to 14 days. Each small cell cluster was transferred to solid 2N6 medium in multi-well plates and continuously cultured for 2 to 3 weeks to grow to 5–6 mm in diameter. 320 and 576 calli from protoplasts originating from bombarded calli in experiment 1 and 2, respectively (Table 1).

A part of grown calli was taken for DNA isolation, Six to 32 peaces of partial calli were collected and treated as a group. DNA isolated from each callus group, was applied to pooled-

Table 1. PCR for the callus group and each callus derived DNA for the NCre-*loxP*-recombination.

Experiment	Total callus* number analyzed (Callus number per single DNA-pool)	PCR positive callus	SelectionFrequency (%)
1	320 (12–16)	1 (E-a)	>0.31
2	576 (12)	1 (E-b)	>0.17

* Each callus of 2 to 3 mm in diameter was derived from a single protoplast. After the bombardment of *NCre* to the calli derived from targeted-*waxy* homozygous seeds, cells were independently grown as protoplasts and applied to PCR screening. NCre–mediated recombinant at *loxP* sites was detected as PCR positive callus.

PCR analysis to detect of NCre-*loxP* recombination at the targeted-*waxy*, as described below (Figure 2). Once the calli, were shown to be PCR positive, the remainder portion was transferred to the plant regeneration medium as according to Terada et al. (2002).

DNA isolation for pooled-PCR

Six to 32 pieces of partial callus from protoplast-derived small callus were brought together for DNA isolation (Figure 2) (Miao and Lam 1994) by the automatic nucleotide isolation system, NA-2000 (Kurabo Co.). The first PCR screening was performed on DNA sample derived from grouped calli. Next, PCR screening was applied on the DNA sample of each callus to identify the one where NCre-*loxP* recombination at the targeted-*waxy* was occurred (Figure 2).

PCR analyses

PCR was performed with LA Taq polymerase (TaKaRa Biomedicals Co.) as follows. The primers were: P1F, 5′-ACACAAATAACTGCAGTCTC-3′ and W2R, 5′-CCGACAT-GGTGGTTGTCTAG-3′ at the sequences of the *Waxy* exon 1 and exon 2, respectively (Figure 1A) (Terada et al. 2002). Initial denaturation was at 94°C for 1 min, with 38 subsequent cycles of denaturation at 94°C for 45 s, annealing and extension at 60°C for 20 min, and final extension at 72°C for 10 min.

Southern blot analysis

All the procedures of Southern blot analysis of genomic DNA samples isolated from leaves of regenerated plants were described before (Terada et al. 2002).

Results

Gene structures in Waxy manipulation and NCre-loxP recombination

The structures of untargeted endogenous *Waxy* (Figure 1A), targeted-*waxy* homozygous (Figure 1C), and heterozygous of recombined *Waxy* by NCre-*loxP* and targeted-*waxy* (Figure 1D) were respectively shown. In *Waxy* targeting, the sequence of 146 bp between the two *Xba*I sites in the intron 1 was replaced with *hpt-ΔEn* (Figures 1A, C) (Terada et al. 2002). In the targeting vector, pRW1, Joint Sequences (JSs) of 1 and 2 (Figure 1B) i.e., 50 bp at the 5′ site and 19 bp at the 3′ site, respectively, were artificially introduced at distal ends of the *loxP* pair through the *Waxy* cloning process.

This 103 bp sequence consisting of a single *loxP* (34 bp) and the 69 bp of JS 1 and JS 2 remained in intron 1 by pCKF1 mediated NCre (see Introduction) site specific recombination is expected to be spliced out (Figure 1D) (Isshiki et al. 1998). NCre site specific recombination at the targeted-*waxy* was detectable as a 1.2 kb PCR fragment amplified with the primers, P1F and W2R (Figure1D) (Terada et al. 2002). The amplified 1.2 kb was further confirmed by restriction enzyme digestions with *Eco*RV and *Hin*dIII, because their recognition sites were remained in intron 1 after the NCre recombination.

Transient expression of NCre in targeted-waxy calli and selection of recombinants

For the transient expression of *NCre* in targeted-*waxy* calli, the seed-derived calli from line E of the targeted-*waxy* homozygote (Terada et al. 2002) were bombarded with pCKF1 encoding *NCre* driven by highly active promoter of CaMV 35S with the castor bean catalase intron 1 (Figure 2). Without a selection marker, a huge number of calli grew, and it was impossible to select the expected recombinants. Therefore, we tried to isolate cells independently as protoplasts (Shimamoto et al. 1889) from bombarded callus surface, where being assumed to receive the *NCre* particles frequently. Such protoplast-derived callus was expected to show clear characterization about DNA recombination. Protoplasts-derived and independently cultured calli were applied to PCR screening as described below (Figures 2, 3 A, B).

A portion (about 0.5 mm in diameter) of callus was isolated from protoplast-derived callus grown independently to about 5–6 mm in diameter and applied for PCR analysis. Remainder callus part was continuously cultured for plant regeneration. Each isolated callus portion was grouped into 6 to 32. Then, DNA was isolated from each callus group and applied to pooled-PCR (Miao and Lam 1995) for the detection of the 1.2 kb recombination fragment (Figure 2).

Because the increase of callus number in single callus group thought to reduce the detection efficiency of the 1.2 kb PCR band, first, we determined the maximum callus number in single group, which permits the detection of the 1.2 kb PCR band. Targeted-*waxy* heterozygous callus was used as a false-positive one for PCR, because it reveals the 1.3 kb band, reflecting the un-modified *Waxy* locus, instead of the 1.2 kb (Figures 1A, 3C). Several pieces of similar sized calli of the

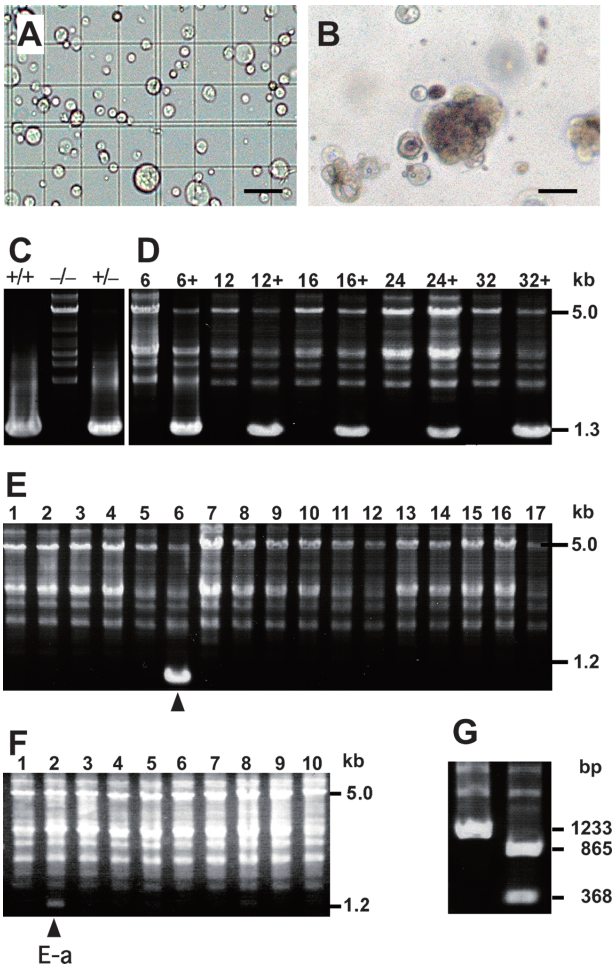

Figure 3. Selection of NCre-*loxP* recombinants from the protoplast-derived calli of the *NCre* bombardment of line E. (A) Protoplasts from the bombarded calli of targeted-*waxy* homozygote line E. Scale bar is 50 μm. (B) Small cell cluster derived from a single protoplast. The scale bar is 1 mm. (C) (D) Estimation of the maximum callus number in a callus group, allows for PCR detection of NCre recombination. (C) +/+, −/−, and +/− indicate the *Waxy* homozygote, targeted-*waxy* homozygote and, targeted-*waxy* heterozygote, respectively. (D) The number on each lane reveals each callus number included in grouped calli of targeted-*waxy* homozygote line E (−/−), and + means the addition of a single false-positive callus of a targeted-*waxy* heterozygote (+/−). (E) Pooled-PCR for NCre-*loxP* recombinant detection in each grouped callus. The number on each lane indicates the each callus group in experiment 1 in Table 1. The arrowhead indicates the group including the NCre-*loxP*recombinant. (F) PCR for each callus in the group No.6 in (E). The number on each lane indicates an independent callus of the No.6 group. The arrowhead indicates the selected line of E-a. (G) *Hin*dIII digests of PCR fragment in (E).

unbombarded line E, which assumed to be un-recombinant false-negative calli, were mixed with a single false-positive callus, and the total DNA was isolated and subjected to pooled-PCR (Figure 1A, D). As shown in Figure 3D, even though 32 pieces of unbombarded calli were included in a single group, the 1.3 kb PCR band was still detectable.

Based on this PCR test, we repeated the following experiments twice using a callus group of 12 to 16 (Table 1). By means of the pooled-PCR of grouped calli (Figures 2, 3E, lane 6) and subsequent PCR of each callus, two independent calli revealing the 1.2 kb band, were obtained and designated as lines E-a (Figure 3F, lane 2) and E-b, respectively. The hit rate of NCre recombination was as low as 0.31% and 0.17% or less in each of the experiments, respectively (Table 1). Because lines E-a and E-b were detected from differently bombarded calli in experiment 1 and 2 (Table 1), at least these two lines were originated from different recombinants. The 1.2 kb fragments from both lines were digested to 865 bp and 368 bp by *Hin*dIII, as expected (Figures 1D, 3G).

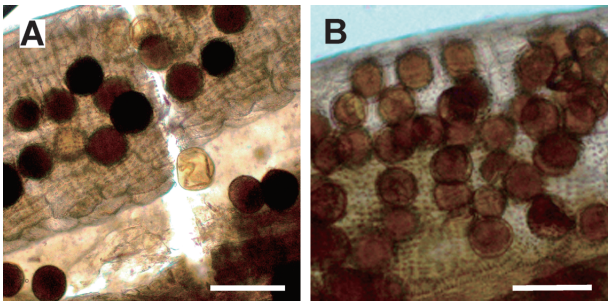

Figure 4. Recovered pollen phenotype in line E-a (A) and line E (B) stained by iodine. The scale bar is 100 μm.

Reactivated Waxy phenotype in pollen of line E-a

The calli of E-a and E-b were regenerated into plants through multiple shoots. Both lines were unfortunately sterile, probably because of protoplast mediation (Terada et al. 2000), however, a few anthers that developed in the E-a plant allowed the observation of *Waxy* reactivation in pollen. As shown in Figure 4A, a potion of pollen was stained dark-blue by iodine (Terada et al. 2000; 2002), showing the reactivated Waxy phenotype, on the other hand, that of the original line E was stained brown (Figure 4B). The segregation ratio, however, was not exactly as 1 : 1 because of the inclusion of unhealthy and unstained-pollen.

Molecular analyses of the NCre-recombinant

To confirm the precision of the recombination, we performed the Southern blot analyses and nucleotide sequencing using leaf DNAs of E-a and E-b. In Southern blot analyses of the DNAs digested by *Hin*dIII and *Eco*RV, bands of all the expected sizes were detected with each probe for the *Waxy* promoter (Figures 1D, 5A, PW4.1), *hpt* (Figures 1D, 5A, Hm 0.9), and the *Waxy* coding region (Figures 1D, 5A, cWx2.7). Especially, NCre-mediated recombination specific fragments of 8.4 kb and 5.6 kb were clearly detected with the cWx2.7 probe digested by *Hin*dIII and *Eco*RV (Figure 5A, lanes 3 and 4), respectively. In addition, all the targeted-*waxy* specific fragments were completely consistent with those

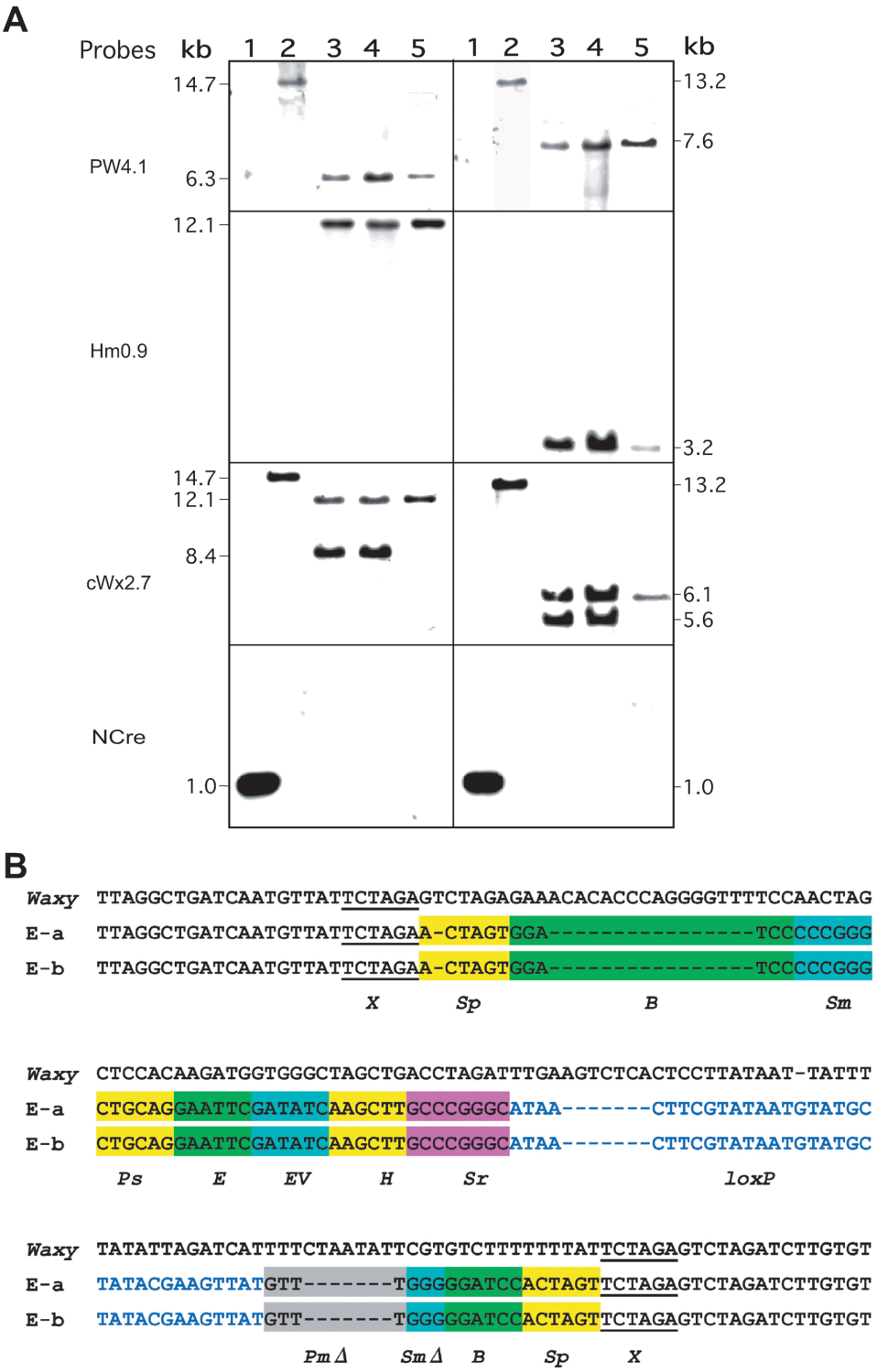

Figure 5. Southern blot and sequence analysis of the original *Waxy*, the recombined *Waxy* by NCre-*loxP*, and the targeted-*waxy* of line E. (A) *Hin*dIII (left) and *Eco*RV (right) digestions of each lane, except for pCKF1 (lane 1), which was digested by *Pst*I and *Eco*RI at both ends of the *NCre* coding region. Lane 2, cv. Nipponbare, Lanes 3 and 4, lines E-a and E-b, respectively. Lane 5, targeted-*waxy* homozygote of line E. The probes are shown for each gene region in Figure 1 (D). (B) Nucleotide sequences of the original *Waxy*, lines E-a and E-b. The abbreviations of the restriction enzyme sites are as below. Sp, *Spe*I, B, *Bam* HI, Sm, *Sme*I, Ps, *Pst*I, E, *Eco*RI, Sr, *Srf*I, Pm Δ, Sm Δ, partial sequences for *Pme*I (4 bp) and *Sma*I (3 bp).

of line E, namely, those of 6.3 kb and 7.6 kb were detected with probe PW4.1, those of 12.1 kb and 3.2 kb, with probe Hm 0.9, and those of 12.1 kb and 6.1 kb, with probe cWx2.7 (Figure 5A, lanes 3, 4, and 5, respectively). These results revealed that NCre-*loxP* recombination had occurred in a heterozygous manner, in agreement with reactivation of the *Waxy* in pollen (Figure 4A) as described above. In Southern blot with

the probe of the *NCre* coding region (*NCre*), there was no band other than its original sequence in the vector pCKF1 (Figures 1D NCre, 5A lane 1). This means that bombarded *NCre* was expressed transiently without integration into the genome of both E-a and E-b.

The nucleotide sequence for recombined junction in the *Waxy* intron 1 revealed the precise replacement of the 146 bp, original region between the two *Xba*I sites into the 103 bp, including a single *loxP* of 34 bp, and 69 bp of the JS 1 (*Spe*I, *Bam*HI, *Sma*I, *Pst*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Srf* I) and JS 2 (partial sequences of *Pme*I (4 bp), *Sma*I (3 bp), and *Bam*HI, *Spe*I) in both lines, as shown in Figures 1D, 5B.

These results revealed that NCre-*loxP* recombination had taken place without any unexpected rearrangement and generated heterozygous plants with an elimination of *hpt*-Δ*En* and reactivation of *Waxy*. Although the original sequence between the two *Xba*I sites in intron 1 had been replaced into the 103 bp, it was still reactive to *Waxy* splicing.

Discussion

We have shown here that the NCre-*loxP* recombination at the targeted-*waxy* locus (Terada et al. 2002) resulted in the elimination of *hpt*-Δ*En* and subsequent *Waxy* reactivation. Since neither random transformation nor ectopic targeting was included in the targeted-*waxy* homozygote (Terada et al. 2002), the recombination was exactly taken place at the pair of *loxP* in targeted-*waxy* locus. Southern blot and sequence analyses clearly revealed precise heterozygous elimination of *hpt*-Δ*En* without *NCre* integration. Because our result is not enough to confirm that the recombination was exactly mediated by NCre, the chemical inducible *Cre* (Zuo et al. 2001; Hare and Chua 2001; Sreekala et al. 2005) is being applied for the gene manipulation to the identically targetred-*adh1* locus by the same strong positive–negative selection mediated rice GT. The elimination of *hpt*-Δ*En* was reproducibly confirmed only with *Cre* induction by PCR and Southern blot (Terada et al. unpublished data). Taken together these observations, it was strongly suggested that NCre-*loxP* mediated gene manipulation at the targeted endogenous gene locus by GT actually work.

Our recombination frequency is quite low but feasible. Because, NCre expression was transient and the recombination was simply detected by PCR without any selection. Whereas random transformation system in higher plants the Cre-*loxP* mediated recombinants have been selected with visual or antibiotic-resistant marker genes of luciferase (Dale and Ow 1990), GUS (Odell et al. 1994a; Srivastava and Ow 2001; Hoa et al. 2002; Cao et al. 2006), and *nptII* (*neomycin phosphotransferase II*) (Odell et al. 1990; Gleave et al. 1999). Among these studies, low frequency of the recombination in transient Cre expression was reported by Gleave et al. (1999), who obtained two Cre-*loxP* excisions of *nptII* from 773 of regenerated tobacco shoots, in spite of using a conditional lethal *codA* (*cytosine deaminase*) gene in the recombination substrate sequence.

Through GT and subsequent NCre-mediated *hpt*-Δ*En* excision, finally the 146 bp between the two *Xba*I sites in the *Waxy* intron 1 (Figure 1A) was replaced to the 103 bp, consisted by a single *loxP* (34 bp) and 69 bp of JS (Figure 1D). In spite of such sequence substitution in the intron 1, *Waxy* reactivation was detected at least in pollen. The result indicates that *Waxy* splicing had taken place, overcoming the sequence substitution of about 100 bp in the intron 1. The splicing efficiency of *Waxy* intron 1 is an important process to determine the rice grain texture through the quantity regulation of *Waxy* transcripts in rice immature seed (Isshiki et al. 1998), although both of line E-a and E-b were unfortunately sterile and have no seed. Therefore, further detailed *Waxy* reactivation to be evaluated by the estimation of *Waxy* transcript and the amylose content in seed of immature and mature, respectively, in the fertile *Waxy* reactivated plants, which will be obtainable more effectively by the inducible Cre mediated recombination as same as our ongoing recombination experiment of targeted-*adh1* rice described above (Terada et al. unpublished data).

Many other effective recombination systems are well established in the GT of mouse and random transformation in higher plants in addition to Cre-*loxP*, such as R-*RS*, FLP-*FRT*, and ϕC31-*att* (Odell and Russell 1994b; Ebinuma et al. 2001; Hare and Chua 2001; Ow 2002; Lyznik et al. 2003). Simultaneously, it has been realized that our strong positive-negative selection-mediated GT in rice is quite effective and widely applicable to any gene region without any other random integration (Terada et al. 2002; 2007; Iida and Terada 2004; 2005) on the basis of acumulative information of the whole genome sequence (IRGSP 2005). We found that our rice GT has an effective potential to introduce a single nucleotide substitution, which had been inserted in the homology arm of the targeting vector (Johzuka-Hisatomi et al. 2008). Subsequent marker elimination (Keenan and Stemmer 2002) mediated by site-specific recombination results in the controlled creation of point mutants. In addition, our GT system depends on the gene transformation of somatic cells of calli (Terada et al. 2002), permitting dual steps of transformations for GT and site-specific recombination in the same tissue culture period. This seems to be a unique property of the GT of a higher plant, rice. In higher plants, the complementation of mutated gene function has been studied by random transformation, which is generally followed by the positional effect. Inactivation and reactivation of the

endogenous gene at that original locus is quite effective for the study of gene function. Although we here reported the reactivation of the targeted-*waxy*, this is the first example of gene manipulation of a higher plant endogenous gene. The combined application of site-specific recombination at the targeted genome locus has no limitation for genome modification, and many ideal mutants can be created for further detailed studies of gene function, which has great potential for the molecular breeding of rice.

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