Co-transformation to tobacco of Cre/*lox* site-specific recombination system and its precise recombination

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Abstract For the temporally and spatially regulated expression of the barnase gene in plant, two kinds of plasmids with cre gene and its directly repeat recognition sites *lox* from bacteriophage P1 were constructed and co-transformed into tobacco by agrobacterium mediated procedure. The transgenic plants were conformed by PCR analysis. The blocking fragment between the two *lox* directly repeat sites was excised by Cre protein in the transgenic plant genome. Cloning and sequencing the DNA fragment from the co-transformed plant DNA showed that the precise DNA excision occurred in transgenic tobacco genome directed by Cre/*lox* site-specific recombination .

Keywords: Cre/*lox* site-specific recombination system, tobacco, co-transformation, precise recombination.

According to the difference of DNA structure as the target, proteins mediating recombination reaction and the principle of recombination, genetic recombination can be divided into three classes: homologous recombination, transposition and site-specific recombination. Among them, based on its ability to mediate *in vivo* DNA precise recombination, the site-specific recombination system is becoming a useful tool for the research on gene expression because the transgene can be precisely controlled by it. Four of these systems are well characterized: the Cre/*lox* system of bacteriophage P1, the R/RS system of the yeast plasmid pSR1, the modified Gin/gix system of bacteriophage Mu and the FLP/FRT system of the yeast 2uM plasmid[1]. The Cre/*lox* recombination system has been applied in eukaryotic organisms. The modification of genome can be achieved through the site-specific DNA excision, inversion and integration by the Cre/*lox* system.

It was confirmed that the Cre recombinase specially recognizes the 34 bp *lox* site and the site-specific recombination reaction was conservative and efficient in yeast[2]. In plants, the Cre recombinase can mediate the excision, inversion and integration of DNA fragment flanking by *lox* site in tobacco protoplast[3]. Excision of DNA fragment between two *lox* sites is almost complete[4].

In controllable site-specific recombination system, the recombinase gene always keep inactive by insertion of a blocking DNA fragment between coding sequence and upstream regulatory region before the recombination reaction takes place. In some cases, recombinase gene and its recognition sites are harboring in different vectors separately. The recombination occurred when the recombinase expression

system and the DNA with recognition sites were introduced into a same organism by co-transformation or sexual hybridization. Recently, co-transformation has been highly estimated for the use of multiple genes transfer in some research groups[5-7].

The barnase gene of Bacillus was placed downstream of another tapetum specific osg6B promoter of rice, inserted the barstar gene flanked by two directly repeat *lox*P sites between the barnase gene and the promoter as a blocking fragment. This structure and P35S/cre have been co-transformed into tobacco genome. The recombination reaction was precise and efficient mediated by Cre recombinase in co-transformed tobacco genome.

1 Materials and methods

(i) Plasmid construction. The 35S/cre construct from pMM23 was inserted into the binary expression vector pBINPLUS containing the selectable marker NPTII gene. The resultant 35S/cre containing binary expression plasmid was named pBin35Scre and was introduced into the Agrobacterium host LBA4404. The 1.6 kb osg6B promoter of rice[8], the 0.33 kb barnase[9] gene and the barstar[10] gene from Bacillus are cloned by PCR and confirmed by sequencing. The barstar coding sequence was inserted into two directly repeat 34 bp *lox*P sites of pG*lox*. Then, the barstar coding sequence flanked by two *lox*P sites was placed between the osg6B promoter and the barnase coding sequence. This structure was inserted into the binary expression vector pGsbar which is with the marker gene 35S/bar. The resultant binary expression plasmid was named pGsbolbsrbn and was introduced into the Agrobacterium LBA4404 to be used in the tobacco genetic transformation experiments in this study (fig. 1).

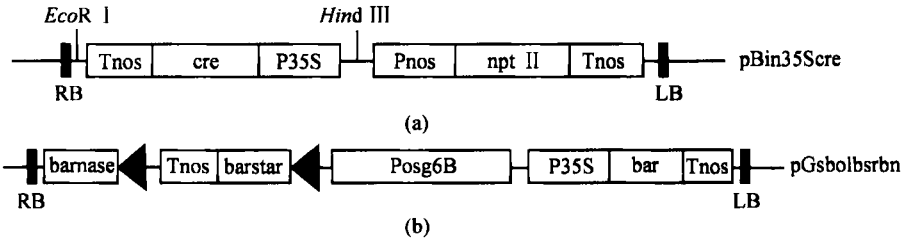

Fig. 1. Schematic representation of recombination binary plasmid pBin35Scre (a) and pGsbolbsrbn (b).

(ii) Tobacco co-transformation and transgenic plant regeneration. *Agrobacterium tumefaciens* LBA4404::pBin35Scre and LBA4404::pGsbolbsrbn were incubated by shaking at 28℃ in YEB containing 100 mg/L Sm, 50 mg/L Rif and 50 mg/L Km, 10 mg/L PPT respectively until the log phase. According to the leaf disc method by Horsch[11], the mixture of same volume of the Agrobacterium suspension was washed and resuspened in 1/2 MS medium. Leaf discs (0.5−1 cm^2) from tobacco (*Nicotiana tabaccum* cv. Wisconsin 38) plants were soaking in this suspension for 5 min. After blotting dry the inoculated discs were co-cultivated on MS solid medium at 25℃, in darkness for 2 days. The discs were then transferred onto regeneration/selection medium containing 500 mg/L Cb, 40 mg/L Km and 10 mg/L PPT (MS supplemented 1.0 mg/L 6-BA) and incubated at 25℃ for 14 h photoperiod until shoot formation. Shoots were transferred into rooting medium (MS with 300 mg/L Cb, 30 mg/L Km and 5 mg/L PPT) to produce putative transgenic plantlets.

(iii) Confirmation of the transgenic status by PCR. CTAB procedure[12] for total DNA extraction was followed. Leaves, 0.5 g per plant, from the putative transgenic and non-transformed control plantlets were ground in liquid nitrogen into powders. Preheated 500 uL 2*CTAB buffer (2% CTAB, 100 mmol/L Tris-Cl, 20 mmol/L EDTA and 1.4 mmol/L NaCl, pH 8.0) was added to the powder. The same volume of CI was added to the mixture to extract DNA. Total DNA was then precipitated with 2/3 volume of isopropanol and washed with 70% ethanol.

In order to detect the transgene, several pairs of primers were designed for the amplification of specific DNA fragments from the putative transgenic and the non-transformed control plantlets. (After pre-denaturing for 10 min at 94℃, 94℃ 1 min, 56℃ 1 min and 72℃ 2 min for 30 cycles, at last 10 min at 72℃).

(iv) Cloning and sequence analysis of the PCR product. The PCR products were run on agarose gel and purified by glass-milk kit and then cloned into pGEM-T vector of Promega company. Sequencing procedure has been followed for the determination of the position where Cre mediated recombination occurred.

2 Results and conclusion

(i) Plasmid construction. Sequences of 0.33 kb barnase gene and 0.26 kb barstar gene of *Bacillus amyloliquefaciens* by PCR were 99.3% and 99% identity respectively to the published sequences[9, 10]. There was no change in amino acid sequences. The sequence of tapetum-specific 1.6 kb osg6B promoter of rice was also identified by sequence analysis. Furthermore, the two directly repeat 34 bp *lox* sites in pG*lox* showed complete identity with the published sequence[11]. The sequence of the structure osg6B/*lox*-barstar-*lox*/barnase in pBsolbsrbn has been confirmed correct by sequence analysis.

(ii) PCR analysis of co-transformed tobacco. The PCR analysis of co-transformed plants was shown in fig. 2. Molecular data from co-transformed tobacco confirmed site-specific recombination at *lox* sites. Fig. 2(b) shows the PCR analysis of the genomes of co-transformed plant and pGsbolbsrbn transformed plant. At first, primers E and F produced a predicted size of 1.0 kb band corresponding to the cre gene from co-transformed plant DNA. Corresponding to the structure shown in fig. 2(a), a fragment of the predicted size of 1.0 kb or 1.9 kb was produced from the co-transformed genome, but not from pGsbolbsrbn transformed plant DNA, using primers B and D or A and C respectively (lanes 5 −8).

To examine whether the site-specific recombination might have occurred, primers outside of the

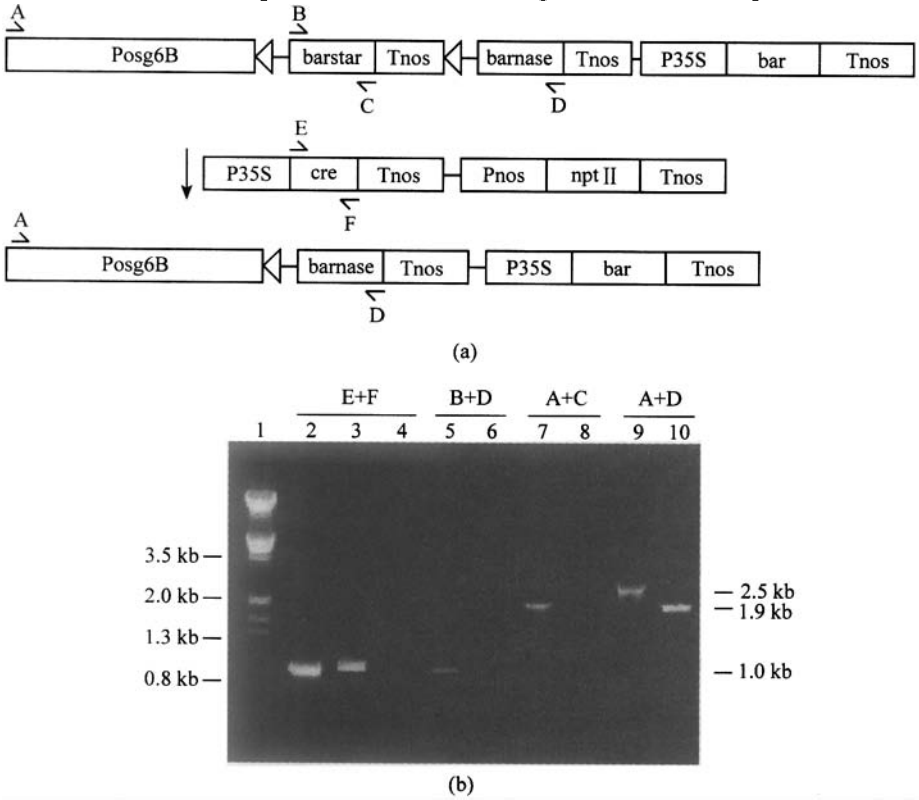

Fig. 2. Agarose gel electrophoresis of the PCR products testing DNA extracted from transformed tobacco by pGsbolbsrbn and co-transformed DNA using several pairs of primers shown in fig. 2(a). The complete structure containing *lox* sites and the predicted excision result, the PCR primers were given by arrowhead. (b) Agarose gel electrophoresis of PCR products testing co-transformed plant genome DNA (lanes 3, 6, 8, 10) and total DNA transformed with pGsbolbsrbn (lanes 4, 5, 7, 9), pBin35Scre (lane 2) as a control for cre gene. Lane 1 is λDNA digested with *Hind* III and *Eco*R I. DNA size markers (in kilobases) are shown to the left.

two *lox* sites were used. Primers A and D indeed produced a fragment identical in size to the predicted 1.9 kb band (lane 10) with co-transformed genome, but a 2.5 kb fragment (lane 9) was observed with pGsbolbsrbn transformed plant DNA, indicating that the blocking fragment between the two *lox* sites was excised.

(iii) Precise recombination in plant genome by the cre/*lox* system. Evidence for a precise recombination reaction was obtained by determining the nucleotide sequence surrounding the *lox* sites of the co-transformed genome. Co-transformed plant-derived fragment produced from PCR by primers A and D was purified and cloned into pGEM-T vector. Fig. 3 shows the sequences of the *lox*-containing regions. The segment between the two *lox* sites indeed was excised and the *lox* regions from primers A and D and pBsolbsrbn share complete identity, indicating that the site-specific recombination was precise and conservative without loss or alteration of the *lox* sequence or its flanking DNA in co-transformed genome.

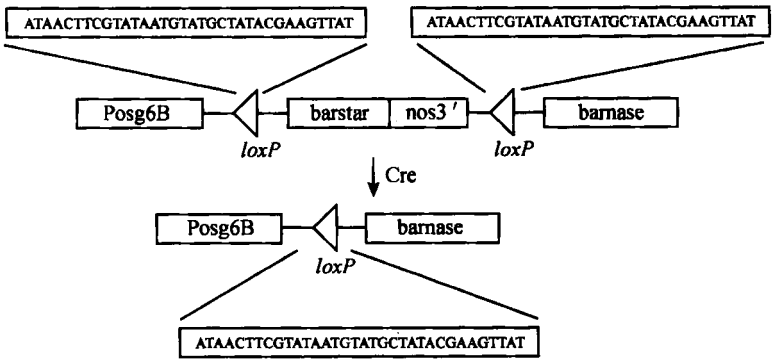

Fig. 3. Precise recombination between *lox* sites in co-transformed tobacco.

Site-specific recombination systems are very useful in the genetic control of transgenes activities. They can be used to determine the functions of special genes during developmental process and applied to chromosome engineering because the genes can be strictly active or inactive at specific tissues or organs at special times by the systems. It is believed that the site-specific systems will play a very important role in many fields of molecular biology.

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