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REGULAR ARTICLE

PREPARATION OF PLANT TRANSFORMATION VECTOR CONTAINING "SELF-EXCISION" CRE/*LOX*P SYSTEM

Zuzana Polóniová, Martin Jopčík, Ildikó Matušíková, Jana Libantová, Jana Moravčíková*

Address: Slovak Academy of Sciences, Institute of Plant Genetics and Biotechnology, , Akademická 2, P.O.Box 39A, 95007 Nitra, Slovak Republic

*Corresponding author: zuzana.poloniova@savba.sk

ABSTRACT

This work is focused on preparation of the plant transformation vector pZP6 containing "self-excision" Cre/*lox*P system. The T-DNA of binary vector consists of the *cre* recombinase gene driven by the *Arabidopsis* DLL promoter and the *npt*II expression unit flanked by two *lox*P sites in direct orientation. The *gus* reporter gene controlled by the double CaMV 35S promoter was placed out of the *lox*P embedded DNA. To confirm functionality of the Cre/*lox*P system, the pZP6 was analyzed for correct removal of the *lox*P embedded sequence in *E. coli.* The pZP6 was transformed into two bacterial strains *A. tumefaciens* AGLO and LBA 4404. Its stability in agrobacteria was evaluated by restriction analyses.

Keywords: Cre/*lox*P system, Marker-free plants, Pollen-specific promoter, Selectable marker genes, Self-excision strategy

INTRODUCTION

Plant transformation is based on the ability to integrate foreign DNA into a host genome and on the efficiency of regeneration of transformed cells. At present, the low transformation efficiency for many crops requires the use of selectable marker genes **(Puchta, 2003)**. These genes are essential for identifying those rare plant cells that had taken up foreign DNA upon transformation **(Bevan *et al.*, 1983)**. Among them, genes encoding

antibiotic or herbicide resistance are widely the most used. Although, there is no evidence about their negative impact on the environment, during recent years some objections concerning their biosafety were raised up **(Ebinuma *et al.*, 2001; Puchta, 2003; Zhang *et al.*, 2006; Darbani *et al.*, 2007)**.

To date, the removal of selectable markers from genome of transgenic plants was accomplished by several approaches as co-transformation **(Depicker *et al.*, 1985; Komari *et al.*, 1996; Tu *et al.*, 2003)**, *Ac* transposition **(Goldsbrough *et al.*, 1993; Ebinuma *et al.*, 1997; Cotsatifs *et al.*, 2002)**, homologous recombination between direct repeats **(Zubko *et al.*, 2000)** or more sophisticated site-specific Cre/*lox*P recombination.

The Cre/*lox*P system was isolated from bacteriophage P1. It consists of the *cre* recombinase gene and two 34 bp *lox*P sites. The Cre recombinase mediates recombination event between two *lox*P sequences in direct orientation. In “self-excision” Cre/*lox*P strategy both the *cre* and selectable marker genes are inserted between two *lox*P sites and upon activation of the *cre* gene, Cre recombinase excises marker gene as well as its own sequence. The activity of the *cre*-driven promoter can be triggered by the external (**Hoff *et al.*, 2001; Zuo *et al.*, 2001; Sreekala *et al.*, 2005; Zhang *et al.*, 2006; Ma *et al.*, 2008, Khattri *et al.*, 2011)** or internal stimuli **(Mlynárová *et al.*, 2006; Verweire *et al.*, 2007; Moravčíková *et al.*, 2008; Kopertekh *et al.*, 2009)**.

The aim of this work was to prepare plant transformation vector containing “self-excision” Cre/*lox*P cassette. In our system, the T-DNA of binary vector pZP6 consists of the *cre* recombinase gene driven by the *Arabidopsis* DLL promoter and the *npt*II expression unit flanked by two *lox*P sites in direct orientation. The *gus* reporter gene controlled by the double CaMV 35S promoter was placed out of the *lox*P embedded DNA. Due to specificity of the *Arabidopsis* DLL promoter, it is expected to control the excision of selectable marker gene from the genome of the transgenic plants during pollen and seed development.

MATERIAL AND METHODS

Vector construct

To prepare plant transformation vector pZP6, the six subcloning steps were used. Firstly, *dCaMV35S/gus/nosT/loxP* sequence as *Hind*III-*Hind*III fragment from pEV2 **(Moravčíková *et al.*, 2008)** was ligated into *Hind*III-digested pBinPlus **(van Engelen *et al.*, 1995)**. For cloning of the DLL promoter, additional *Pac*I cloning site was incorporated using

PCR approach with the combination of primers P1(5´-AGTTAATTAAAAGCTTTGGTTT TGAGGCAACTCCCCTT-3´) and P2 (5´-TTTGATATCTCCGCCTCCGCTCGTCGAGTG A-3´). The PCR reactions was carried out in 25 µl mixture containing 100 – 200 ng of DNA template, 20 pmol of each primer, 200 µM dNTPs, 1 × PCR buffer and 1 unit of *Taq* DNA polymerase (Expand High Fidelity PCR System, Roche). The first PCR step of 94 °C for 2 min. was followed by 35 cycles of 94°C for 45 s, 64°C for 30 s and 72°C for 2 min. The last step was performed at 72°C for 10 min. The PCR product (*1. part of the DLL promoter*) was ligated into pGem-T® Easy (Promega) to yield pZP2. Subsequently, the 1300 bp *Pac*I-*EcoR*V fragment from pZP2 and *2.part of DLL promoter/cre^{INT}/nosT* as 2313 bp *EcoR*V-*Acc65*I fragment from pZP3 were ligated into *Pac*I, *Acc65*I-digested pUN **(Vaculková *et al.*, 2007)** (pZP4). The plasmid pZP5 was created by cloning of *dCaMV35S/gus/nosT/lox*P as 3000 pb *Asc*I-*Pac*I fragment from pZP1 and *DLL/cre^{INT}/nosT* as 3600 pb *Pac*I-*Acc*65I fragment from pZP4 into *Asc*I, *Acc65*I-digested pUN. To obtain *Asc*I-*Acc65*I *dCaMV35S/gus/nosT/loxP* fragment, the plasmid pZP5 was firstly partially digested with *Acc65*I and then as *Asc*I-*Acc65*I fragment used in the next cloning step. The plasmid pZP6 was obtained by ligation of *dCaMV35S/gus/nosT/lox*P/*DLL/cre^{INT}/nosT* as 6600 bp *Asc*I-*Acc*65I fragment and *nosP/nptII/nosT/loxP* as 2200 bp *Acc65*I-*Pac*I fragment from pJL22 (unpublished) into *Asc*I, *Pac*I-digested pUN. After verification of pZP6 by restriction analyses and DNA sequencing, the binary vector pZP6 was introduced into *Agrobacterium* strains LBA 4404 and AGLO using "heat shock" method **(Höfgen and Willmitzer, 1988)**.

Excision of *lox*P embedded sequence in *Escherichia coli*

The plasmid pZP6 was evaluated for recombination events according to the method described by **Mlynárová and Nap (2003)**. The plasmids pZP6 and pMH303, containing the *cre* gene under control of the CaMV 35S promoter, were co-transformed into *E. coli* DH5αF´. The correct excision of the *lox*P embedded sequence was confirmed by restriction analyses.

Stability of pZP6 in *Agrobacterium tumefaciens*

The stability of the binary vector pZP6 in *A. tumefaciens* LBA 4404 and AGLO was verified by restriction analyses after re-transformation of pZP6 isolated from agrobacteria into *E. coli*.

RESULTS AND DISCUSSION

To evaluate the feasibility of the tissue-specific *Arabidopsis* DLL promoter in marker free Cre/*lox*P strategy, plant transformation vector pZP6 was constructed using standard cloning techniques. The cloning strategy is designed in the figure 1. The T-DNA of resulting plasmid pZP6 contained the reporter *gus* gene under control of the double CaMV 35S promoter and one pair of *lox*P sites flanking intron-containing *cre* gene (cre^{INT}) driven by the DLL promoter and selectable *npt*II gene controlled by the nos promoter (Figure 2a). Based on our previous experiences with instability of long DNA sequences in bacteria, the low copy number binary vector pUN was used. The vector pUN comprises of broad host range RK2 replicon from pBin19 and of multiple cloning site (MCS) and T-DNA region, both from a pBINPLUS-derived pLV06 vector. It was shown that absence of the ColE1 replicon in the backbone of the binary vector could contribute to stability of hardly clonable DNA sequences **(Vaculková *et al.*, 2007)**.

Before transformation of plasmid pZP6 into agrobacteria, the recombination event was tested in *E. coli*. Due to the presence of a plant intron in the *cre* gene, the pZP6 was co-transformed with plasmid containing *cre* gene driven by the CaMV 35S promoter. It was shown that, the *CaMV35S-cre* cassette has sufficient activity to efficiently excise DNA between *lox*P sites in *E. coli* **(Mlynárová and Nap, 2003).** The identity and stability of pZP6 was verified by restriction analyses (Figure 3).

Following co-transformation (3 repeats), totally 72 clones (24 clones/repeat) were isolated and subjected to restriction analyses with *Xho*I. The position of *Xho*I sites in the T-DNA of pZP6 before and after recombination event is indicated in the figure 2a and figure 2b, respectively. The correct excision was verified in all 72 analyzed clones. An example of restriction analyses of pZP6 before and after recombination event is shown in the figure 4.

In another experiments, the stability of pZP6 in agrobacteria was tested. Therefore, the pZP6 was transformed into two bacterial strains *A. tumefaciens* LBA 4404 and AGLO (separately). After re-transformation of pZP6 from both bacterial strains into *E. coli*, totally 48 clones (24/strain) were isolated and digested with *Xho*I. Restriction analyses of all 24 clones (AGLO) demonstrated correct *Xho*I-restriction pattern (Figure 4a). However, 1 out of 24 clones (LBA 4404) differed from expected (Figure 4b, lane 8) indicating instability of pZP6 in strain LBA 4404. Therefore, *A. tumefaciens* AGLO/pZP6 was chosen for further plant transformation experiments.

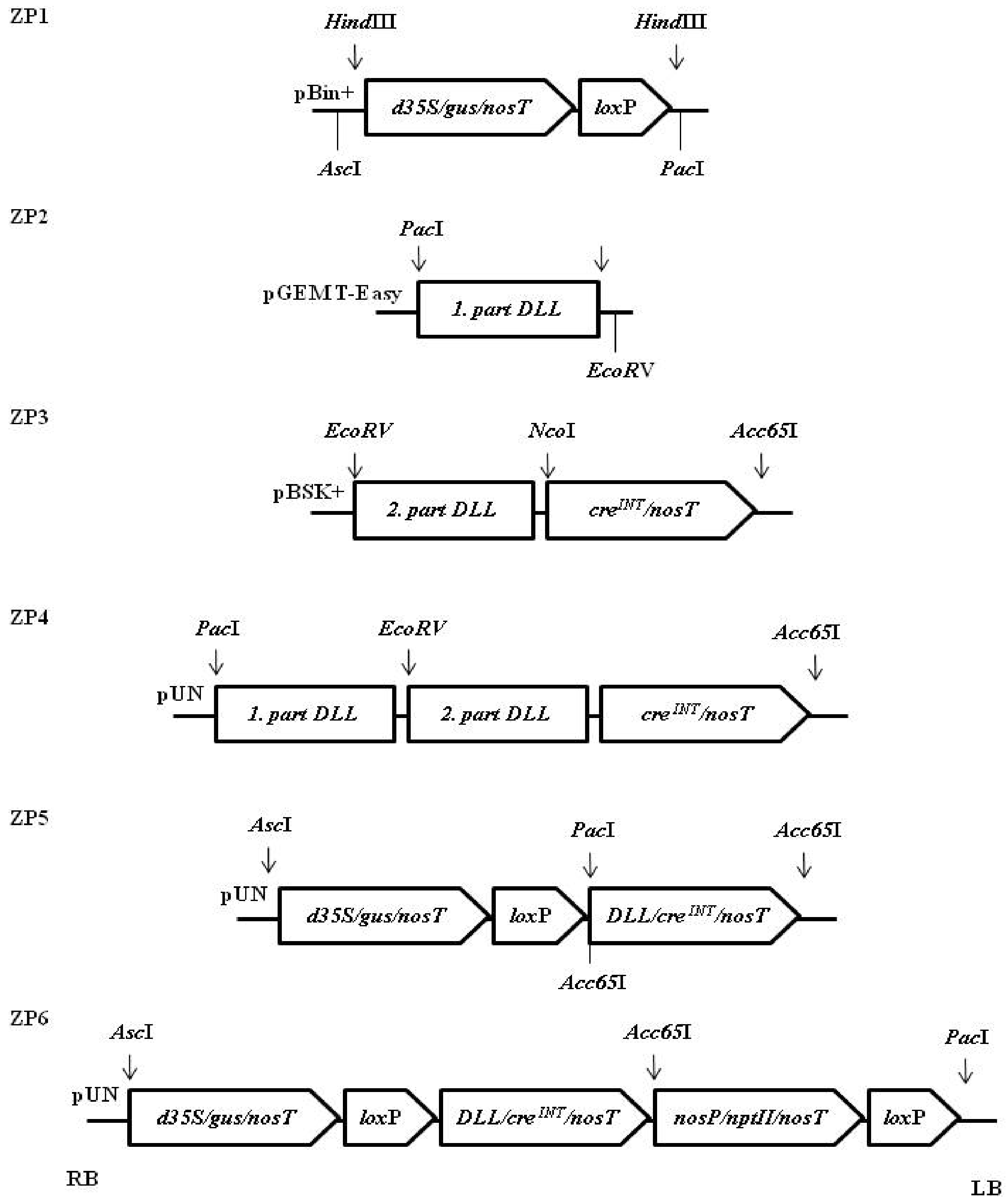

Figure 1 Cloning strategy used to prepare plant transformation vector pZP6 (The arrows indicate restriction enzymes used in individual cloning steps)

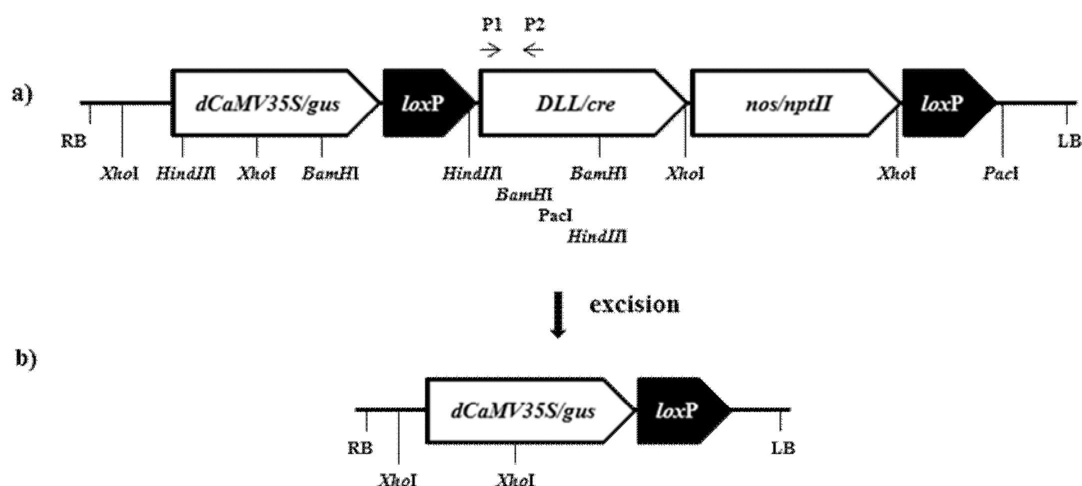

Figure 2 T-DNA structure of plant binary vector pZP6

a) The T-DNA consists of the reporter gene (*gus*) under control of the double CaMV 35S promoter (*dCaMV35S*), the intron-containing *cre* recombinase gene (*cre*) driven by the *Arabidopsis* promoter (DLL) and the neomycin phosphotransferase gene (*npt*II) regulated by the nos promoter. All genes are terminated by the nos terminator. Black arrows indicate presence and orientation of the *lox*P sites. The primers used for PCR analyses are indicated as P1, P2. **b)** The T-DNA configuration generated after excision of the *lox*P embedded DNA. Other abbreviations used: RB, LB – right and left borders of T-DNA. The restriction sites used for restriction analyses are indicated.

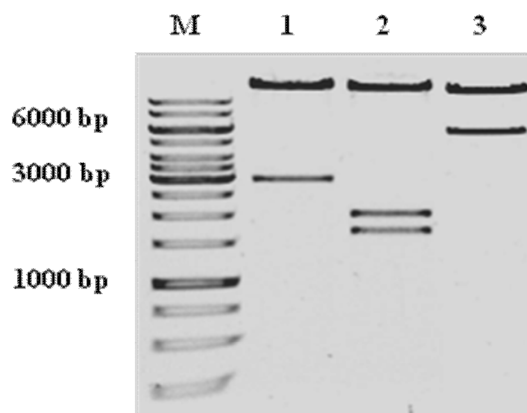

Figure 3 Restriction analyses of pZP6

Photograph of the ethidium bromide-stained 1 % agarose gel, **lane M** – 1 kb DNA ladder (Fermentas)**, lanes 1-3** pZP6/*Hind*III**,** pZP6/*BamH*I, pZP6/*Pac*I, respectively.

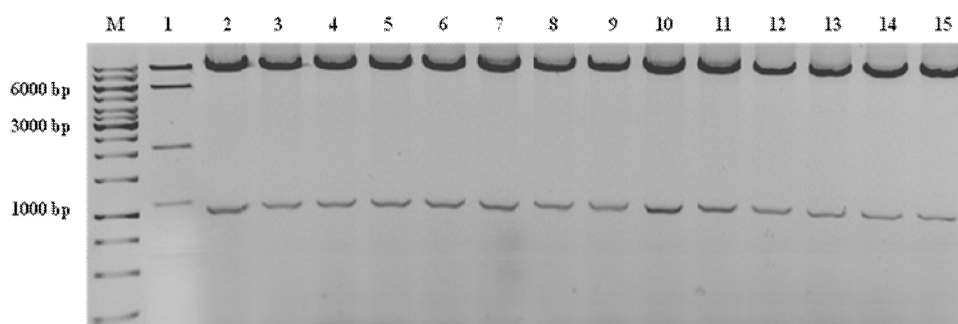

Figure 4 Restriction analyses of plasmid pZP6 after recombination event in *E.coli*
Photograph of the ethidium bromide-stained 1 % agarose gel carrying in **lane M** – 1 kb DNA ladder (Fermentas)**, lane 1** – pZP6/*Xho*I**, lanes 2-15** – pZP6 clones digested with *Xho*I after Cre-mediated recombination.

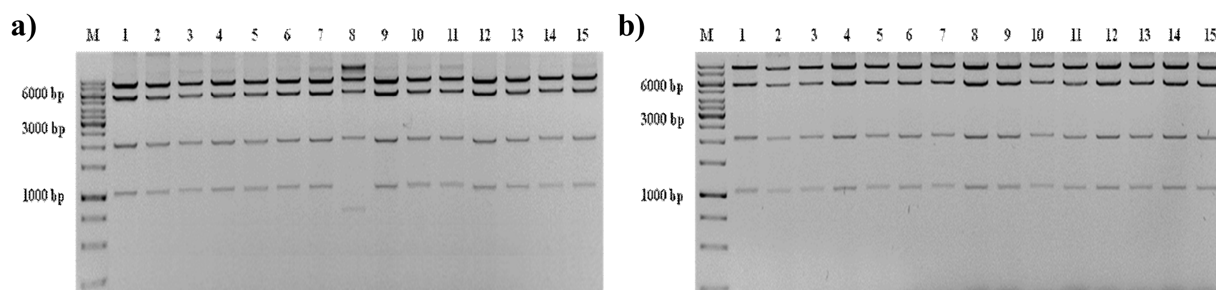

Figure 5 Restriction analyses of pZP6 after re-transformation from *A. tumefaciens* LBA 4404 (a) and AGLO (b) into *E.coli*
Photograph of the ethidium bromide-stained 1 % agarose gel carrying in **lane M** – 1 kb DNA ladder (Fermentas)**, lane 1** – pZP6/ *Xho*I**, lanes 2-15** – pZP6 clones digested with *Xho*I after re-transformation from *A. tumefaciens* into *E. coli.*

CONCLUSION

With the aim to generate marker-free transgenic plants using the "self-excision" Cre/*lox*P system controlled by the pollen and seed-specific DLL promoter, the plant binary vector pZP6 was prepared and introduced into agrobacteria. The analyses focused on removal of the *lox*P embedded sequence in *E.coli* and on stability of pZP6 in two bacterial strains *A. tumefaciens* (AGLO and LBA 4404) confirmed functionality of Cre/*lox*P system and stability of pZP6 in *A. tumefaciens* AGLO, respectively. *A. tumefaciens* AGLO carrying binary vector

pZP6 will be used for tobacco plant transformation and the excision efficiency of selectable marker gene removal from the transgenic tobacco genome during pollen and seed development will be evaluated.

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