PREPARATION OF PLANT TRANSFORMATION VECTOR CONTAINING “SELF-EXCISION” CRE/LOXP 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/loxP system. The T-DNA of binary vector consists of the cre recombinase gene driven by the Arabidopsis DLL promoter and the nptII expression unit flanked by two loxP sites in direct orientation. The gus reporter gene controlled by the double CaMV 35S promoter was placed out of the loxP embedded DNA. To confirm functionality of the Cre/loxP system, the pZP6 was analyzed for correct removal of the loxP 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/loxP 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/loxP recombination.

The Cre/loxP system was isolated from bacteriophage P1. It consists of the cre recombinase gene and two 34 bp loxP sites. The Cre recombinase mediates recombination event between two loxP sequences in direct orientation. In “self-excision” Cre/loxP strategy both the cre and selectable marker genes are inserted between two loxP 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/loxP 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 nptII expression unit flanked by two loxP sites in direct orientation. The gus reporter gene controlled by the double CaMV 35S promoter was placed out of the loxP 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 HindIII-HindIII fragment from pEV2 (Moravčíková et al., 2008) was ligated into HindIII-digested pBinPlus (van Engelen et al., 1995). For cloning of the DLL promoter, additional PacI cloning site was incorporated using
PCR approach with the combination of primers P1 (5’-AGTTAATTAAAGCTTTGGT TTGAGGCAAACTCCCTTT-3’) and P2 (5’-TTTGATATCTCCGCTGGCTGAGTGA TGA-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 PacI-EcoRV fragment from pZP2 and 2. part of DLL promoter/creINT/nosT as 2313 bp EcoRV-Acc65I fragment from pZP3 were ligated into PacI, Acc65I-digested pUN (Vaculková et al., 2007) (pZP4). The plasmid pZP5 was created by cloning of dCaMV35S/gus/nosT/loxP as 3000 pb AccI-PacI fragment from pZP1 and DLL/creINT/nosT as 3600 pb PacI-Acc65I fragment from pZP4 into AccI, Acc65I-digested pUN. To obtain AccI-Acc65I dCaMV35S/gus/nosT/loxP fragment, the plasmid pZP5 was firstly partially digested with Acc65I and then as AccI-Acc65I fragment used in the next cloning step. The plasmid pZP6 was obtained by ligation of dCaMV35S/gus/nosT/loxP/DLL/creINT/nosT as 6600 bp AccI-Acc65I fragment and nosP/nptII/nosT/loxP as 2200 bp Acc65I-PacI fragment from pJL22 (unpublished) into AccI, PacI-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 loxP 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’.

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/loxP 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 loxP sites flanking intron-containing cre gene (cre\textsuperscript{INT}) driven by the DLL promoter and selectable nptII 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 loxP 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 *XhoI*. The position of *XhoI* 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 *XhoI*. Restriction analyses of all 24 clones (AGLO) demonstrated correct *XhoI*-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 (nptII) regulated by the nos promoter. All genes are terminated by the nos terminator. Black arrows indicate presence and orientation of the loxP sites. The primers used for PCR analyses are indicated as P1, P2. b) The T-DNA configuration generated after excision of the loxP 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/HindIII, pZP6/BamHI, pZP6/PacI, 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/XhoI, lanes 2-15 – pZP6 clones digested with *XhoI* 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/ Xhol, lanes 2-15 – pZP6 clones digested with *XhoI* after re-transformation from *A. tumefaciens* into *E. coli*.

**CONCLUSION**

With the aim to generate marker-free transgenic plants using the “self-excision” Cre/loxP 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 loxP embedded sequence in *E.coli* and on stability of pZP6 in two bacterial strains *A. tumefaciens* (AGLO and LBA 4404) confirmed functionality of Cre/loxP 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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REFERENCES


