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REGULAR ARTICLE

PLANT TRANSFORMATION VECTORS AND THEIR STABILITY IN *AGROBACTERIUM TUMEFACIENS*

Zuzana Polóniová[*1], Pavol Dubnický[2], Zdenka Gálová[2,3], Jana Libantová[1], Ildikó Matušíková[1], Jana Moravčíková[1]*

***Address*:** Ing. Zuzana Polóniová, Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademická 2, P.O.Box 39A, 95007 Nitra, Slovak Republic, phone number: +421376943332

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

[2]Department of Biochemistry and Biotechnology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Tr. A. Hlinku 2, 94976 Nitra, Slovak Republic

[3]Institute of Chemistry, Center of excellence for white-green biotechnology, Slovak Academy of Sciences, Tr. A.Hlinku 2, 94976 Nitra, Slovak Republic

*Corresponding author: zuzana.poloniova@savba.sk

ABSTRACT

The stability of the plant transformation vectors pTS2, pEV2 and pEV8 was tested in two *Agrobacterium tumefaciens* strains LBA 4404 and C58C1 that differed in chromosomal background. The T-DNAs of all binary vectors contained the β-glucuronidase reporter and the neomycin phosphotransferase selectable marker genes. In addition, the plasmids pEV2 and pEV8 contained the Cre/*lox*P recombination system. The binary vectors were transformed into *A. tumefaciens* strains and their stability was evaluated by restriction analyses after retransformation of plasmids from *A. tumefaciens* into *E. coli*. Our results showed that plasmids pTS2 and pEV2 were stable in both bacterial strains while the pEV8 exhibited instability in *A. tunmefaciens* LBA 4404.

Keywords: *Agrobacterium tumefaciens*, plant transformation vector

INTRODUCTION

Agrobacterium-mediated transformation is one of the most used gene delivery system in many plant species. Compared with the biolistic transformation, this method has several advantages. It is inexpensive method that uses intrinsic property of agrobacteria to attack wounded plant cells while the T-DNA is predominantly integrated into the transcriptionally active part of the plant chromosome **(Kuta and Tripathi, 2005)**. Thetransferandintegration ofthe transgene/sinto the nucleus is carried out mainly with help ofplant transformation vectors called "binaryvectors" (**Lee and Gelvin, 2008**). The genes of interest are placed on the T-DNA between the left (LB) and right border (RB) sequences. Till now many binary and superbinary vectors were successfully used in plant transgenosis **(Komori *et al.* 2007, Lee and Gelvin 2008)**. They are different in presence/absence of the selectable marker genes or in replication functions in *E. coli* and *A. tumefaciens*. For example, the binary vectors pBin19 **(Bevan 1984)** or pUN **(Vaculková *et al.*, 2007)** posse an origin RK2 replicon and produce 4-5 copies in *E. coli* while the binary vector pBinPlus **(van Engelen *et al.*, 1995)** contains except for RK2 also ColE1 replicon that ensured increased number of copies (40 per cell). However, increased number can results in increased metabolic cost to the host cell **(Goss and Peccoud, 1999)** and/or instability of some cloned (longer, AT-CT rich or prone to secondary structures) DNA sequences **(Pierson and Barcak, 1999, Frariand and Hamilton, 2001)**. Several authors have reported structural instability of high copy number plasmids **(Ordway and Detloff 1996, Pierson and Barcak 1999, Vaculková *et al.*, 2007)**.

The objective of our study was to test the stability of three plant transformation vectors pTS2, pEV2 and pEV8 in bacterial cells. The T-DNAs of all binary vectors contained the β-glucuronidase(*gus*) reporter and the neomycin phosphotransferase (*npt*II) selectable marker genes. In addition, the plasmids pEV2 and pEV8 contained the Cre/*lox*P recombination system. The Cre/*lox*P system consists of *cre* recombinase gene and two *lox*P sites. Upon activation, the Crerecombinase mediates recombination event between two *lox*P sequences in direct orientation.Stability of the plasmids in *A. tumefaciens* was verified by restriction analyses after retransformation of plasmids from *A. tumefaciens* into *E. coli*.

MATERIAL AND METHODS

Vector constructs

Plasmid pTS2 **(Salaj *et al.*, 2009)** is a derivate of the pBinPlus **(Van Engelen *et al.*, 1995)** that in the T-DNA region contains the *gus* gene under control of the dCaMV35S promoter and the *npt* II gene (Figure 1a).

Plasmid pEV2 **(Moravčíková *et al.*, 2008)** is a derivate of the pUN **(Vaculková *et al.*, 2007)**. The T-DNA region contains the *gus* gene under control of the dCaMV35S promoter and one pair of the *lox* P sites flanking the *cre* recombinase and *npt* II genes (Figure 2a).

Plasmid pEV8 **(Moravčíková *et al.*, 2011)** is a derivate of the pUN **(Vaculková *et al.*, 2007)**. The T-DNA region contains the *gus* gene driven by the light-sensitive Lha3St1 promoter and one pair of the *lox* P sites flanking the *cre* and *npt* II genes (Figure 3a).

Bacteria

Escherichia coli DH5αF´ F´/ gyrA96 (Nal^r) recA1 relA1 endA1 thi-1 hsdR17 (r_k-m_k+) glnV44 deoRΔ(*lacZ*YA-argF) U169 [Φ80dΔ(*lacZ*)M15] **(Woodcock *et al.*, 1989)**.

Agrobacterium tumefaciens LBA4404[Ach 5, pTiAch 5, occ^-, ape^-, vir, +::Tn 904, (Rif^R), (Sm^R)] **(Hoekema*et al.*, 1983)**.

Agrobacterium tumefaciens C58C1(Rif^R) contains modified Ti plasmid **(Goodner *et al.*, 2001)**.

Bacteria were cultivated at 37 ºC (*E. coli*) or 28 ºC (*A. tumefaciens*) in the liquid or solid Luria-Bertani (LB) medium **(Sambrook *et al.*, 1989)**supplemented with antibiotics 50 µl .ml^{-1} kanamycin (Duchefa) or 50 µl . ml^{-1} kanamycin (Duchefa) and 50 µl . ml^{-1} rifampicin (Duchefa), respectively.

Stability of the plant transformation vectors in *Agrobacterium tumefaciens*

The plasmids pTS2, pEV2 and pEV8 were transformed into *A. tumefaciens* LBA 4404 and C58C1 (separately) using the heat shock methods described by **Höfgen and Willmitzer (1988)**. Following isolation of plasmids from agrobacterial cells, the plasmid DNAs were re-transformed into *E. coli* using the heat shock methods described by **Froger and Hall (2007)**.

The stability of the binary vectors in *A. tumefaciens* was verified by restriction analyses of individual clones (24 per transformation event) isolated from *E. coli* after retransformation.

RESULTS AND DISCUSSION

Inour experiments, thebinaryvectors pTS2, pEV2 and pEV8(Figure 1a, 2a, 3a)carrying in their T-DNA regions *gus* reporter and *npt* II selectable marker genes were used. In addition, the plasmids pEV2 and pEV8 contained Cre/*lox*P recombination system consisting of the *cre*recombinaseandtwo34pb*lox*Psequences. The identity of plasmids was verified by restriction analyses (data not shown). The stability of the vector constructsin *A. tumefaciens* was tested by transformation of the individual plasmids pTS2, pEV2 and pEV8into two *Agrobacterium* strains (octopine LBA 4404 and nopaline C58C1). Then,the individual plasmids were isolated from overnight *Agrobacterium* cultures (LBA4404/pTS2, LBA4404/pEV2, LBA4404/pEV8, C58C1/pTS2, C58C1/pEV2 and C58C1/pEV8) and retransformed into *E. coli*. From each retransformation event (6), 144 clones (24 per event) were isolated and subjected to the restriction analyses with *Hind* III. The position of the restriction enzyme in the T-DNA regions of individual plasmids is indicated in Figure 1a, 2a, 3a.

Based on the restriction map of the pTS2, the fragment of size 3 kb (Fig. 1b) is expected to detect after separation of excised fragments on a 1% agarose gel. Correct fragments were detected in all analysed clones (LBA4404/pTS2 and C58C1/pTS2).

For the plasmid pEV2, the presence of 4 fragments from 900 bp to 3000 bp indicates correct *Hind* III-restriction pattern (Figure 2b). Our results showed that restriction analyses of all 48 clones (LBA4404/pEV2 and C58C1/pEV2) were consistent with expected (Figure 2c, 2d).

Similarly to the restriction map of the pEV2, the digestion of the plasmid pEV8 with *Hind* III results in excision of the 4 fragments from 900 bp to 3000 bp (Figure 3b). The correct *Hind* III-restriction pattern was found in all 24 (LBA4404/pEV8) clones (Figure 3c), However, analyses of (C58/pEV8) clones revealed that the sizes of 2 fragments did not correspond to expected in 1 out of 24 clones (Figure 3c, lane 4) indicating an instability of cloned DNA sequences in bacterial cells. It could be related with presence of the Cre/*lox*P recombination system in T-DNA of the binary vector. It was shown that such sequences could be source of instability in bacteria **(Vaculková *et al.*, 2007)**. Similarly, in our previous experiments (**Polóniová *et al.*, 2012**) the binary vector pZP6 containing Cre/*lox*P sequence

exhibited instability in *A. tumefaciens* LBA 4404 while nodeletion or rearrangements were revealed in *A. tumefaciens* AGLO. It could coincide with different chromosomal background of bacterial strains used in our experiments **(Hellens *et al*., 2000)**.

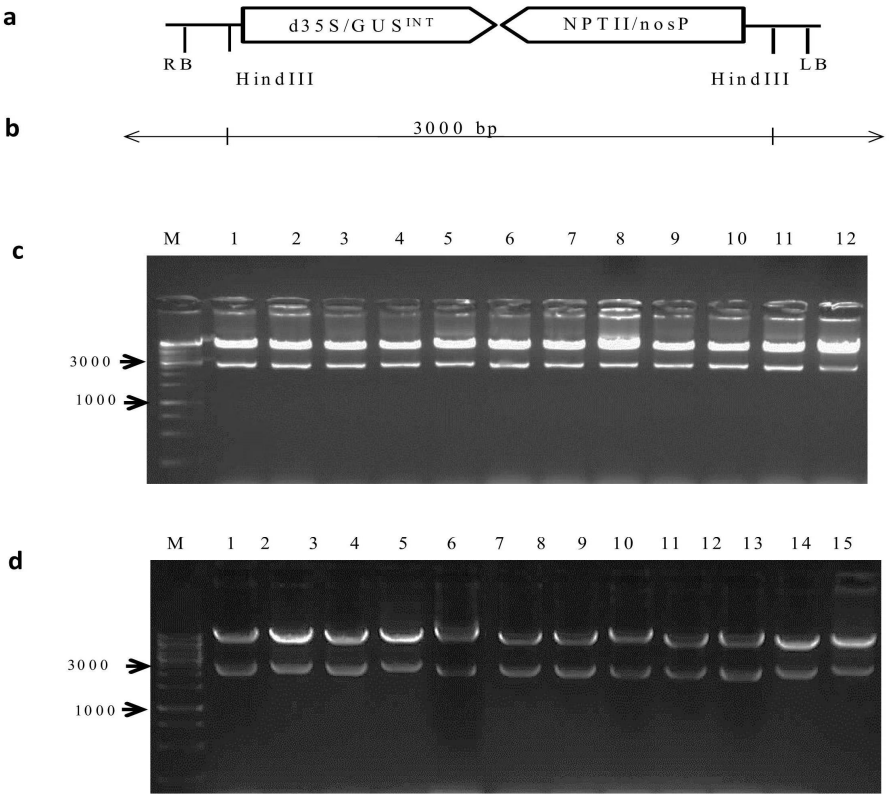

Figure 1 Restriction analyses of plasmid pTS2 after retransformation from *A. tumefaciensin* to *E. coli*

(a) T-DNA structure ofpTS2. The T-DNA consists of an intron-containing β-glucuronidase (GUS^{INT}) gene under control of the dCaMV35S (d35S) and neomycin phospotranseferase (NPTII) gene. Both genes are terminated by *nosT*. (b) The size of individual fragments expected after *Hind* III-digestion of pTS2.**(c)** Photograph of ethidium bromide stained 1% agarose gel carrying *Hind* III-digested pTS2 clones (lanes 1-12) after retransformation of pTS2 from *A. tumefaciens* LBA4404 into *E. coli*. **(d)** Photograph of ethidium bromide stained 1% agarose gel carrying *Hind* III-digested pTS2 clones (lanes 1-12) after retransformation of pTS2 from *A. tumefaciens* C58C1 into *E. coli*. Lane M – 1 kb DNA marker (Fermentas).

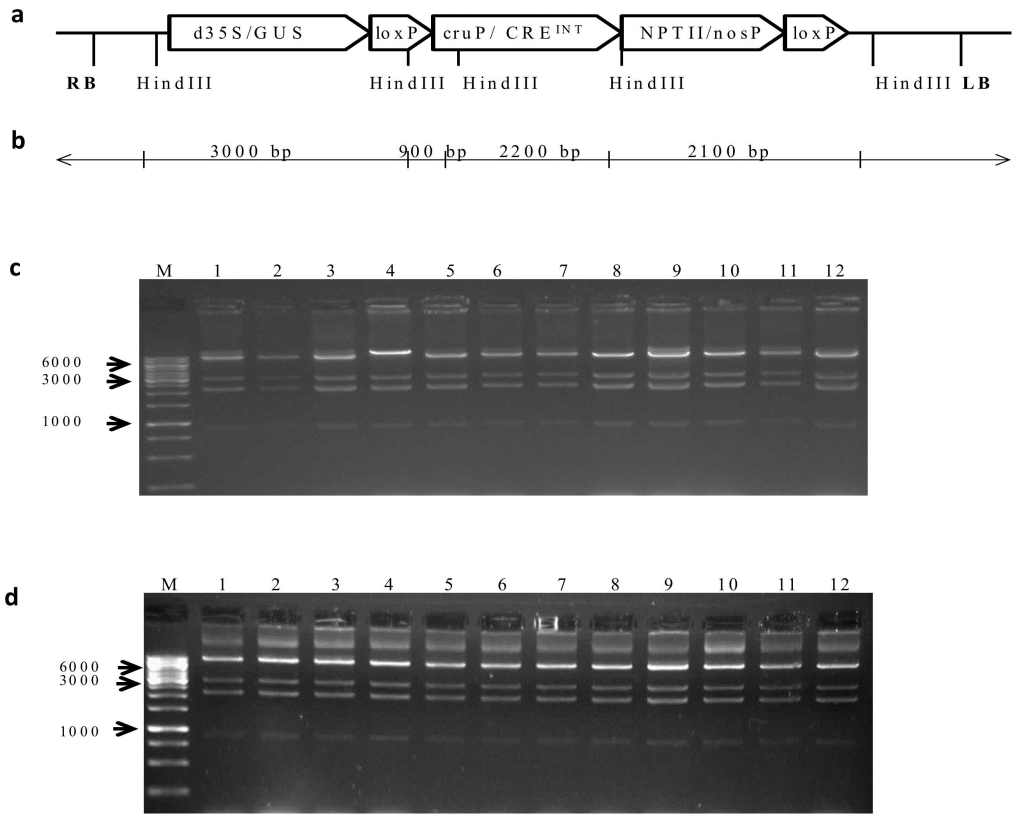

Figure 2 Restriction analyses of plasmid pEV2 after retransformation from *A. tumefaciens*into *E.coli*

(a) T-DNA structure ofpEV2. The T-DNA consists of the β-glucuronidase (GUS) gene under control of the dCaMV35S (d35S) and *lox* P-embedded cassette consisting of the intron-containing *cre* recombinase (CRE^{INT}) gene controlled by the *Arabidopsis*cruciferin (*cruP*) promoter and neomycin phospotranseferase (NPTII) gene. All genes are terminated by *nosT*. **(b)** The size of individual fragments expected after *Hind* III-digestion of pEV2. **(c)** Photograph of ethidium bromide stained 1% agarose gel carrying *Hind* III-digested pEV2 clones (lanes 1-12) after retransformation of pEV2 from *A. tumefaciens* LBA4404 into *E. coli*. **(d)** Photograph of ethidium bromide stained 1% agarose gel carrying *Hind* III-digested pEV2 clones (lanes 1-12) after retransformation of pEV2 from *A. tumefaciens* C58C1 into *E. coli*. Lane M – 1 kb DNA marker (Fermentas).

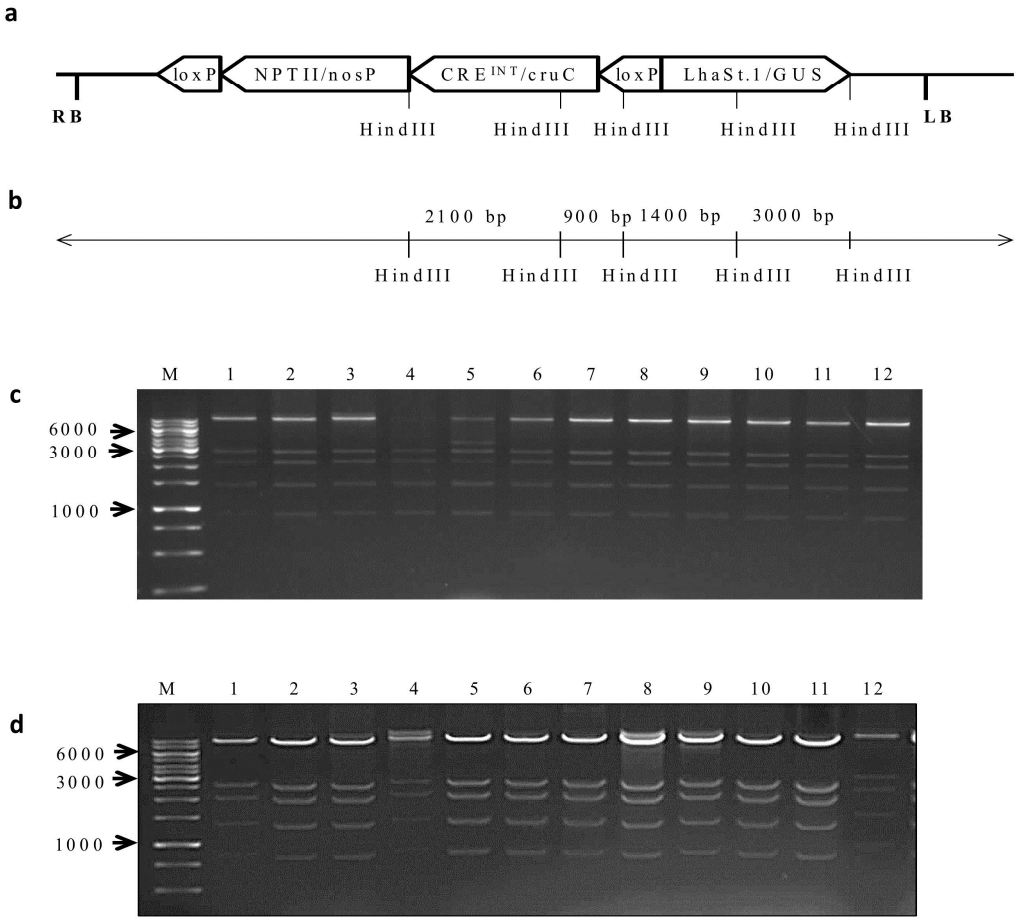

Figure 3 Restriction analyses of plasmid pEV8 after retransformation from *A. tumefaciensin* to *E. coli*

(a) T-DNA structure ofpEV8. The T-DNA consists of an intron-containing β-glucuronidase (GUS) gene under control of the light-sensitive Lha3St1 (LhaSt1) and *lox* P-embedded cassette consisting of the intron-containing *cre*recombinase (CRE^{INT}) gene controlled by the *Arabidopsis* cruciferin (*cruP*) promoter and neomycin phospotranseferase (NPTII) gene. All genes are terminated by *nosT*. **(b)** The size of individual fragments expected after *Hind* III-digestion of pEV8. **(c)** Photograph of ethidium bromide stained 1% agarose gel carrying *Hind* III-digested pEV8 clones (lanes 1-12) after retransformation of pEV8 from *A. tumefaciens* LBA4404 into *E. coli*. **(d)** Photograph of ethidium bromide stained 1% agarose gel carrying *Hind* III-digested pEV8 clones (lanes 1-12) after retransformation of pEV8 from *A. tumefaciens* C58C1 into *E. coli*. Lane M – 1 kb DNA marker (Fermentas).

CONCLUSION

Prior plant transformation experiments, the binary vectors pTS2, pEV2 and pEV8 were tested for their stability in *A. tumefaciens* LBA 4404 and C58C1. Our results showed that plasmids pTS2 and pEV2 were stable in both bacterial strains while the pEV8 was shown to be instable in LBA 4404. Therefore, *A. tumefaciens* LBA 4404/pTS2, *A. tumefaciens* LBA 4404/pEV2 and *A. tumefaciens* C58C1/pEV8 will be used for tobacco transformation.

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