BIOLOGIA PLANTARUM 51 (3): 538-540, 2007

BRIEF COMMUNICATION

# A modified low copy number binary vector pUN for *Agrobacterium*-mediated plant transformation

E. VACULKOVÁ*, J. MORAVČÍKOVÁ*, I. MATUŠÍKOVÁ*, M. BAUER** and J. LIBANTOVÁ*[1]

*Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, P.O. Box 39A, SK-95007 Nitra, Slovak Republic**
*Research Institute of Animal Production, Hlohovska 2, SK-94992 Nitra, Slovak Republic***

## Abstract

A modified low copy number plant binary vector pUN has been constructed and successfully used to clone unstable DNA sequences. The vector pUN comprises of low copy number, broad host range RK2 replicon from pBin19 and of multiple cloning site (MCS) and T-DNA region, both from a pBINPLUS-derived pLV06 vector. The absence of the ColE1 replicon in the backbone of the binary vector significantly contributed to stability of hardly clonable DNA sequences and enabled their transfer into the tobacco plants through *Agrobacterium*-mediated transformation.

*Additional key words*: ColE1 replicon, Ti vector, unstable DNA sequences.

The most efficient system for gene transfer into plants is *Agrobacterium*-mediated transformation that exploits the natural ability of the soil microorganism to transform wide range of plant species (for review see Hansen and Wright 1999, Cheng *et al*. 2004, Uranbey *et al.* 2005). The genes to be transferred *via* bacterial Ti plasmid are cloned between the borders of its T-DNA region. The majority of binary vectors harbour an origin of replication from a broad host range plasmid that ensures replication in both *Escherichia coli* (cloning procedure) and *Agrobacterium* (transformation of plants). For example, one of the first binary vectors pBin19 (Bevan 1984) possesses an origin RK2 and produce 4 - 5 copies in *E. coli* (Kahn *et al*. 1979). To facilitate DNA manipulation in *E. coli*, the copy number and therefore plasmid yields, have been increased (up to 40 copies per cell) in many other binary vectors by incorporating a ColE1 replicon into the backbone of the vectors (for review see Hellens *et al*. 2000). On the other hand, increased copy number (due to presence and utility of two active independent origins) can result in increased metabolic cost to the host cell (Goss and Peccoud 1999) and/or instability of some cloned DNA sequences. Several authors have reported structural instability of moderate or high copy number plasmids encountered in heterologous cloning (Trinh *et al*. 1994, Ordway and Detloff 1996, Pierson and Barcak 1999). In addition to copy number of the plasmid and metabolic burden of plasmid maintenance, there are some other factors that influence stability of the plasmid. These include, for example, host strain genotype or recombinogenic potential of plasmid sequences.

The aim of our work was to prepare the binary transformation vector construct pEV2 comprising the Cre/*loxP* site-specific recombination system to be used in plant transformation experiments. We came out from the prediction that a low copy vector may overcome the deleterious effects of unstable DNA fragments cloned into *E. coli* (Pierson and Barcak 1999). As the majority of current binary vectors contain moderate or high copy number origin of replication we decided to prepare low copy number binary vector pUN, derivate of the pLV06 (Mlynarova *et al.* 2002) that contains, in contrast to pLV06 vector, only low copy number replicon RK2

*Received* 12 September 2005, *accepted* 2 March 2006.
*Abbreviations*: GUS - β-glucuronidase; MCS - multiple cloning site; NPTII, NPTIII - neomycin phosphotransferase II and III.
*Acknowledgements*: Authors thank Dr. Imrich Barák for valuable advices, Ivana Galbičková and Anna Fábelová for *in vitro* plant care; Dr. Jan Peter Nap and Dr. Ludmila Mlynarová for indispensable scientific support. This work was supported by UNESCO scholarship to J.M, by program 2003 SP 27/028 OD 01/028 OD01 of Slovak Ministry of Agriculture and by Slovak Grant Agency APVT 51-00-56-02.
[1] Author for correspondence; fax (+421) 37 7336660, e-mail: nrgrliba@savba.sk

(Fig. 1*A*). The vector lacks the selectable marker gene in T-DNA. This coincides with the current trend to clone selectable marker gene independently based on the marker genes available and intended experiment.

Subsequently, under the same experimental conditions, the 2 kb *Asc*I-*Asp*718 fragment of *nos/nptII-lox*P and the 6 kb *Pac*I-*Asc*I fragment of *dCaMV35S/gus-loxP-cru/cre*$^{INT}$ were cloned into polylinker of T-DNA of binary vector pUN (*Pac*I-*Asp*718) (Fig. 1*B*). From the first set of 12 recombinant plasmids analysed 4 recombinants revealed correct full-length inserts without any rearrangements (Fig. 2). The resulting pUN-based binary vector construct pEV2 appeared to be stable in *Agrobacterium tumefaciens* LBA 4404. This was verified after retransformation to *E. coli* and restriction mapping of isolated corresponding plasmids. Cloning success we got by using the binary vector pUN suggests that a reduction in copy number may alleviate certain instability problems encountered in heterologous cloning. This vector construct was successfully used in tobacco explants transformation (data not shown).

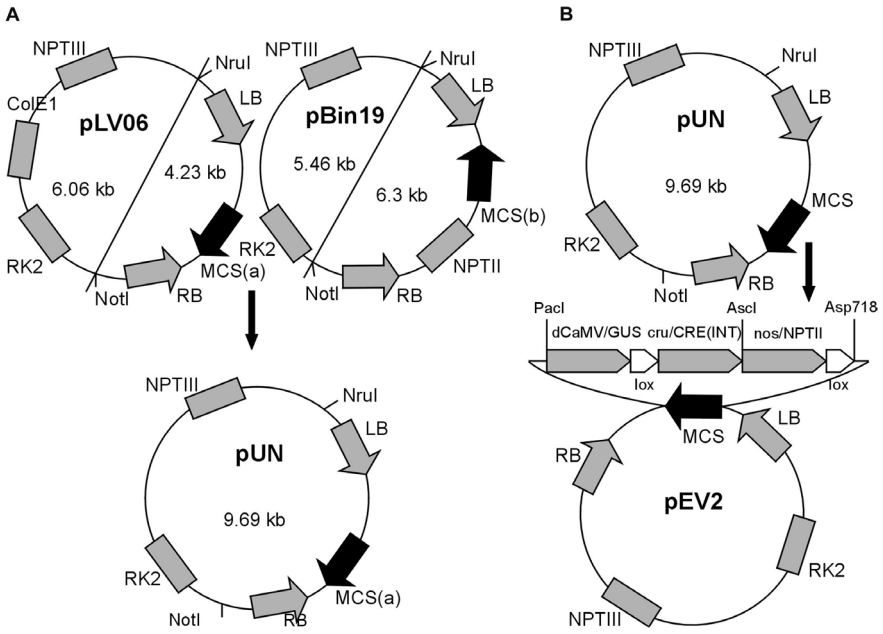


Fig. 1. Strategy for preparation of plant binary construct pEV2. *A* - The construction of plant transformation vector pUN. The 4.23 kb fragment of pLV06 was ligated to a 5.46 kb (*Not*I- *Nru*I) fragment of p Bin19. The unique restriction sites in the MCS are: *Pvu*I (*Pac*I (351), *Eco*RI (355), *Sac*I (365), *Xma*I (371), *Kpn*I (371), *Sma*I (373), *Bam*HI (376), *Xba*I (382), *Sal*I (388), *Sph*I (404), *Hind*III (406), *Asc*I (413), *Cla*I (714), *Nhe*I (719), *Xho*I (725), *Sca*I (733), *Spe*I (737). *B* - The construction of plant binary vector pEV2. The 6 kb *Pac*I - *Asc*I fragment of dCaMV 35S/GUS-lox-Cru/Cre and 2 kb *Asc*I - *Asp*718 fragment of nos/nptII - lox were cloned into *Pac*I - *Asp*718 sites of pUN vector. Abbreviations used: RB, LB - right and left borders of T-DNA; MCS multiple cloning site, NPTII, NPTIII - neomycin phosphotransferase marker genes providing the selection in *Agrobacterium* or *E. coli*, respectively, containing the plasmid, dCaMV - double CaMV 35S promoter; GUS - glucuronidase gene; cru - cruciferin promoter; CRE$^{INT}$- Cre-recombinase gene comprising intron; nos - nopaline synthase promoter; lox - 34 bp loxP sites (to be used in Cre-lox recombination assays).

In our hands, the cassette incorporating *dCaMV35S/gus-loxP-cru*/*cre*$^{INT}$*-nos/nptII-loxP* had presented cloning problems in ColE1-containing binary vector pLV06 prior to the use of pUN as a cloning vector. It has been shown, that AT- and GC-rich sequences, the sequences with strong secondary structure or large inserts are unstable in high copy vectors for reasons less well understood so far (Tao and Zhang 1998, Pierson and Barcak 1999, Frary and Hamilton 2001). It can be due to various causes occurring in the course of replication (topological barriers), transcription (strong promoters), and/or translation (gene product itself interferes with normal metabolism). These difficulties can be successfully overcome by using bacterial host strains (*SURE* cells from *Stratagene*, La Jolla, USA, *STBL2*$^{TM}$ and *STBL4*$^{TM}$ cells from *Life Technologies*, Carlsbad, USA) that were designed specifically for increasing stability of problematic plasmids. These engineered strains can be very helpful in cases when causes of instability are predicted. For example *STBL2*$^{TM}$ and *STBL4*$^{TM}$ cells are recommended for stable cloning direct repeats and tandem array genes. *SURE* cells can stabilize DNA sequences with non-standard secondary and tertiary structures. However these strains have some limitations. For example *STBL2*$^{TM}$ does not contain the Δ*M15lac*Z domain for blue-white screening of recombinants. *SURE* carries Tn5 transposon fragment encoding the kanamycin resistance gene and therefore is not convenient to be transformed by the most plant binary vectors that contain neomycin phosphotransferase selectable gene.

Low copy number plasmids represent an alternative recommended for cloning and maintaining unstable or long DNA sequences (Tao and Zhang 1988, Lernar and Inouye 1990, Mead *et al.* 2005). The binary vector pUN presented here effectively increased the structural stability of the cassette mentioned above. In contrast to the original pLV06, lack of the ColE1 origin appeared to be sufficient for stabilizing the inserted DNA above mentioned. Although problematic eukaryotic DNA inserts do not have to be exclusively contingent on single copy status, low copy number of recombinant plasmid in bacterial cells is one of factors significantly contributing to stability of cloned inserts.

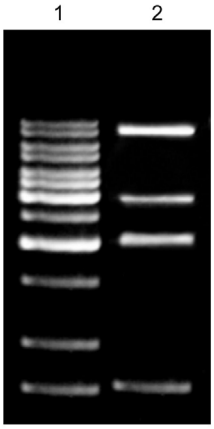


Fig. 2. The restriction pattern of vector construct pEV2 restricted by *Xho*I restriction enzyme. *Lane 1* - 1 kb DNA ladder, *lane 2* - pEV2/*Xho*I.

In case of binary vectors utilised for plant transformation a new low copy number vector pUN enriched the quite short list of similar plant binary vectors available so far.

In the following experiments pEV2 vector construct was successfully used in *A. tumefaciens* LBA 4404 tobacco transformation. Comparing to the other binary vectors with GUS expression unit and comprising in the backbone of the vector except for RK2 also ColE1 replicon, no significant differences in the transformation efficiency and GUS activity of the transformants were observed (Moravčíková *et al*. 2003).

## References

Bevan, M.: Binary *Agrobacterium* vectors for plant transformation. - Nucl. Acids Res. **12**: 8711-8721, 1984.

Cheng, M., Lowe, B.A., Spencer, M., Ye, X., Amstrong, C.L.: Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. - *In Vitro* cell. dev. Biol. Plant **40**: 31-45, 2004.

Frary, A., Hamilton, C.M.: Efficiency and stability of high molecular weight DNA transformation: analysis in tomato. - Transgenic Res. **10**: 121-132, 2001.

Goss, P.J.E., Peccoud, J.: Analysis of the stabilizing effect of Rom on the genetic network controlling *ColE1* plasmid replication. - In: Altman, P., Dunker, A.K., Hunter, L., Klein T.E. (ed.): Pacific Symposium on Biocomputing. Pp. 65-76. World Scientific, Hawaii 1999.

Hansen, G., Wright, M.S.: Recent advances in the transformation of plants. - Trends Plant Sci. **4**: 226 - 231, 1999.

Hellens, R., Mullineaux, P., Klee, H.: A guide to *Agrobacterium* binary Ti vectors. - Trends Plant Sci. **5**: 446-451, 2000.

Kahn, M., Kolter, R., Thomas, C., Figurski, D., Meyer, R., Remaut, E., Helinski, D.R.: Plasmid cloning vehicles derived from plasmids ColE1, F, R6K, and RK2. - Methods Enzymol. **68**: 268-280, 1979.

Lerner, C.G., Inouye, M.: Low copy number plasmids for regulated low-level expression of cloned genes in *Escherichia coli* with blue/white insert screening capability. - Nucl. Acids Res. **18**: 4631, 1990.

Mead, D., Patterson, M, Godiska, R.: High stability for cloning deleterious genes and genomes. - In: Plant & Animal Genomes XIII Conference, San Diego 2005.

Mlynarová, L., Libantová, J., Vrba, L., Nap, J.P.: The promiscuity of heterospecific *lox* sites increases dramatically in the presence of palindromic DNA. - Gene **296**: 129-137, 2002.

Moravčíková, J., Libantová, J., Nap, J.P., Mlynarová, Ľ.: [The influence of the type of the binary vector and the concentration of *Agrobacterium* inoculum on the expression of reporter gene in transgenic plants.] - In: Užík, M. (ed.): Nové Poznatky z Genetiky a Šľachtenia Poľnohospodárskych Rastlín. Pp. 105-106. Výskumný Ústav Rastlinnej Výroby, Piešťany 2003. [In Slovak.]

Ordway, J.M., Detloff, P.J.: *In vitro* synthesis and cloning of long CAG repeats. - BioTechniques **21**: 609-610, 1996.

Pierson, V.L., Barcak, G.J.: Development of *E. coli* strains tolerating unstable DNA sequences on Col E1 vectors. - Focus **21**: 18-19, 1999.

Tao, Q., Zhang, H.B.: Cloning and stable maintenance of DNA fragments over 300 kb in *Escherichia coli* with conventional plasmid-based vectors. - Nucl. Acids Res. **26**: 4901-4909, 1998.

Trinh, T., Jessee, J., Bloom, R.F., Hirsch, V.: STBL2TM: an *Escherichia coli* strain for the stable propagation of retroviral clones and direct repeat sequences. - Focus **16:** 78-80, 1994.

Uranbey, S., Sevimay, C.S., Kaya, M.D., Ipek, A., Sancak, C., Basalma, D., Er, C., Özcan, S.: Influence of different co-cultivation temperatures, periods and media on *Agrobacterium tumefaciens*-mediated gene transfer. - Biol. Plant. **49**: 53-57, 2005.