Journal of Plant Biology, December 1999, 42(4) : 317-320 **SHORT COMMUNICATION**

Construction of Small Binary Vectors for *Agrobacterium*-Mediated Transformation in Plants

Jeong Hwa Lim[1], Young Na Kang[2], Young A Kim[2], Dae Heon Kim[2], and Inhwan Hwang[1]*

[1]Department of Life Science and Center for Plant Intracellular Trafficking,
Pohang University of Science and Technology (POSTECH), Pohang 790-784, Korea
[2]Department of Molecular Biology, Gyeongsang National University, Chinju 660-701, Korea

Despite the popular use of the *Agrobacterium*-mediated method for transforming plants, only a very limited number of binary vectors are available. In addition, those binary vectors are rather inconvenient for plasmid manipulation because of their large size. Here, we report the construction of small binary vectors, pBmin1 and pBminGFP1, for *Agrobacterium*-mediated transformation. The sizes of pBmin1 and pBminGFP1 are approximately 5.0 kb and 6.5 kb, respectively. These vectors contain one kanamycin resistance gene, with a double promoter for expression in both bacteria and plant cells. Vector pBminGFP1 has the 35S-GFP chimeric gene as a reporter. These small binary vectors would greatly facilitate plasmid manipulation and plant transformation.

Keywords: *Agrobacterium*, binary vector, GFP, transformation

Since the successful transformation of plant cells using T-DNA of *Agrobacterium*, numerous attempts have been made to develop suitable binary vectors that can grow in *Escherichia coli* as well as in *Agrobacterium* (Otten et al., 1981; Bevan, 1984; Wang et al., 1984; An et al., 1985; Olszewski et al., 1988; Zambryski, 1988; Becker et al., 1992; Fuse et al., 1995; Kim et al., 1996). Among the most popular binary vectors used for plant transformation are Bin19 and its derivatives (Bevan, 1984).

Although these vectors have been widely used for transformation experiments, they are large molecules and are inconvenient for plasmid manipulation. The large inserts can also show marked instability at times. Therefore, numerous attempts have been made to construct a small binary vector (Alliotte et al., 1988; McBride and Summerfelt, 1990; Hajdukiewicz et al., 1994). Frisch et al. (1995) reported that the complete nucleotide sequence of Bin19 was 11,777 bp. This analysis revealed that many regions appear to be unnecessary for the binary vector. Therefore, we attempted to construct a smaller binary vector that is easy to manipulate and provides higher transformation efficiency.

MATERIALS AND METHODS

*Corresponding author; fax +82-562-279-2199
e-mail ihhwang@postech.ac.kr

Recombinant Technology

Standard recombinant techniques were used, as described previously (Maniatis et al., 1982; Ausubel et al., 1989)

Plant Growth and Transformation

Arabidopsis plants were grown in MS medium (pH 5.7) containing 2% sucrose and 0.5 gL^{-1} MES. Cultures were kept under continuous light, at 20°C and 70% relative humidity. The transformations were carried out according to the protocol of Valvekens et al. (1988). Constructs were introduced into *Agrobacterium* (LBA4404) by electroporation, and the transgenic plants were then generated from C24 root explants.

RESULTS AND DISCUSSION

The minimum requirements for producing a binary vector are an origin of replication, the *trfA* gene for the replication factor, left and right T-DNA-border regions, and selection marker genes for bacteria and plant cells. Therefore, to generate a small binary vector we first attempted to reduce the size of the origin, *oriV*, in Bin19. A 0.7-kb DNA fragment containing the *oriV* was isolated from Bin19 by digestion with the restriction enzymes RsaI and AvaI. To determine whether the 0.7-kb fragment was sufficient to func-

tion as an origin in both *E. coli* and *Agrobacterium*, we ligated the 0.7-kb fragment to a 2.7-kb DNA fragment containing the *trfA* and *nptIII* genes. Ligated DNA gave kanamycin-resistant colonies in both *E. coli* and *Agrobacterium*, which confirmed that the 0.7-kb DNA fragment contained all the necessary elements for an origin.

The next step was to isolate a minimal DNA fragment of the *trfA* gene for replication. The *trfA* in Bin19 appears to be polycistronic with the *nptIII* gene. Therefore, a promoter region was necessary for expressing the *trfA* gene. The promoter of the *nptIII* gene was chosen for the *trfA* gene, and was isolated by PCR from the *nptIII* gene in BIN 19. We used oligonucleotide primers (5′ GGCCGGATCCTACGCCAATTTGAAAACAAC 3′; 5′ CCATATGTATTTCCTTCCTCTTTTC 3′) that corresponded to the −1 and −200 regions from the promoter of the *nptIII* gene. The 200-bp DNA fragment (named $nptIII_p$) was ligated to the promoterless *trfA* gene from Bin19. The resulting chimeric gene then was able to support replication of *oriV* in both *E. coli* and *Agrobacterium*.

We then introduced a T-DNA region into the small plasmid. To transfer a DNA fragment into plant cells by *Agrobacterium*, the plasmid must have left- and right-border regions, with a selection marker gene. Again, we reduced the size of the two border regions in Bin19. An approximately 300-bp fragment was isolated for the left-border region by digesting Bin19 with Sau3A1 and PvuII. For the right-border region, we isolated a 200-bp DNA fragment by digesting Bin19 with BglII and SacII.

As a selection marker for plant cells we chose the *nptII* gene. In the case of Bin19, two selection marker genes, *nptII* and *nptIII* for plant and bacterial cells, respectively, were used. However, we attempted to use only one kanamycin resistance gene for selection in both bacteria and plant cells to reduce the size of the binary vector. To express one kanamycin gene, *npt*II, in both prokaryotic and eukaryotic cells, a double promoter was necessary.

For bacterial cells, the 200-bp *nptIII* promoter was fused to the *nptII*-coding region isolated from Bin19. This generated a chimeric construct, the $nptIII_p$-*nptII*-*nos* terminator. The *nptII*-coding region, together with the terminator region, was isolated by using PCR. A primer (nptII-5: 5′ CCATATGATTGAACAAGATGGA 3′) corresponding to the 5′ end of the *nptII*-coding region and the T3 primer were used for PCR amplification, with a pBluescript clone containing the *nptII* gene as a template. The chimeric $nptIII_p$-*nptII* gene gave kanamycin-resistant colonies in both *E. coli* and *Agrobacterium*.

To express the *nptII* gene in plant cells, we used the same *nos* promoter of the chimeric *nptII* gene in Bin19. This promoter was also isolated by PCR, using the T7 primer and a specific primer (nos-3: 5′ GGATCCGGTGCAGATTAT 3′) that corresponded to the -1 region of the *nos-nptII* gene in Bin19. A pBluescript clone containing the *nos-nptII* gene was the template. The 200-bp *nos* promoter was fused to the $nptIII_p$-*nptII* chimeric gene to generate nos_p-$nptIII_p$-

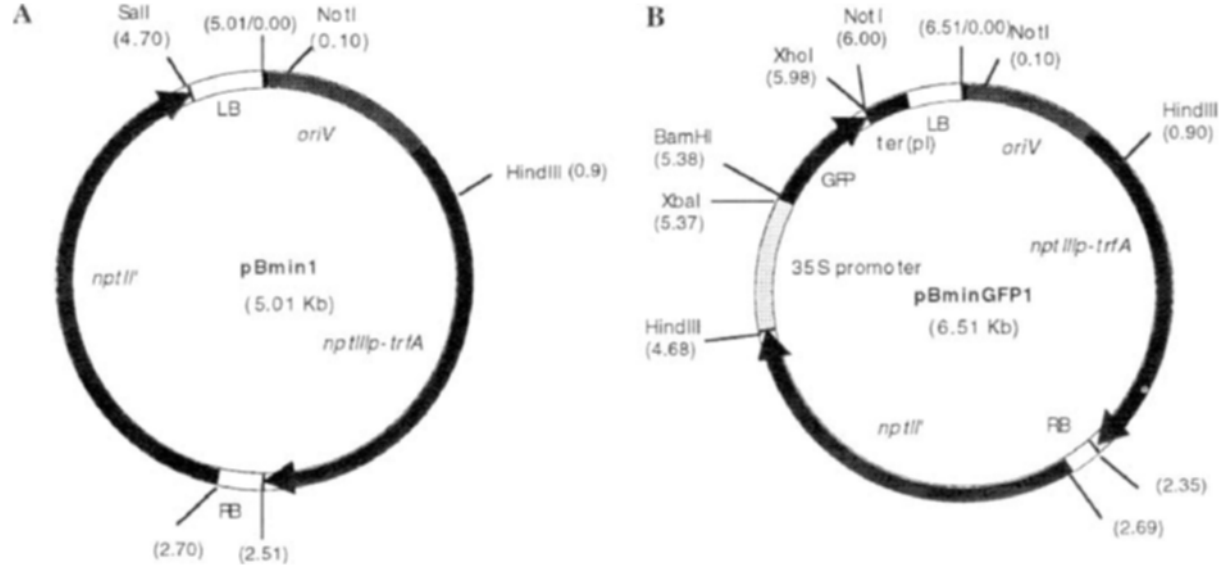

Figure 1. Restriction maps for plasmids of pBmin1 (A) and pBminGFP1 (B). *oriV*, broad-host-range origin; *nptIII*p-*trfA*, the chimeric *trfA* gene with *nptIII* promoter; RB, right-border region; LB, left-border region; *nptII'*, the chimeric *nptII* gene with *nptIII* promoter and *nos* promoter; GFP, green fluorescent protein gene; ter(pI), 3′ region of the potato protease inhibitor II gene.

nptII (named *nptII'*). Thus, the *nptII'* gene had a double promoter consisting of the *nptIII* and *nos* promoters for expression in bacteria and plant cells, respectively.

The *nptII'* gene was then inserted into pBluescript containing the two border regions to generate pBlueT-DNA. The T-DNA region was re-isolated from pBlueT-DNA and, subsequently, ligated to the DNA fragment containing the *oriV* and $nptIII_p$-*trfA* chimeric gene to give pBmin1. Thus, the final version of the new binary vector, pBmin1, which is approximately 5 kb in size, consists of two border regions, the selection marker *nptII'* gene, *oriV*, and the $nptIII_p$-*trfA* gene for replication (Fig. 1A).

We also constructed an expression vector based on pBmin1. As a reporter gene, the 35S-GFP chimeric gene was isolated from pBI121mGFP (a gift from J. Haseloff, MRC, UK). The *nos* terminator of the 35S-GFP chimeric gene was replaced with a 300-bp DNA fragment (ter(pI)) from the 3' end of the protease inhibitor II gene of potato (Keil et al., 1986). This was done because the *nos* terminator was used as a terminator for the *nptII'* gene. The new 35S-GFP-ter(pI) chimeric gene was introduced into the SalI site, which is located downstream of the *nptII'* gene of pBmin1, to generate pBminGFP1 (Fig. 1B).

Because we modified many regions, including the promoter region of the *nptII* gene, we examined the transformation efficiency of these new vectors. The new binary vector, pBminGFP1, was able to generate kanamycin-resistant calli from *Arabidopsis* root explants. Green calli appeared approximately one week after the root explants were cultivated on shoot-induction media. Interestingly, the green calli were generated slightly more rapidly using pBminGFP1 than pBI121. More importantly, pBminGFP1 generated more green calli than did pBI121 (Table I), which indicates that the transformation efficiency of the new small vector is better than that of pBI121.

We also examined the expression of green fluorescent protein (GFP) in the leaf tissues of regenerated plants after three weeks in culture. The leaf tissues showed bright green fluorescence when examined under a fluorescence microscope (data not shown). This indicates that the cells transformed with pBminGFP1 express GFP.

Table 1. Transformation efficiency of pBminGFP1.

Construct	1 week	2 weeks	4 weeks
pBI121	0	30%	60%
pBminGFP1	5%	50%	80%

The transformation efficiency was calculated as the number of green calli per 100 root explants. Growth was on 100 μg mL^{-1} kanamycin. The percentages are the average of four identical plates.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ministry of Science and Technology (UR). We thank Dr. J. Haseloff for donating the plasmid pBI121mGFP.

Received November 22, 1999; accepted November 29, 1999.

LITERATURE CITED

Alliotte T, Zhu LH, van Montagu M, Inzé D (1988) Plant expression vectors with the origin of replication of the W-type plasmid Sa. Plasmid 19: 251-254

An G, Watson BD, Stachel S, Gordon MP, Nester EW (1985) New cloning vehicles for transformation of higher plants. EMBO J 4: 277-284

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1989) Current Protocol in Molecular Biology. New York: Greene Publishing Associates/Wiley-Interscience

Becker D, Kemper EK, Schell J, Masterson R (1992) New plant binary vectors with selectable markers located proximal to the left T-DNA border. Plant Mol Biol 20: 1195-1197

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 12: 8711-8721

Frisch DA, Harris-Haller LW, Yokubaitis NT, Thomas TL, Hardin SH, Hall TC (1995) Complete sequence of the binary vector Bin 19. Plant Mol Biol 27: 405-409

Fuse T, Kodama H, Hayashida N, Shinozaki K, Nishimura M, Iba K (1995) A novel Ti-plasmid-convertible lambda phage vector system suitable for gene isolation by genetic complementation of *Arabidopsis thaliana* mutants. Plant J 7: 849-856

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol 25: 989-994

Keil M, Sanchez-Serrano J, Schell J, Willmitzer L (1986) Primary structure of a proteinase inhibitor II gene from potato (Solanum tuberosum). Nucleic Acids Res 14: 5641-5650

Kim M-S, Park J-K, Eum J-S, Sim W-S (1996) Effective combination of *Agrobacterium tumefaciens* strains and Ti plasmids for the construction of plant vector system. J Plant Biol 39: 179-184

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Laboratory, MA

McBride KE, Summerfelt KR (1990) Improved binary vectors for *Agrobacterium*-mediated plant transformation.

Plant Mol Biol 14: 269-276

Olszewski N, Martin FB, Ausubel FM (1988) Specialized binary vector for plant transformation: expression of the Arabidopsis AHAs gene in Nicotiana tabaccum. Nucl Acids Res 16: 10765-10782

Otten L, de Greve H, Hernalsteens JP, van Montagu M, Schieder O, Straub J, Schell J (1981) Mendelian transmission of genes introduced into plants by the Ti plasmids of *Agrobacterium tumefaciens*. Mol Gen Genet 183: 209-213

Valvekens D, van Montagu M, van Lijsebettens M (1988) *Agrobacterium tumefaciens*- mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci USA 85: 5535-5540

Wang K, Herrera-Estrella L, van Montagu M, Zambryski P (1984) Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. Cell 38: 455-462

Zambryski P (1988) Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. Ann Rev Genet 22: 1-30