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Full Length Research Paper

An efficient marker-free vector for clean gene transfer into plants

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A marker-free vector, pBINMF, for clean gene transfer was constructed based on the binary vector pBINPLUS. Vector pBINMF, carrying only a multiple cloning site (MCS) between the left and the right T-DNA border, was suitable to directly generate marker-free transgenic plants (MFTPs) without any vector sequences inserted into plant genomes. Transformation efficiency of pBINMF was tested by transferring *gus*A reporter gene and *npt*II gene into tomato, respectively. The results from three experiments show that 4.4% of regenerated shoots were transgenic by polymerase chain reaction (PCR) test, and the phenotype expression percentage of PCR positive transformants was 57.4% as tested by beta-glucuronidase (GUS) assay. Moreover, 24 out of 27 phenotype positive transformants were homogeneous as confirmed by blue staining over whole leaves and roots in GUS staining. The vector might also be used in tobacco marker-free gene transfer as was confirmed by transient expression assay of *gus*A. The results indicate that pBINMF was an efficient and reliable vector to directly generate complete MFTPs in plant species, and might be especially suitable for vegetatively propagating species.

Key words: Marker-free vector, GUS assay, tomato, tobacco.

INTRODUCTION

Agrobacterium-mediated plant transformation commonly occurs at a very low frequency. Hence, selection marker genes usually encoding antibiotic and herbicide resistance (Bevan et al., 1983; Shah et al., 1986) are widely used to identify rare plants carrying foreign DNA. Nowadays, concern is growing that antibiotic- or herbicide-resistance genes could spread into genomes of wild-type plants or microorganisms, with a potential problem to human health and the ecosystem (Dale et al., 2002). To overcome this problem, efforts were made to generate transgenic plants without selectable marker gene, for example by co-transformation (Komari et al., 1996; Zubko et al., 2000; Zuo et al., 2001; Ma et al., 2008; Sun et al., 2009; Cui et al., 2011). However, these methods were all based on the assumption that it was not feasible to isolate transgenic plants without selectable markers (Ow, 2001; Leclercq et al., 2010; Sengupta et al., 2010), thus marker genes will be transferred into plant cells first and then removed by tedious procedures, which make these strategies time-consuming and inefficient.

The best solution to get marker-free transgenic plants (MFTPs) is to avoid the use of selectable marker genes in the beginning of transformation by a marker-free vector. De Vetten et al. (2003) and Jia et al. (2007) obtained successfully MFTPs by this method. However, the vectors they used still carried partial sequences of disabled marker gene cassette (*npt*II and its regulatory elements) and other vector sequences between the left and right T-DNA border. These redundant sequences were transferred along with the genes-of-interest into plant genome, which may be harmful to human health and the environment (Yoder and Goldsbrough, 1994).

Advances in clean gene transformation technology allow introduction of genes-of-interest into plants without antibiotic resistance marker genes or vector sequences (Yoder and Goldsbrough, 1994). Here we described the construction and application of an improved marker-free

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Abbreviations: MFTPs, Marker-free transgenic plants; **MCS,** multiple cloning site; **GUS,** beta-glucuronidase; **PCR,** polymerase chain reaction.

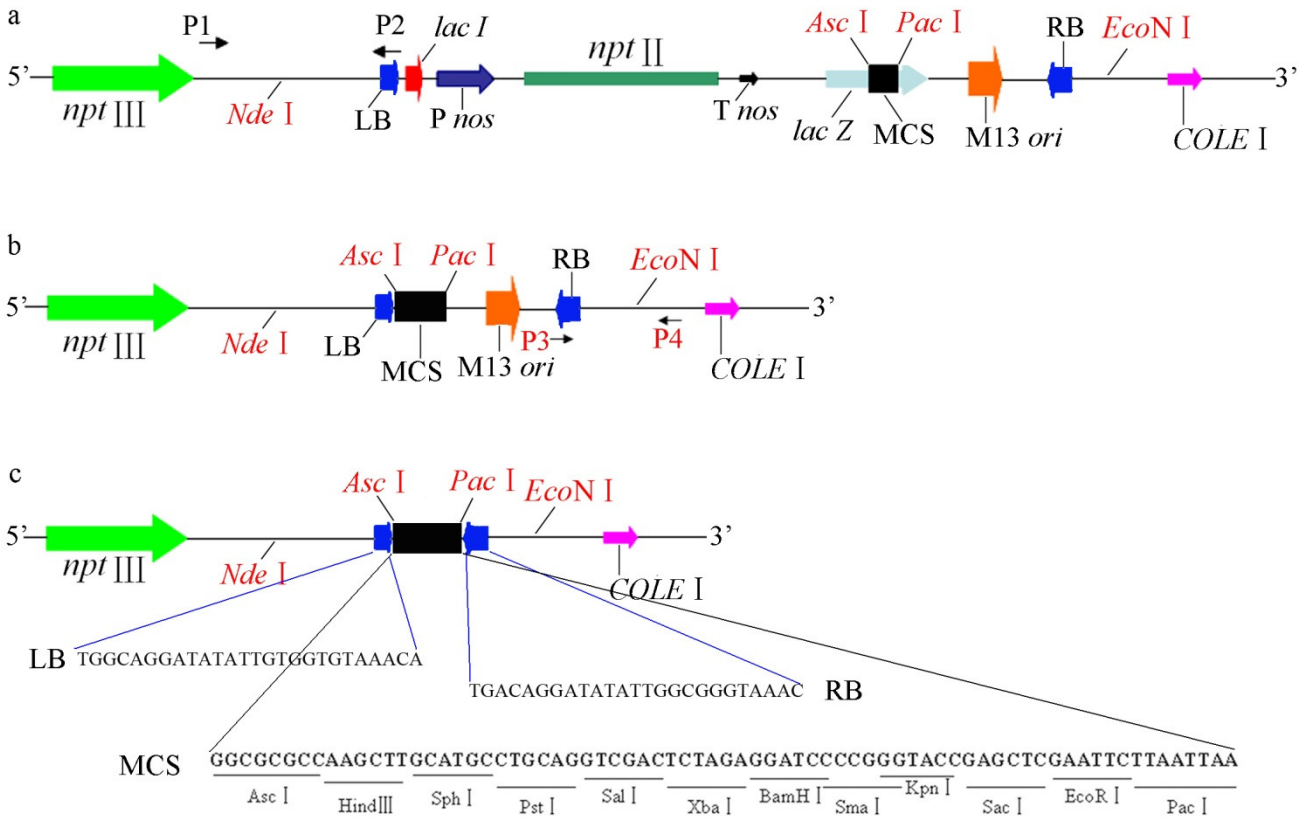

Figure 1. Schematic presentation of three binary vectors. (a) The vector pBINPLUS. (b) The vector pBINLD, in which a 2749-bp fragment containing the *npt*II gene under the control of Pnos and other ancillary sequences was deleted from pBINPLUS. (c) The marker-free vector pBINMF carrying only a MCS between LB and RB. Compared to pBINLD, in pBINMF a 309-bp fragment was deleted. *npt*II, a plant selective marker gene, codes for neomycin phosphotransferase II. Pnos: Promoter of nopaline synthase gene (*nos*); Tnos: terminator of nopaline synthase gene (*nos*); LB and RB, the left and right borders of T-DNA; MCS, multiple cloning sites. *npt*III, Encoding neomycin phosphotransferase III, confers resistance to kanamycin; colE1, a region coming from colE1 plasmid, involved in DNA replication; M13 ori, an origin of replication derived from M13 phage; lacZ, encoding bacterial enzyme β-galactosidase, a reporter gene for selection; lacI; encoding *lac* repressor which is a DNA-binding protein which inhibits the expression of genes coding for proteins involved in the metabolism of lactose in bacteria. *In vitro*, Isopropyl β-D-1-thiogalactopyranoside (IPTG) is a commonly-used allolactose mimic which can be used to induce transcription of genes being regulated by *lac* repressor.

transformation system for this purpose. Due to the absence of any vector sequences between the left and the right T-DNA border in pBINMF, only the gene-of-interest was integrated into the plant genome, the transgenic plants by this vector were safe to human health and environment.

MATERIALS AND METHODS

Construction of marker-free vector pBINMF

A fragment of approximately 3 kb containing the left T-DNA border was amplified from the DNA of plasmid pBINPLUS (van Engelen et al., 1995) (Figure 1a) using primers P1(5′-ACCAGGCGG-GTCAAATCAGG-3′) and P2 (5′-TTggcgcgccAAATTA-AAAACGTCCG CAATGTGTATTAAG-3′), the lower case letters represent an *Asc*I site. Then the polymerase chain reaction (PCR) product was digested with *Asc*I (New England Biolab, NEB) and *Nde*I (NEB) and subsequently inserted into pBINPLUS digested with the same enzymes to produce the vector pBINLD (Figure 1b). An approximately 1-kb fragment containing the right T-DNA border was amplified from pBINPLUS using primers P3 (5′-CCttaattaaGGAAACTATCAGTGTTTGACAGGATATATTGGC-3′) and P4 (5′-CGACAATCCCGCGAGTCCC-3′), the lower case letters represent a *Pac*I site. Then the PCR product digested with *Pac*I (NEB) and *Eco*NI (NEB) was ligated into pBINLD digested with the same enzymes, thus resulting in the final marker-free vector, pBINMF (Figure 1c). After two PCR amplifications combined with two enzyme digestions, the *npt*II gene and the other vector sequences between left border and right border of T-DNA in pBINPLUS were removed completely. To test whether DNA sequences of PCR products inserted into vectors were right, DNA sequencing of pBINMF was performed with primers P1 and P4. The PCR amplifications were performed using *pfu* Ultra™ High-Fidelity DNA polymerase (Stratagene, Germany). Primer synthesis and DNA sequencing were performed at Shanghai Sangon Biological Engineering Technology and Services Co.,Ltd.

Generation of expression cassette

To test the transformation efficiency of the pBINMF, *gus*A and *npt*II genes were cloned into pBINMF, respectively. A fragment containing the cauliflower mosaic virus 35S promoter, *gus*A gene and nos terminator was excised with *Eco*RI plus *Hin*dIII from pBI121 (Amicis et al., 2007) and inserted into the same sites of pBINMF, thus resulting in pBINMF-*gus*A plasmid. An 800-bp PCR product (*npt*II) was amplified from pBI121 using the following primers PN1: 5'-TCGtctagaATGATTGAACAAGATGGATTGCACGCAGGTTC-3' and PN2: 5'-GCTggatccGTCATTTCGAACCCCAGAGT-CCCGCTCAG-3'; the lower case letters are *Xba* I and *Bam*H I sites, respectively, and then the PCR product was ligated into *Bam*HI and *Xba*I sites of pSN1301 (Li et al., 2006) to construct pSN1301- *npt*II. The fragment containing the cauliflower mosaic virus 35S promoter, *npt*II gene and CAMV polyA was excised with *Eco*RI plus *Hin*dIII from pSN1301- *npt*II and inserted into the same sites of pBINMF, thereby resulting in the recombinant vector pBINMF- *npt*II. The two recombinant vectors were introduced into supervirulent *Agrobacterium tumefaciens* strain, AGL0 (Lazo et al., 1991) respectively, by triparental mating method. Recombinant *Agrobacterium* cells containing pBINMF-*gus*A and pBINMF- *npt*II, named *Agro*:*gus*A and *Agro*: *npt*II respectively, were used for the genetic transformation, and the transformation efficiency was calculated by the number of independent, confirmed transgenic shoots / the total number of explants inoculated × 100 (in %).

Tomato transformation and regeneration

Seeds of tomato cultivar 'Moneymaker' were surface-sterilized in 95% (v/v) ethanol for 30 s and in 20% (v/v) sodium hypochlorite for 10 min consecutively, followed by three rinses with sterilized double-distilled water. Seeds were germinated in a plant box with 40 ml of 1/2 MS medium (Murashige et al., 1962), 30 g L^{-1} sucrose and 5.5 g L^{-1} agar. Plants were maintained in culture room at 24°C under a 16-h photoperiod provided by cool-white fluorescent tubes at a photon flux density of approximately 70 µE $m^{-2}s^{-1}$. The seedlings were used in genetic transformation when the cotyledons just emerged from the seed coats but the first true leaf had not yet emerged.

Prior to the transformation, *Agro*:*gus*A and *Agro*: *npt*II were grown in 3 ml yeast extract and beef (YEB) medium (5 g L^{-1} beef extract, 1 g L^{-1} yeast extract, 5 g L^{-1}peptone, 5 g L^{-1} sucrose and 2 mM $MgSO_4$) with appropriate antibiotics at 28°C respectively. Then 2 ml of overnight culture was inoculated into 100 ml YEB medium, and the inoculum was grown at 240 rpm to log phase (OD_{600} = 0.8), centrifuged, and resuspended in full strength liquid Murashige and Skoog (MS) medium without sucrose to a final OD_{600} of 0.5 for transformation. Cotyledon explants were immersed in the above *Agrobacterium* suspension supplemented with 200 µM acetosyringone (AS) respectively for 10 min. After inoculation, the treated explants were blotted dry on sterile filter paper and then placed on co-culture medium with the abaxial side down, and incubated in the dark for two days. The explants were then transferred on shoot inducing medium (MS medium supplemented with 20 g L^{-1} sucrose, 100 mg L^{-1} inositol, 0.5 mg L^{-1} folic acid and 5.5 g L^{-1} agar) containing 2 mg L^{-1} trans-Zeatin-riboside (Sigma Z3541) and 200 mg L^{-1} timentin. After culture on shoot-inducing medium for about three weeks, many regenerated shoots were found growing on the cotyledon segments. Shoots were excised from the explants and rooted on MSSV medium (MS medium containing 30 g L^{-1} sucrose, 0.5 mg L^{-1} folic acid and 5.5 g L^{-1} agar) with 0.5 mg L^{-1} indole-3-butyric acid (IBA) and 200 mg L^{-1} timentin. After two to three weeks, the rooted shoots were transferred into soil for further analysis.

Polymerase chain reaction analysis

Genomic DNA of all plants were extracted by hexadecyltrimethyl ammonium bromide (CTAB) method according to Smith (2005). Transgenic plants were identified by specific genomic DNA PCR analysis individually. The primers used to amplify the g*us*A gene were PG1 (5′-TCCTGTAGAAACCCCAACCCG-3′) and PG2 (5′-CCATCAGCACGTTATCGAATC-C-3′). Another pair of primers PN1 and PN2 earlier mentioned were used to amplify an 800-bp fragment for the *npt*II gene.

Histochemical assay of beta-glucuronidase (GUS) activity

GUS assay was carried out according to the method described by Jefferson (1987). Leaves and roots of two-week-old transgenic tomatoes were immersed in a solution of 2 mM 5-bromo-4-choro-3-indolyl-β-D-glucuronic acid (X-Gluc) in 50 mM sodium phosphate (pH7.0) for 10 h at 37°C. The stained tissue was then transferred into 70% (v/v) ethanol for 24 h to remove chlorophyll. Wild-type tomato was used as control.

Tobacco transient expression

Four to eight-week old tobacco plants grown in standard greenhouse conditions were used in the experiments. Liquid cultures of *A. tumefaciens* for leaf infiltration were prepared following the method of Wroblewski et al. (2005). Infiltration was performed as described by Schob et al. (1997). GUS activity was evaluated at the fourth, eighth, tenth and twelfth days after inoculation (DAI) following the method of Jefferson (1987).

RESULTS AND DISCUSSION

A marker-free vector, pBINMF, was constructed based on pBINPLUS by removal of unwanted sequences including the marker gene in pBINPLUS, and the new construct was verified by combination of restriction enzyme digestion and sequencing. Comparisons of restriction patterns of pBINLD and pBINMF with *Asc*I/*Nde*I and *Pac*I/*Eco*NI respectively, were consistent with the theoretical prediction (Figure 2). The fragment of about 3-kb which was removed first contained the selectable marker gene (*npt*II) and other vector sequences between left and right borders of T-DNA. The sequencing result (Figure 1c) indicates that pBINMF contained only a multiple cloning site between the left border and the right border of T-DNA and thus confirmed the restriction pattern. 12 restriction enzyme sites (Figure 1c) are available in the multiple cloning site of pBINMF, which facilitate the insertion of genes-of-interest. Thus, the vector pBINMF should provide a useful tool for directly obtaining MFTPs.

Three changes were found from the sequencing results. Two pairs of thymine (TT) and guanines (GG) upstream and downstream the multiple cloning site in pBINMF were introduced by protective bases for restriction enzyme in primers in the process of construction. A mutant base from adenine (A) to guanine (G) also was found upstream the right T-DNA border for an unknown reason. Those

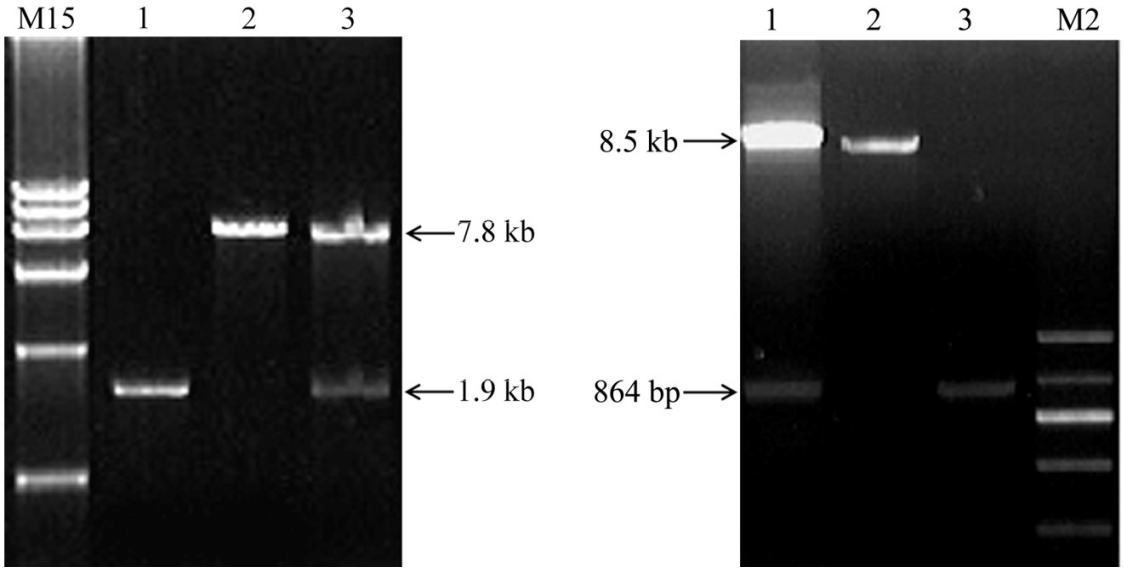

Figure 2. Restriction patterns of pBINLD (left) and pBINMF (right) with *Asc* Ⅰ/*Nde* Ⅰ and *Pac* Ⅰ/*Eco*N Ⅰ, respectively. Left panel: lanes 1 and 2, the large fragments purified from digested PCR product and pBINPLUS, respectively; lane 3, the enzyme digestion of the vector pBINLD with *Asc*I/*Nde* I; M15, DNA marker DL15000. Right panel: lane 1, the enzyme digestion of the vector pBINMF with *Pac*I/*Eco*NI; lanes 2 and 3, the large fragments purified from digested pBINLD and PCR product, respectively; M2, DNA Marker II. PCR, Polymerase chain reaction.

changes seemed not to affect the usability of pBINMF as shown in the following experiments. Furthermore, in order to test whether foreign genes could be efficiently introduced into plant genome by pBINMF, *gus*A gene was inserted into pBINMF to construct pBINMF- *gus*A. pBINMF- *gus*A was transferred into *A. tumefaciens* strain AGL0 (de Vetten et al., 2003) to transform tomato cotyledon explants. Strain AGL0 exhibits high transformation efficiency because it contains a DNA region originating from the virulence region of Ti plasmid pTiBo542 of the supervirulent strain A. *tumefaciens* A281. In the following experiments, only timentin was added to the medium to suppress *Agrobacterium* growth without any antibiotic selection for transgenic plants. PCRs were conducted as described by de Vetten et al. (2003) to detect the *gus*A transgenic plantlets using primers of PG1 and PG2.

Histochemical GUS staining was used to confirm the PCR results in our experiments. *Gus*A encoding GUS can be detected directly by histochemical assay and is commonly used as a convenient and visible reporter for the identification of transformed plants. First, 1044 regenerated plantlets were identified by PCR analysis individually (Figure 3 left). Subsequently, to make sure that PCR positive plantlets were also positively expressed, leaves of these plantlets were dipped in X-Gluc staining solution to detect GUS activity (Figure 3 right). The 47 PCR positive plantlets were verified by GUS assay and 27 of them showed blue coloration (Table 1). The efficiency of GUS-positive plantlets varied from 40 to 75% in three experiments, with the average positive efficiency of 57.4% (Table 1). The absence of GUS expression in the rest of the plantlets was probably caused by gene silencing of the *gus* A transgene, or GUS expression level was too low to be detected (Matzke and Matzke, 1998). The 57.4% average efficiency of phenotypic plantlets was higher than the previously reported 45.1% (de Vetten et al., 2003) and 47.6% (Jia et al., 2007). Higher efficiency might be due to reduction of homology-based gene silencing resulting from the absence of redundant sequences between the left and right border of T-DNA in pBINMF. Our results therefore suggested that pBINMF was a useful vector for directly generating complete-marker-free transformants.

The 27 *gusA* positive transgenic plantlets were further analyzed by GUS staining to determine the homogeneity of the transformants. In this case, homogeneous transgenic lines were identified by totally blue leaves and roots in GUS staining (Figure 3 right, C), whereas heterogeneous transgenic lines were identified by partially blue leaves and roots in GUS staining (Figure 3 right, A). As shown in Table 1 of the 27 GUS positive transformants from the three experiments, 24 were completely GUS stained, whereas three were partially GUS stained. These observations indicated that chimerism occurred at a low frequency (11%). To compare the transformation efficiency of pBINMF (transformation without selection) with that of conventional vectors (transformation with selection), we cloned *npt*II gene into pBINMF and infected tomato cotyledon explants with AGL0 carrying the recombinant vector pBINMF-*npt*II. The explants of tomato cotyledons after inoculation were transferred on solid cul-

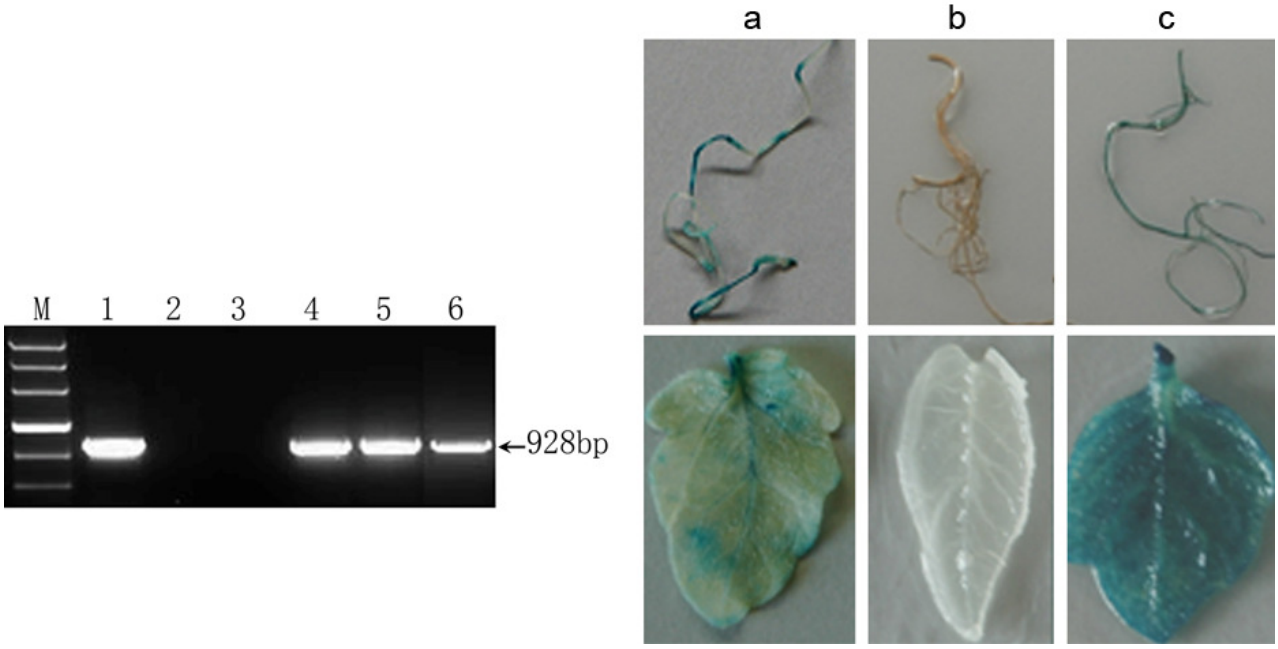

Figure 3. PCR amplification of *gus*A gene (left) and GUS assay (right) for transgenic tomatoes by pBINMF transformation. Left panel: lane 1, pBI121 carrying the *gus*A gene; lanes 2 and 3, the wild type control and pseudo-transformant; lanes 4 to 6, positive transgenic tomatoes; M, DNA Marker III. Right panel: roots (top) and leaves (down) of heterogeneous transgenic plant (a), non-transgenic control (b) and homogenous transgenic plant (c) respectively. PCR, Polymerase chain reaction; GUS, beta-glucuronidase.

Table 1. Efficiency of pBINMF Transformation Mediated with *A .tumefaciens* strain AGL0.

Experiment no.	No. of PCR *npt*II^{+}shoot/ tested (%)		No. of GUS^{+} line (%)		
	With-selection	Without-selection	GUS^{+b}/GUS^{+a}	GUS^{+c}/GUS^{+b}	GUS^{+a}/GUS^{d}
1	2/3 (66.7)	25/431 (5.8)	9/12 (75.0)	7/9 (88.9)	12/221 (5.4)
2	5/6 (83.3)	20/357 (5.6)	10/15 (66.7)	9/10 (90.0)	15/350 (4.3)
3	2/4 (50.0)	12/518 (2.3)	8/20 (40.0)	8/8 (87.5)	20/473 (4.2)
Total no.	9/13 (69.2)	57/1306 (4.4)	27/47 (57.4)	24/27 (88.9)	47/1044 (4.5)

[a]Number of positive transformants confirmed by PCR; [b]number of regenerates showing GUS activity; [c]number of homogeneous regenerates tested by GUS histochemical staining; [d]number of regenerated plantlets tested in *gus*A transformation. GUS, beta-glucuronidase; PCR, polymerase chain reaction.

ture mediums with and without kanamycin, respectively. After two to four weeks, shoots appeared on the explants. When the shoot grew to about 1 cm, we cut them off from explants and transferred into rooting medium for further PCR analysis using gene-specific primers, PN1 and PN2. As shown in Table 1, the transformation efficiency of pBINMF ranged from 2.3 to 5.8% in three experiments, and the average frequency of 4.4% agreed well with the previously reported efficiency of 4.5% (de Vetten et al., 2003). Although the transformation efficiency of conventional vectors was 69.2% with kanamycin selection, the shoots generated from the explants appeared later and in much lesser numbers than those generated from the explants without selection due to kanamycin suppression of plant regeneration. A large number of shoots obtained from explants without kanamycin selection made it possible to easily obtain MFTPs, even though the transformation efficiency of pBINMF was lower than that of conventional vector.

In order to test whether pBINMF can also be used for transformation of other plant species than tomato, the leaves of four- to eight-week old tobacco were agro-injected for monitoring transient expression. According to Janssen and Gardner, (1990), GUS expression at 10 to 14 DAI was due to the stable integration of the T-DNA, whereas a peak of GUS expression in leaf disc at three to four DAI was caused by the transient expression of the un-integrated T-DNA. Therefore, GUS staining in the treated leaves in our experiments was investigated at the fourth, eighth, tenth and twelfth (Figure 4) DAI and GUS

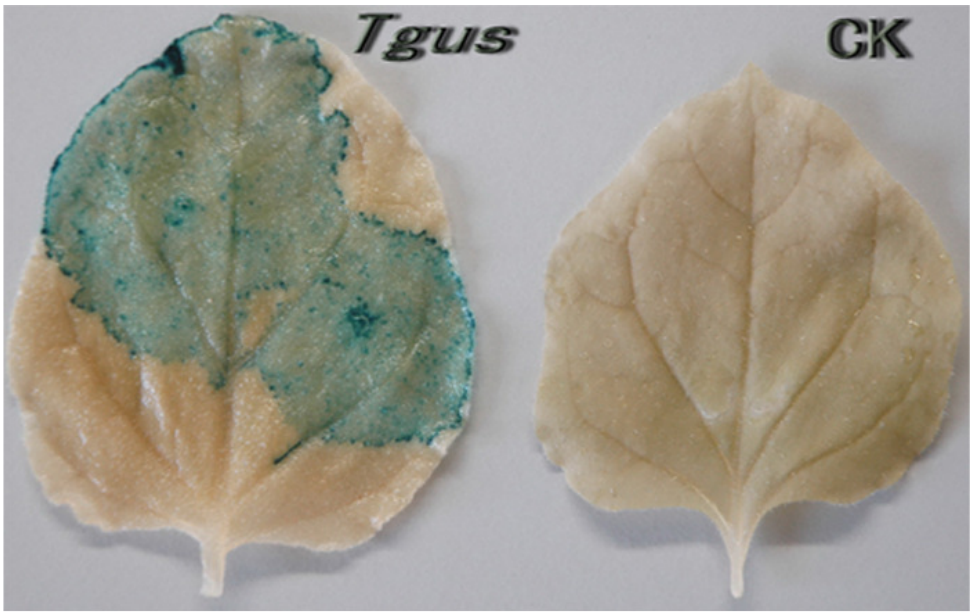

Figure 4. Staining results of GUS transient assay 12 DAI in tobacco by marker-free vector pBINMF. Tgus and CK indicate the leaves of tobacco injected AGL0 with pBINMF-*gus*A and pBINMF, respectively. Three independent experiments were carried out. GUS, β-Glucuronidase; DAI, days after inoculation.

activities were present in all, although the blue coloration varied to some degree. This result indicates that pBINMF could be used in another species for plant marker-free transformation.

In summary, pBINMF is an efficient marker-free vector with a number of useful features. First, pBINMF is applicable for generating marker-free transgenic plants directly. It does not require genetic segregation, which is an advantage especially in the case of vegetatively propagated plants like potato, cassava, banana and grape. Secondly, pBINMF is different from the previous maker-free vectors (de Vetten et al., 2003; Jia et al., 2007) in that antibiotic gene and other vector sequences were completely removed between the left and right border of T-DNA. Without genes encoding antibiotic resistance and other unwanted sequences, pBINMF is safer to create transgenic plants with lower risks to human health and the environment.

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