Botanical Studies (2011) 52: 239-248.

Construction of the binary vector with bi-selectable markers for generating marker-free transgenic plants

Ching-Yi LIN[1], Hsin-Mei KU[2], Chee-Wee TAN[1,2], Shyi-Dong YEH[1], and Fuh-Jyh JAN[1,*]

[1]*Department of Plant Pathology, National Chung Hsing University, Taichung 402, Taiwan*
[2]*Department of Agronomy, National Chung Hsing University, Taichung 402, Taiwan*

(Received January 22, 2010; Accepted November 5, 2010)

ABSTRACT. Plant transformation typically involves antibiotic or herbicide resistance genes as selection markers to identify the transformed plants. However, there have been public concerns over the safety of the marker genes that remain in transgenic plants. It is therefore desirable to remove marker genes prior to the release of transgenic plants. In this study, we used the co-transformation strategy to develop a binary vector with bi-selectable markers, pGA2TNH. Such strategy enables the generation of marker-free transgenic plants and increases utilization of additional plant species, especially crops with natural resistance or tolerance to kanamycin. One plasmid carrying two separated T-DNAs was adopted in order to construct multiple selectable markers in one of the T-DNAs. Markers used were the *hpt* gene for hygromycin resistance and the *npt*II gene for kanamycin resistance. pGANP-CP1/pBin19 and pGA2T-CP1 were constructed to evaluate and compare their efficacy in generating marker-free transgenic plants. The former consisted of two individual plasmids carrying separate T-DNA of the target and marker genes, while the latter contained a single plasmid carrying two T-DNAs for the target and marker genes. In pGA2TNH system, the co-transformation frequency of the R_0 transgenic *Nicotiana benthamiana* plants with both selection markers and target gene was 50%, which was as efficient as the other two systems. As expected, segregation of the two T-DNAs was observed in progeny. In one marker gene copy of transgenic plants, the elimination of marker gene was found at a ratio of 17.5% in pGA2TNH system, and 18.6% and 24.1% in pGA2T-CP and pGANP-CP1/pBin19, respectively. This demonstrated that the binary vectors we constructed were efficient and feasible in eliminating marker genes. It also provides a practical and simple tool for generating marker-free transgenic crops, which will have a significant impact on their acceptance by the public.

Key words: Co-transformation; Marker gene-free; Transgenic plant.

INTRODUCTION

Selectable marker genes are indispensable for the identification of transformed cells among a large number of untransformed ones during the plant transformation process. Antibiotic and herbicide resistance genes are the most efficient and widely-used selectable markers (Miki and McHugh, 2004). Although selectable marker genes play no role in genetically modified plants after transformation, their presence has caused increasing concerns, especially around the issues of environmental safety and public health (Goldstein et al., 2005). Thus, there is an imperious need to eliminate selectable marker genes, especially those with antibiotic and herbicide resistance, for environmental and public safety. Moreover, limited suitable marker genes are available for recalcitrant plants, while removal of marker genes could allow the successive transformations to continue recycling the same selectable marker (Jacob and Veluthambi, 2002).

To date, several methods: co-transformation, site-specific recombination (i.e., Cre/*loxP*, FLP/*FRT* and R/*RS*), transposon-based expelling systems (i.e., Ac transposon) and antibiotic or herbicide gene replacement (metabolic chemical or enzyme), have been developed to remove selectable marker genes (for reviews see Yoder and Goldsbrough, 1994; Ebinuma et al., 2001; Hare and Chua, 2002; Puchta, 2003; Darbani et al., 2007). Among these, co-transformation seems the simplest approach, since development or application of additional selectable markers or DNA excision systems is not required (Daley et al., 1998).

Co-transformation regularly relies on *Agrobacterium*-mediated transformation. This involves the introduction of two separate T-DNAs into the same plant cell, one of which carries the selection marker and the other carrying the gene of interest. If these two different T-DNAs are integrated into unlinked loci, the marker-free transgenic plants are segregated at the progeny level (Ebinuma et al., 2001). In principle, co-transformation consists of three methods depending on where the desired gene and the

*Corresponding author: E-mail: fjjan@nchu.edu.tw; Fax: +886-4-22854145; Tel: +886-4-22840780 ext. 360.

selectable marker are inserted. They can be inserted in (1) two T-DNAs within the same binary vector (two-T-DNA/one-plasmid) (Depicker et al., 1985; Komari et al., 1996; Xing et al., 2000; Lu et al., 2001; Matthews et al., 2001; Miller et al., 2002; Xue et al., 2003; Zhou et al., 2003; Breitler et al., 2004; Chen et al., 2005), (2) two binary vectors within the same *Agrobacterium* strain (two-plasmid/one-strain) (de Framond et al., 1986; Daley et al., 1998; Jacob and Veluthambi, 2002; Permingeat et al., 2003; Higgins et al., 2006) or (3) two binary vectors with two different *Agrobacterium* strains (two-plasmid/two-strain) (Depicker et al., 1985; McKnight et al., 1987; De Block et al., 1991; Komari et al., 1996; De Neve et al., 1997).

Different approaches for the elimination of selectable marker genes have been developed over the last several years, but most of them have been patented. Since the co-transformation system is the simplest strategy and can be applied to a wide range of plant species, we have focused on using it to establish an efficient marker-free transformation system. Meanwhile, there are crops such as orchids that are naturally resistant or tolerant to kanamycin and require more than 500 mg/l kanamycin for selection (Chia et al., 1994). Kanamycin is also considered ineffective for several legumes and gramineae (Ziemienowicz, 2001). This characteristic limits the use of the marker-free system to those carrying the *npt*II gene as the selection marker. In this study, we adopted a one plasmid with two T-DNAs system to develop a marker-free binary vector carrying multiple selectable markers in one T-DNA and enabled the binary vector to alleviate the limitation of the usage of *npt*II. Co-transformation of *Nicotiana benthamiana* using single *A. tumefaciens* strain was adapted to allow the use of a selection marker during plant regeneration followed by recovery of progeny carrying the desired gene but not the marker gene. Our results indicated that the system developed in this study can be useful in generating marker-free transgenic plants for commercial applications, especially those plants having natural resistance or tolerance to kanamycin.

MATERIALS AND METHODS

Vector construction

The pGA482G carrying pRK2 replication origin and the gentamycin resistance gene for bacterial selectable marker (Jan, 1998) was digested using *Bam*HI/*Hin*dIII and a 2 kb fragment, including the plant selection marker gene *npt*II, was excised from the T-DNA region of the vector. The digested vector was then ligated with annealed polylinker, which was prepared by annealing two complementary oligonucleotide primers FJJ 2003-3/FJJ 2003-4 (Table 1). The resulting plasmid, with pRK2 replication origin and the gentamycin resistance gene for bacterial selectable marker, was designated as pGANP (Figure 1A). The expression cassette containing the *Tomato leaves curl virus* (ToLCV) partial coat protein (CP1) gene under the *Cauliflower Mosaic Virus* (CaMV) 35S promoter was digested using *Kpn*I/*Hind*III and inserted into the binary vector, pGANP. The plasmid carrying the CP1 gene as the target gene and without the selectable marker was named pGANP-CP1 (Figure 1C).

The vector with two T-DNAs was designed as follows: the marker-free binary vector pGANP (Figure 1A) was modified to contain two separate T-DNAs, one containing a selection marker (*npt*II) and the other carrying the CP1 gene. The other set of right and left borders was prepared as follows: the multiple cloning sites in pBin19 (Frisch et al., 1995) were removed by digesting with *Eco*RI/*Hin-d*III and the nick was ligated with an annealed polylinker, which was prepared by annealing two complementary oligonucleotide primers FJJ2004-70/FJJ2004-71 (Table 1). The inter-medium vector was designated as pBin20. The T-DNA region containing *npt*II from pBin20 was amplified by polymerase chain reaction (PCR) (with LA-*Tag* polymerase, TaKaRa) with primers FJJ2004-76/FJJ2004-77 (Table 1). The amplified 3.4 kb fragment was digested with *Apa*I/*Eco*NI and ligated with pGANP, digested with the same enzymes. The vector harboring two separate T-DNAs and carrying the gentamycin resistance gene for bacterial selectable marker was designated as pGA2T (Figure 1B). The expression cassette containing CP1 gene under the CaMV 35S promoter was inserted into the pGA2T by *Kpn*I/*Hind*III sites. The plasmid carrying two T-DNAs, one for the CP1 gene as target gene and the other for *npt*II as selectable marker, was named pGA2T-CP1 (Figure 1D).

The binary vector of bi-selectable markers for generating marker-free transgenic plants was derived from pGA2T and was constructed as follows: an intact expression cassette of hygromycin resistance (*hpt*) gene driven by CaMV 35S promoter was amplified from pCAMBIA1302 (Hajdukiewicz et al., 1994) using primers FJJ2006-29/FJJ2006-30 with *Asc*I site at 5′ end, and *Spe*I site at 3′ end (Table 1). The intact cassettes were digested with the respective enzymes and inserted into the pGA2T digested by the same restriction enzymes. The resulting plasmid harbored two selectable markers including *hpt* and kanamycin resistance (*npt*II) in one of the two T-DNAs and was designated as pGA2TNH (Figure 2A).

The pGA2TNH and pGA2T-CP1were separately introduced into *A. tumefaciens* LBA4404 (Hoekema et al., 1983) by electroporation (BIO-RAD, Inc.). The two plasmids in the single-strain co-transformation system were also established by electroporation and introduced into *A. tumefaciens* LBA4404 (Hoekema et al., 1983) of two type plasmid DNAs, i.e., pBin19 (Frisch et al., 1995) containing the pRK2 replication origin and *npt*II as selectable marker gene along with pGANP-CP1 carrying the pRK2 replication origin and target gene (CP1). The desired bacterial colonies for each vector system were selected on the medium containing 50 mg/l gentamycin and 50 mg/l streptomycin. The constructs were confirmed by PCR using specific primer pairs for the *npt*II gene (FJJ 2002-14/FJJ 2002-15) and the *hpt* gene (FJJ 2007-11/FJJ 2007-12) (Table 1).

Table 1. Sequences of the primers used in this paper.

Primer number	Primer sequence	Size
Specific primers for 35S terminator and 35S promoter, respectively		
FJJ 97-16	5′-AGAGATAGATTTGTAGAGA-3′	
FJJ 99-12	5′-ATATGGATATCTCCACTGACGTAAGGGATG-3′	
Specific primers for *npt*II gene		1.1 kb
FJJ 2002-14	5′-CCCCTCGGTATCCAATTAGAG-3′	
FJJ 2002-15	5′-CGGGGGGTGGGCGAAGAACTCCAG-3′	
Specific primers for ToLCV partial CP1 gene		460 bp
FJJ 2004-1	5′-CAGATTCTTCGACCTCACATCCCCAACCAG-3′	
FJJ 2004-2	5′-CACGTTCTCGGCAACCCATTCTACAAGTTC-3′	
Specific primers for *Hpt* gene		900 bp
FJJ2007-11	5′-CAGCGTCTCCGACCTGATGCAG-3′	
FJJ2007-12	5′-GTACTTCTACACAGCCATCGGT-3′	
Specific primers for T-DNA (for target gene) of pGA2TNH		200 bp
FJJ99-8	5′-AAGCTGTGACCGTCTCC-3′	
FJJ2005-218	5′-TCTGCAATGGCAATTACCT-3′	
Oligonucletide primers		
FJJ 2003-3	5′-GATCCCTCGAGCTTAAGTGTACAA-3′	
FJJ 2003-4	5′-AGCTTTGTACACTTAAGCTCGAGG-3′	
FJJ2004-70	5′-AATTACTAGTATTTAAATGGCGCGCC-3′	
FJJ2004-71	5′-AGCTGGCGCGCCATTTAAATACTAGT-3′	
Primers for T-DNA region from pBin19		3.4 kb
FJJ2004-76	5′-TATAGGGCCCTGCCGCCTTACAACGGCTCT-3′	
FJJ2004-77	5′-TTAATACCTCGCAGAGGAATCTTGCTCGTCTCGCT-3′	
Primers for expression cassette containing *Hpt* gene from CAMBIA1302		2.1 kb
FJJ2006-29	5′-ATGGCGCGCCTAATTCGGGGGATC-3′	
FJJ2006-30	5′-GGACTAGTATGGTGGAGCACGA-3′	

*The restriction enzyme sequences used are underlined.

Tobacco transformation

Transformation of *N. benthamiana* was carried out using the leaf disk method (Horsch et al., 1985). The explants were screened on the medium containing 500 mg/l carbenicillin and 300 mg/l kanamycin or 50 mg/l hygromycin. Regenerated shoots were rooted and transferred to soil for further analysis.

Polymerase chain reaction (PCR)

Genomic DNA was isolated from wild-type and transgenic plants as described by Fulton et al. (1995) with some modification (Jan et al., 2000). One µl (0.5-1.0 µg) of the isolated DNA was used in PCR amplification. The PCR mixture contained 200 ng of downstream and upstream primers, 1X *Taq* DNA polymerase reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100 and 1.75 mM $MgCl_2$), 2.5 U *Taq* DNA polymerase and 2.5 mM dNTP. The reaction was performed with an initial cycle at 93°C for 5 min; followed by 34 cycles at 93°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final cycle at 72°C for 5 min (Jan, 1998). Details of the primers and the sizes of PCR amplified products are given in Table 1. The PCR products were monitored by electrophoresis on 0.8% agarose gel.

Southern blot analysis

Genomic DNA was isolated as previously described. Fifteen µg genomic DNA were digested with either *Eco*RI

or *Spe*I, separated by electrophoresis on 1% agarose gel in 1X TAE buffer, and transferred to a nylon membrane (Gene Screen Plus membrane, NEN life science). Blots were hybridized with ^{32}P-dATP- (Pharmacia, Feinberg and Vogelstein, 1983) labeled probes prepared from selection marker gene and T-DNA fragments or the CP1 gene amplified by PCR from the plasmids. The membrane was autoradiographed by exposure to Kodak X-ray film for the appropriate length of time at -80°C.

Progeny segregation analysis

Seeds from R_0 self-pollinated co-transformed plants were surface-sterilized in 50% Clorox containing 0.1% Tween-20 and stirred with a Vortex Mixer (Model G560) for 10 min, followed by triple rinsing with sterile distilled water. The surface-sterilized seeds were germinated on the antibiotic-free MS medium [1 liter contained 4.4 g MS salt (Duchefa Biochemie, the Netherlands), 3% sucrose and 0.8% agar]. The R_1 seedlings were cultured on antibiotic-free MS medium for l week, and then transferred to greenhouse. Genomic DNA was isolated and used for PCR amplification with specific primers of T-DNA fragment for target gene and *npt*II to detect the marker-free transgenic plants (Table 1).

RESULTS

Construction of co-transformation vectors

To develop the marker-free co-transformation system with bi-selectable marker genes, two intermediary vectors, pGANP (Figure 1A) and pGA2T (Figure 1B), were constructed. The pGANP consisted of multiple cloning sites for the gene of interest in the T-DNA region. Another T-DNA region containing *npt*II as selectable marker modified from pBin19 was inserted into pGANP to obtain a second recombinant binary vector with multiple T-DNAs named pGA2T (Figure 1B). The pGA2TNH (Figure 2A) was derived from pGA2T by insertion of an intact expression cassette of hygromycin resistance (*hpt*) gene from pCAMBIA1302, situated upstream the *npt*II gene to lead one of the T-DNA region harboring two selectable marker genes.

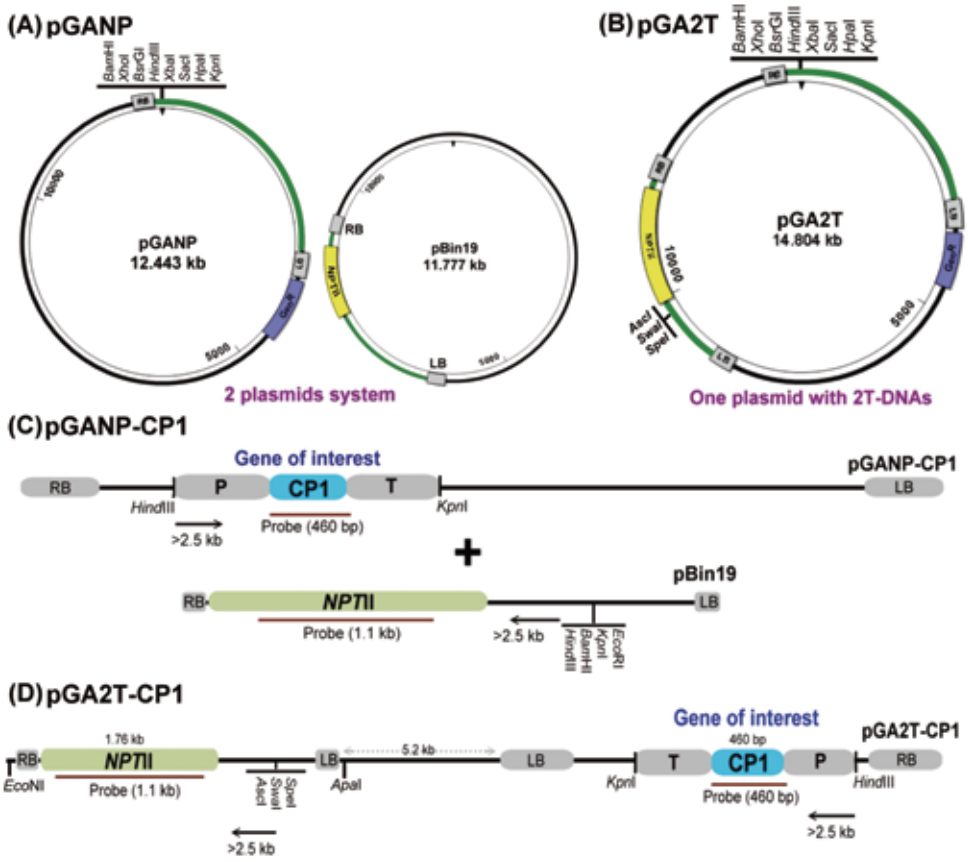

Figure 1. Constructions of marker-free binary vectors. (A) Circular map of pGANP and pBin19 for 2 plasmids system; (B) Circular map of pGA2T, harboring two separated T-DNAs. Partial CP gene of ToLCV was inserted into pGANP and pGA2T as the target gene and the resulting plasmids were named pGANP-CP1 (C) and pGA2T-CP1 (D), respectively. The schematic representation of T-DNA regions in the major vectors used for two plasmids system (C) and one plasmid with 2 T-DNAs system (D). Green line of circular map indicate T-DNA region; Gen^R: gentamycin-resistance gene; the arrows indicate the right border junction fragments, CP1 and *npt*II sequence regions were used as probes for Southern blotting. CP1, partial ToLCV coat protein gene; *npt*II, kanamycin resistance gene; LB, left border; RB, right border; P, 35S promoter; T, 35S terminator.

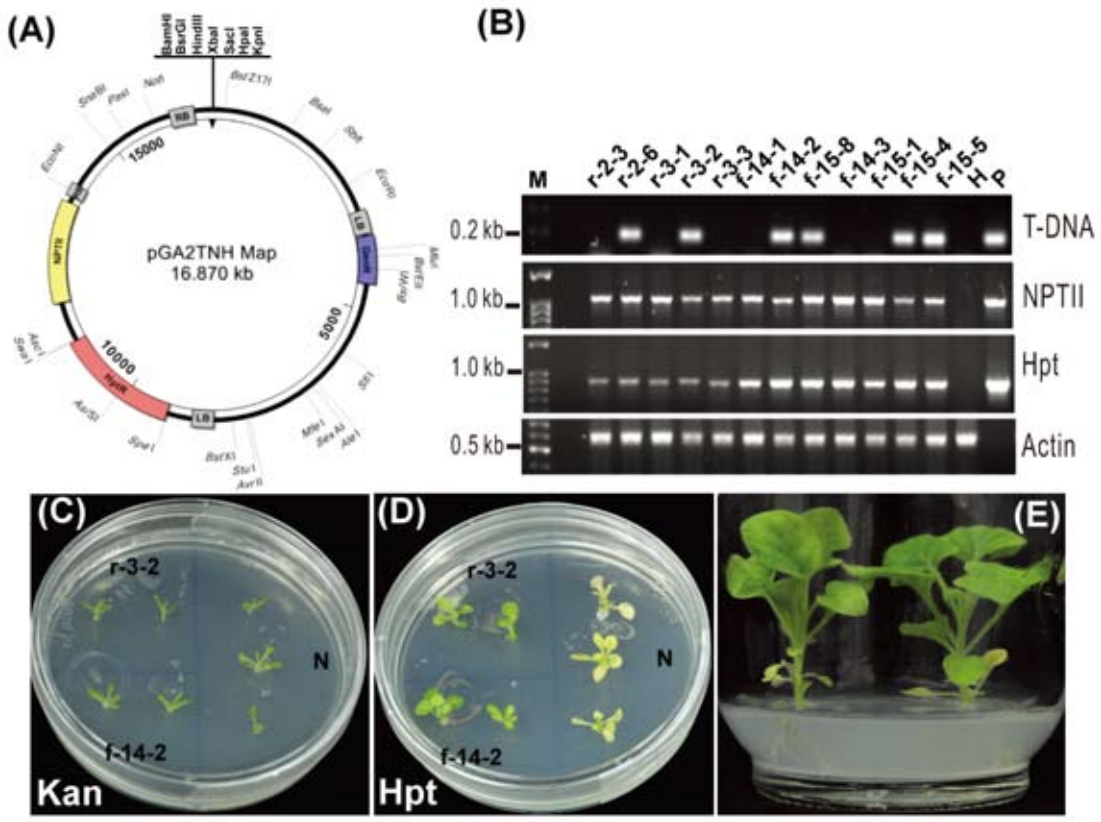

Figure 2. Bi-selectable marker-genes system. (A) The map of 2 T-DNAs with bi-selectable marker vector, pGA2TNH. The unique restriction enzyme sites were indicated on the map. (B) PCR analysis of the integration of T-DNA (for target gene) and selectable marker genes (*Hpt* and *npt*II). H: non-transformed plant; P: plasmid of pGA2TNH used as positive control; Actin: actin gene used as internal control. The agro-infected explants were screened on the medium containing kanamycin (300 mg/l) to obtain regenerated shoots. Propagated shoots of transgenic R_0 lines were screened on the medium containing kanamycin (300 mg/l) first for 3 weeks (C). The top shoots were then cut from the propagated lines and transferred to the medium containing hygromycin (50 mg/l) for another 3 weeks (D). (E) Transgenic plants growing on rooting medium. The photographs were taken 15 days after the plantlets were placed onto the medium. f, r: transgenic lines, f-14-2 and r-3-2; N: *npt*II transgenic plants (transformed with pGA482G).

The co-transformation efficiency of pGA2TNH was compared with two other vector systems within one *Agrobacterium* strain. Two plasmids carrying two different T-DNAs named pGANP-CP1 (Figure 1C) and one plasmid carrying two T-DNAs named pGA2T-CP1 (Figure 1D) were constructed by insertion of the target gene into pGANP and pGA2T, respectively. The pGANP-CP1 and pBin19, which contained a kanamycin resistance gene (*npt*II) as selectable marker, were introduced into the *A. tumefaciens* strain and the resulting clone was used to investigate the efficiency of the 2 plasmids/one strain system (Figure 1A). The multiple T-DNAs binary vector pGA2T-CP1 (Figure 1D) was used to analyze the delivery of two T-DNAs from one vector system in plant cells.

Frequency of co-transformation and molecular analysis of R_0 plants

The co-transformation efficiency of pGA2TNH was analyzed by *N. benthamiana* plant transformation. The transformed explants were initially screened on selection medium containing kanamycin. Each transgenic R_0 shoot was propagated as 3-6 plantlets and maintained on selection medium containing kanamycin. To evaluate the feasibility of both marker genes on pGA2TNH, some propagated R_0 lines were screened on the medium containing kanamycin. Initially, this was done for 3 weeks (Figure 2C), the top shoots were then cut from the propagated lines and transferred to the medium containing hygromycin (Figure 2D) for another 3 weeks. Some other propagated R_0 lines were initially screened on the medium containing hygromycin. After three weeks, the top shoots were cut and transferred to the medium containing kanamycin for an additional 3 weeks. Twelve lines revealed resistance to both kanamycin and hygromycin (Table 2). The integration of the genes of interest and the selection markers (*npt*II and *hpt*) was studied by PCR to determine the co-transformation frequency. Six out of twelve (50.0%) R_0 plants transformed with pGA2TNH contained both selectable markers and genes of interest (Table 2, Figure 2B). The other two systems, pGANP-CP1/pBin19 and the pGA2T-CP1, were analyzed by *N. benthamiana* plant transformation. The regenerated R_0 plants were screened on selection medium containing kanamycin and used to study the integration of the CP1 gene and *npt*II by PCR

to determine the co-transformation frequency. Ten out of twenty-three (43.5%) R_0 plants from pGANP-CP1/pBin19 and nine out of eighteen (50%) R_0 plants from pGA2T-CP1 contained both selectable markers and genes of interest (Table 2).

Southern blot analysis was carried out to investigate the integration pattern of marker and target genes in the R_0 transgenic plants to predict their genotype segregation frequency. The copy numbers of target genes in transgenic plants were estimated based on the number of either the *Spe*I or *Eco*RI digested fragments that hybridized with the probes encoding the sequence of selection marker genes and the sequence of T-DNA (or CP1 gene), respectively. In pGA2TNH systems, the border fragments hybridized with the sequence of T-DNA and selection marker genes expect to be longer than 3.5 kb and 4 kb, respectively (Figure 3A). The sizes of hybridization bands from all analysis lines were as predicted except line f-15-8 with a hybridization band about 3 kb, which may have been due to the incomplete integration of the T-DNA region (Figure 3A, top). In the pGANP-CP1/pBin19 and the pGA2T-CP1 systems, the fragments at the borders that were hybridized to the CP1 gene and to *npt*II, respectively, were longer than 2.5 kb (Figure 1). Additionally, the R_0 transgenic plants showed either single or multiple copy signals for both selection marker genes and for the sequence of T-DNA (or CP1 gene), which indicated their presence (Figure 3B).

Segregation of marker-free transgenic plants

The transgenic plants carrying the gene of interest and selection marker genes were maintained in the greenhouse for self-pollination and seeds were collected to screen the marker-free transgenic plants at the progeny level. Seeds were surface-sterilized and germinated on MS medium. If the two co-transformed T-DNAs were integrated into unlinked loci, selectable marker and target genes would segregate at the progeny level. The expected four genotypes of progeny were: *npt*II (+)/target (+), *npt*II (+)/target (-), *npt*II (-)/target (+), and *npt*II (-)/target (-).

In the bi-selectable markers system, three selected pGA2TNH-transformed R_0 lines, f-14-2, f-15-5 and r-2-6, were examined by PCR for segregation of marker-free transgenic plants (Table 3). Among these three R_0 lines, lines f-14-2 and f-15-5 exhibited all four classes of R_1 plants expected for the unlinked integration of two separate T-DNAs. Of the 45 R_1 plants of line f-14-2, 12 plants tested positive for the gene of interest but lacked the selection marker gene, showing a marker gene elimination frequency of 26.7%. The marker gene elimination frequency of line f-15-5 was 17.5% (Table 3). Southern blot analysis

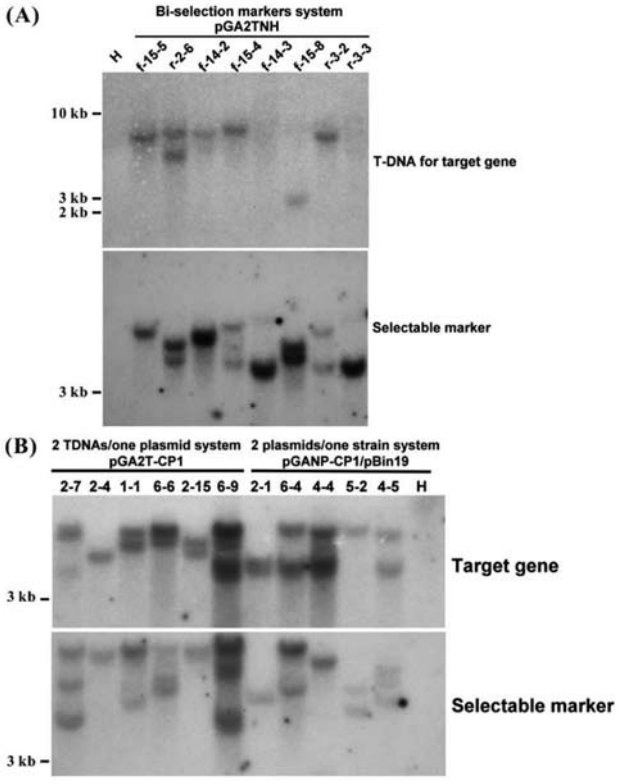

Figure 3. Southern blot analysis of the R_0 transgenic plants. (A) 15 μg genomic DNAs of pGA2TNH lines were analyzed. Total DNA digested with *Eco*RI and probed with the sequence of T-DNA for target gene (top), while total DNA digested with *Spe*I was probed with selection marker gene (bottom). Lane H: non-transformed plant. (B) In pGA2T-CP1, 15 μg genomic DNA of lines 2-7, 2-4, 1-1, 6-6, and 2-15 were digested with *Hind*III and probed with CP1 coding sequence, while total DNA digested with *Spe*I was probed with *npt*II coding sequence. In pGANP-CP1/pBin19, total DNA (15 μg) of lines 2-1, 6-4, 4-4, 5-2, and 4-5 were digested with *Hind*III and the sequence of CP1 (up) and *npt*II gene (down) were used as probes. Fragments hybridized with the CP1 and *npt*II were expected to be longer than 2.5 kb as shown (Figure 1C, D). Lane H: non-transformed plant.

Table 2. Frequencies of co-transformation in R_0 lines.

Constructs transformed	Number of R_0 (Kan^R) lines		
	Total	Co-transformed R_0 lines (Kan^R+target gene)	Co-transformation frequency (%)
pGA2TNH	12[a]	6[b]	50.0
2T-DNA (PGA2T-CP1)	23	10	43.5
2-plasmid (pGANP-CP1/pBin19)	18	9	50.0

[a]Number of R_0 lines containing Hygromycin resistant (Hpt^R) gene and Kanamycin resistant (Kan^R) gene.
[b]Co-transformed R_0 lines containing of Hpt^R gene, Kan^R gene and the other T-DNA for gene of interest.

Table 3. Segregation of selectable marker and interested gene in R_1 progeny.

Constructs transformed	Line (R_0)	Copy number (R_0)[c]		Number of R_1 plants							
		T	N	Total	*npt*II/ target +/+	*npt*II/ target +/-	*npt*II/ target -/+	*npt*II/ target -/-	Expected ratio for the four genotypes	Marker-free transgenic plants (%)	Linked *npt*II/target[d]
pGA2TNH[a]	f-14-2	1	1	45	21	7	12	5	9:3:3:1	26.7	In[e]
	f-15-5	1	1	40	19	10	7	4	9:3:3:1	17.5	0
	r-2-6	2	2	38	30	7	1	0	225:15:15:1	2.6	1
2T-DNA[b] (PGA2T-CP 1)	2-4	1	1	43	27	6	8	2	9:3:3:1	18.6	0
	6-6	2	3	47	43	1	3	0	945:63:15:1	6.4	1
	1-1	2	2	45	40	1	4	0	225:15:15:1	8.9	0
2-plasmids[b] (pGANP-CP 1/pBin19)	2-1	2	1	58	43	0	14	1	45:3:15:1	24.1	0
	6-4	2	2	46	40	2	4	0	225:15:15:1	8.7	0

[a]The T-DNA region for interested gene and *npt*II fragments of R_1 plants were amplified by PCR.
[b]The CP1 gene and *npt*II fragments of R_1 plants were amplified by PCR.
[c]The copy number was analysis by Southern blot; T: target gene (CP1); N: *npt*II.
[d]Phenotypic linkage behavior was determined by the smallest chi-square analysis (X^2). The critical value for 1 *df* at a significant level of 0.05 is 3.841. The chi-square value of more than 3.841 was designated as linked loci. $X^2 = \Sigma(\text{Observed-Expected})2/\text{Expected}$.
[e]In: incompletely linked. The event was not accepted as unlinked but still produced recombinant R_1 phenotype.

of the R_1 progeny of line f-15-5 confirmed the integration of the target gene and selection marker of the plant genome (Figure 4).

In the 2 T-DNAs/one plasmid system, three selected pGA2T-CP1 transformed R_0 lines, 2-4, 6-6 and 1-1, were examined for the segregation of marker-free transgenic plants. Of these three lines, only lines 2-4 exhibited all four classes of R_1 plants expected for the unlinked integration of two separate T-DNAs (*npt*II and CP1). Out of the 43 R_1 plants analyzed, 8 plants tested positive for the partial CP1 gene fragment but lacked the *npt*II fragment, showing a marker gene elimination frequency of 18.6% (Table 3). The two other R_0 lines, 6-6 and 1-1, showed only three classes of R_1 plants [without *npt*II (-)/target (-)], indicating a marker-free elimination frequency of 6.4% and 8.9%, respectively (Table 3).

When two pGANP-CP1/pBin19 co-transformed R_0 lines, 2-1 and 6-4, of the 2 plasmids/one strain system were used to analyze the segregation frequency, both lines showed the production of marker-free transgenic progeny.

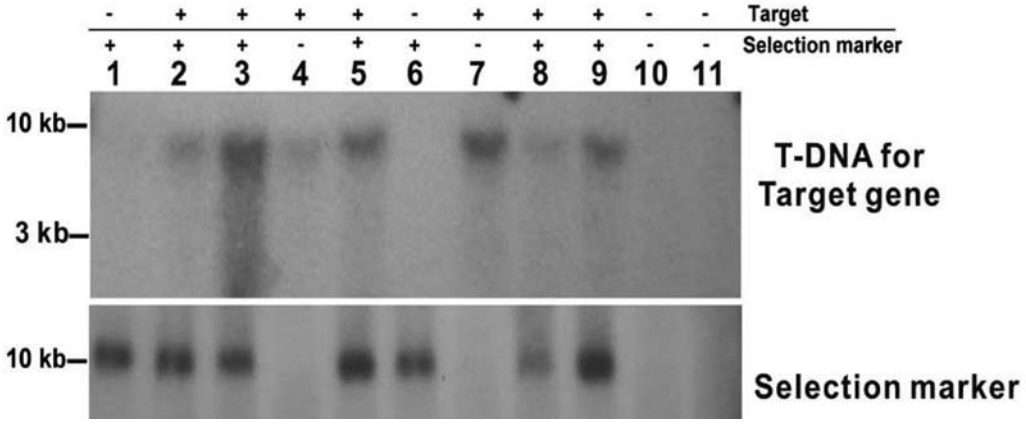

Figure 4. Southern blot analysis of the progeny of pGA2TNH transgenic line f-15-5. Genomic DNA (15 μg) of R_1 progeny were digested with *Eco*RI and probed with the sequence of T-DNA for target gene (top), while total DNA digested with *Spe*I was probed with selection marker gene (bottom). R_1 progeny of selection markers (+)/target (+) (lanes 2, 3, 5, 8 and 9), selection markers (+)/target (-) (lanes 1 and 6), selection markers (-)/target (+) (lanes 4 and 7), selection markers (-)/target (-) (lane 10) and non-transformed control (lane 11).

Lines 2-1 and 6-4 produced only three classes of R_1 plants and R_0 plants of line 2-1 lacked the genotype of *npt*II (+)/target (-). Of the 58 R_1 plants, 14 plants exhibited *npt*II (-)/target (+), indicating a marker gene elimination frequency of 24.1% (Table 3). R_0 plants of line 6-4 lacked the genotype of *npt*II (-)/target (-) and of 46 R_1 plants, 4 plants exhibited *npt*II (-)/CP1 (+), giving a marker-free elimination frequency of 8.7% (Table 3).

DISCUSSION

The current trend to eliminate selectable markers from transgenic plants is the result of environmental and public health concerns. Several strategies for greater efficiency and/or convenience have been successfully implemented. (Verweire et al., 2007; Charng et al., 2008). Though *npt*II, which confers resistance to the antibiotic kanamycin, is the most widely-used selectable marker, it is ineffective when used as a selection agent for plants like orchids (Chia et al., 1994), cereals, several legumes and gramineae (Ziemienowicz, 2001). This characteristic limits the use of the marker-free system for plants carrying the *npt*II gene as the selection marker. In this study, we show how we developed a new bi-selectable marker binary vector called pGA2TNH, not only to generate marker-free transgenic plants but also to alleviate the limitations of *npt*II.

Multiple selection marker genes were inserted into one of the two T-DNAs in one plasmid and introduced into *N. benthamiana* plants. In the first selection, the regenerated shoots grew well on the kanamycin- and hygromycin-containing mediums, indicating that both selection marker genes were functionally expressed (Figure 2C, D). Previous studies indicated that high transformation frequencies can be obtained by utilizing a single *Agrobacterium* strain to deliver two T-DNAs. Komari et al. (1996) observed that the co-transformation frequency in a single strain method was more than 47%, while the frequency was less than 37% when a mixed-strain method was used. Similar results were obtained by Miller et al. (2002) and Chen et al. (2005). In this study, the co-transformation frequency using the bi-selectable marker system (pGA2TNH) was 50% as compared to 50% using 2 plasmids/one strain (pGANP-CP1/pBin19) and 43.5% with the traditional 2 T-DNAs/one plasmid (pGA2T-CP1) system. These results showed that the co-transformation frequencies were almost the same among the three systems (Table 2) indicating that multiple selectable markers in one T-DNA had no impact on the transformation frequency. This suggests that the transfer and the interaction of each T-DNA are independent events, even when multiple T-DNAs are in the same plasmid.

A high efficiency of co-transformation and unlinked integration of T-DNAs are two important parameters for the elimination of selectable markers in the progeny (Komari et al., 1996). The copy number of marker gene integration was only one or two copies in bi-selectable markers system developed in this study (Figure 3A). However, in the other two systems there were often more than two copies (Figure 3B). Low copies of marker genes increased the marker-free segregation proportion indicating that the bi-selectable marker system may be an easier system for obtaining marker-free transgenic plants. For instance, a high ratio of marker-free transgenic plant was observed on transgenic plants carrying one copy of selection marker genes in this study. While co-transformed transgenic plants carrying one copy of target gene and marker gene, respectively, and the two genes were integrated into unlinked loci of plant genome, the expected ratio for the four genotypes in R_1 progeny was 9:3:3:1. The ratio of marker-free transgenic plants (target gene only) in R_1 was 3/16 (18.75%) close to the results observed in pGA2TNH (17.5%) and pGA2T-CP1 (18.6%) (Table 3). The same, co-transformed transgenic plants carrying two copy of target gene and one copy of marker gene, the expected ratio for the four genotypes in R_1 progeny was 45:15:3:1. The ratio of marker-free transgenic plants in R_1 was 15/64 (23.4%) close to the results observed in pGANP-CP1/pBin19 (24.1%) (Table 3). Though few lines showed phenotypic linkage behavior, they were observed in transgenic lines carrying multiple copies of trans- and marker genes (Table 3). One special case occurred on line f-14-2 of pGA2TNH. The event was not accepted as unlinked but still produced high ratio of marker-free segregation proportion (Table 3). This may be due to the recombination of transgene and marker genes after the integration of the plant genome.

We have succeeded in developing an efficient bi-selectable marker system capable of generating marker-free transgenic plants. The binary vector pGA2TNH can be used for crop improvement and more conveniently, for experimental or commercial needs. In practical application, binary vector pGA2TNH has the potential to transform a wide range of plant species, including di- and monocotyledonous plants, and even flower crops. It also is less tedious, since genetic manipulation involves only one plasmid. Moreover, unlike some double T-DNA vectors constructed only by inserting a small "right border and left border" section into the polylinker of a standard binary vector (Matthew et al., 2001), the distance between the 2 T-DNA was more than 2 kb in the pGA2TNH vector which may reduce the frequency of T-DNA integration into linked loci. Developing marker-free transgenic plants not only decreases the risk of harm to the environment and to the public, but also boosts public acceptance of transgenic crops.

Acknowledgements. We are grateful to Dr. Wen-Hsiung Ko, Professor Emeritus, University of Hawaii at Manoa and Dr. Chung-Jan Chang, Professor, University of Georgia for their critical review of this manuscript. This study was partially supported by a grant (NSC93-2752-B-005-002-PAE) of "Program for Promoting Academic Excellence of Universities" from the National Science Council and a grant (97AS-1.1.1-FD-Z1) from the Agriculture and Food Agency, Council of Agriculture, Executive Yuan, Taiwan, ROC.

LITERATURE CITED

Breitler, J.C., D. Meynard, J. van Boxtel, M. Royer, F. Bonnot, L. Cambillau, and E. Guiderdoni. 2004. A novel two T-DNA binary vector allows efficient generation of marker-free transgenic plants in three elite cultivars of rice (*Oryza sativa* L.). Transgenic Res. **13:** 271-287.

Charng, Y.C, K.T. Li, H.K. Tai, N.S. Lin, and J. Tu. 2008. An inducible transposon system to terminate the function of a selectable marker in transgenic plants. Mol Breed. **21:** 359-368.

Chen, S., X. Li, X. Liu, H. Xu, K. Meng, G. Xiao, X. Wei, F. Wang, and Z. Zhu. 2005. Green fluorescent protein as a vital elimination marker to easily screen marker-free transgenic progeny derived from plants co-transformed with a double T-DNA binary vector system. Plant Cell Rep. **23:** 625-631.

Chia, T.-F., Y.-S. Chan, and N.-H. Chua.1994. The firefly luciferase gene as a non-invasive reporter for *Dendrobium* transformation. Plant J. **6:** 441-446.

Daley, M., V.C. Knauf, K.R. Summerfelt, and J.C. Turner. 1998. Co-transformation with one *Agrobacterium tumefaciens* strain containing two binary plasmids as a method for producing marker-free transgenic plants. Plant Cell Rep. **17:** 489-496.

Darbani, B., A. Eimanifar, C.N., Stewart, and W.N. Camargo. 2007. Methods to produce marker-free transgenic plants. Biotechnol. J. **2:** 83-90.

De Block, M. and D. Debrouwer. 1991. Two T-DNA's co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. Theor. Appl. Genet. **82:** 257-263.

de Framond, A.J., E.W. Back, W.S. Chilton, L. Kayes, and M.D. Chilton. 1986. Two unlinked T-DNAs can transform the same tobacco plant cell and segregate in the F1 generation. Mol. Gen. Genet. **202:** 125-131.

De Neve, M., S. De Buck, A. Jacobs, M. van Montagu, and A. Depicker. 1997. T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. Plant J. **11:** 15-29.

Depicker, A., L. Herman, A. Jacobs, J. Schell, and M. van Montagu. 1985. Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction. Mol. Gen. Genet. **201:** 477-484.

Ebinuma, H., K. Sugita, E. Matsunaga, S. Endo, K. Yamada, and A. Komamine. 2001. Systems for the removal of a selection marker and their combination with a positive marker. Plant Cell Rep. **20:** 383-392.

Feinberg, A.P. and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6-13.

Frisch, D.A., L. W. Harris-Haller, N.T. Yokubaitis, T.L. Thomas, S.H. Hardin, and T.C. Hall. 1995. Complete sequence of the binary vector pBin 19. Plant Mol. Biol. **27:** 405-409.

Fulton, T.M., J. Chunwongse, and S.D. Tanksley. 1995. Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol. Biol. Rep. **13:** 207-209.

Goldstein, D.A., B. Tinland, L.A. Gilbertson, J.M. Staub, G.A. Bannon, R.E. Goodman, R.L. McCoy, and A. Silvanovich. 2005. Human safety and genetically modified plants: a review of antibiotic resistance markers and future transformation selection technologies. J. Appl. Microbiol. **99:** 7-23.

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

Hare, P.D. and N.H. Chua. 2002. Excision of selectable marker genes from transgenic plants. Nat. Biotechnol. **20:** 575-580.

Higgins, J.D., H.J. Newbury, D.J. Barbara, S. Muthumeenakshi, and I.J. Puddephat. 2006. The Production of Marker-Free Genetically Engineered Broccoli with Sense and Antisense ACC synthase 1 and ACC oxidases 1 and 2 to Extend Shelf-Life. Mol. breed. **17:** 7-20.

Hoekema, A., P.R. Hirsch, P.J.J. Hooykaas, and R.A. Schilperoort. 1983. A binary plant vecter strategy based on sepration of vir- and T-region of the *Agrobacterium tumefaciens* Ti plasmid. Nature **303:** 179-180.

Horsch, R.B., J.E. Fry, H.L. Hoffmann, D. Eichholtz, S.G. Rogers, and R.T. Fraley. 1985. A simple and general method for transferring genes into plants. Science **227:** 1229-1230.

Jacob, S.S. and K. Veluthambi. 2002. Generation of selection marker-free transgenic plants by cotransformation of a cointegrate vector T-DNA and a binary vector T-DNA in one *Agrobacterium tumefaciens* strain. Plant Sci. **163:** 801-806.

Jan, F.-J. 1998. Roles of nontarget DNA and viral gene lengh in influencing multi-virus resistance through homology-dependent gene silencing. Ph. D. Dissertation, Department of Plant Pathology, Cornell University, Ithaca, New York, USA.

Jan, F.-J., F. Fagoaga, S.-Z. Pang, and D. Gonsalves, 2000. A minimum length of N gene sequence in transgenic plants is required for RNA-mediated tospovirus resistance. J. Gen. Virol. **81:** 235-242.

Komari, T., Y. Hiei, Y. Saito, N. Murai, and T. Kumashiro. 1996. Vectors carrying two separate T-DNAs for co-transformation of higher plant mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J. **10:** 165-174.

Lu, H.-J., C.R. Zhou, Z.-X. Gong, and N.M. Upadhyaya. 2001. Generation of selectable marker-free transgenic rice using double right border (DRB) binary vectors. Aust. J. Plant Physiol. **28:** 241-248.

Matthew, P.R., M.-B. Wang, P.M. Waterhouse, S. Thornton, S.J. Fieg, F. Gubler, and J.V. Jacobsen. 2001. Marker gene elimination from transgenic barley, using co-transformation with adjacent "twin T-DNAs" on a standard *Agrobacterium* transformatiom vector. Mol. Breed. **7:** 195-202.

McKnight, T.D., M.T. Lillis, and R.B. Simpson. 1987. Segregation of genes transferred to one plant cell from two separate *Agrobacterium tumefaciens* strains. Plant Mol. Biol. **8:** 439-445.

Miki, B. and S. McHugh. 2004. Selection marker genes in transgenic plants: applications, alternatives and biosafety. J. Biotechnol. **170:** 193-232.

Miller, M., L. Tagliani, N. Wang, B. Berka, and D. Bidney. 2002. High efficiency transgene segregation in co-transformed maize plants using an *Agrobacterium tumefaciens* 2 T-DNA binary system. Transgenic Res. **11:** 381-396.

Permingeat, H.R., M.L. Alvarez, G.D. Cervigni, R.A. Ravizzini, and R.H. Vallejos. 2003. Stable wheat transformation obtained without selectable markers. Plant Mol. Biol. **52:** 415-419.

Puchta, H. 2003. Marker-free transgenic plants. Plant Cell Tiss. Org. Cult. **74:** 123-134.

Verweire, D., K. Verleyen, B.de Sylvie, M. Claeys, and G. Angenon. 2007. Marker-Free Transgenic plants through genetically programmed auto-excision. Plant Physiol. **145:** 1220-1231.

Xing, A., Z. Zhang, S. Sato, P. Staswick, and T. Clement. 2000. The use of two T-DNA binary system to derive marker-free transgenic soybeans. *In Vitro* Cell Dev. Biol. Plant **36:** 456-463.

Xue, G.P., M. Patel, J.S. Johnson, D.J. Smyth, and C.E. Vickers. 2003. Selectable marker-free transgenic barley producing a high level of cellulase (1,4-beta-glucanase) in developing grains. Plant Cell Rep. **21:** 1088-1094.

Yoder, J.I. and A.P. Goldsbrough. 1994. Transformation systems for generating marker-free transgenic plants. Bio/Technology **12:** 263-267.

Ziemienowicz, A. 2001. Plant selectable markers and reporter genes. Acta Physiol. Plant. **23:** 363-374.

Zhou, H.-Y., S.-B. Chen, X.-G. Li, G.-F. Xiao, X.-L. Wei, and Z. Zhu. 2003. Generation marker-free transgenic tobacco plants by *Agrobacterium*-mediated transformation with double T-DNA binary vector. Acta Bot. Sin. **45:** 1103-1108.

生產無篩選標示轉基因植物的多功能轉殖載體之開發應用

林靜宜[1] 古新梅[2] 陳志威[1,2] 葉錫東[1] 詹富智[1]

[1] 國立中興大學 植物病理學系

[2] 國立中興大學 農藝學系

植物轉殖通常需要利用抗生素或殺草劑的抗性基因作為選擇性標示基因以區分其中少數轉殖成功的植物。然而存在轉基因植物中的選擇性標示基因之安全性近來已成為大眾關注的話題，因此自轉基因植物中移除選擇性標示基因乃為當務之急。本研究中，我們以共轉型法發展了一套生產無篩選標示轉基因植物的多功能轉殖載體，pGA2TNH，並且可適用更多的植物種類，尤其是對 kanamycin 具有天然耐 / 抗性的作物。我們利用單載體攜帶兩組 T-DNA 為策略，並於其中一組 T-DNA 中構築兩種不同的選擇性標示基因，其一為 *hpt* 而另一為 *npt*II 基因。為了作為對照，我們也構築了兩組載體 pGANP-CP1/pBin19 與 pGA2T-CP1，藉以比較三者生產無篩選標示基因轉基因植物的效率。前者為單一菌株內兩個獨立的質體各自攜帶有目標基因或標示基因的 T-DNA，後者為同一質體中同時帶有目標基因及標示基因的 T-DNA。經 pGA2TNH 系統再生之轉基因菸草的共轉型效率為 50%，其效率和其他兩組系統相當。至於兩組 T-DNA 於子代的分離現象亦符合預期，於單一標示基因套數的轉基因植物中，移除標示基因的比例可達 17.5%，而 pGA2T-CP 與 pGANP-CP1/pBin19 則分別為 18.6% 與 24.1%。結果證實我們所發展之系統確實可行且能有效率地移除選擇性標示基因，進一步可提供簡便及實用性兼具的工具應用至更為廣泛的物種上生產無篩選標示轉基因植物，且有助於提升大眾對轉基因作物的接受度。

關鍵詞：共轉型法；無篩選標示；選擇性標示基因；轉基因植物。