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Improved Co-transformation of Maize with Vectors Carrying Two Separate T-DNAs Mediated by *Agrobacterium tumefaciens*

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Abstract

Vectors with two separate T-DNAs for co-transformation mediated by *Agrobacterium tumefaciens* were tested in maize inbred A188. Although the frequency of transformation during the initial trials was very low, it was remarkably improved by the vector with a phosphinothricin resistance gene (*bar*) using a modified transformation protocol, which is characterized by the presence of silver nitrate and carbenicillin in the selection medium. The vector with hygromycin resistance gene (*hpt*) showed low frequency even with the improved protocol, but the use of maize ubiquitin gene promoter elevated the frequency. Both co-transformation and segregation of the progeny free from the selection markers frequencies were reasonably high and similar to those previously observed in rice and tobacco. The two-T-DNA vectors will be a useful tool in molecular biology and biotechnology studies of maize.

Key words: *Agrobacterium tumefaciens*, co-transformation, maize.

Abbreviations

bar, phosphinothricin resistance gene; GUS, β-glucuronidase; HPT, hygromycin resistance gene; HYG, hygromycin; PPT, phosphinothricin.

Selection markers are usually essential components in vectors for the transformation of higher plants. However, once the transformants are obtained, their presence is not only unnecessary but also problematic. They hinder the re-transformation of the plants with the same markers, however, available markers are limited. Noteworthy, selection markers are not desired for regulatory complications in transgenic plants to be commercialized except for the cases in which target traits, such as herbicide tolerance, serve as selection markers. Various trials have been carried out for removal of the selection marker genes from transformed plants either physically with site specific recombinases (Odell *et al.*, 1990) or genetically following co-transformation with marker genes and other genes being placed on different DNA molecules (Depicker *et al.*, 1985; Scocher *et al.*, 1986; De Block and Debrouwer, 1991; Komari *et al.*, 1996; Ebinuma *et al.*, 1997; Daley *et al.*, 1998; McCormac *et al.*, 2001).

An advantage of co-transformation is that the construction of molecules may be simplified. Komari *et al.* (1996) created vectors that carried two separate transfer DNAs (T-DNAs) for the co-transformation of higher plants mediated by *Agrobacterium tumefaciens*. They observed that both the frequencies of co-transformation and segregation of the marker-free progeny were reasonably high in tobacco and rice. Because various genes of interest can easily be inserted into these vectors, co-transformation became a sensible option in the *Agrobacterium*-mediated transformation of higher plants.

Maize is one of the most important crops in the world, and the commercialization of transgenic maize was started years ago. However, studies of the removal of selection markers from maize transformants were very limited. Recently, Miller *et al.* (2002) examined the two-T-DNA vector during the transformation of a particular genotype, Hi-II, and observed high frequencies of co-transformation and marker-free progeny. In maize, transformation techniques are often highly genotype dependent, and many procedures need to be worked out for each genotype. We started the development of co-transformation methods for inbred A188. Although initial tests of the two-T-DNA vectors in the

inbred A188 were inefficient, a significant improvement has been made and reported here.

The two-T-DNA vectors employed in the present study are illustrated in **Fig. 1.** They were constructed according to the procedures described by Komari *et al.* (1996) and Kuraya *et al.* (in preparation). As selection markers, pSB424 and pSB4U24 have a hygromycin resistance gene (*hpt*) and pSB624 has a phosphinothricin (PPT) resistance gene (*bar*).

Initially, immature embryos of A188 were infected with *A. tumefaciens* LBA4404(pSB424) and LBA4404(pSB624) according to the procedures described by Ishida *et al.* (1996, original protocol). No transformants were obtained with LBA4404 (pSB424) and only 22 PPT resistant plants were obtained from a total of 388 immature embryos in 3 experiments with an overall frequency of 5.7% with LBA4404(pSB624) (**Table 1**). Ishida *et al.* (1996) were able to transform immature embryos of A188

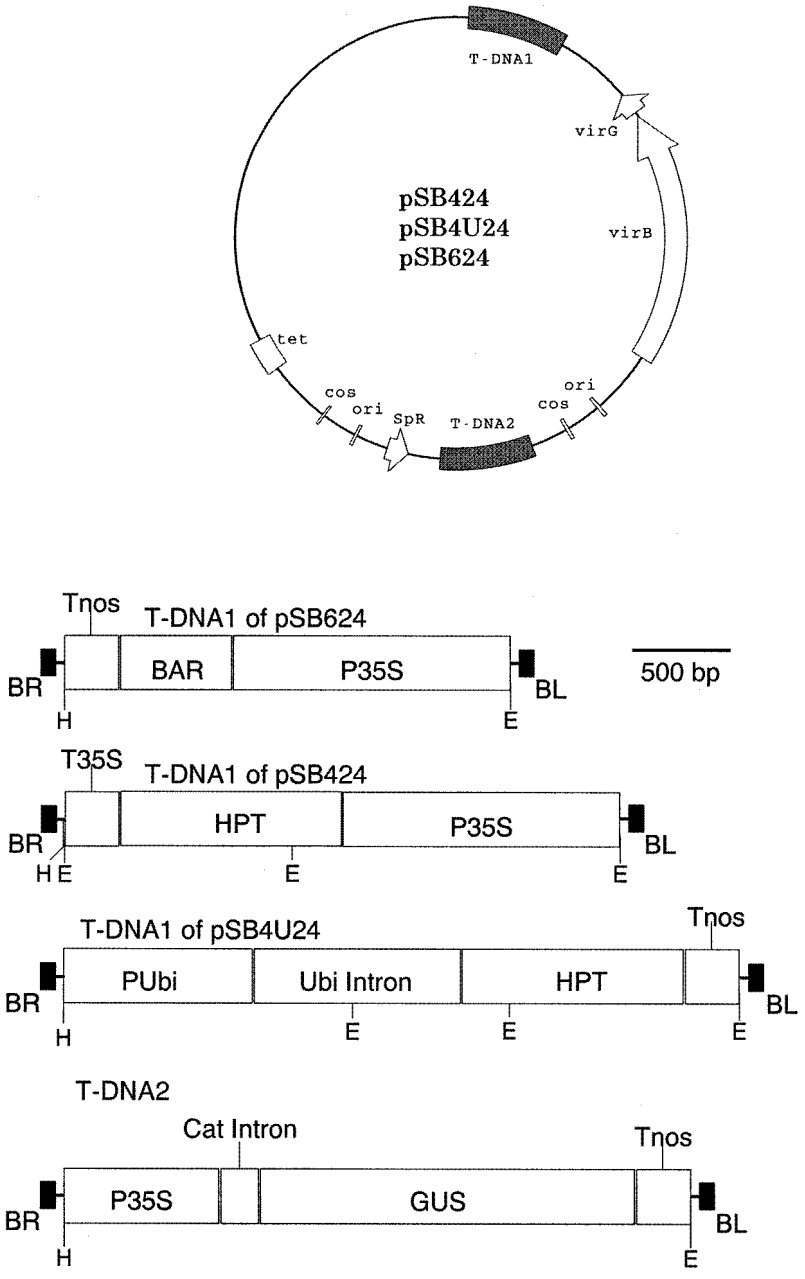


Fig. 1 Map of plasmid and structure of T-DNAs.
Abbreviations: BR, right border; BL, left border; SpR, spectinomycin-resistance gene; tet, tetracycline-resistance gene; ori, origin of replication of ColE1; cos, cos site of phage λ ; virB, *virB* gene from pTiBo542; virG, *virG* gene from pTiBo542; P35S, 35S promoter of cauliflower mosaic virus; Tnos, 3' signal of nopaline synthase gene; T35S, 3' signal of 35S RNA; HPT, hygromycin-resistance gene; BAR, phosphinothricin-resistance gene; PUbi, polyubiquitine promoter of maize; Ubi intron, intron of maize polyubiquitine; Cat intron, intron of castor bean catalase; H, *Hin*dIII; E, *Eco*RI

with LBA4404(pSB131) and LBA4404(pTOK233), which have the same selection markers as pSB624 and pSB424, respectively, at higher frequencies (25% and 10%, respectively). Thus, the co-transformation vectors were unexpectedly inefficient. Such phenomena, in which the transformation frequencies for similar vectors are different, often observed, but the reasons are unknown.

Ishida *et al.* (2003) described an improved protocol for maize transformation, which is characterized by the presence of 10 μM silver nitrate and 250 mg l^{-1} carbenicillin in place of cefotaxime in the selection medium. A188 was transformed with LBA4404(pSB624) and LBA4404(pSB424) accord ing to the improved protocol. With LBA4404 (pSB624), 81 PPT resistant plants were obtained from a total of 190 immature embryos with an average frequency of 42.6%. Expression of β-glucuronidase (GUS) was examined by a calorimetric assay using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) according to the procedure described by Hiei *et al.* (1994), and 41 out of 81 were GUS positive. However, LBA4404(pSB424) produced only 3 hygromycin resistant plants from 147 immature embryos (2.0%) even with the improved protocol (**Table 1**). Therefore, the new protocol significantly improved the production of PPT resistant plants by pSB624, but the transformation by pSB424 was still inefficient.

pSB4U24 was then tested. The *hpt* was driven by the promoter for a maize ubiquitin gene in pSB4U24 whereas it was driven by the 35S promoter of cauliflower mosaic virus in pSB424. With pSB4U24, 27 hygromycin resistant plants were obtained from a total of 192 immature embryos (14.1%), and 17 out of 27 were GUS positive (**Table 1**). Therefore, the use of the ubiquitin promoter, which is very strong constitutive promoter in cereals, was very effective. The higher expression of the marker gene probably contributed to the higher frequency of hygromycin resistant plants.

Table 1 Efficiency of transformation

Vector	Selection medium[1)]	Experiment no.	Number of immature embryos		B/A (%)	Number of immature embryos produced antibiotic resistant, GUS+ plants (C)	C/B (%)
			Inoculated (A)	Produced antibiotic resistant plants (B)			
pSB624	Original	1	123	1	0.8	1	100.0
		2	133	17	12.8	7	41.2
		3	132	4	3.0	1	25.0
		Total	388	22	5.7	9	40.9
	Improved	1	45	21	46.7	13	61.9
		2	50	17	34.0	7	41.2
		3	95	43	45.3	21	48.8
		Total	190	81	42.6	41	50.6
pSB424	Original	1	67	0	0.0	0	0.0
		2	60	0	0.0	0	0.0
		Total	127	0	0.0	0	0.0
	Improved	1	83	0	0.0	0	0.0
		2	64	3	4.7	2	66.7
		Total	147	3	2.0	2	66.7
pSB4U24	Improved	1	84	6	7.1	3	50.0
		2	63	12	19.0	8	66.7
		3	45	9	20.0	6	66.7
		Total	192	27	14.1	17	63.0

[1)] Improved selection medium: Original LSD1.5 medium (Ishida *et al.*, 1996) without cefotaxime, plus 250 mg l^{-1} carbenicillin and 10 μM $AgNO_3$.

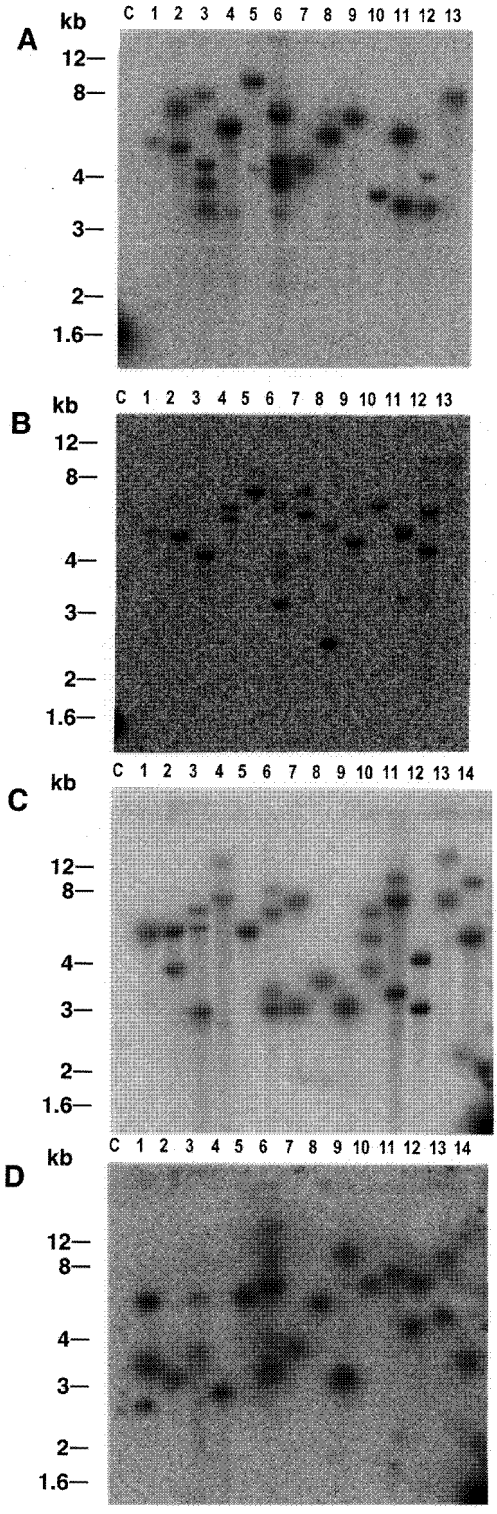


Fig. 2 Southern blot analysis of transformed plants (T0 generation). DNA extracted from PPT-resistant and GUS-positive (A and B) or hygromycin-resistant and GUS positive (C and D) was digested with *Eco*RI (A and B) or *Hin*dIII (C and D), and hybridized to the *gus* (A, C), *bar* (B) or *hpt* (D) probe. Lane C, non-transformed control plant; lanes 1-13 (A and B) or 1-14 (C and D), transformed plants regenerated from independent immature embryos infected with LBA4404 (pSB624) (A and B) or LBA4404 (pSB4U24) (C and D).

Southern hybridization was carried out using 21 independent PPT resistant and GUS positive plants produced by pSB624, and 14 independent hygromycin resistant and GUS positive plants produced by pSB4U24 according to the procedure described by Komari *et al.* (1989). The probes for the *bar* and GUS were the fragments amplified by the polymerase chain reaction from pSB25 and pBI221, respectively, (Ishida *et al.*, 1996). The probe for the *hpt* was the 1.1-kb *Bam*HI fragment from pGL2-IG (Hiei *et al.*, 1994). All of these plants were positive for both the resistance marker gene and the GUS gene in the Southern hybridization (**Fig. 2**).

The fact that all analyzed plants were different in hybridization patterns confirmed that the transgenes were randomly integrated into plant chromosomes (**Fig. 2**). Seventeen out of 21 plants transformed with pSB624 had 1 or 2 copies of the GUS gene. The copy number of the *bar* gene was 1 or 2 in all analyzed plants (**Table 2**). Ten of 14 plants transformed with pSB4U24 had 1 or 2 copies of the GUS gene while 12 plants had 1 or 2 copies of the *hpt* gene (**Table 2**). The ratio of plants that had a single copy of both the selection marker gene and the GUS gene was 46.2%, six (A9, 15, 39, 40, 41 and 52) out of 13 lines analysed for pSB624 and 14.3%, two (D9 and 13) out of 14 lines analysed for pSB4U24.

The progeny of the transgenic plants was obtained by self-pollination or cross with non-transformed A188. Resistance to antibiotics of detached leaves was assayed using a modified method of Wang and Waterhouse (1997). Leaves were excised from 10-day old seedlings and placed on a medium consisted of MS inorganic salts (Murashige and Skoog, 1962), 0.5 mg l^{-1} 6-benzylaminopurine (BA), 0.5 g l^{-1} 2-[N-Morpholino] ethanesulfonic acid (MES) and 8 g l^{-1} agar, and was supplemented with either 0.01% Basta (Hoechst, Frankfurt, Germany) for PPT resistance or 100 mg l^{-1} hygromycin for hygromycin resistance. The explants were cultured under constant illumination (50 μmol $m^{-2}s^{-1}$) at 25℃, and resistance to PPT or hygromycin was scored after 4 days of incubation.

The T1 progeny of 21 independent plants transformed with pSB624 was assayed for resistance to PPT and expression of GUS. All lines except A40 showed typical Mendelian segregation for both traits. The progeny that was GUS positive and PPT sensitive was obtained in 9 out of 21 lines (**Table 2**). Likewise, the GUS-positive, hygromycin-sensitive progeny was obtained from 3 out of 11 plants transformed with pSB4U24 (**Table 2**).

T2 seeds were obtained by self-pollination from four phenotypes of T1 plants of lines in which the GUS expression and resistance were unlinked, and

Table 2 Segregation of antibiotic resistance and GUS expression in the T1 progeny of transformants infected with LBA4404 (pSB624) or (pSB4U24) and estimation of the number of loci in the T0 plants

Line no. (T0) [1]	Copy number (T0)		Pollination [2]	Number of T1 plants				Number of loci in T0		
	GUS	bar or HPT		GUS+ PPT R or HYG R	GUS+ PPT S or HYG S	GUS- PPT R or HYG R	GUS- PPT S or HYG S	Unlinked GUS	Unlinked bar or HPT	Linked GUS-bar or GUS-HPT
A9	1	1	self	49	0	0	14	0	0	1
A13	3	1	self	33	10	17	4	1	1	0
A24	4	1	self	22	0	0	8	0	0	1
A28	2	2	self	43	17	4	0	2	1	0
A33	1	NT	self	28	0	0	11	0	0	1
A35	2	NT	self	34	0	0	14	0	0	1
A38	1	2	self	29	10	5	2	1	1	0
A39	1	1	self	6	8	6	0	1	1	0
A40	1	1	self	36	24	9	4	1	1	0?
A41	1	1	self	35	0	0	8	0	0	1
A43	2	NT	self	18	0	0	6	0	0	1
A44	2	2	self	44	14	3	2	2	1	0
A46	2	NT	self	33	0	0	8	0	0	1
A49	1	NT	self	25	11	5	1	1	1	0
A50	1	NT	self	35	0	0	12	0	0	1
A52	1	1	self	28	0	0	9	0	0	1
A54	5	1	self	32	0	0	9	0	0	1
A56	3	NT	self	17	0	0	14	0	0	1?
A7	2	2	female	15	3	13	1	1	2	0
A15	1	1	female	11	24	20	9	1	1	0
A25	2	NT	female	26	0	0	21	0	0	1
Control	-	-	-	0	0	0	63	-	-	-
D1	1	3	self	35	0	0	13	0	0	1
D2	2	1	self	23	0	0	9	0	0	1
D6	3	2	self							
D7	2	2	self	28	3	0	1	1	0	1
D9	1	1	self							
D11	4	3	self	41	0	6	0	0	1	1
D12	2	1	self	21	0	0	14	0	0	1
D13	1	1	self							
D16	1	2	self							
D21	3	1	self							
D23	3	1	self							
D24	2	2	self							
D25	2	2	self	36	7	0	5	1	0	1
D26	2	1	female	15	0	0	27	0	0	1
D28	NT	NT	self	36	5	0	7	1	0	1
D29	NT	NT	self	18	0	10	3	0	1	1
D30	NT	NT	self	16	0	0	0	0	0	1
D31	NT	NT	self	0	0	25	7	0	?	?
Control	-	-	-	0	0	0	32	-	-	-

[1] Line A was inoculated with LBA4404 (pSB624) and selected with phosphynothricin, line D was inoculated with LBA4404 (pSB4U24) and selected with hygromycin.

[2] self, T1 plants were obtained by self pollination; female, T1 plants were obtained by crossing pollen of non-transformed A188.

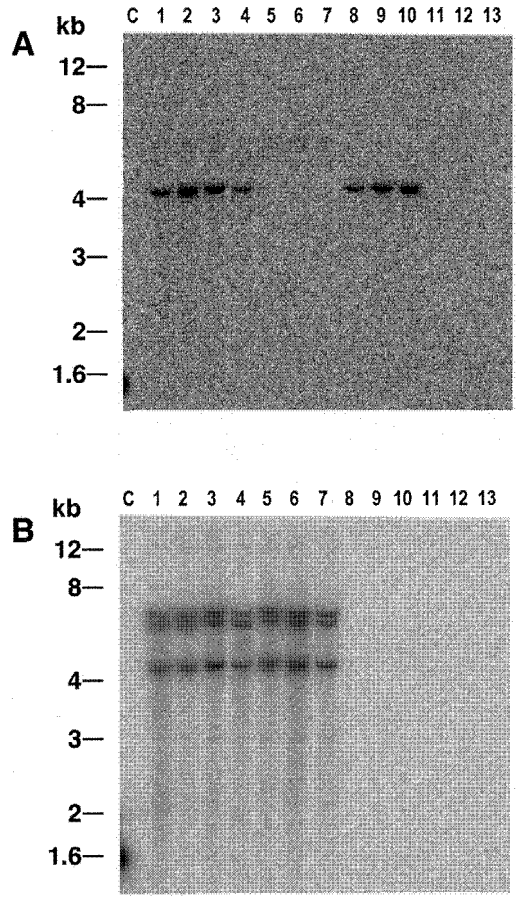
NT: not tested

bar, phosphinothricin resistance gene; HPT, hygromycon resistance gene

GUS+, GUS positive; GUS-, GUS negative

PPT, phosphinothricin; HYG, hygromycin

R, resistant; S, sensitive ? Strange segregation ratio.



assayed for the transgene expression. As an example, results from line A13, are shown **Table 3**. The GUS expression and resistance were inherited as two independent Mendelian loci.

The T1 progeny was also analyzed by Southern hybridization. For example, T1 plants of four phenotypes in line A13 were examined with the GUS and *bar* probes. The hybridization patterns and phenotypes were well correlated (**Fig. 3**).

We have demonstrated that co-transformation and segregation of marker-free transformants are

Fig. 3 Southern blot analysis of the T1 progeny of trasnformed plants A13.
DNA from T0 plant (lane 1), PPT-resistant, GUS-positive T1 progeny (lanes 2-4), PPT-sensitive, GUS-positive T1 progeny (lanes 5-7), PPT-resistant, GUS-negative T1 progeny (lanes 8-10) and PPT-sensitive, GUS-negative T1 plants (lanes 11-13) were digested with *Eco*RI and hybridized to the *bar* (A) and *gus* (B) probe.

Table 3 Segregation of GUS expression and phosphinothricin-resistance in the T2 progeny of transformant A13, which had unlinked gene of *GUS* and *BAR*, and estimation of the genotype of the T1 plants

T1 phenotype	Number of T2 plants				T1 genotype	
	GUS+, PPT R	GUS+, PPT S	GUS-, PPT R	GUS-, PPT S		
GUS+, PPT R	34	0	10	0	*GUS*/-	*BAR*/*BAR*
GUS+, PPT R	47	0	0	0	*GUS*/*GUS*	*BAR*/*BAR*
GUS+, PPT R	29	18	0	0	*GUS*/*GUS*	*BAR*/-
GUS+, PPT R	36	11	0	0	*GUS*/*GUS*	*BAR*/-
GUS+, PPT R	47	0	0	0	*GUS*/*GUS*	*BAR*/*BAR*
GUS+, PPT R	24	11	12	1	*GUS*/-	*BAR*/-
GUS+, PPT R	12	9	2	2	*GUS*/-	*BAR*/-
GUS+, PPT R	28	18	0	0	*GUS*/*GUS*	*BAR*/-
GUS+, PPT R	27	7	10	3	*GUS*/-	*BAR*/-
GUS+, PPT R	16	7	7	2	*GUS*/-	*BAR*/-
GUS+, PPT S	0	40	0	8	*GUS*/-	-/-
GUS+, PPT S	0	39	0	8	*GUS*/-	-/-
GUS+, PPT S	0	43	0	0	*GUS*/*GUS*	-/-
GUS+, PPT S	0	48	0	0	*GUS*/*GUS*	-/-
GUS-, PPT R	0	0	32	13	-/-	*BAR*/-
GUS-, PPT R	0	0	33	13	-/-	*BAR*/-
GUS-, PPT R	0	0	47	0	-/-	*BAR*/*BAR*
GUS-, PPT R	0	0	47	0	-/-	*BAR*/*BAR*
GUS-, PPT R	0	0	30	0	-/-	*BAR*/*BAR*
GUS-, PPT S	0	0	0	29	-/-	-/-
GUS-, PPT S	0	0	0	32	-/-	-/-
GUS-, PPT S	0	0	0	32	-/-	-/-

GUS+, GUS positive; GUS-, GUS negative
PPT, phosphinothricin
R, resistant; S, sensitive

now efficient processes in maize inbred A188. It is evident that the improved protocol of transformation was effective in solving the initial difficulties in transformation. The frequencies of both the co-transformation and unlinked integration were high and similar to the ones reported by Komari *et al.* (1996) in rice and tobacco. Therefore, the two-T-DNA vectors will likely be a useful tool in the studies of molecular biology and biotechnology in maize. Because pSB624 appeared to be better than both pSB424 and pSB4U24 in the overall performance, the use of the vectors that carry the *bar* gene as a selection marker may be the first choice for various applications in co-transformation in maize. On the other hand, because the *hpt* is the marker most frequently employed in rice transformation (Hiei *et al.*, 1994; Komari *et al.*, 1996; Abedinia *et al.*, 1997; Itoh *et al.*, 1997; Li *et al.*, 1997; Yokoi *et al.*, 1997), pSB4U24 will be useful when genes are examined in both rice and maize.

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