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ORIGINAL PAPER

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Co-transformation using a negative selectable marker gene for the production of selectable marker gene-free transgenic plants

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Abstract A negative selectable marker gene, *codA*, was successfully co-transformed with a GUS reporter gene to develop selectable marker gene-free transgenic plants. The pNC binary vector contained a T-DNA harboring the *codA* gene next to the *nptII* gene, while a second binary vector, pHG, contained a GUS reporter gene. Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were co-transformed via the mixture method with *Agrobacterium tumefaciens* LBA4404 strains harboring pNC and pHG, respectively. Seeds harvested from the co-transformants were sown on germination media containing 5-fluorocytosine (5-FC). Analysis of the progeny by GUS staining and PCR amplification revealed that all of the 5-FC-resistant R_1 plants were *codA* free, and that the *codA* gene segregated independently of the GUS gene. Because *codA*-free seedlings developed normally on 5-FC-containing medium, we suggest that co-transformation with negatively selectable markers is a viable method for the production of easily distinguished, selectable marker gene-free transgenic plants.

Introduction

Agrobacterium-mediated gene-transfer methods have the advantages of high transformation efficiency, limited transfer of T-DNA sequences, and relatively stable transfer of long T-DNA inserts. Researchers are able to easily handle transgenes in small binary vectors, in which selectable marker genes and target genes are usually loaded side by side between the T-DNA borders. Once transgenic plants are produced and selected according to the appropriate antibiotic or herbicide resistances, the selectable resistance gene becomes superfluous, and may cause problems such as limitations on the use of the same selectable marker genes for the addition of further transgenic traits, or even raise concerns regarding the release of the selectable products into the environment following commercialization of the transgenic plants (Puchta et al. 2000).

Several methods have been developed for the elimination of selectable marker genes in the final plant product. One promising method involves site-specific recombination-based systems such as the phage-derived *cre*/loxP system, the yeast derived *R*/RS system, and maize Ac/Ds system. However, at present their applications are limited to a few model plants, such as *Arabidopsis* (Gleave et al. 1999; Endo et al. 2002; Goldsbrough et al. 1993).

Another possible method for eliminating selectable marker genes is based on co-transformation. In these systems, selectable marker genes and target genes are not loaded between the same pair of T-DNA borders. Instead, they are loaded into separate T-DNAs, which are expected to segregate independently in a Mendelian fashion (Framond et al. 1986; McKnight et al. 1987; Vain et al. 2003). The advantages of co-transformation methods include the high adaptability of conventional, unmodified *Agrobacterium*-mediated gene-transfer methods and easier handling of the binary vectors because the two T-DNA are separated and, hence, target gene T-DNA can be handled independently of selectable marker gene T-DNA. This method depends on co-transformation efficiency and the independent integration of T-DNA into the plant genome. Generally, the co-transformation efficiency is in the range of 30–50%, which is acceptable for practical applications (Depicker et al. 1985; McCormac et al. 2001; Komari et al. 1996).

Negative selectable marker genes such as *iaaH*, *codA*, *argE*, and $P450_{SU1}$ can be used to kill or inhibit the growth

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of the transgenic plants. Isopentinyl transferase converts naphthalene acetamide to naphthalene acetic acid, resulting in the inhibition of normal plant growth (Karlin-Neumann et al. 1991; Eklof et al. 2000). Cytosine deamidase (CodA), ArgE, and $P450_{SU1}$ convert non-toxic 5-fluorocytosine (5-FC), acetyl phosphinothricin, and proherbicide R4702 into toxic 5-FU, phosphinothricin, and the herbicide R4702 metabolite, respectively (Schlaman and Hooykaas 1997; Koprek et al. 1996; O'Keefe et al. 1994; Werck-Reichhart et al. 2000). So, they can be used to easily sort transgenic plants (or seeds) from non-transgenic ones.

In this study, we combined negative selection with a co-transformation system with the aim of developing a practical method for identifying transgenic plants without large-scale PCR. We used the mixture method of transformation, despite its slightly lower co-transformation efficiency versus the single-strain method, because the mixture method is simpler and practically applicable. We transformed tobacco (*Nicotiana tabacum* cv. Samsun NN) with a mixture of two *Agrobacterium* strains: one containing the *nptII* gene and *codA* gene and the other containing a GUS reporter gene. Co-transformation efficiencies among R_0 plants transformed with different concentrations of the two *Agrobacterium* strains were compared by GUS staining and PCR. R_1 seeds from each co-transformant were germinated on medium containing 5-FC. The surviving R_1 seedlings were transferred to pots, and their PCR and GUS staining patterns were analyzed. With this associated system, selectable marker gene-free transgenic plants were successfully isolated through negative selection.

Materials and methods

Construction of vectors pNC and pHG

Unless otherwise mentioned, vectors were constructed using the standard techniques described by Sambrook et al. (1989). The *codA* gene was amplified from *Escherichia coli* JM109 genomic DNA, and the *mas* promoter was amplified from pTiAch5 of *A. tumefaciens*, using the following primers:

- *codA* forward: GTGGATCCATGTCGAATAACGC
- *codA* reverse: GTGAGCTCTCAACGTTTGTAATCGATGGC
- *Pmas* forward: GGTCTAGAGGCCAACAGAGCCTGGC
- *Pmas* reverse: GAGGATCCCGATTTGGTGTATCGAGATT

PCR products were cloned into pGEMT-easy vectors (Promega), and the sequences were verified by direct sequencing. The respective primers introduced *Bam*HI and *Sac*I sites at both ends of the *codA* PCR product and *Xba*I and *Bam*HI sites at both ends of the *mas* promoter product. To produce pNC, the 1.3-kb *Bam*HI/*Sac*I *codA* fragment was ligated between the *Bam*HI and *Sac*I sites of pBI121, resulting in pBI121codA. The 2.3-kb *Hin*dIII fragment from pMAT21, including a kanamycin resistance gene, was ligated into the *Hin*dIII sites of the pCB301 mini-binary vector, resulting in pN (Xiang et al. 1999). Three-point ligation was performed with the *Bam*HI/*Eco*RI fragment of pBI121codA, the *Xba*I/*Bam*HI fragment of the *mas* promoter, and the *Xba*I/*Eco*RI fragment of pN, resulting in pNC. For production of pHG, the *Pvu*II fragment of pINDEX3, which contains an enhanced hygromycin resistance gene, was ligated to the *Pvu*II fragment of pBluescript SK+ to generate the pH binary vector. The *Eco*RI/*Hin*dIII fragment of pBI121, which includes the GUS gene, was then ligated to the *Eco*RI/*Hin*dIII fragment of pH, resulting in pHG.

Agrobacterium handling

Competent *A. tumefaciens* LBA4404 was transformed with pNC and pHG binary vectors by the freeze-thaw method (Chen et al. 1994), and transformants were selected on YEP media containing kanamycin (25 mg/l). Transformation was confirmed with restriction analysis and PCR amplification of plasmids mini-prepped from each *Agrobacterium* strain (An et al. 1988).

Tobacco transformation and negative selection

Tobacco plants were transformed by the leaf-disk method (Horsch et al. 1985). Liquid *Agrobacterium* cultures were quantified by spectrophotometry at λ=600 nm and 0.5, 1, 2, 5, and 10 ml of the pNC-harboring strain were mixed with 1-ml aliquots of the pHG-harboring strain. Each harvested mixture was resuspended in 20 ml liquid MS medium and used directly for co-cultivation. Shoots were regenerated on shoot-inducing medium containing kanamycin (100 mg/l).

Negative selection was performed with R_1 seeds on MS medium containing 1% sucrose and 250 mg/l 5-fluorocytosine (Sigma, added directly to autoclaved, warm medium). After 7 days of seeding, resistant seedlings were transferred to pots and grown in a greenhouse.

GUS assay of transformants

Detached leaves or seedlings were infiltrated with GUS reaction solution [100 mM NaH_2PO_4, 10 mM Na_2EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% Triton X-100 (pH 7.0), and 40 μg/ml X-Gluc], using a laboratory aspirator (Jefferson et al. 1987). After incubation overnight at 37°C, chlorophyll was removed by bleaching in absolute ethanol.

PCR analyses of transformants

PCRs were performed with the AccuPower PCR Premix (Bioneer). Each reaction mixture (20 μl) consisted of 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 40 mM KCl, 250 μM dNTPs, and 1 U *Taq* DNA polymerase. Primers for *codA*-specific PCR were as listed above, and the GUS primers are as follows:

– GUS forward: ATGTTACGTCCTGTAGAAACC
– GUS reverse: CCTGTTGATCCGCATCACG-CAGTTC

Amplifications were carried out in a GeneAmp 2400 as follows: pre-cycling for 2 min at 94°C, followed by 35 cycles of 20 s at 94°C, 20 s at 50°C, and 40 s at 94°C. PCR products were run in 0.7% agarose gel, stained with ethidium bromide dye, and visualized with a UV illuminator.

Southern hybridization analysis

Total DNA was extracted from 0.5 g of young leaves, using the method of Dellaporta et al. (1983). Ten micrograms of genomic DNA were digested with *Eco*RI, electrophoresed, and transferred to Hybond-N^+ nylon membranes (Amersham), using the capillary transfer method. Fixing DNA to the membrane was done by baking for 2 h at 80°C. The membrane was hybridized overnight at 42°C with the 1.2-kb [^{32}P]-labeled GUS gene fragment amplified by PCR using specific primers 5′-TCATTGTTTGCCTCCCTGCTGCGG-3′ and 5′-GGAT-CAACAGGTGGTTGCAACTGG-3′.

Results

Transformation of tobacco with binary vectors and analysis of R_0 plants

Vector pNC contained a single T-DNA harboring the *nptiI* gene, and the *codA* gene fused with the *mas* promoter (Fig. 1). Vector pHG contained the GUS reporter gene.

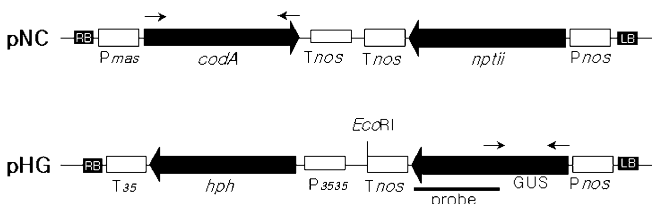

Fig. 1 Schematic maps of selectable marker gene T-DNA regions of pNC and pHG. *Arrows* indicate PCR primers used to confirm transgenic plants. The *underlined* portion of GUS gene, the 1.2-kb fragment amplified by PCR, was used as a probe for Southern blot analysis. *RB* Right border; *LB* left border; *Pmas* promoter of the mannopine synthase gene from pTiAch5; *Pnos* and *Tnos* promoter and terminator, respectively, of the nopaline synthase gene; *hph* hygromycin phosphotransferase; *P3535* and *T35* doubly-enhanced CaMV 35S promoter and terminator, respectively

Table 1 Co-transformation efficiency depended upon concentration ratio of LBA4404(pNC) to LBA4404(pHG). Data are mean values of three replications

pNC/pHG (ml)	0.5/1	1/1	2/1	5/1	10/1	5/0
GUS+/GUS−	43/36	35/45	22/58	5/75	1/79	0/81
co-tf (%)	54[a]	44[a]	28[b]	6[c]	1[c]	0[c]

[a,b] Values are significantly different at 5% level by Duncan's multiple range test

The mixture method, which uses a mixture of *Agrobacterium* strains to deliver multiple T-DNAs to plants cells, was used to introduce pNC and pHG binary vectors into the tobacco plants (Komari et al. 1996; Depicker et al. 1985; Petit et al. 1985). The highest co-transformation efficiency was achieved with an equal mixture of the two *Agrobacterium* strains. For the experimental transformations, 1 ml pNC and 1 ml pHG (Ab_{600}=1) were mixed and used to infect the tobacco plants. Consistent with previous reports, the maximal co-transformation efficiency was 40–50% of the total kanamycin-resistant (Km^R) regenerated tobacco shoots (Table 1). Co-transformants were GUS positive and yielded *codA*-specific fragments, following PCR amplification (data not shown). Among 21 co-transformed R_0 plants infected with mixture of two *Agrobacteria* [of which ratio was 0.5/1(pNC/pHG)], ten were randomly selected for further characterization of their segregation properties and negative selection patterns. Among these ten Km^R R_0 plants, seven lines (nchg1–7) contained both the GUS and *codA/nptII* genes, two lines (nchg8 and -9) contained the GUS and *nptII* genes, and one line (nchg10) had the *codA/nptII* gene alone. The latter three R_0 plants were included as controls (Table 2).

T-DNA segregation and analysis of selectable marker gene-free seedlings after negative selection

All ten transgenic plants were morphologically normal and showed full fertility. Seeds were harvested from them and

Table 2 Segregation of 5-fluorocytosine (5-FC) resistance and GUS expression in the R_1 progenies of tobacco transformants. Data are mean values of three replications

Line number (R_0)	5-FC^R		5-FC^S		5-FC^R:5-FC^S	GUS+:GUS−
	GUS +	GUS −	GUS +	GUS −		
nchg1	11	11	6	20	22:26	17:31
nchg2	18	5	30	9	23:39	48:14
nchg3	18	0	28	0	18:28	46:0
nchg4	16	4	17	4	20:21	33:8
nchg5	0	7	0	67	7:67	0:74
nchg6	0	0	15	5	0:20	15:5
nchg7	11	4	31	13	15:44	42:17
nchg8	32	7	0	0	39:0	32:7
nchg9	34	12	0	0	46:0	34:12
nchg10	0	5	0	50	5:50	0:55

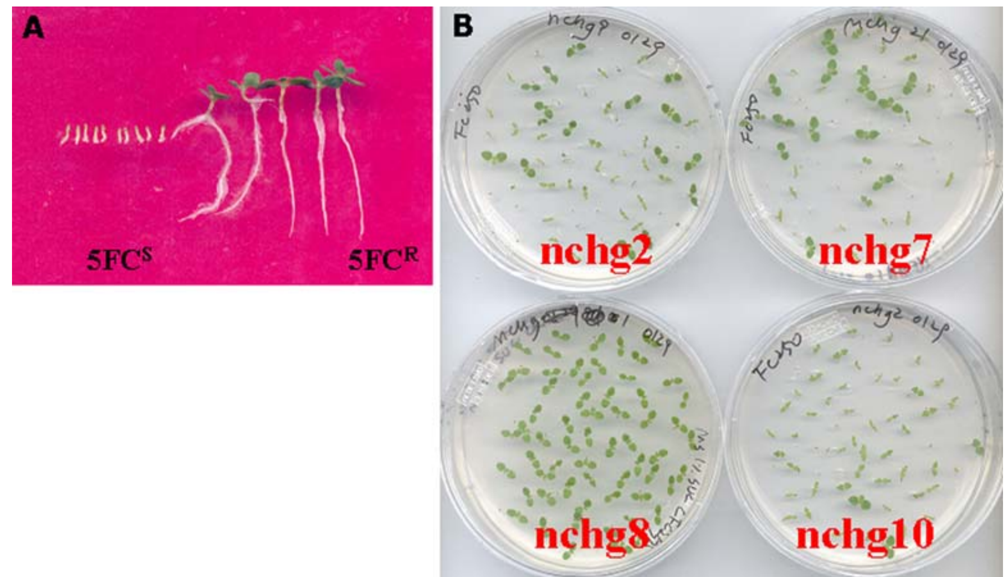

Fig. 2 Discrimination of R_1 seedlings germinated on negative selection medium containing 5-fluorocytosine (*5-FC*) (250 mg/l) (**A, B**)

germinated on media containing the negative selection agent, 5-FC. Because 5-fluorouracil (5-FU), a deaminated product of 5-FC by the CodA enzyme, inhibits normal development of seedlings, only transgenic plants harboring the *codA* gene showed abnormal seedling development (Fig. 2a, b). Following negative selection, the segregation ratios for GUS staining (GUS+:GUS−) and resistance to 5-FC [sensitive(S):resistant(R)] varied from 1 to 3 in most cases (Table 2). The ratios of GUS+ to GUS− among 5-FC^R seedlings and 5-FC^S seedlings were similar except in line nchg1, which was thought to be the result of linked integrations of each T-DNAs or multiple integrations of each T-DNAs. These results showed that the *codA* and GUS genes segregated as independent genetic loci. The *codA* gene seemed to be solely responsible for abnormal seedling growth, because all seedlings of lines nchg8 and nchg9, which lacked the *codA* gene, survived normally on media containing 5-FC.

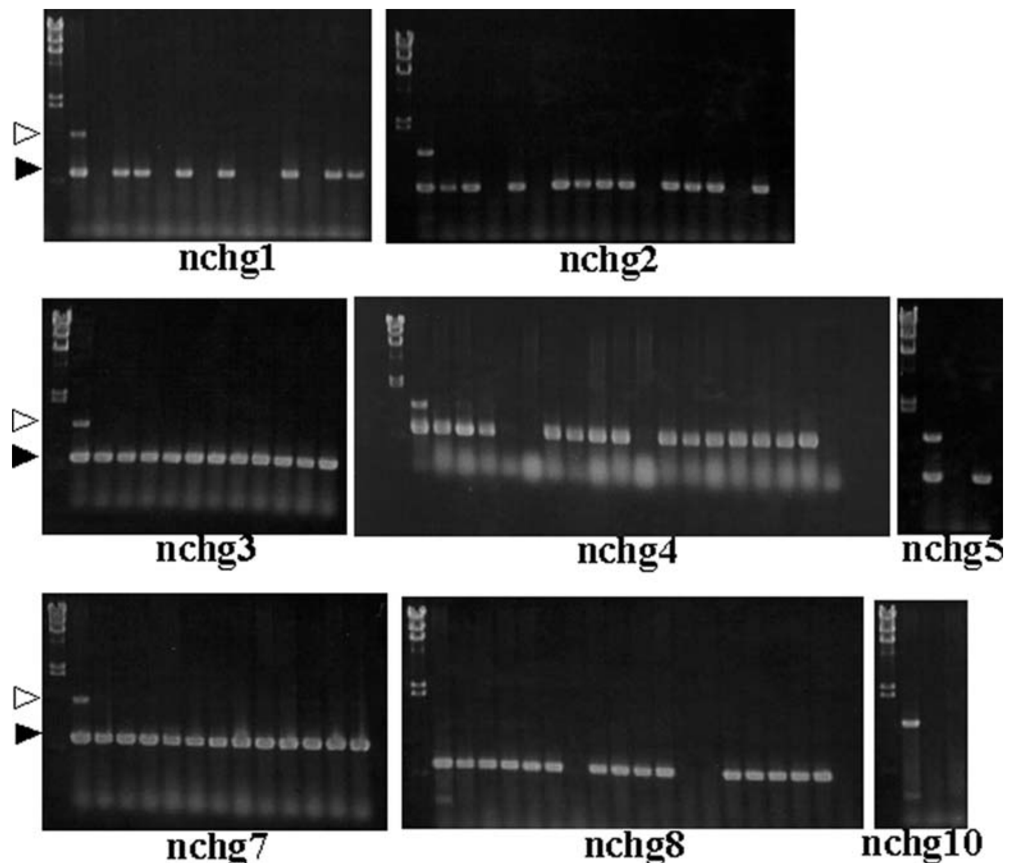

Fig. 3 PCR analysis of 5-FC^R R_1 plants. Genomic DNA from arbitrary 5-FC^R R_1 plants was used as templates for *codA*- and GUS-specific PCR amplification. *Lane 1* DNA size marker, *lane 2* PCR products of each R_0 plant, *lane 3–end lane* those of R_1 plants. *Open arrowheads* indicate *codA*-specific amplification, and *closed arrowheads* indicate GUS-specific amplification

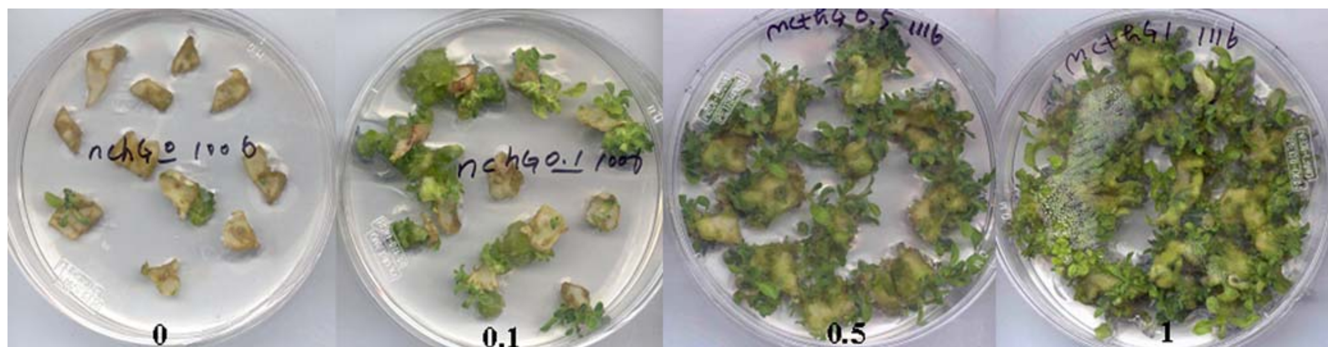

Fig. 4 Shoot regeneration from tobacco leaflets transformed with indicated ratios of LBA4404(pNC) and LBA4404(pHG). Infection mixtures (20 ml) contained 0, 0.1, 0.5, or 1 ml of pNC (Ab_{600}=1), mixed with 1 ml of pHG (Ab_{600}=1)

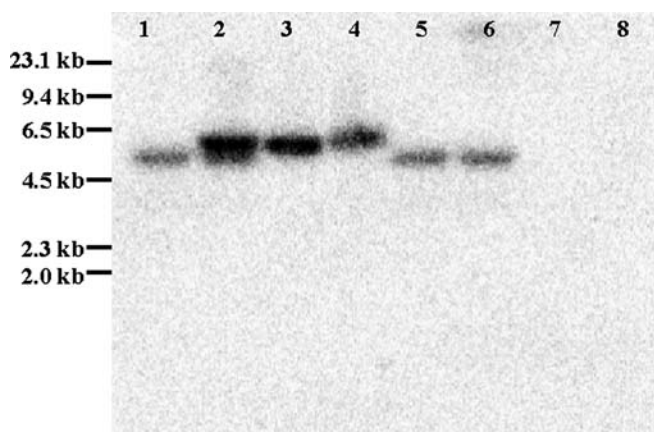

Fig. 5 Southern hybridization analysis of 5-FC^R nchg7 tobacco transformants. The genomic DNA from 5-FC^R nchg7 transgenic tobacco was used for Southern blot analysis. *Eco*RI digest of 10 μg genomic DNA was hybridized with the 1.2-kb [^{32}P]-labeled coding region of GUS gene amplified by PCR. *Lanes 1–6* GUS+ transgenic plants, *lanes 7 and 8* GUS− transgenic plants

After negative selection, transgenic plants showing normal growth were PCR-tested for the *codA*- and GUS-specific fragments. All normal survivors lacked *codA*-specific amplification and also appeared to lack the *nptII* gene, which was next to the *codA* gene in pNC. The transgenic plants that showed positive GUS staining also showed GUS-specific amplification.

In all transgenic plants, 5-FC-resistant R_1 plants were free of the *codA* gene. GUS gene segregation in R_1 seeds of lines nchg2, -4, -6, and -7 appeared Mendelian, irrespective of the negative selection (Fig. 3).

For further affirmation of segregation data, several of the 5-FC-resistant R_1 plants from nchg7 were subjected to Southern hybridization analysis using PCR-amplified fragments as a probe (Fig. 1). Six GUS+ transgenic plants (Fig. 5, lanes 1–6) showed one or two bands but two GUS − transgenic plants no bands (Fig. 5, lanes 7 and 8). Two different sizes of bands indicated more than two different insertions of T-DNAs. No band signals were detected by Southern hybridization of the same nylon membrane, using the *codA* gene-specific probe (data not shown).

Discussion

In this study, we showed that selectable marker gene-free R_1 seeds could be successfully discriminated from total R_1 seeds by negative selection using the *codA* gene from *E. coli*. Co-transformation methods have been posited as possible strategies for the elimination of selectable marker genes (Komari et al. 1996; McKnight et al. 1987), and several such methods have been reported. However, this is the first report in which negative selection is combined with co-transformation for the purpose of simplifying the identification of selectable gene-free transgenic plants. Negative selection tends to focus on the target gene, because the selection strategy seeks to rule out the progeny with T-DNAs harboring selectable marker genes. Each T-DNA integrated by co-transformation behaves as a Mendelian locus during inheritance (Framond et al. 1986; McKnight et al. 1987), as confirmed by our observation that the target genes in R_0 plants segregated similarly in both 5-FC^R and 5-FC^S R_1 plants.

Because co-transformation depends on the establishment of an effective *Agrobacterium*/plant cell interaction, the relative amounts of each *Agrobacterium* strain in the infection mixture was expected to influence the co-transformation efficiency (Depicker et al. 1985). We found that the co-transformation efficiency was highest at similar concentrations of each *Agrobacterium*, and as their difference increased, the efficiency of co-transformation diminished dramatically (Table 1). In our experiments, up to 50% of selected T_0 plants were co-transformed when 0.5 ml of pNC (Ab_{600}=1) was mixed with 1 ml of pHG (Ab_{600}=1) in a total infection mixture of 20 ml. In contrast, a mixture of less than 0.1 ml pNC with 1 ml of pHG resulted in a drastically decreased number of co-transformed T_0 plants (Fig. 4). This may result from infective competition between the two *Agrobacterium* strains, or from the low absolute concentration of the *Agrobacterium* strain harboring the selectable marker binary vectors. Our results suggest that concentration ratios in the range of 0.5–1 are optimal for co-transformation via the mixture method.

This negative selection method by *codA* gene will allow researchers to easily narrow their search for selectable marker-free transgenic plants without massive amounts of

PCR analyses. In addition, because the viability is equal to the inverse of the selectable marker T-DNA segregation ratio, analysis of negative selection results may provide clues about the molecular arrangement of selectable marker T-DNA in transgenic plants. Indeed, the GUS staining and PCR results suggested that our segregations were nearly Mendelian in nature.

Taken together, our results suggest that negative selection and co-transformation may provide a simple, effective method for developing selectable marker gene-free transgenic plants that may be identified as such, without extensive, time-consuming assays.

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