A conditional negative selection for *Arabidopsis* expressing a bacterial cytosine deaminase gene

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ABSTRACT

The enzyme activity for cytosine deaminase, which converts cytosine to uracil in bacteria, is usually undetected in higher plants and animals. The enzyme also catalyzes conversion of non-toxic 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), a toxic compound for plant growth. The gene encoding cytosine deaminase (*codA*) from *Escherichia coli* was fused to cauliflower mosaic virus (CaMV) 35S promoter (P35S), and cloned into a binary vector pLABR101. The resulting plasmid pLABR102 contained two marker genes for plants: a positive marker gene, bialaphos resistance (*bar*) gene driven by the promoter from nopaline synthase gene (Pnos) and a negative one, P35S-*codA*. The binary vector pLABR102 was transformed into *Arabidopsis thaliana* via *Agrobacterium*-mediated transformation. In transgenic progenies (T3) of the second (T2) generation heterozygous for a single T-DNA insertion, a 3 : 1 segregation ratio was observed on both bialaphos (resistance to sensitive) and 5-FC (sensitive to unaffected). From T2 plants homozygous for the T-DNA insert, on the other hand, no segregation was detected: all the T3 seedlings were resistant to bialaphos and sensitive to 5-FC. PCR and Northern analyses showed that the 5-FC sensitivity in transgenic descendants was caused by the integration and expression of the chimeric *codA* gene in the *Arabidopsis* genome. The results indicated that cytosine deaminase from *E. coli* is functional and useful for negative selection in *Arabidopsis*, and that sensitivity to 5-FC as well as the positive bialaphos resistance are dominant traits in *Arabidopsis*.

1. INTRODUCTION

In plants dominant positive markers have been widely used for selection and screening of transformants. Examples of such markers are the kanamycin resistance coded by the neomycin phosphotransferase (*nptII*) gene (Bevan et al., 1983b; Herrera-Estrella et al., 1983), hygromycin resistance coded by the hyg-

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romycin phosphotransferase (*hpt*) gene (van den Elzen et al., 1985; Waldron et al., 1985) and bialaphos resistance coded by the phosphinothricin acetyltransferase (*PAT* or *bar*) gene (De Block et al., 1987). The products of positive marker genes convert toxic compounds such as antibiotics and herbicides to nonpoisonous ones. Following the expression of negative marker genes, cytotoxic compounds are produced directly (nonconditional markers) or innocuous compounds are metabolized into cytotoxic ones causing cell death (conditional markers). The negative genetic markers have been useful for "gene targeting" mediated by homologous recombination especially in mouse (Capecchi, 1989). The gene targeting has been demonstrated to be effective for the elucidation of the function of given genes. In addition to the positive selection with a neomycin resistance (*neo^r*) gene, the negative selection with a herpes simplex virus thymidine kinase (*HSV-tk*) gene (Mansour et al., 1988) or a diphtheria toxin A-fragment (*DT-A*) gene from corynebacteriophages (Yagi et al., 1990) is used to eliminate nonhomologous recombination (random insertion) and to enrich homologous recombination using the positive and negative selection (PNS) strategy (Capecchi, 1989). The PNS strategy would be widely applicable to any other species since it is independent of the function of the target genes.

Several negative-selection markers have already been demonstrated to be functional in plants. Those include such suicide genes (nonconditional negative markers) as yeast *RAS2* (Hilson et al., 1990), ribonuclease genes from *Aspergillus oryzae* (*RNase-T1*) and from *Bacillus amyloliquefaciens* (*barnase*) (Mariani et al., 1990), *DT-A* (Koltunow et al., 1990; Czako and An, 1991; Thorsness et al., 1991) and *Pseudomonas aeruginosa* exotoxin A gene (Koning et al., 1992). The anti-*nptII* gene in combination with a constitutive *nptII* gene would also have potential as negative markers in the PNS procedure (Xiang and Guerra, 1993).

The conditional negative markers would be more useful not only for gene targeting but for screening of altered phenotype(s) caused by the expression of a gene of interest. Amidohydrolase coded by *tms2* gene from T-DNA of *Agrobacterium tumefaciens* converts indole-3-acetamide into indole-3-acetic acid (Depicker et al., 1988; Karlin-Neumann et al., 1991). Transgenic plants with a constitutive nitrate reductase (NR), consisted of an NR cDNA driven by CaMV 35S promoter, are selectively killed on medium containing chlorate and ammonium since the endogenous *NR* gene is not expressed under nitrate-free conditions (Nussaume et al., 1991). Very recently it has been demonstrated that the *HSV-tk* gene would work as a negative marker gene in *Arabidopsis* (Czakó and Márton, 1994), and also that transgenic tobacco plants with chloroplast-targeted expression of the *Streptomyces* cytochrome P450 gene were selectively susceptible to R7402 (sulfonylurea pro-herbicide) (O'Keefe et al., 1994).

Cytosine deaminase in bacteria catalyzes conversion of cytosine to uracil. The enzyme is also capable of conversing 5-FC, a nontoxic compound, into 5-FU, a cytotoxic metabolite which is a precursor of metabolic inhibitors for both RNA

and DNA synthesis (Mullen et al., 1992; Stougaard, 1993). Since the cytosine deaminase activity is not usually detectable in higher organisms (Mullen et al., 1992; Stougaard, 1993), the cytosine deaminase (*codA*) gene was cloned into an expression vector and introduced into mouse fibroblast cells. Transfected cell populations exhibited significant cytosine deaminase activity and were selectively killed in the presence of 5-FC (Mullen et al., 1992). In plants a chimeric *codA* gene under the control of CaMV 35S promoter was introduced into tobacco and *Lotus japonicus* (Stougaard, 1993). The results revealed that the *codA* marker is useful for substrate-dependent negative selection and segregates as a dominant marker. The lack of cytosine deaminase activity in many plant species including *Arabidopsis*, pea, barley, etc. indicated that the *codA* gene is widely applicable as a negative selective marker (Stougaard, 1993).

In this paper, we introduced a chimeric *codA* gene as a negative marker and a chimeric *bar* gene as a positive marker into *Arabidopsis* to investigate the effectiveness of those marker genes in *Arabidopsis*, a model plant for molecular genetics. The results indicated that in *Arabidopsis* the *codA* and *bar* genes function well as a negative marker and a positive marker, respectively. In the course of this study, Perera et al. (1993) transformed *codA* gene and Pnos-*nptII* gene (a positive marker) into *Arabidopsis* and reported that the *codA* gene works in *Arabidopsis* as a negative selective marker.

2. MATERIALS AND METHODS

Plant Growth

Sterilized seeds of *Arabidopsis* (Wassilewskija ecotype) were germinated and grown on germination medium [GM: Murashige-Skoog (MS) medium (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 20 g/l of sucrose, 0.5 g/l of 2-(*N*-morpholino)ethanesulfonic acid (MES), 2.5 g/l of Gelrite, pH 5.7]. Plants including shoots regenerated from root explants in vitro were grown under white light at ca. 100 $\mu\text{mol}/\text{m}^2/\text{sec}$, 12 h light/12 h dark cycle at 22°C, and root explants were cultured under white light at ca. 50 $\mu\text{mol}/\text{m}^2/\text{sec}$ if not specifically mentioned. 5-FC and 5-FU (Wako Pure Chemical, Japan) were dissolved in 10 mM MES (pH 5.7), sterilized by filtration and added after autoclaving of GM. The concentration of 5-FC was 250 mg/l for standard negative selection.

Agrobacterium-mediated transformation of Arabidopsis

The transformation procedure for *Arabidopsis* was according to the root-transformation method (Valvekens et al., 1988) in principle using the *bar* gene which confers bialaphos resistance. Root explants cut out from in vitro-grown plants were cultured on callus-inducing medium [CIM: GM + 0.5 mg/l of 2, 4-dichlorophenoxyacetic acid (2, 4-D) + 0.05 mg/l of kinetin] for three days in the dark. The explants were then cocultivated with *Agrobacterium* (strain EHA101)

containing an appropriate binary vector for 2–3 days on CIM in darkness. After washing the root explants with liquid GM containing 100 mg/l of carbenicillin (Cb), the explants were cultured on shoot-inducing medium [SIM: GM + 0.15 mg/l of indole-3-acetic acid (IAA) + 5.0 mg/l of N^6 -(2-isopentenyl)adenine (2ip)] containing 100 mg/l of Cb and 300 mg/l of vancomycin (Vm) for two days and were transferred to selection medium (SIM + Cb + Vm + 1 mg/l of bialaphos) for obtaining transgenic shoots (T1 generation). The regenerated shoots with bialaphos resistance were transferred to GM containing 1 mg/l of bialaphos for shoot elongation, flowering and T2 seed setting in vitro. In control experiments no shoot regeneration was observed from root explants without *Agrobacterium* treatment on SIM containing bialaphos at the concentration of 1.0 mg/l or more.

DNA isolation

Isolation of genomic DNA preparation from transgenic or nontransgenic *Arabidopsis* plants was performed according to Edwards et al. (1991) with some modifications. Fifty to 80 mg of leaf tissue was ground with 500 μ l of extraction buffer [0.2 M Tris-HCl (pH 7.5), 0.25 M NaCl, 25 mM EDTA and 0.5% SDS] and one droplet of 2-mercaptoethanol in an Eppendorf tube and was centrifuged at 13,000 rpm for one min. The supernatant was extracted once with phenol and once with phenol-chloroform. This additional step improved the purity and solubility of the DNA sample. Equal volume of isopropanol was added to the water phase and mixed. After centrifugation at 13,000 rpm for 10 min, the pellet containing DNA was washed once with 70% ethanol, dried under vacuum and dissolved at 60°C in 100 μ l of TE buffer.

PCR amplification

Two sets of oligonucleotide primers for PCR were designed so that either a 0.53 kb or a 1.0 kb DNA fragment, which contains the *codA* sequence from position 2,081 to 2,609 in *codBA* operon (Danielsen et al., 1992) or contains both the *codA* sequence and the poly (A) signal of nopaline synthase gene (pAnos; Bevan et al., 1983a) in a binary vector pLABR102 (Fig. 1) respectively, could be amplified specifically from genomic DNAs of transgenic plants by PCR. The 0.53 kb fragment also contains a *Cla*I restriction site to bring about 0.22 kb and 0.31 kb fragments. The forward and reverse primers used for amplification of the 0.53 kb *codA*-containing sequence were *codA*-1 with the sequence 5'-GGATTGATCTGCAAATCGTC-3' (20 bases) and *codA*-2 with the sequence 5'-AGCGGATACACGGATCGAA-3' (20 bases). Those used for amplification of the latter 1.0 kb sequence were *codA*-1 and *Tnos*-1R with the sequence 5'-TATCCTAGTTTGC GCGCT-3' (18 bases). PCR amplification was carried out using approximately 100 ng of genomic DNA, Taq DNA polymerase (AmpliTaq[®], Perkin Elmer Cetus) and the two primers in a reaction volume of 50 μ l. The reaction conditions were as follows: denaturation at 94°C for 1.5 min, annealing at

55°C for 2.0 min, polymerization at 72°C for 3.0 min. These steps were repeated for 30 cycles followed by a 7 min extension at 72°C. Each of the PCR products was analyzed on a 1.5% agarose gel.

RNA isolation and Northern blot analysis

Total RNA was isolated individually from expanded leaf tissue (ca. 100 mg) of each T3 plant according to Fromm et al. (1985). Each total RNA sample (10 µg) was glyoxalated, separated on a 1.4% agarose gel and transferred to a nylon membrane according to Sambrook et al. (1989). A 1.3 kb *SacI*-*XbaI* DNA fragment containing *codA*-coding sequence (Fig. 1) was labeled with [α -³²P] dCTP (Amersham) by random priming and used as a probe in Northern hybridization.

3. RESULTS

Construction of a binary vector containing the chimeric *codA* gene

pLABR101 (Fig. 1) was constructed from pLAN421 (Uematsu et al., 1991) and the *Pnos-bar* gene in pGPTV-BAR (Becker et al., 1992). The *Pnos-nptII* gene in pLAN421 was replaced with the *Pnos-bar* gene taken from pGPTV-BAR. The T-DNA region of the resulting plasmid pLABR101 contained the *Pnos-bar* gene and a P35S-*GUS* (β -glucuronidase) reporter gene. A P35S-*codA* chimeric gene derived from pNE3 (Fig. 1; Stougaard, 1993) was cloned into the *EcoRI*/*HindIII* sites of pLABR101 to construct pLABR102. Consequently, pLABR102 contained the *Pnos-bar* gene as a positive selective marker and P35S-*codA* gene as a putative marker for negative selection in *Arabidopsis* cells (Fig. 1).

Figure 1. Schematic representation of pNE3 containing the P35S-*codA* gene and T-DNA regions in the binary vectors pLABR101 and pLABR102. RB and LB, right and left border of T-DNA; Tnos and Tg7, polyadenylation signal of *nos* gene and gene 7 of the Ti plasmid, respectively. Ω , 5' leader sequence of tobacco mosaic virus RNA which functions as an enhancer of translation (Gallie et al., 1987).

Effect of 5-FC and 5-FU on wild-type Arabidopsis

Germination and growth of *Arabidopsis* seedlings were not inhibited visually on medium containing various concentrations of 5-FC (100–500 mg/l) as shown in Fig. 2A, though plant growth was slightly suppressed with 5-FC at 1,000 mg/l (data not shown). In contrast to the effect of 5-FC, 5-FU was toxic to *Arabidopsis* growth at all the concentrations tested from 25 to 200 mg/l (Fig. 2B). Not only the growth of *Arabidopsis* but its germination was inhibited severely in the presence of 5-FU at 50 mg/l or more. The control experiments indicated that cytosine deaminase activity in *Arabidopsis* should be sufficiently low to apply negative selection with *codA* gene expression.

Figure 2. Response of wild-type *Arabidopsis* plants grown on germination medium (GM) containing 5-FC (A) or 5-FU (B). Photographs were taken after two weeks of growth on each medium.

Effect of 5-FC on offsprings of transgenic plants

Seeds (T2) from some of the transgenic *Arabidopsis* lines transformed with pLABR102 were germinated on medium containing 1 mg/l of bialaphos. When more than 20 T2 seeds in total were obtained from a T1 transformant, the growth response of *Arabidopsis* seedlings on medium containing 250 mg/l of 5-FC was also tested. In each line tested for both drugs, the segregation ratio of T2 seedlings for bialaphos resistance (resistant to sensitive) was similar to that for 5-FC sensitivity (sensitive to unaffected), suggesting that the *codA* gene would be functional and dominant in *Arabidopsis*. Each T2 plant showing bialaphos resistance was transplanted and grown in soil to obtain independent T3 seed pools. The response of each offspring (T3) was scored on bialaphos and 5-FC. A typical growth response of T3 transgenic *Arabidopsis* to each drug is shown in Fig. 3A and 3B. A compilation of the segregation data from T3 lines on bialaphos or 5-FC

Figure 3. Growth response of T3 transgenic *Arabidopsis* seedlings transformed with pLABR102 on GM containing 1 mg/l of bialaphos or 250 mg/l of 5-FC. Photographs show two-week-old transgenic seedlings. A, W102A8-4; B, W102A8-3.

is presented in Table 1. For example, as shown in Fig. 3A and Table 1, about 3 : 1 was the segregation ratio of T3 seeds of W102A8-4 derived from a T2 line (W102A8) selected for bialaphos resistance. Similarly, the ratio of those for 5-FC sensitivity was about 3 : 1 for sensitive to unaffected. The other T3 line W102A8-3, which was also obtained from W102A8, showed no segregation for bialaphos resistance or 5-FC sensitivity (Fig. 3B and Table 1). In W102A8-3, all the seeds tested were bialaphos resistant and 5-FC sensitive. Accordingly the line W102A8 was suggested to possess a single insertion of T-DNA per haploid genome or T-DNA insertion(s) closely linked on a single chromosome. The similar segregation ratios for these two phenotypes were observed also in the other independent transgenic lines (W102A2 and W102A7). Each ten of the transgenic seedlings (T3) with bialaphos resistance or 5-FC resistance in vitro were transferred to soil. 1.0 g/l of Herbiace® (Meiji-seika, Japan) containing 20% bialaphos was applied by spraying to the T3 transgenic plants. All the T3

Table 1. Segregation of T3 transgenic seedlings on the sensitivity of bialaphos and 5-FC

Transgenic line	Growth on bialaphos		Growth on 5-FC	
	resistant : sensitive	χ^2 ^a	sensitive : unaffected	χ^2 ^a
W102A2-1	37 : 19	2.38	44 : 10	1.20
W102A2-2	44 : 14	0.02	42 : 16	0.20
W102A7-1	45 : 14	0.05	48 : 17	0.05
W102A7-2	57 : 0	–	61 : 0	–
W102A8-1	41 : 13	0.03	48 : 9	2.57
W102A8-2	39 : 17	0.86	42 : 14	0
W102A8-3	50 : 0	–	48 : 0	–
W102A8-4	41 : 13	0.03	37 : 13	0.03
W102A8-5	40 : 15	0.15	38 : 11	0.17
W102A8-6	39 : 15	0.22	39 : 12	0.06

^a χ^2 was calculated based on an expected ratio of 3 : 1 ($P > 0.05$).

individuals with bialaphos-resistance in vitro showed herbicide resistance and all the 5-FC resistant seedlings were sensitive to bialaphos like wild-type plants (Fig. 4A). On the contrary, the growth of bialaphos-resistant plants were severely inhibited after transfer from bialaphos-containing medium to that with 5-FC (Fig. 4B). These results demonstrate that the P35S-*codA* chimeric gene is functional as a conditional negative-selection marker in the presence of 5-FC in *Arabidopsis* and that the phenotype on 5-FC sensitivity is a dominant trait.

Molecular analysis of transgenic plants

PCR analysis was performed to examine whether the *codA*-coding sequence is present or not in the genomes of individual bialaphos-resistant plants or in those of individual 5-FC-resistant plants. The DNA preparations of individual T3 seedlings with bialaphos resistance contained a 0.5 kb DNA fragment which was specifically amplified by PCR with *codA*-1 and *codA*-2 as primers (Fig. 5A) and had a single *Cla*I restriction site to bring about 0.3 and 0.2 kb fragments (data not shown). No amplified DNA fragment, however, was observed in the genomic DNA from each T3 plant with 5-FC resistance. An expected 1.0 kb fragment was also specifically amplified by PCR with *codA*-1 and *Tnos*-1R as primers in each DNA sample from bialaphos-resistant T3 individuals but not in that from 5-FC-resistant seedlings (data not shown). These results indicated that the 5-FC-resistant phenotype observed among the T3 seedlings was caused by the segregation of the negative marker gene and consequently the absence of foreign *codA* gene in the genome. Northern analysis with *codA* coding sequence as a probe indicated that the T3 bialaphos-resistant individuals expressed the *codA* gene and that 5-FC-resistant ones showed no expression of the gene (Fig. 5B).

These results showed that the 5-FC sensitivity in transgenic descendants was

Figure 4. Response of T3 transgenic seedlings scored for bialaphos resistance or 5-FC resistance in vitro, after application of Herbiace® (A) or transfer to GM containing 5-FC (B). In vitro the T3 transgenic seeds were germinated and grown for 2 weeks on GM containing 250 mg/l of 5-FC or 1 mg/l of bialaphos. A, T3 plants (line W102A9-1) showing resistance to bialaphos (left) or 5-FC (right), in vitro, were transferred to soil, grown for 10 days, sprayed all over with 1 g/l of Herbiace® containing 20% (w/v) of bialaphos and grown for two weeks in a closed greenhouse. B, Plants (line W102A9-5) resistant to bialaphos (left) or 5-FC (right), in vitro, were also transferred to new GM+5-FC and grown for 13 days.

caused by the integration of chimeric *codA* gene into the plant genome and by the expression of the integrated gene. Consequently cytosine deaminase from *E. coli* is functional and useful for negative selection not only in tobacco and *L. japonicus* but also in *Arabidopsis*.

4. DISCUSSION

We constructed a binary vector with the *bar* gene as a positive selectable marker and the *codA* gene as a negative selectable marker (Fig. 1). Chimeric *bar* genes driven by CaMV 35S promoter or by the promoter of a *RbcS* (small subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase) gene from *Arabi-*

Figure 5. Molecular analysis of the chimeric *codA* gene in individual T3 transgenic plants. A, PCR analysis with *codA*-1 and *codA*-2 as primers. Lanes at both ends indicate molecular markers. Bi⁺ and 5-FC⁺, individuals resistant to bialaphos and 5-FC, respectively. wt, wild type individual. B, Northern blot hybridization with *codA* coding sequence as a probe. Lanes 1 and 2: W102A8-1 with bialaphos resistance (1) and with 5-FC resistance (2); 3 and 4: W102A8-4 with bialaphos resistance (3) and 5-FC resistance (4). Each DNA or RNA sample was prepared from a single plant.

dopsis have been transformed into *Arabidopsis*. Both chimeric *bar* genes conferred bialaphos resistance on transgenic *Arabidopsis* by herbicide spray tests (H. Ichikawa et al., unpublished results). We confirmed in this paper that in *Arabidopsis* the *bar* gene is also useful for selection of transformants with bialaphos resistance in vitro. To our knowledge this is the first report in *Arabidopsis* indicating that the Pnos-*bar* gene can be used as a positive marker for the selection of bialaphos-resistant regenerants in vitro by *Agrobacterium*-mediated transformation (D'Halluin et al., 1992).

Since wild type *Arabidopsis* (Wassilewskija ecotype) showed resistance to 5-FC (Fig. 2A), the endogenous cytosine deaminase activity should be absent or very low in *Arabidopsis*. The result confirmed the 5-FC resistance of the RLD ecotype of *Arabidopsis* (Perera et al., 1993) and the absence of cytosine deaminase activity in *Arabidopsis* (Stougaard, 1993). Transgenic *Arabidopsis* plants with the P35S-*codA* gene were changed to be 5-FC sensitive (Fig. 3 and Table 1). All the bialaphos-resistant individuals tested so far were sensitive to 5-FC (Fig. 4), possessed the *codA* transgene and expressed the *codA* gene (Fig. 5). T3 segregants showing 5-FC-resistance were bialaphos sensitive, and did not possess and express the *codA* gene. The 5-FC-insensitive plants in T3 generation turned out to be wild-type plants segregated from a T2 plant heterozygous for a single T-DNA insertion. Genetic analysis of *codA* transgenic progenies indicated that the 5-FC sensitivity caused by the P35S-*codA* gene was a dominant trait (Table 1). Consequently, our results clearly demonstrated that *codA* gene is a useful

dominant-negative marker in *Arabidopsis*.

Perera et al. (1993) described that three out of six transgenic lines (T3) with 35S-*codA* transgene and kanamycin resistance showed insensitivity to 5-FC. They also suggested that relatively high copy number (four to five) of the *codA* gene in the 5-FC-insensitive transgenic lines would have caused the partial reduction in *codA* gene expression (epigenetic gene silencing). We have analyzed our transgenic plants (T3) with special emphasis on genetics and at the individual level for responses to 5-FC and bialaphos, presence of transgene (*codA* sequence) and *codA* gene expression. Six independent transgenic lines including three lines shown in Table 1 were subjected to genetic analysis. The results demonstrated that each line possessed a single T-DNA insertion per haploid genome as deduced from the segregation ratios on responses to bialaphos and 5-FC (Table 1 and data not shown). Expression of the transgenes (*bar* and *codA*) was stable in the T3 generation and no genetic discrepancy was observed between the ratios on sensitivity to bialaphos and to 5-FC in each T3 transgenic line. Moreover our *codA* gene contained between CaMV 35S promoter and the *codA* gene the 5'-leader sequence of tobacco mosaic virus RNA [Ω (omega) sequence; Fig. 1] which is a general enhancer of translation (Gallie et al., 1987). If the *CodA* phenotype would be more affected by partial gene silencing than kanamycin phenotype as suggested by Perera et al. (1993), the frequency of *codA*-transgenic plants (T3) unaffected by 5-FC but resistant to kanamycin could be reduced to some extent by the insertion of Ω sequence in the chimeric *codA* gene. Accordingly, our transformation system using the *bar* gene as a positive selectable marker and P35S-" Ω "-*codA* gene as a negative marker would be more suitable for the selection of transgenic *Arabidopsis* plants with a single T-DNA insertion and stable expression of the *codA* gene when compared with the system by Perera et al. (1993), and for the genetic analysis of effect of *codA* negative marker gene.

Homologous DNA recombination is a prerequisite for gene targeting or gene knock-out experiments designed to elucidate the function of the specific genes. In such experiments part of the target gene is replaced with another DNA sequence for example the positive marker gene, *nptII*, and is inactivated completely. Though the homologous DNA recombination has been observed in plants, almost all the reported experiments were designed with primary transgenic plants carrying a defective copy of positive selective marker genes (Paszkowski et al., 1988; Baur et al., 1990; Lee et al., 1990; Offringa et al., 1990; Peterhans et al., 1990; Halfter et al., 1992). Those strategies in plants are dependent on the function of the marker genes in contrast to the PNS strategy which is independent of the function of the marker gene (Capecci, 1989). Very recently Miao and Lam (1995) reported the first successful disruption in plants of a non-selectable marker gene, the *TGA3* locus of *Arabidopsis*, using the β -glucuronidase (*GUS*) reporter gene instead of a negative marker gene. Their study demonstrated the feasibility of targeting of non-selectable locus in plants

with the negative selection strategies. The combination of the *bar* (a positive marker) gene and the *codA* (a negative marker) gene could be a good choice in applying the PNS procedure for gene targeting in plants including *Arabidopsis*.

Besides the homologous recombination a conditional negative-selection is also useful for the detection of gene silencing by mutation. For example, a homozygous transgenic line transformed with the *codA* gene fused to a promoter of a given gene is treated with a mutagen (M1 generation). It will be possible to screen M2 progenies without expression of the chimeric *codA* gene by using 5-FC resistance. Those mutant lines might contain both cis and trans mutations. The combination of the *codA* gene and such a reporter gene as the *GUS* gene each fused independently with the identical promoter region will detect cis mutations in M2 lines. Silencing of both chimeric genes at the same time in a single plant would discriminate trans from cis mutants which would be expected to have a mutation in only one of the two chimeric genes. The molecular genetic analysis of such trans mutants would be useful in the isolation and characterization of transcription factors or signal transduction components involved in the expression of gene(s) of interest. The effectiveness of the negative selection with the *codA* gene, however, would be dependent on the permeability and transport of the substrate 5-FC in plant tissues, and on the intensity of expression, tissue specificity and temporal specificity of the promoter fused to the *codA* gene. Since CaMV 35S promoter is sufficiently active in root cells (Benfey et al., 1989) and the root tissues can be in contact directly with the substrate in vitro, the P35S should be a suitable promoter in the *codA* negative selection system. To isolate and characterize transcription factors and signal transduction components related to the gene of interest, we are currently investigating whether our negative selection system using the *codA* marker gene would be applicable and useful for promoters not usually expressed in root cells such as *RbcS*, *Cab*, etc.

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