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Cotransformation frequencies of foreign genes in soybean cell cultures

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Summary. Through the use of electroporation and a soybean (*Glycine max* L.) protoplast system, we generated stably transformed cell lines expressing a number of foreign genes (neomycin phosphotransferase, β-glucuronidase, chloramphenicol acetyl transferase, and phosphinothricin acetyl transferase). Selected and unselected marker genes were cointroduced either linked on a single plasmid or as separate plasmids. Calli expressing multiple genes were recovered, and cotransformation frequencies were established for both cases. Our results show a 50% cotransformation frequency in the case of linked genes. In situations in which two genes are introduced on independent plasmids, cotransformation frequencies are 18%–27%. Similar rates of cotransformation were observed among various marker pairs.

Key words: Soybean – *Glycine max* – Tissue culture – Electroporation – Transgenic tissue

Introduction

Successful application of genetic engineering to soybean has recently been accomplished. Bacterial marker genes including neomycin phosphotransferase and β-glucuronidase have been successfully introduced into both callus cultures (Christou et al. 1988) and intact plants (McCabe et al. 1988). Improving the agronomic value of major crops such as soybean is likely to involve the introduction of multiple genes, many of which will not provide selectable or directly screenable phenotypes among the initial products of transformation. The objective of the present study was to determine the efficiency of cotransformation with unselected markers in soybean. We anticipate the results will contribute to a strategy for multigene transformation that promises a reasonable probability of success.

A previously developed soybean protoplast system (Christou et al. 1987) was employed in our experiments. This system is reliable, large numbers of protoplasts can easily be prepared and treated, transformation frequencies are relatively high, and results are obtained within a reasonably short time. Moreover, the characteristics of transformation events recovered from electroporation and electric-discharge particle acceleration (McCabe et al. 1988; Christou et al. 1987, 1988, 1989) appear very similar. Thus, the protoplast system provides a useful model for establishing conditions under which one or more foreign genes can be stably incorporated and expressed in plant tissues. Five plasmids comprising four different marker genes were used: a *NOS-NPT II* (nopaline synthase – aminoglycoside phosphotransferase II) gene conferring resistance to kanamycin was used as the selected marker, and CaMV 35S (cauliflower mosaic virus)-CAT (chloramphenicol acetyl transferase), -Bar (phosphinothricin acetyl transferase), and -GUS (β-glucuronidase) genes as unselected markers. Cotransformation frequencies were determined for selected and unselected genes present on the same plasmid as well as for genes present on separate plasmids.

Materials and methods

Vector construction

Plasmids, pCMC1021 (Christou et al. 1988) and pCMC1100 (McCabe et al. 1988), have been described. pCMC1220 is a CaMV 35S-chloramphenicol acetyl transferase expression plasmid derived from pCMC1201 (Umbeck et al. 1987), by substi-

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Table 1. Cotransformation frequency

Plasmid/Enzyme activity	Exper. no. 1	Exper. no. 2	Exper. no. 3		% cotransformation frequency		
					Exper. no. 1	Exper. no. 2	Exper. no. 3
pCMC1021 (4.5 n*M*):pCMC1220 (5.5 n*M*)							
APHII	11	13	5	APH-CAT	18	23	27
CAT	2	3	4				
pCMC1021 (22.5 n*M*):pCMC1220 (5.5 n*M*)							
APHII	42	22	32	APH-CAT	14	22	32
CAT	6	3	3				
pCMC1021 (4.5 n*M*):pCMC1100 (4.1 n*M*)							
APHII	15	12	25	APH-GUS	13	17	8
GUS	2	2	2				
pTVGUS (0.6 n*M*)							
APHII	18	12	24	APH-GUS	67	46	54
GUS	12	6	13				
pTVBAR (0.6 n*M*)							
APHII	6	12	14	APH-BAR	33	33	26
BAR	2	4	4				
pTVGUS (0.6 n*M*)+pCMC1220 (5.5 n*M*)							
APHII	8	9	12	APH-GUS	100	100	75
GUS	8	9	9	APH-CAT	0	11	8
CAT	0	1	1	GUS-CAT	0	11	11
pTVGUS (2.9 n*M*)+pCMC1220 (5.5 n*M*)							
APHII	26	36	20	APH-GUS	46	39	45
GUS	12	14	9	APH-CAT	7	8	10
CAT	2	3	2	GUS-CAT	10	25	22
pTVBAR (0.6 n*M*)+pCMC1220 (5.5 n*M*)							
APHII	5	7	ND	APH-BAR	80	57	ND
BAR	4	4	ND	APH-CAT	20	14	ND
CAT	1	1	ND	BAR-CAT	25	25	ND
pTVBAR (3.1 n*M*)+pCMC1220 (5.5 n*M*)							
APH	7	ND	ND	APH-BAR	43	ND	ND
BAR	3	ND	ND	APH-CAT	14	ND	ND
CAT	1	ND	ND	BAR-CAT	33	ND	ND

tuting a CaMV 35S promoter fragment (nucleotides 7013–7440 of the CaMV sequence; Gardner et al. 1981) for the NOS promoter. The pTV plasmids (Barton et al. 1987) are vectors based on pRSF1010 (Bagdasarian et al. 1981) that include a selectable *NOS-NPT II* chimeric gene and a polylinker for insertion of additional markers, flanked by *Agrobacterium* T-DNA borders. In pTVGUS and pTVBAR the engineered T-DNA regions contain a CaMV 35S-GUS gene (Jefferson et al. 1986) or CaMV 35S-BAR gene (Thompson et al. 1987), respectively, as well as the beta-lactamase and replication-origin regions from pBR322 (Bolivar et al. 1977). The *NOS-NPT II* gene and CaMV 35S-GUS or-BAR genes are immediately adjacent to each other and directed in opposite orientations in these plasmids. The latter region is common to all five plasmids. pCMC 1021, pCMC 1100, and pCMC 1220 range in size from 3.5 to 4.5 kb, whereas pTVBAR and pTVGUS are approximately 15 and 16 kb in size, respectively.

Protoplast isolation

Protoplasts were isolated from cultivar Williams 82, and cultured as described (Christou et al. 1987). Following their isolation and purification, protoplasts were diluted with KAO protoplast media to the desired density for electroporation.

Electroporation

Protoplasts were electroporated at a density of $2-4 \times 10^6$ per ml in KAO protoplast media supplemented with 40 m*M* NaCl and supercoiled plasmid DNA. No carrier DNA was used in these experiments. The concentrations of plasmid DNA employed are indicated in Table 1. Conditions for the electroporation were as described (Christou et al. 1987).

Culture of protoplasts and selection of stable transformations

Following electroporation, protoplasts were cultured in Corning 75-cm^2 tissue culture flasks containing 10 ml of KAO medium as described elsewhere (Christou et al. 1987). Kanamycin-resistant colonies appeared 2–3 weeks following plating on low melting point agarose solid medium supplemented with kanamycin at 50 mg/l. The transformation frequencies for kanamycin-resistant colonies ranged between 10^{-4} and 10^{-5}. Lower transformation frequencies were observed than those previously reported, due to the use of Williams 82 rather than Mandarin Ottawa. Transformed calli were amplified under selection and assayed for enzyme activities. Several of the amplified calli were also analyzed by Southern blots.

NPT II assays

Callus extracts were prepared as described by Platt and Yang (1987) and assayed for NPT II activity by the procedure of Reiss et al. 1984).

β-Glucuronidase assays

Twenty to fifty milligrams of callus tissue was incubated for 1 h at 37 °C in GUS assay buffer (Jefferson 1987). Samples were scored visually for the production of the dimeric indigo dye.

CAT assays

Cell-free extracts were prepared for CAT assays as described by Herrera-Estrella et al. (1983), and activity was determined by the method of Gorman et al. (1982).

BAR assays

Phosphinothricin acetyl transferase activity was determined as described by De Block et al. 1987.

Results

Cotransformation was analyzed for several different marker genes. Three independent repetitions of each experiment were performed in order to assess the reproducibility of our results. All transformed calli were amplified under selection and assayed for the appropriate enzyme activities. Results from each analysis are shown in Table 1.

Plasmid pCMC1021, conferring kanamycin resistance, was used in conjunction with plasmids pCMC1220 and pCMC1100 to establish cotransformation frequencies of unlinked genes. For unlinked *NPT II* and *CAT* genes, cotransformation frequencies ranged from 18% to 27%. Increasing the concentration of pCMC1021 fivefold, and thus the ratio of pCMC1021 : pCMC1220 from 1:1 to 5:1, resulted in an increase in the number of transformants obtained, but did not change the number of CAT-expressing colonies recovered. Consequently, the fraction of transformants expressing CAT was reduced approximately 50%. Similar results were obtained when pCMC1100 was used as the unselected marker. In the latter case, cotransformation frequencies of 8% to 17% were obtained when the two genes were used in a ratio of 1:1.

Cotransformation frequencies involving linked genes were determined using pTVGUS and pTVBAR, allowing selection on kanamycin and screening for either β-glucuronidase or phosphinothricin acetyl transferase activity as the unselected markers. Cotransformation frequencies were 46%–67% with pTVGUS and 26%–33% with pTVBAR.

Plasmid pCMC1220 was also used in conjunction with pTVGUS and pTVBAR to analyze cotransformation of both linked and unlinked unselected markers in the same population of transformants. Surprisingly, addition of pCMC1220 with either pTVGUS or pTVBAR resulted in an increase of the cotransformation frequency of the two linked genes by approximately 50%. The reason for such an increase is unknown. The enhancement of cotransformation is lost upon increasing the molar ratio of the pTV plasmids to the unlinked *CAT* gene from 0.1:1 to 0.5:1, suggesting that pCMC1220 may be acting as carrier DNA. In this regard it should be noted that the pTV plasmids are approximately five times the size of pCMC1220. Cotransformation of the unselected *CAT* gene was found to be 9% and 17% in experiments with pTVGUS and the pTVBAR, respectively. Examining frequencies of cotransformation between the linked unselected and the unlinked unselected genes, GUS-CAT and BAR-CAT, we found that both were between 10% and 25%. No significant difference was observed between the 0.1:1 and the 0.5:1 molar ratios.

Southern blot analysis of DNA isolated from selected transformants (data not shown) indicated that the organization of the transgenes is similar to that previously observed in this (Christou et al. 1987) and other systems (Schocher et al. 1986; Krens et al. 1985; Paszkowski et al. 1984; Riggs and Bates 1986). All transformed calli exhibited hybridization signals for the selected *NPT II* gene, and those calli showing enzyme activity for the unselected markers also exhibited hybridization signals for the corresponding genes. The pattern of fragments displayed by calli exhibiting hybridization signals indicates transformants frequently contain rearranged as well as intact copies of the transgenes. Transformed calli that do not exhibit expression of an unselected gene usually do not show hybridization signals for that gene.

Discussion

Using the above plasmids we were able to investigate the effect of different markers in linked and unlinked configurations as well as in various combinations. Effects of DNA dose were also preliminary investigated. The results indicate that linkage of the selected and unselected markers on a single DNA molecule leads to significantly higher cotransformation frequencies than those observed with unlinked markers. Linked unselected markers were expressed in 50%–100% of transformants, whereas unlinked unselected markers were coexpressed in approximately 20%–25% of transformants.

Some marker-specific effects were observed. Most apparent are the consistently lower cotransformation frequencies obtained with pTVBAR relative to pTVGUS. The chimeric GUS and BAR genes in these two plasmids are completely analogous and the enzyme assays are comparably sensitive, so it does not seem likely that differences in expression level account for the observed differences in cotransformation frequency. Both cases also

involve unselected genes introduced on the same plasmids as the selected *NPT II* gene, making it unlikely that the observed differences are due to contamination differences between the plasmid preparations. On the other hand, pTVBAR also gave consistently lower overall transformation efficiencies, thereby reducing the sample size and the statistical significance of the observed cotransformation frequencies.

Enhanced cotransformation of linked markers was observed when unlinked plasmids were included in the transformation mixture. Previous reports have indicated that the presence of carrier DNA can enhance transformation frequency (Schocher et al. 1986; Jongsma et al. 1987; Peerbolte et al. 1985). A carrier effect may contribute to our observations but does not fully explain the results, because the overall transformation efficiency is reduced when the unlinked plasmid is included.

The cotransformation frequencies we report for active, unlinked markers are similar to those reported previously for other systems (Uchimiya et al. 1986; Schocher et al. 1986; Tagu et al. 1988; Damm et al. 1989). Thus, in several plant systems, coexpression of unlinked genes occurs at a frequency of approximately 20%–25%. It is important to recognize that this consensus derives from quite different plant systems and transformation protocols and is, therefore, probably generalizable. Not surprisingly, we find a higher frequency for coexpression of unselected genes when they are included on the same plasmid with the selected marker than when the two genes are on separate plasmids. More important is the finding that the frequency of coexpression of genes linked on a single plasmid can be very high-approaching 100%.

In contrast to previous reports (Schocher et al. 1986) we do not frequently observe Southern signals for genes or gene fragments in calli that do not show expression of the corresponding marker enzyme. Transformants that do not give positive enzyme assays for unselected markers usually do not exhibit detectable Southern hybridization signals. These contrasting data may be due to differences in the plant systems employed and possibly reflect the dominance of various recombination pathways in the different systems.

In particle-acceleration transformations of soybean (Christou et al. 1989), where plants rather than calli are produced, we have been able to follow transformation events by general genetic methods. Results from such investigations are consistent with those described above: i.e., linked markers are coexpressed at a high frequency, and nonexpressing plants usually do not exhibit detectable Southern hybridization signals. Furthermore, genetic analyses demonstrate that all copies of the integrated transgenes in soybean plants are usually tightly linked.

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