

## Transformation of 12 different plasmids into soybean via particle bombardment

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**Abstract.** Particle bombardment offers a simple method for the introduction of DNA into plant cells. Multiple DNA fragments may be introduced on a single plasmid or on separate plasmids (co-transformation). To investigate some of the properties and limits of co-transformation, 12 different plasmids were introduced into embryogenic suspension culture tissue of soybean [*Glycine max* (L.) Merrill] via particle bombardment. The DNAs used for co-transformation included 10 plasmids containing RFLP markers for maize and 2 plasmids separately encoding hygromycin-resistance and  $\beta$ -glucuronidase. Two weeks following bombardment with the 12 different plasmids, suspension culture tissue was placed under hygromycin selection. Hygromycin-resistant clones were isolated after an additional 5 to 6 weeks. Southern hybridization analysis of 26 hygromycin-resistant embryogenic clones verified the presence of introduced plasmid DNAs. All of the co-transforming plasmids were present in most of the transgenic soybean clones and there was no preferential uptake and integration of any of the plasmids. The copy number of individual plasmids was approximately equal within clones but highly variable between clones. While some clones contained as few as zero to three copies of each plasmid, others clones contained as many as 10 to 15 copies of each of the 12 different plasmids.

**Key Words:** *Glycine max* - Recombination - Soybean - Transformation

### Introduction

Production of transgenic plants has become routine for many plant species, permitting modifications to basic metabolism and introduction of characteristics that could not be incorporated using conventional breeding techniques. Although different procedures exist for the intro-

duction of foreign DNA, particle bombardment has become the method of choice for transformation of many plant tissues. With particle bombardment, the difficulties of using fragile protoplasts and host-range limitations associated with *Agrobacterium* can be avoided.

Although success in particle bombardment-mediated transformation of plants has increased tremendously in recent years, there is limited information available on the fate of introduced DNA. In order for the DNA to be integrated into the genome, the introduced DNA must be physically modified. These modifications are important in understanding the organization, integration and expression of the introduced DNA.

Southern hybridization analysis (Southern 1975) of DNA from stably transformed plants produced by naked DNA-mediated transformation reveals complex hybridization patterns (Bates *et al.* 1990). These hybridization patterns give clues as to the nature of plasmid DNA processing following introduction into the cell. Although integration at single sites in the genome is most common (Christou *et al.* 1989), multiple integration sites have also been observed (Potrykus *et al.* 1987). Southern hybridization analysis of DNA from transgenic tobacco obtained by electroporation of plant protoplasts (Riggs *et al.* 1986), and from transgenic cotton and soybean (Finer *et al.* 1990, 1991) obtained via particle bombardment, indicates that the introduced plasmid forms head-to-tail concatemers. This suggests that the plasmids underwent homologous recombination during the transformation process. In addition to homologous recombination, plasmid DNA(s) could also recombine via illegitimate recombination or, alternatively, the plasmid(s) could be linearized and ligated to other plasmid fragments during the integration process. The high efficiency of co-transformation and the observation of a genetically-linked integration of calf thymus DNA used as a carrier provides evidence for this type of recombination process in electroporated plant protoplasts (Peerbolte *et al.* 1985).

In order to gain an understanding of plasmid DNA integration and determine some of the limitations of particle bombardment in plants, we co-transformed 12 different plasmids (as a single cocktail) into embryogenic soybean tissues. One of these plasmids contained a scorable  $\beta$ -glucuronidase gene, another contained a selectable hygromycin-resistance gene, and ten other plasmids contained different RFLP markers from maize. Southern hybridization analyses of stably transformed tissue revealed the following: 1) All 12 of the plasmids could be taken up and incorporated, 2) There was no preferential uptake or integration of any one of the plasmids, 3) Plasmid amplification may have occurred in some clones, 4) Concatemer formation, indicative of homologous recombination, was observed to a limited extent, 5) Ligation of plasmid fragments also occurred at a high rate.

## Materials and Methods

**Plasmid DNAs.** The plasmid pUCGUS (Finer *et al.* 1990), which encodes  $\beta$ -glucuronidase (GUS), and pCIB 709 (Rothstein 1987), which encodes resistance to the antibiotic hygromycin-B, were used as the scorable and selectable marker genes, respectively. The 5' regulatory element for both genes was the CaMV35S promoter. Plasmids bnl 14.28, and umc 29, 34, 38, 39, 82, 84, 107, 115, and 119 (obtained from David Hoisington, University of Missouri, Columbia, MO) contained RFLP markers for maize. Plasmid DNAs were prepared using the alkaline lysis method and were purified by centrifugation in cesium chloride-ethidium bromide gradients as described by Sambrook *et al.* (1975).

**Soybean Transformation.** Embryogenic suspension cultures of soybean [*Glycine max* (L.) Merrill] cv Fayette were initiated and maintained as described previously (Finer *et al.* 1988). Cultures were bombarded according to Finer *et al.* (1991). Briefly, 1 g of embryogenic suspension culture was transferred to a 3.5 cm Petri dish. The excess medium was removed with a pipet tip and the tissue was allowed to air dry in a laminar flow hood for 10 to 15 min. Immediately prior to bombardment, the tissue was covered with a 500  $\mu$ m pore size nylon screen (Tetko Inc. Elmsford, N.Y.).

The twelve plasmids were mixed together in equal amounts (equal mass) to obtain a final total DNA concentration of 1  $\mu$ g/ $\mu$ l in TE. As the plasmids were 4-5 kb, equal mass was closely equivalent to equimolar. Plasmid DNAs were precipitated onto tungsten M10 particles (1.1  $\mu$ m; Sylvania Chemical and Metals, Towanda, PA) using a calcium chloride precipitation method (Finer *et al.* 1990). Each sample was bombarded once using a Biolistics™ Particle Delivery System (model BPG).

**Selection for Transformed Tissues.** One to 2 h. following bombardment, embryogenic tissue was resuspended in liquid FN maintenance medium (Finer and Nagasawa 1988). After 2 weeks, embryogenic tissue was then transferred to fresh FN medium containing 50  $\mu$ g/ml hygromycin-B (Calbiochem, LaJolla, CA). Tissues were placed in fresh hygromycin-containing maintenance medium every week for 3 additional weeks.

Seven to 8 weeks post bombardment, brown clumps of nontransformed embryogenic tissue containing green lobes of hygromycin-resistant tissue were removed and separately cultured in fresh hygromycin-containing maintenance medium. Tissues were removed from these cultures for histochemical GUS assays (Jefferson 1987), DNA extraction and Southern hybridization analysis (Southern 1975).

**Molecular Analyses.** DNA was extracted from transgenic embryogenic tissue 1 year after selection using the CTAB procedure (Saghei-Marooof *et al.* 1984). DNA concentration was determined using a TKO 100 minifluorometer (Labarca *et al.* 1980) and equal amounts of genomic DNA were digested with the appropriate restriction enzymes. DNAs were

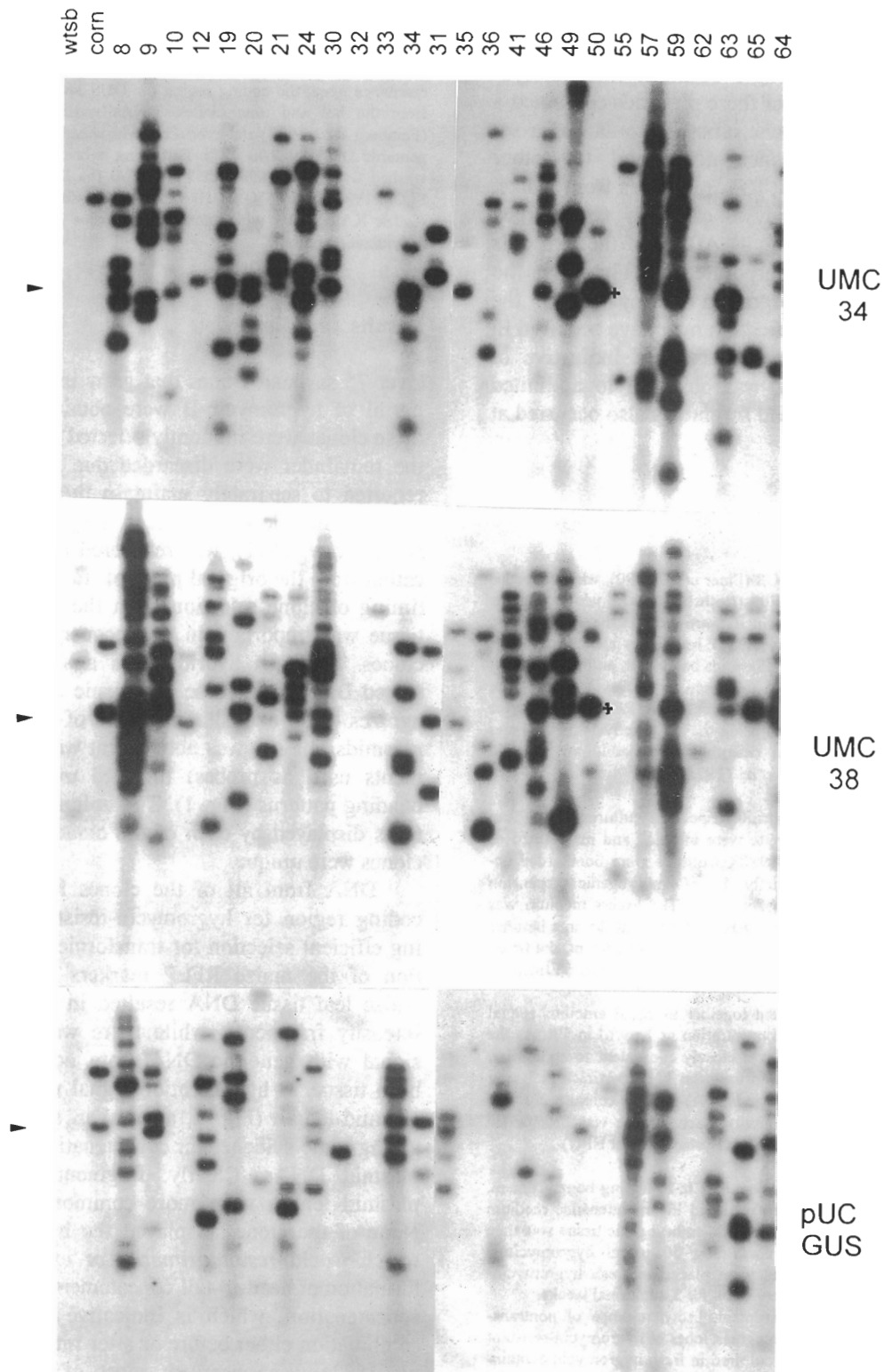
then electrophoresed through either a 0.4% or 0.8% agarose gel following digestion with either one or two restriction enzymes, respectively. The DNA was transferred from the gels to nylon membranes as described previously (Finer *et al.* 1991).

DNA probes consisted of the 1 kb coding region of the hygromycin-resistance gene, the coding region for GUS and the maize DNA inserts from the bnl and umc clones. DNAs were random primer labeled (Feinberg *et al.* 1983) and hybridized with membranes containing soybean genomic DNA for 24 - 48 h. Following hybridization, membranes were washed five times each with 2 X SSC/0.1% SDS and then with 0.1 X SSC/0.1% SDS at 65°C for 10 min each. Membranes were then placed on Kodak XAR-5 film with intensifying screens at -70°C to visualize the hybridization pattern.

## Results and Discussion

Over 75 soybean clones that grew in the presence of 50  $\mu$ g/ml of hygromycin-B were obtained. Twenty six of these clones were randomly selected for further analysis; the remainder were discarded due to the large efforts required to separately maintain this amount of tissue. Each clone, derived from a single transformed cell (Finer *et al.* 1991), was recovered prior to clone duplication from the original piece of transformed tissue. The timing of clone selection from the original bombarded tissue was important in the recovery of non-duplicated clones. Southern hybridization analysis of *Hind*III-digested DNA from these transgenic clones (*Hind*III recognizes only one site on each of the 12 introduced plasmids, the site was not present within the DNA fragments used as probes) revealed unique and complex banding patterns (Fig. 1). The unique hybridization patterns displayed by each of the clones indicated that all clones were unique.

DNA from all of the clones hybridized with the coding region for hygromycin-resistance gene, indicating efficient selection for transformed tissue. Hybridization of the maize RFLP markers to *Hind*III-digested maize leaf tissue DNA resulted in 1 to 4 single copy intensity fragments, while there was no hybridization signal with genomic DNA from non-transformed soybean tissue. A hybridization signal was present at unit-plasmid-length (Fig. 1, arrows) in some of the clones, giving some evidence for concatenation of the introduced plasmid DNAs. Clearly, fragments other than unit plasmid length were more common than unit length. None of the clones displayed the hybridization pattern which would result primarily or exclusively from the formation of head-to-tail concatemers. Although plasmid concatenation, which is indicative of homologous recombination either before or after integration (Folgers *et al.* 1982) was observed, most of the recombination events (as indicated by the complex banding pattern) appear to be illegitimate. Homologous recombination between plasmids can only occur if a region of homology exists between those plasmids. Because all of the plasmids used in this study were pUC based, they contain approximately 2680 bp of common sequence, where ho-



**Figure 1** Representative samples of autoradiographs of Southern hybridization analysis. *Hind* III-digested genomic DNA (10  $\mu$ g) from non-transformed soybean (wtsb), corn, and 26 transformed soybean clones hybridized with DNAs from *umc34* (top panel), *umc38* (middle panel) and pUCGUS (bottom panel). The arrows designate unit-length of the plasmid DNA which contained the region being used as a probe. The “+” sign designates clear unit-length fragments for Clone 50.

mologous recombination could take place. Plasmid concatenation has been observed with electroporated tobacco cells (Riggs *et al.* 1986) and bombarded cotton and soybean embryogenic cultures (Finer *et al.* 1990, 1991). In these studies, as well as most others involving DNA introductions, only one or two different plasmids are typically used. Homologous recombination may be more common between identical plasmids than with similar plasmids. The use of 12 different plasmids, as reported here, makes analysis of recombination events more difficult.

The copy number of the introduced plasmids was estimated based exclusively on the number of bands on the autoradiographs using signal from maize leaf DNA as a single copy reference (Table 1). Copy number was determined from the original film as some signal definition was lost in the photographic copies (Fig. 1). There was low variability in plasmid copy number within clones but higher variability among clones. The copy number for individual plasmids varied greatly, from zero in some clones to 16 for plasmid umc 38 in clone 30. All of the clones contained at least some introduced plasmid DNA. Clones 32, 33, 35 and 50 were classified as low copy number clones and contained an average of 2 or fewer copies of each plasmid. Clones 9, 24, 57, 59, 63 and 64 were high copy number clones and these clones contained an average of 10 or more copies of each intro-

duced plasmid. Most of the clones that contained high copies of one plasmid had high copy numbers for all other plasmids, while the low copy number clones generally had low copy numbers for all plasmids. Four clones (clones 8, 12, 30 and 65) that varied more widely in copy number of introduced plasmid DNAs, were termed variable copy clones, while the remaining clones were intermediate copy number clones. The complex hybridization patterns observed in all of the clones indicate that the plasmids primarily recombined with each other at random. The consistency of plasmid copy number suggests that there was no preferential uptake of any of the plasmids.

The presence of intense hybridization signals at positions other than unit-length indicates that plasmid DNA was amplified in some clones. Amplification of introduced plasmid DNAs was clearly observed in clones 9, 24, 34, 49, and 63. Amplification of plasmid DNA results in either partial or full amplification of the hybridization signal. This is noticeable as some of the hybridization signals are intense while others are present at low intensity. Amplification of resistance genes in response to both antibiotic (Czernilofsky *et al.* 1986) and herbicide (Wang *et al.* 1991) selection has been reported. Selection of transformed clones using high levels of hygromycin-B may have contributed to amplification of the DNA (hygromycin resistance gene along with the

Clone Number	Plasmids												GUS		
	umc 29	umc 34	umc 38	umc 39	umc 82	umc 84	umc 107	umc 115	umc 119	bnl 14.28	pCIB 709	pUC GUS	Activity		Intact unit
	29	34	38	39	82	84	107	115	119	14.28	709	GUS	8 wks	16 wks	
⊕ 8	3	6	2	4	5	9	1	4	11	8	2	2	+	+	
↑ 9	11	11	14	11	13	14	11	10	14	13	6	13	-	-	YES
10	9	6	9	0	5	9	10	5	12	5	4	6	-	-	
⊕ 12	0	1	2	0	1	2	0	2	2	1	1	0	-	-	
19	7	10	7	4	8	10	9	9	10	9	5	9	+	+	YES
20	8	6	5	2	4	8	9	3	4	9	5	5	+	+	YES
21	6	6	7	2	8	7	7	5	9	5	4	3	-	-	
⊕ 24	9	9	9	10	10	12	10	11	11	12	5	8	-	-	YES
⊕ 30	7	7	16	7	7	13	7	14	8	7	8	5	-	-	
↓ 32	4	0	1	3	3	3	2	4	3	1	2	1	-	-	
↓ 33	1	1	2	1	3	1	0	1	0	1	1	0	-	-	
34	7	5	6	4	6	9	5	6	9	7	6	7	+	-	YES
31	5	3	3	6	5	7	3	9	6	5	2	3	+	+	YES
↓ 35	0	2	3	1	1	2	2	2	3	3	2	4	+	+	YES
36	1	5	6	5	5	7	5	7	9	6	3	0	-	-	
41	6	5	11	5	6	10	5	6	6	7	6	4	-	-	
46	13	11	10	9	4	10	5	9	11	9	8	2	-	-	YES
⊕ 49	4	5	10	5	6	12	7	10	12	10	5	4	-	-	
50	2	2	3	1	1	2	1	4	3	3	2	1	-	-	YES
55	3	2	5	2	4	1	3	4	3	3	2	1	-	-	
↑ 57	10	13	12	12	13	12	9	12	11	15	10	13	+	-	YES
↑ 59	9	10	12	9	11	11	12	13	13	13	7	7	+	-	YES
62	2	3	2	5	2	4	2	7	7	4	2	0	-	-	
↑ 63	10	8	6	12	13	11	9	9	11	13	13	7	-	-	
↑ 64	8	8	10	12	8	10	9	9	11	12	12	7	+	+	YES
65	3	2	4	5	4	11	4	11	9	9	4	6	+	-	YES

Table 1. Plasmid copy number, GUS activity and intactness of the GUS gene in transformed soybean tissue (↑ a indicates high copy number clone; ↓ indicates a low copy number clone; ⊕ indicates a variable copy number clone; clones not labeled are intermediate copy number) following co-transformation with 12 different plasmids.

recombinant integration unit) in some of the clones.

When the copy number and size of the introduced plasmids are considered, approximately 600 kb of plasmid DNA was introduced into the soybean genome for high copy clones. The amount of introduced plasmid DNA in this report is considerably higher than reported earlier for other bombardment and naked DNA-mediated transformation systems. Around three to five plasmid copies were introduced with a liposome mediated transfer to protoplasts (Deshay *et al.* 1985). Transgenic soybean obtained via particle bombardment contained approximately five copies of a single plasmid (McCabe *et al.* 1988). While in transgenic tobacco obtained via microinjection, some cell lines contained less than one copy of the plasmid (Crossway *et al.* 1980). The large amount of introduced DNA in this report may result from either the use of a large number of different plasmids or an enhanced "transformation competence" (the ability to incorporate foreign DNA) of these soybean cells. The results of an 11-way co-transformation in embryogenic cotton cultures gave similar hybridization patterns to those reported here (data not shown). Although extensive analyses were not undertaken, approximately 10 copies of each of the 11 plasmids were introduced into these embryogenic cotton cultures (Finer, unpublished).

In general, particle bombardment results in low numbers of integration events in soybean (Christou *et al.* 1989). The number of integration events (separate sites of integration) was not determined in the present study. Although plants were recovered from some of the cultures obtained in this study (unpublished), they showed severely reduced fertility and progeny analysis could not be used to determine the number of integration events. This reduced fertility was a result of the extended cell culture period and was not related to the introduction of large amounts of DNA. Plants recovered from non-transformed cultures showed the same phenotype as the transformed plants.

GUS assays were performed on embryogenic cultures that were morphologically similar and were grown under identical conditions. All the clones were tested for GUS activity once at 8 weeks and again at 16 weeks after transformation. The presence of the intact GUS coding unit did not correlate with the expression of the GUS gene. Of the 13 clones that contained the intact expression unit for GUS, 4 clones never showed histochemical GUS activity and 4 clones displayed GUS activity only after 8 weeks (and not at 16 weeks). Only six of the 26 clones showed GUS activity at both time points (Table 1). Four of the clones that were GUS-positive at the 8 week period tested negative at the 16 week test. All clones that were initially GUS-negative remained GUS-negative at the 16 week point. Southern hybridization analysis indicated the presence of an intact GUS expression unit in all but one clone that at some point expressed GUS. Clone 8, the single clone that did not con-

tain the intact expression unit (Table 1) but expressed GUS, may have lost non-essential regions of the expression unit during recombination (Finer and McMullen 1991) or the coding region may have integrated into a nonhomologous sequence, resulting in transcriptional and/or translational fusion between the GUS coding region and an endogenous gene (Halfter *et al.* 1992, Koncz *et al.* 1989, Mayerhofer *et al.* 1991). In addition, some of the clones that never expressed GUS also contained the proper size restriction length fragment associated with the intact GUS expression unit.

In clones that contained the intact expression unit but did not stably express GUS, there was no relationship between copy number and expression. Co-suppression, which is a suppression of gene expression resulting from introduction of multiple copies of the same gene (Napoli *et al.* 1990), was not clearly observed in this study. Co-suppression has been suggested to occur in transformed soybean tissue (Finer *et al.* 1991), where multiple copies of the intact GUS gene were present but the gene was not expressed. The tandemization of DNA giving rise to more than one promoter per integration site could lead to transcriptional interference (Czernilofsky *et al.* 1986), resulting in the absence of GUS activity.

In most cases of naked DNA-mediated transformations, only one or two different plasmid DNAs are utilized. Multiple copy integrations, resulting from the introduction of one or two different plasmids, can lead to formation of large concatemers, where the plasmid DNAs can lie directly adjacent to one another. The proximity of these introduced plasmid DNAs to one another or their position within the genomic DNA may influence expression of the genes carried on this DNA (position effect). In the case of multiple plasmid co-transformations, the introduced plasmid DNAs are separated by each other, thus creating a type of "buffer" region for gene expression. If the introduced genes are adjacent to or associated with regions of introduced DNA that do not reduce or possibly enhance gene expression, it should be possible to modify or stabilize transgene expression thereby reducing position effect. The use of RFLP markers, which are typically low copy number and unmethylated, for co-transformation experiments may actually enhance gene expression, transformation efficiencies and serve as an additional tool for detecting the introduced DNA in the progeny. Scaffold Attachment Regions (Breyne *et al.* 1992) and Transformation Booster Sequences (TBS) (Meyers *et al.* 1988), which enhance transformation rates and gene expression, may act in somewhat similar ways.

Studies of co-transformation can provide much information on the process of recombination. Co-transformation can also be used to introduce large numbers of genes on different plasmids without the labor intensive and inefficient process of repeated transformations. If these plasmids recombine to form large chains of mixed

plasmid DNAs, multiple co-transformations may also be useful for construction of subchromosomal regions where the genes of interest could be flanked by expanses of defined DNAs.

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