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Development of the particle inflow gun for DNA delivery to plant cells *

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Summary. A simple and inexpensive particle bombardment device was constructed for delivery of DNA to plant cells. The Particle Inflow Gun (PIG) is based on acceleration of DNA-coated tungsten particles using pressurized helium in combination with a partial vacuum. The particles are accelerated directly in a helium stream rather than being supported by a macrocarrier. Bombardment parameters were partially optimized using transient expression assays of a ß-glucuronidase gene in maize embryogenic suspension culture and cowpea leaf tissues. High levels of transient expression of the ß-glucuronidase gene were obtained following bombardment of embryogenic suspension cultures of corn and soybean, and leaf tissue of cowpea. Stable transformation of embryogenic tissue of soybean has also been obtained using this bombardment apparatus.

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid, PCV = packed cell volume, GUS = ß-glucuronidase, NOS = nopaline synthase.

Introduction

Particle bombardment offers a rapid method for delivery of DNA to plant cells for both transient gene expression (Klein *et al*, 1987) and stable transformation studies (Klein *et al.*, 1988). The main benefit of this method is that intact plant tissues can serve as the target. Most of the reports on particle bombardment utilize the same basic concept for particle acceleration and delivery: a force provided by either an explosion or expansion of compressed gas propels a macrocarrier holding particles towards an immobile object which retains the macrocarrier but permits the particles to pass. This force can be generated by a 22 caliber power load (Sanford *et al.*, 1987), a high voltage electric discharge (McCabe *et al.*, 1988), and a release of either compressed air (Oard *et al.*, 1990) or helium (Johnston *et al.*, 1991). The immobile object can be a plate with a small orifice or a screen. Recently, devices that accelerate particles directly in a stream of helium (Takeuchi *et al.*, 1992) or carbon dioxide or nitrogen (Sautter *et al.*, 1991) have been described. Although the flowing helium device (Takeuchi *et al.*, 1992) is very simple and inexpensive, severe tissue damage can result from the manual release of the particles without the benefit of a vacuum. Micro-targeting (Sautter *et al.*, 1991) has much potential but the components of this device may be difficult to obtain.

The major factor limiting particle gun technology is accessibility of devices due to high cost and complexity. The development of an inexpensive and efficient device that is simple to build and operate would aid in the distribution and utilization of this technology.

Materials and Methods

Particle Inflow Gun Design: The Particle Inflow Gun (PIG) was constructed using equipment and supplies that were readily available from equipment supply companies. The vacuum chamber (Fig. 1a) was welded together from 6.4 mm steel plate and measured 16.5 x 16.5 x 30.5 cm. The vacuum chamber was painted to prevent oxidation of the metal. The front of the vacuum chamber was left open and ground smooth to provide a good seal with the door. The door was constructed from 2.5 cm thick plexiglass and a 6.4 mm thick neoprene rubber gasket was glued to and recessed in the door. The plexiglass door was attached to the steel box with two hinges. Two collars were welded into holes drilled in the top and side of the box. All of the fittings used in construction of the PIG were 1/4 inch (6.4 mm) I.D. National Iron Pipe. The vacuum/gauge/vent assembly, which consisted of two high pressure needle valves (Trerice; Detroit, MI, #735-2) and a vacuum gauge (Marshall town, Hastings, NE, #G14489), was connected to the collar in the side of the box (Fig 1a) using a cross fitting. The needle valves were rated to tolerate 140 PSI while the vacuum gauge displayed the vacuum settings down to 30 in. Hg. A 2-way solenoid (ASCO, Florham Park, NJ, Red Hat II, #JKF8262G22, with type I splice box) was connected to the collar on the top of the chamber (Fig 1b). The solenoid was controlled by a timer relay (National Controls Corp., #Q2F00001321, timer duration of 50 msec to 2 sec) set for the minimum timer duration of 50 msec. A copper line from a helium tank set at 40-80 PSI was connected to the other opening of the solenoid.

On the inside top of the vacuum chamber, a stainless steel, male Leur-lok needle adaptor (Clay Adams, Parsippany, NJ, #7553) was connected to the collar using a compression fitting (Fig 1b). A 13 mm stainless steel (Fisher Scientific, Pittsburgh, PA, #09-753-10A) or plastic (Gelman, Ann Arbor, MI, #4317) syringe filter unit could be readily attached to and removed from the device using the Leur-lok needle adaptor. A 9.5 mm plexiglass insert was designed to fit just inside of the vacuum chamber and grooves for a plexiglass shelf were cut into the left and right walls of the insert at every 1.5 cm.

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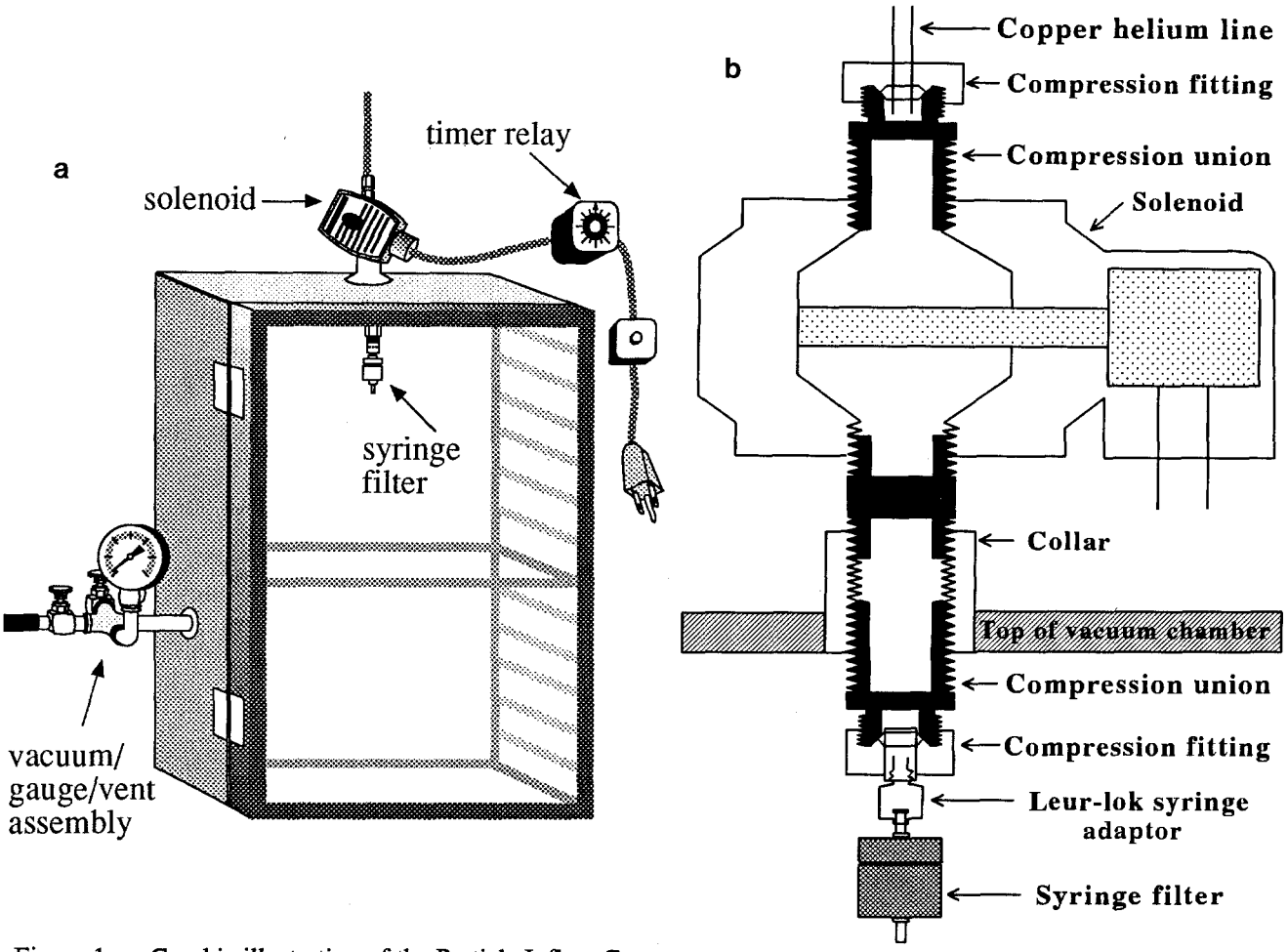

Figure 1. a. Graphic illustration of the Particle Inflow Gun.
b. Schematic showing connections from the helium line through the syringe filter. Prior to bombardment, 2 μl of DNA-coated particles was placed on the screen in the syringe filter.

Plant Tissue Preparation: Embryogenic suspension culture tissue of soybean (*Glycine max* Merrill cv "Fayette") was prepared as previously described (Finer and McMullen, 1991). One gram of tissue was placed in the center of a 3.5 cm Petri dish and the excess medium was removed. The tissue was placed uncovered in a hood for 15 min to facilitate partial drying.

Type II embryogenic callus cultures of corn (Zea mays A188 x B73) were initiated and maintained on $AgNO_3$-containing medium as described previously (Vain *et al*, 1989). Embryogenic suspension cultures were initiated from type II embryogenic callus in a medium containing MS salts (Murashige and Skoog, 1963), B5 vitamins (Gamborg *et al.*, 1968), 2% sucrose, and 1.5 mg/l 2,4-D (pH 5.7). The suspension cultures were maintained in 125 ml DeLong flasks by weekly subculture of 10-20 μl PCV of tissue into 30 ml of fresh medium. Corn suspension cultures were maintained in the light (30 $\mu Em^{-2}s^{-1}$; 16 hr day) at 150 rpm. Prior to bombardment, embryogenic corn cells (100-300 μl PCV) were filtered through either a 500 μm or 1 mm filter and evenly dispersed on a 7 cm filter paper disc (Whatman #4). Discs were stored on the maintenance medium solidified with agarose for short periods of time.

Leaf tissue of cowpea (*Vigna unguiculata*) was obtained from greenhouse-grown plants. Plants were grown under natural lighting from April to October and with supplemental lighting (12 hr day) from November to March.

DNA Constructions: For transient expression studies of soybean and cowpea, pUCGUS (CaMV35S promoter:GUS coding region: NOS terminator) was utilized (Finer and McMullen, 1990). For optimization of transient expression in embryogenic corn cells, pGB5 (CaMV35S promoter:*Sh-1* intron:GUS coding region:NOS terminator) was constructed. This plasmid was made by insertion of 1028 bp of the *Sh1* first intron/exon junctions (Vasil *et al.*, 1989) into the *Bam*HI site of pUCGUS after both the intron fragment (plus linker sites) and *Bam*HI-cleaved pUCGUS ends were made blunt with T4 polymerase.

The plasmid pHygr which contained a gene for resistance to the antibiotic hygromycin was constructed by first ligating the CaMV35S promoter as a HindIII-BamHI fragment from pBI121 (Jefferson *et al.*, 1987) into *Hin*dIII/*Bam*HI cleaved pUC119 to generate pCaMV. The NOS terminator was then ligated into pCaMV as a *Sst*I/*Eco*RI fragment from pBI121 to generate pCaMV-NOS. Finally, the *Bam*HI fragment from pLG90 (Gritz and Davies, 1983) that contained the coding region for the hygromycin resistance gene was ligated into the BamHI site of pCaMV-NOS. For stable transformation of soybean, pUCGUS was mixed with pHygr at 9:1 prior to DNA precipitation.

Particle Bombardment using the PIG: Tungsten particles (M10; provided by Sylvania Chemicals/Metals, Towanda, PA) were sterilized in ethanol and the DNA was precipitated according to Finer and McMullen (1991) for soybean and cowpea. For corn, 20 μl of DNA (20 μg), 10 μl of tungsten (1 mg), 25 μl of 2.5 M $CaCl_2$, and 10 μl of 100 mM spermidine were mixed and placed at 4°C. After 5 min, 45 μl of supernatant were removed and discarded.

For bombardment, 2 μl of the particle suspension was placed in the center of the screen in a disassembled syringe filter unit. The syringe filter unit was reassembled and then screwed into the needle adaptor. Plant tissue, in Petri dishes, was placed on the adjustable shelves at distances of 14, 17, 20, and 23 cm from the screen in the

syringe filter unit. The tissue was bombarded either unprotected or covered with a baffle. The baffles were made of either 1 mm or 500 μm nylon screens (Tetko, Inc., Elmsford, NY) and were placed either directly on top of the tissue or at a distance of 9 cm above the tissue. The baffle that was positioned 9 cm above the tissue was made by cutting off the bottom of a 400 ml disposable polypropylene beaker and attaching a screen to the bottom of the beaker. The beaker was then inverted and placed over the tissue prior to bombardment. A vacuum of 28-30 in Hg was applied and the particles were discharged when the helium (at 40-80 PSI) was released following activation of the solenoid by the timer relay.

Post-Bombardment Treatments: Embryogenic soybean and maize suspension cultures and cowpea leaf tissue were stained for transient GUS activity 2 days following bombardment according to Jefferson (1987). Selection for stably-transformed soybean lines, DNA extraction from those lines, and Southern hybridization analyses were performed as described previously (Finer and McMullen, 1991).

Results and Discussion

Particle Inflow Gun Development:

The PIG contained the following features that made it much easier to construct and operate than other devices and contributed to consistent results. 1) Expansion of compressed helium was used to propel the particles (Johnston *et al.*, 1991). This method is superior to other propulsion methods because helium is inert, leaves no residue, and gives reproducible acceleration. 2) A timer relay-driven solenoid (Morikawa *et al.*, 1989) was used to release the helium. The solenoid was more accurate than a syringe stopcock (Takeuchi *et al.*, 1992) and required no setup compared to a membrane rupture system (Johnston *et al.*, 1991). Moreover, the solenoid functioned at low helium pressures, which allowed good particle penetration and may have been less damaging to the target tissue. 3) The particles were directly accelerated in the helium stream (Sautter *et al.*, 1991, Takeuchi *et al.*, 1992) rather than being supported by a macrocarrier. Specially-constructed macrocarriers and stopping plates or screens were therefore not necessary. In the absence of such consumables, the cleanup time and therefore cycle time for each bombardment (2-3 min.) was reduced. 4) A vacuum chamber was used to hold the target tissue. The vacuum reduced the drag on the particles and lessened tissue damage by dispersion of the helium gas prior to impact. The vacuum also contributed to the pressure differential, which may have been responsible for the efficient particle acceleration.

Particle Distribution:

In order to measure effective particle penetration and transient expression, leaf tissue of cowpea was subjected to particle bombardment. Cowpea leaf tissue was selected because it gave more consistent transient expression than leaf tissue of other plants. The thousands of GUS-positive foci obtained with cowpea leaf tissue were not quantified due to confluence of the spots.

With no baffle, cells exhibiting transient GUS expression were mostly restricted to a 3.5 cm diameter ring that was 3-4 mm thick (Fig 2a). The only tissue that expressed GUS within this ring was veinal tissue and the expression was deep within the tissue, indicating subsurface penetration of particles and damage to the surface tissues. Most of the non-veinal tissue in the center of this ring appeared to be damaged. This central necrotic area is similar to that reported by Klein *et al.* (1988) for tobacco leaves and suspension culture cells.

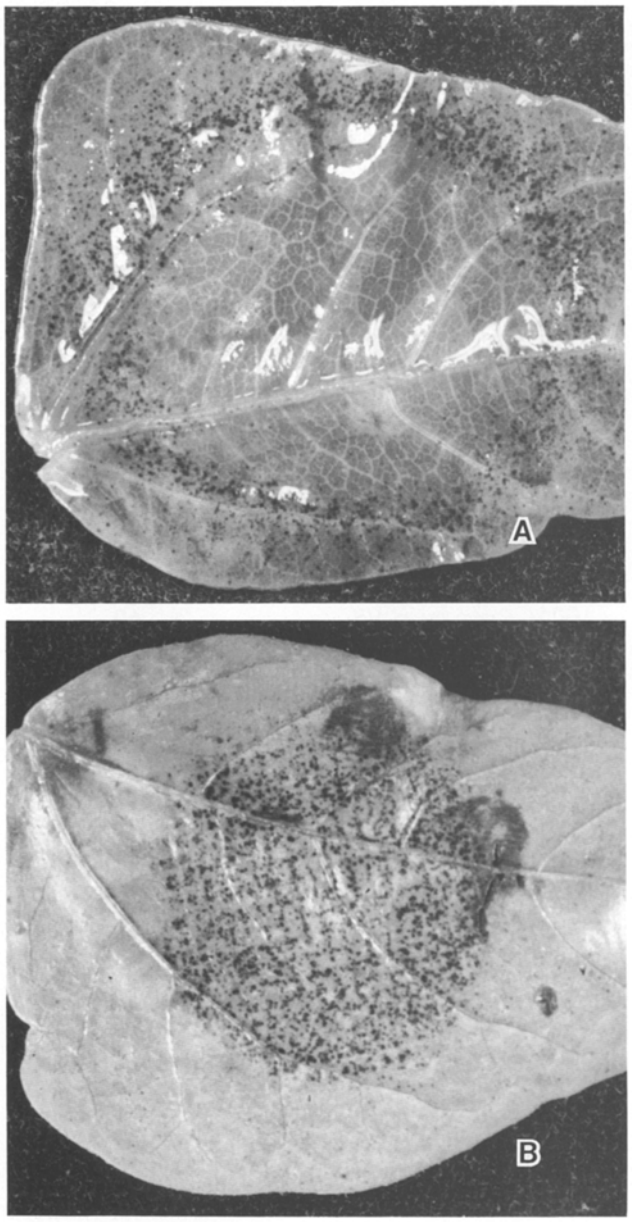

Figure 2. a. Transient expression of the GUS gene in leaf tissue of cowpea bombarded with no baffle (80 PSI, tissue 17 cm from syringe filter). b. Transient expression with 1 mm baffle placed directly on the leaf tissue. Note the differences in GUS activity staining patterns on the periphery and interior of the bombarded leaf.

Use of the 1 mm baffle directly on top of the cowpea leaf tissue during bombardment resulted in a striking alteration in distribution of GUS expression (Fig. 2b). In the centermost portion of the blast, GUS-expressing cells reflected a positive impression of the screen while on the periphery of this area, the GUS-expressing cells gave a negative impression. This distribution indicated that in the center of the bombarded area, the particles moved around the fibers of the screen and penetrated the cells that were protected or shielded

PCV (μl)	filtration (μm)	pressure (PSI)	distance (cm)	baffle (500 μm)	blue foci[1]
300	<1000	**40**[2]	17	-	781 [b3]
300	<1000	**50**	17	-	1031 [b]
300	<1000	**60**	17	-	1105 [b]
300	<1000	**70**	17	-	1258 [ab]
300	<1000	**80**	17	-	1890 [a]
100	<1000	70	17	-	480 [b]
300	<1000	70	17	-	1258 [a]
100	**<1000**	60	17	-	522 [b]
100	**<500**	60	17	-	1258 [a]
100	<500	60	**14**	-	935 [a]
100	<500	60	**17**	-	1302 [a]
100	<500	60	**20**	-	1236 [a]
100	<500	60	**23**	-	1344 [a]
100	<500	60	17	-	1516 [a]
100	<500	60	17	+	1440 [a]

Table I. Effect of factors on transient expression of the GUS gene in embryogenic suspension culture tissue of corn. [1]Each value represents the mean of from 4 - 11 bombardments. [2]Parameters in bold type were varied within experiments. [3]Values followed by different letters are significantly different at P=0.05 by one way analysis of variance.

from the blast by the screen. The leaf cells in the areas between the fibers of the screen were apparently insufficiently protected and did not survive the impact of the blast. On the periphery of the ring, the GUS-positive regions were located between the fibers while the GUS-negative regions were shielded from the particles by the screen. It was not determined if the damage in the center of the blast resulted from the particles, the solution carrying the particles, or the helium burst. Although baffles have been used to obtain stable transformation in cotton (Finer and McMullen, 1990), corn (Gordon-Kamm *et al.*, 1990), and soybean (Finer and McMullen, 1991), the specific effect of these baffles were not well-documented.

Optimization of Bombardment Parameters:

Due to the simplicity of the PIG, it was necessary to modify only a few parameters for optimization. Bombardment parameters were optimized using embryogenic maize suspension cultures and the initial conditions were as follows: 60 PSI helium, 17 cm distance from syringe filter to tissue, 300 μl PCV of cells, cells filtered through 1 mm filter, no baffle, and 2 μl of the particle preparation.

The effect of different pressures of helium is shown in Table I. Transient expression of the GUS gene increased with increasing pressure up to a maximum average of 1,890 blue foci per shot with 80 PSI. However, at the highest pressure, many cells were dislodged from the Petri dish and a pressure of 60-70 PSI was selected for future experiments.

In comparing 100 μl versus 300 μl PCV of target cells (Table I), the number of blue foci increased in proportion to the PCV. At a PCV of 500 μl, 2,698 blue loci were obtained from a single shot. This largest PCV gave the greatest confluence of cell clumps and the most surface area for targeting. Because small amounts of embryogenic tissue are generally more suitable for liquid culture (Finer and McMullen, 1990, 1991), we examined the use of smaller clumps of tissue, which could provide a large surface area for targeting while maintaining a low PCV. Filtration of the suspension culture tissue through a 500 μm instead of a 1 mm filter led to a 2.6-fold increase in transient expression of the GUS gene (Table I). In addition to providing a large target area for bombardments, the small clumps of tissue were less likely to be dislodged from the filter disc from the impact of the bombardment. Large clumps of tissue protrude further from the filter paper disc and may therefore be more vulnerable to displacement by the helium burst.

Evaluation of various distances between the syringe filter and the tissue surprisingly revealed no significant differences in bombardment efficiency (Table I). This is in contrast to earlier experiments, using larger cell clumps (< 1 mm), where cell loss was greater at shorter distances (unpublished). Also surprising was the absence of a beneficial effect from use of a 500 μm baffle placed 9 cm above the tissue (Table I). It was anticipated that the baffle would give an effect similar to that seen with the cowpea leaf tissue. However, parameters such as the size and location of the baffle, as well as the pressure utilized for the corn bombardments were different from those used for the cowpea leaves.

A comparison between the stainless steel and plastic syringe filter units (Takeuchi *et al.*, 1992) gave insignificant differences in the number of blue foci obtained per bombardment (unpublished). Plastic syringe filters were preferred because of lower cost and lower reactivity of the plastic with solutions. Upon repeated

usage of the stainless steel filter units, oxidation of the metal was apparent in the funnel area below the screen. This oxidation may affect particle acceleration and helium flow. Although the plastic filters units were only rated to withstand 40 PSI (stainless steel units were rated at 100 PSI) such pressures were never present in the filter unit itself as it was simply a holding or flow-through device for the particles.

Transformation of Soybean:

An average of 11.5 stably-transformed, hygromycin-resistant clones were obtained for each bombardment of embryogenic soybean tissue. Every hygromycin-resistant soybean clone that has been obtained and analyzed to date (Finer and McMullen, 1991; and unpublished) contained the introduced hygromycin resistance gene. This indicated good efficacy in the hygromycin selection scheme. The number of clones obtained using the PIG was over 3 times that reported earlier for soybean (Finer and McMullen, 1991) using an older gunpowder version of the commercially-available particle gun (Biolistics, Model BPG). Transient expression of the GUS gene in embryogenic soybean tissue increased 2.5-fold, from an average of 709 blue foci with the gunpowder version to 1,812 with the PIG.

Southern hybridization analysis of a hygromycin-resistant, GUS-positive clone that was obtained using the PIG confirmed the presence of the intact expression unit for both the GUS and hygromycin resistance genes (Fig. 3). Based on the number of hybridization bands obtained with *Hin*dIII-digested DNA and the intensity of the hybridization signals, we estimate the presence of 3-4 copies of the GUS gene and only one copy of the hygromycin resistance gene. A higher copy number for the GUS gene was expected because pHygr and pUCGUS plasmids were mixed at 1:9 prior to DNA precipitation for bombardment and more of the pUCGUS plasmid was present for recombination and integration. Digestion of genomic DNA with *Bgl*II-restricted DNA (plasmids do not contain *Bgl*II restriction sites) revealed a high molecular weight band, indicating integration of the introduced DNAs into plant genomic DNA.

Conclusion

We report here on the development and partial optimization of the Particle Inflow Gun. This device was simple to operate, inexpensive and performed comparably to commercial devices. After preliminary optimization of this device for transient GUS expression, the factors that had the greatest influence on transient expression of the GUS gene in embryogenic corn tissue were the cell preparation methods. Stable transformation of embryogenic soybean has been obtained using this device and efforts are underway to generate transgenic corn plants.

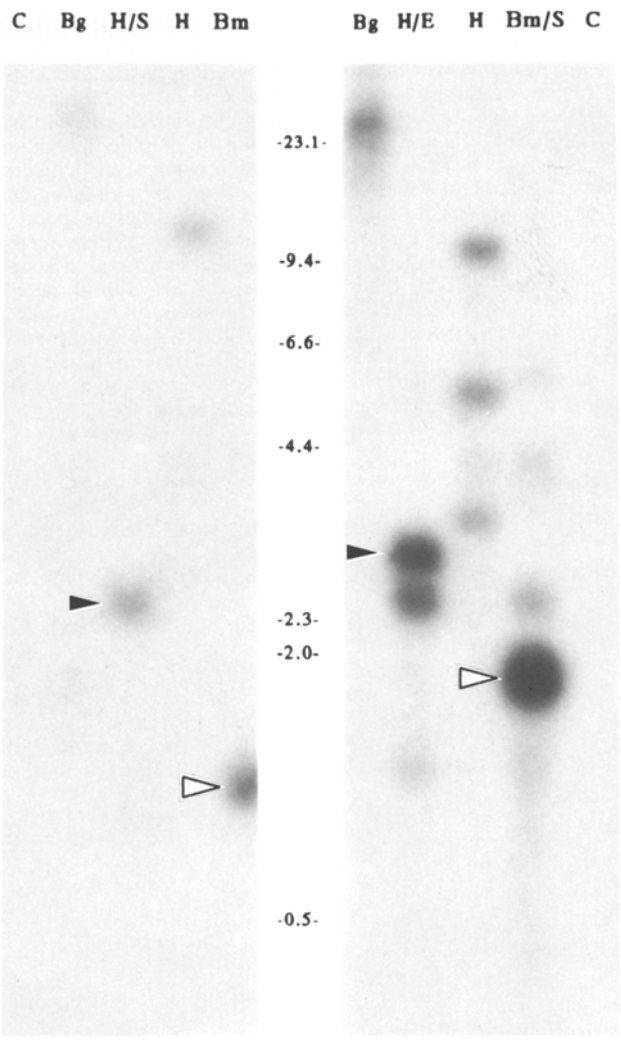

Figure 3. Southern hybridization analysis of a transformed soybean clone. DNA from nontransformed soybean cultures (C) was digested with *Hin*dIII. DNA from transformed soybean (all other lanes) was digested with various combinations of *Hin*dIII (H), *Bgl*II (Bg), *Sst*I (S), *Bam*HI (Bm), and *Eco*RI (E). *Hin*dIII digests pHygr (5.2 kb) and pUCGUS (6.1 kb) once and there are no restriction sites for *Bgl*II. *Hin*dIII/*Eco*RI cleaves the intact 2.9 kb expression unit for the GUS gene (right black arrow) and *Hin*dIII/*Sst*I cleaves the intact 2.2 kb expression unit for the hygromycin resistance gene (left black arrow). Digestion with *Bam*HI/*Sst*I and *Bam*HI releases the intact coding unit for the GUS gene (1.8 kb, right white arrow) and the hygromycin resistance gene (1.0 kb, left white arrow) respectively. The DNA on the right side of the figure was probed with the coding unit for the GUS gene and the DNA on the left side of the figure was probed with the coding unit for the hygromycin resistance gene.

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