

## TRANSFORMATION OF SOYBEAN [*GLYCINE MAX* (L.) MERRILL] USING PROLIFERATIVE EMBRYOGENIC TISSUE MAINTAINED ON SEMI-SOLID MEDIUM

ELIANE R. SANTARÉM<sup>1</sup> AND JOHN J. FINER<sup>2</sup>

*Department of Horticulture and Crop Science, OARDC, The Ohio State University, Wooster, Ohio 44691*

(Received 17 March 1999; accepted 8 July 1999; editor, Jack Widholm)

### SUMMARY

Transgenic soybean can be efficiently produced by particle bombardment of embryogenic suspension culture material. Unfortunately, the time required to obtain a transformation-competent soybean suspension culture line is often lengthy and can result in reduced fertility of regenerated plants. In addition, establishment and maintenance of embryogenic suspension cultures can be very difficult. The objective of this work was to minimize the time required to obtain transformation-competent embryogenic tissue and optimize DNA delivery into that tissue. Somatic embryos were induced from immature cotyledons of soybean [*Glycine max* (L.) Merrill cv 'Jack'] by placement of cotyledons, adaxial side up, on a MS-based induction medium containing 40 mg (181  $\mu$ M) 2,4-dichlorophenoxyacetic acid (2,4-D) per l and 6% sucrose. Embryogenic tissues, which formed from the surface of the cotyledons within 2–4 wk, were transferred to an embryo proliferation medium containing 20 mg (90  $\mu$ M) 2,4-D per l and 3% sucrose. After 4 wk, proliferative embryogenic tissue could be used for transformation via particle bombardment. Desiccation of target tissue, period of subculture prior to bombardment, and the number of bombardments per target tissue were evaluated for enhancement of transient  $\beta$ -glucuronidase (GUS) expression. The highest number of blue foci was observed when the target tissue was desiccated for 10 min in an uncovered Petri plate containing proliferation medium, subcultured on the same day of bombardment, and bombarded three times on a single day. For stable transformation, selection was started 20 d after bombardment using 9 mg hygromycin per l for 4 wk, and 18 mg per l thereafter. Stably transformed clones were obtained from tissue bombarded once and twice on a single day. GUS assays and Southern hybridization analysis of DNA from putative clones confirmed stable integration of the introduced genes. Fertile transgenic plants were obtained in 11–12 mo following culture initiation.

**Key words:** D20 medium; embryogenesis; hygromycin resistance; particle bombardment.

### INTRODUCTION

Since the first reports of particle bombardment-mediated plant transformation, a large number of species, previously considered recalcitrant for transformation, have been transformed with this procedure (Klein et al., 1989; Christou et al., 1991; Ellis et al., 1993; McCabe and Martinelli, 1993). For particle bombardment-mediated transformation to be successful, selection of the proper target tissue is critical. In the case of soybean, both shoot apices (McCabe et al., 1988) and proliferative embryogenic cultures (Finer and McMullen, 1991; Hadi et al., 1996; Stewart et al., 1996) have been transformed with this method. Soybean shoot apices present a more difficult target as the meristem can be obscured by leaf primordia. Proper control of the depth of particle penetration is difficult but extremely important. With proliferative embryogenic tissue, new embryos are derived from single epidermal cells (Finer, 1988) which are more accessible for transformation. Proliferative embryogenic cultures of soybean may also be more receptive to transformation because of the rapid proliferation of surface cells. Unfortunately, most stable transforma-

tion studies of proliferative embryogenic soybean tissue use suspension cultures (Finer and Nagasawa, 1988), which take 6 mo or more to become transformation-competent (Hazel et al., 1998), and the plants regenerated from these lines often show reduced or complete loss of fertility (Hadi et al., 1996). In addition, embryogenic suspension cultures of soybean can be very difficult to establish and maintain.

A procedure for rapid induction of soybean somatic embryos has recently been described (Santarém et al., 1997). Embryogenic tissue, induced from immature cotyledons with a medium containing 40 mg (181  $\mu$ M) 2,4-D per l, proliferated on semi-solid medium containing reduced levels of 2,4-D (Bailey et al., 1993, Stewart et al., 1996). This proliferative embryogenic tissue, obtained as soon as 4 wk after induction, could be used as a target for gene transfer in soybean cells, avoiding the manipulations and problems associated with long-term suspension cultures.

For each different target tissue, the culture conditions and physiological stage of the tissue must be optimized. Tissue damage must be minimized and cell viability must be improved for maximum recovery of stable transformants. The objective of this work was to obtain transformation-competent embryogenic tissue of soybean in the least amount of time and to optimize the conditions for efficient delivery of DNA to that tissue.

<sup>1</sup>Present address: Universidade de Cruz Alta, Laboratório de Cultura de Tecidos Vegetais in vitro, Campus Universitário, Caixa Postal 858, Cruz Alta—RS 98025-810, Brasil at E-mail: fine.1@osu.edu.

<sup>2</sup>To whom correspondence should be addressed.

## MATERIALS AND METHODS

**Initiation and maintenance of embryogenic tissue.** Somatic embryos were induced from immature cotyledons of soybean [*Glycine max* (L.) Merrill] cv 'Jack.' Immature cotyledons were cultured abaxial side facing D40 medium (Santarém et al., 1997), which contained MS salts (Murashige and Skoog, 1962), B<sub>5</sub> vitamins (Gamborg et al., 1968), 40 mg (181  $\mu$ M) 2,4-dichlorophenoxyacetic acid (2,4-D) per l, and 6% sucrose, and solidified with 0.2% Gelrite™ (Merck & Co., Rahway, NJ), pH 7. After 3 wk on D40 medium, embryogenic cotyledons were transferred to D20 medium containing MS salts, B<sub>5</sub> vitamins, 20 mg (90  $\mu$ M) 2,4-D per l, 3% sucrose, and 0.2% Gelrite™, pH 5.7. Embryogenic tissue, containing masses of globular proliferative embryos, was selectively subcultured every 2 wk. Before bombardment, 10 clusters of proliferative embryogenic tissue ( $\approx$ 100 mg) were placed in the middle of 90-mm Petri dishes containing D20 medium. Tissues were maintained under a 16-h photoperiod (30  $\mu$ Em<sup>-2</sup>s<sup>-1</sup>) at 27° C throughout the culture period.

**Particle bombardment.** The plasmid pHG1 (Finer and McMullen, 1991), which encodes both hygromycin resistance and  $\beta$ -glucuronidase, each under the transcriptional control of CaMV 35S promoter, was used for transient and stable transformation studies. DNA was precipitated onto tungsten M10 particles (1.1  $\mu$ m; Sylvania Chemicals and Metals, Towanda, PA) with a CaCl<sub>2</sub>-spermidine precipitation procedure (Finer and McMullen, 1990). The mixture containing the precipitated DNA was resuspended after precipitation and 2  $\mu$ l was removed for bombardment. Tissue was bombarded using a particle inflow gun (Finer et al., 1992).

**Desiccation of target tissue.** Before bombardment, proliferative embryogenic tissue was either dried in uncovered Petri dishes containing D20 medium in a laminar flow hood (physical desiccation) for 5, 10, 15, and 30 min or transferred to D20 medium containing 0.2 M mannitol and 0.2 M sorbitol (osmotic desiccation) for 15, 30, 60, and 120 min. Where the embryogenic tissue was dried in a hood, the relative humidity in the laboratory was approximately 50%, the temperature was 27° C, and the air flow in the hood was 27 m/min.

**Time of bombardment.** Proliferative embryogenic tissue was bombarded 0, 1, 2, 3, 4, 5, 7, 10, and 15 d after subculture to fresh medium. At Day 0, the tissue was in contact with fresh medium for approximately 3 h before bombardment. For all time points, the tissue was subjected to physical desiccation for 10 min.

**Number of shots.** The optimum number of shots per bombardment was evaluated by bombarding embryogenic tissue once, twice, or three times on the same day (Day 0), or on both Day 0 and Day 3. For each time point, the tissue was subjected to physical desiccation for 10 min.

**Histochemical GUS assay.** Histochemical  $\beta$ -glucuronidase (GUS) assays were performed on 100 mg of embryogenic tissue 2 d after bombardment. Samples were incubated at 37° C in GUS assay mix (Jefferson, 1987). GUS expression was scored visually for the production of dimeric indigo dye. Results were expressed as the average number of GUS-expressing foci per cluster (~10 mg) of proliferative embryogenic tissue. Each treatment consisted of three replications of 100 mg of tissue. Means were analyzed by analysis of variance. Treatment means were separated by Fisher's least significant difference test ( $\alpha = 0.05$ ).

**Stable transformation.** For studying stable expression of the introduced gene, embryogenic proliferative tissue was partially dehydrated for 10 min in open Petri dishes containing D20 medium and bombarded once, twice, or three times on the same day.

Selection for transformed tissue was started 20 d after bombardment on D20 medium containing 9 mg hygromycin per l. After 4 wk, tissues were transferred to D20 medium containing 18 mg hygromycin per l. Tissue was subcultured every 14 d. Putative transgenic clones were obtained after 2 to 3 mo of selective culture.

**Plant regeneration.** Plants were regenerated from embryogenic tissue as described previously (Finer and McMullen, 1991). The T<sub>0</sub> plants were grown in the greenhouse under a 24-h photoperiod for 2 mo to prevent premature flowering and subsequently grown under a 16-h photoperiod to induce flowering.

**Molecular analysis.** DNA was extracted from transformed embryogenic clusters, leaves, and seeds with the CTAB procedure (Saghai-Marouf et al., 1984). Total DNA (10  $\mu$ g) was digested with HindIII and electrophoresed through a 0.8% agarose gel. DNA was transferred to GeneScreen Plus membrane (NEN Research Products, Boston, MA). Digestion of pHG1 with HindIII releases a 2.3-kb fragment, which contains the expression unit for

the hygromycin resistance gene. Total RNA was isolated from in vitro-transformed plantlets with a modified lithium chloride procedure (Hadi, 1996). Twenty micrograms of total RNA was electrophoresed through a 1.5% formaldehyde/formaldehyde agarose gel and transferred to a GeneScreen Plus membrane. Blotting and hybridization were performed according to the manufacturer's instructions. The probe consisted of the coding region from the hygromycin resistance gene, random prime labeled with <sup>32</sup>P (Feinberg and Volgestein, 1983).

## RESULTS AND DISCUSSION

Somatic embryos were induced on D40 induction medium within 3 wk and transferred to D20 medium for proliferation. After approximately 2 wk on this medium, compact embryogenic clusters, defined as proliferative embryogenic tissue, were obtained. This tissue consisted of masses of globular embryos (Fig. 1 a) and was yellow-green to green. This type of tissue was used for all bombardments.

**Desiccation of target tissue.** Transient expression of the GUS gene was influenced by desiccation of target tissue. Physical desiccation of proliferative embryogenic tissue for 15 min or longer increased the number of GUS-expressing foci in each cluster of tissue more than did desiccation for 0 and 5 min; however, no significant difference was observed among treatments of 10, 15, and 30 min (Table 1). This treatment is comparable to the 15-min drying used to enhance transient expression in embryogenic suspension culture tissue of soybean (Vain et al., 1993b). Although embryogenic suspension cultures were dried in empty Petri dishes (Vain et al., 1993b), the proliferative embryogenic tissues used in the present study were dried in Petri dishes containing D20 medium. As more extensive drying of tissue in empty Petri dishes has resulted in rapid water loss and tissue damage (Finer, unpublished), the use of Petri dishes containing media apparently extended the time that the tissue could be exposed to prebombardment drying by buffering the water loss.

Preculture of embryogenic tissue for 15 or 30 min on D20 medium containing 0.2 M mannitol and 0.2 M sorbitol did not enhance transient expression, when compared to the 10-min physical desiccation, used here as control. However, preincubation on this osmotic medium for longer than 30 min decreased transient expression (Table 1). In maize, extended preculture of embryogenic tissue on an osmotic conditioning medium also resulted in reduced transient expression (Vain et al., 1993a). In the case of osmotic enhancement, the increase of transient expression has been attributed to cell plasmolysis, whereby the bombarded plasmolyzed cells are less likely to lose protoplasm through bombardment wounds in the cell wall. The reduction in transient expression resulting from extended preincubation reported here may have resulted from osmotic adjustment, which may have allowed the cells to return to a hypertonic state. On the basis of our results, 10 min of physical desiccation in uncovered Petri plates containing D20 medium was used as a treatment before bombardment.

**Time of bombardment.** When the time period from the latest subculture to bombardment was evaluated, the peak of transient GUS activity was observed with tissue bombarded 4 d after subculture (Table 2). This time after subculture corresponds to the peak mitotic index observed previously for soybean embryogenic suspension cultures (Hazel et al., 1998). In that report, peak GUS expression was observed at Day 2 following subculture, 2 d before the maximum mitotic index. In alfalfa, GUS expression was highest 3 to 6 d after the tissue was transferred to fresh medium (Brown et al., 1994), a period that was also previously identified as having the highest frequency of cells undergoing division (Atanassov and Brown, 1984).

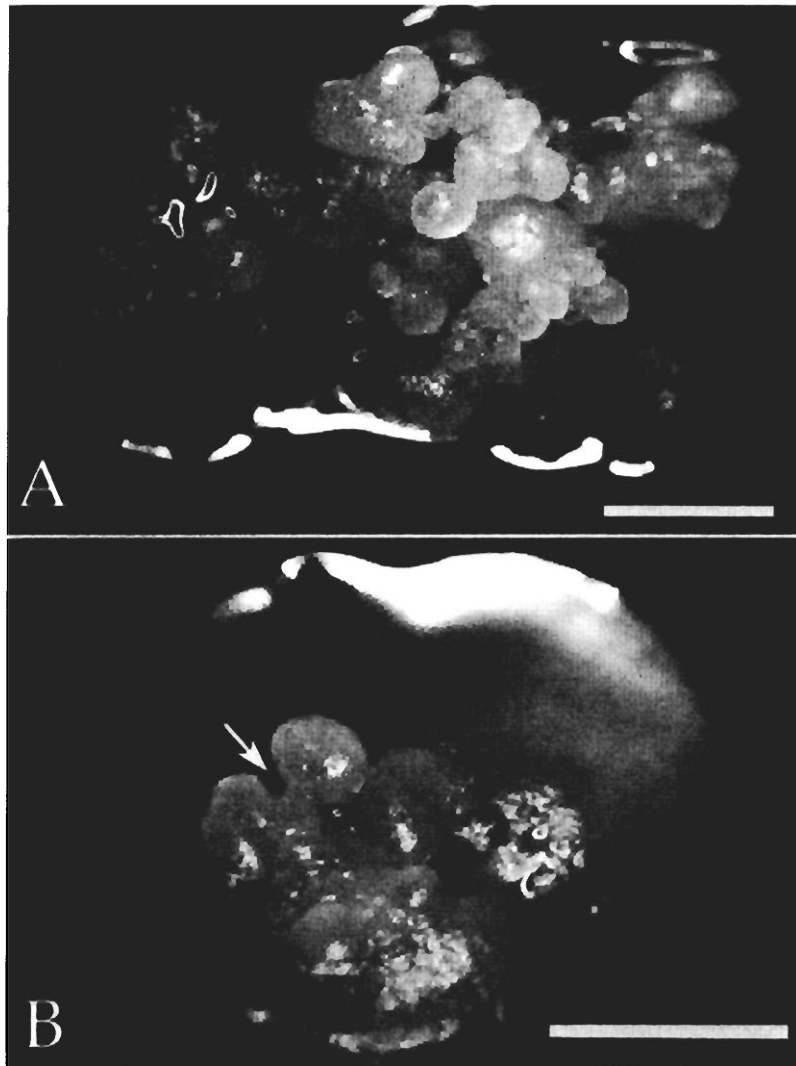


FIG. 1. A, Proliferative embryogenic tissue of soybean maintained on D20 medium. B, Growth of nodulated hygromycin-resistant tissue (arrow) from amorphous hygromycin-sensitive tissue on semi-solid medium. Bar, 1 mm.

TABLE 1

EFFECT OF PHYSICAL AND OSMOTIC DESICCATION ON TRANSIENT EXPRESSION OF GUS IN SOYBEAN EMBRYOGENIC TISSUES BOMBARDED WITH pHG1

Physical desiccation		Osmotic desiccation	
Time (min)	Transient expression <sup>a</sup>	Time (min)	Transient expression <sup>a</sup>
0	51.3 y <sup>b</sup>	Control <sup>c</sup>	60.3 z
5	45.3 y	15	62.1 z
10	65.9 yz	30	54.3 z
15	75.9 z	60	31.7 y
30	66.5 yz	120	30.2 y

<sup>a</sup>Average number of GUS foci expressed per cluster of embryogenic tissue.

<sup>b</sup>Different letters indicate a significant difference among means at  $\alpha = 0.05$ .

<sup>c</sup>The control treatment for osmotic drying was 10 min of physical desiccation.

TABLE 2

EFFECT OF TIME AFTER SUBCULTURE ON GUS EXPRESSION OF EMBRYOGENIC BOMBARDED TISSUE OF SOYBEAN

Days after subculture	Transient expression <sup>a</sup>
0	63.0 y <sup>b</sup>
1	57.5 y
2	63.9 y
3	72.3 y
4	151.8 z
5	80.2 y
7	70.2 y
10	65.8 y
15	70.0 y

<sup>a</sup>Average number of GUS foci expressed per cluster of embryogenic tissue.

<sup>b</sup>Different letters indicate a significant difference among means at  $\alpha = 0.05$ .

TABLE 3

EFFECT OF NUMBER OF SHOTS AND DAY OF BOMBARDMENT ON TRANSIENT EXPRESSION OF GUS IN SOYBEAN

Transient expression <sup>a</sup> as affected by:		
Number of shots per day	Day(s) of bombardment after subculture	
	Day 0	Day 0,3
1	56.9 y <sup>b</sup>	76.9 y
2	61.2 y	92.8 y
3	75.2 z	126.8 z

<sup>a</sup>Average number of GUS foci expressed per cluster of embryogenic tissue.

<sup>b</sup>Different letters in the columns indicate a significant difference among means at  $\alpha = 0.05$ .

It appears that maximum transgene expression in cells can be influenced by the specific cell cycle stage.

**Number of bombardments.** Transient expression of GUS was affected by the number of bombardments and the day of bombardment (Table 3). However an interaction between the two factors was not detected at  $\alpha = 0.05$  ( $F > 0.0508$ ). Regardless of the day of bombardment, three bombardments resulted in higher transient GUS expression (Table 3). Although this treatment yielded the highest transient expression, the damage caused to the tissue as a consequence of the repetitive bombardment was also increased. Lonsdale et al. (1990) found that double bombardments increased transient expression of the GUS gene introduced into germinating wheat embryos, whereas subsequent bombardments resulted in greater tissue damage with no increase in transformation efficiency. Klein et al. (1988) also reported an increase in transient expression events in maize suspension cells following three successive bombardments.

**Stable transformation.** Clones were initially recovered 2–3 mo following selection on hygromycin-containing medium. Hygromycin-resistant tissues were easily identified as yellow to green proliferative embryogenic material that arose from white to brown amorphous hygromycin-sensitive tissue (Fig. 1 b). Among the tissues that were bombarded once, twice, or three times on a single day, partial browning was observed on the tissue bombarded three times and no clones were recovered with this treatment. Five independent clones were recovered from the other treatments, representing production of transgenic clones from 4.2% of the initially bombarded clumps of embryogenic tissue. Three of the five clones originated from tissue that was bombarded twice. Southern hybridization analysis (Fig. 2 a) clearly showed five different hybridization patterns from these five clones, confirming they were derived from independent transformation events. With the transformation efficiency reported here, it is very unlikely that any of these clones represent mixtures or chimeras of different transformed clones. The complex hybridization patterns observed with the clones obtained in this study are typical of transformants obtained from particle bombardment-mediated transformation (Pawlowski and Somers, 1996). A 2.3-kb band representing the intact expression unit for the hygromycin resistance gene was observed in only three of the five clones. Clones 3 and 5 displayed hybridization signals larger than the expected 2.3-kb band, which could have resulted from the loss of one and/or both of the *Hind*III sites which bordered the hygromycin resistance gene. Clone 3 did not express GUS, indicating either recombination and loss of a por-

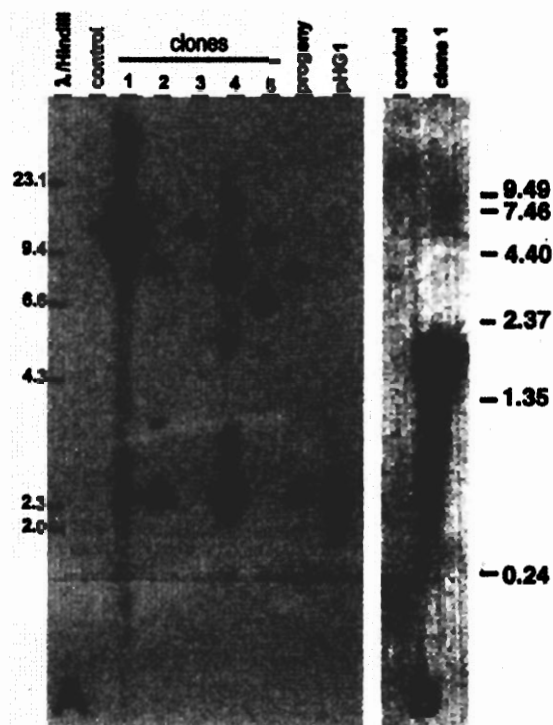


FIG. 2. A, Southern hybridization analysis of clones transformed with pHG1. Genomic DNA was isolated from nontransformed embryogenic tissue (control), transformed embryogenic tissue from clones 1–5, and progeny from clone 1. B, Northern blot analysis. Total RNA was isolated from control and transgenic plantlets regenerated from nontransformed embryogenic tissue and clone 1, respectively.

tion of the introduced plasmid, gene methylation, or gene silencing in this low-copy transformant.

Ten *in vitro* plantlets, obtained from clone 1, all strongly expressed GUS in all tissues (data not shown). Northern hybridization analysis of tissue from clone 1 (Fig. 2 b) confirmed the expression of the hygromycin resistance gene in transgenic plantlets. Southern hybridization analysis of progeny from pooled immature embryos from clone 1 T<sub>0</sub> plants confirmed stable integration and transmission of the hygromycin resistance gene (Fig. 2 a).

This paper reports the use of an alternative target tissue coupled with selection on semi-solid medium for soybean transformation. Proliferative embryogenic tissue, which could be obtained 2 mo after culture induction, was suitable for stable transformation. This is a significant improvement over bombardment of embryogenic suspension culture tissue of soybean, in which transformability (transformation competency) was first observed 6 mo after culture initiation (Hazel et al., 1998) and in which problems of sterility in the regenerated plants are common (Hadi et al., 1996). Using this new target tissue (proliferative embryogenic soybean tissue, maintained on D20 medium) with selection on semi-solid media, we have introduced different DNAs into embryogenic tissue of a few different soybean cultivars, with similar efficiency of clone recovery as that reported here (Finer, unpubl.). Factors which increased transformation in this study included physical desiccation of freshly subcultured tissue for 10 min before bombardment and bombardment of the target tissue

twice on the same day. Fertile transgenic plants were recovered 8–10 mo after bombardment.

## ACKNOWLEDGMENTS

The authors are grateful to Barbara Norris, Juliane Essig, and Lloyd Ringley, Jr. for technical assistance. We also thank Bernard Pelissier, Masood Z. Hadi, Harold Trick, Tilak Ponappa, and Holly Franz for their valuable comments. Salaries and research support were provided by the United Soybean Board, State and Federal funds appropriated to The Ohio State University/Ohio Agricultural Research and Development Center, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. Mention of trademark or proprietary products does not constitute a guarantee or warranty of the product by OSU/OARDC, and also does not imply approval to the exclusion of other products that may also be suitable. This is Journal Article Number HCS 98-4.

## REFERENCES

- Atanassov, A.; Brown, D. C. W. Plant regeneration from suspension culture and mesophyll protoplasts of *Medicago sativa*. *Plant Cell Tissue Organ Cult.* 3:149–162; 1984.
- Bailey, M. A.; Boerma, H. R.; Parrott, W. A. Genotype effects on proliferative embryogenesis and plant regeneration of soybean. *In Vitro Cell. Dev. Biol.* 29P:102–108; 1993.
- Brown, D. C. W.; Tian, L.; Buckley, D. J.; Lefebvre, M.; McGrath, A.; Webb, J. Development of a simple particle bombardment device for gene transfer into plant cells. *Plant Cell Tissue Organ Cult.* 37:47–53; 1994.
- Christou, P.; Ford, T. L.; Kofron, M. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Bio/Technology* 9:957–962; 1991.
- Ellis, D. D.; McCabe, D. E.; McInnis, S.; Ramachandran, R.; Russell, D. R.; Wallace, K. M.; Martinelli, B. J.; Roberts, D. R.; Raffa, K. F.; McCown, B. H. Stable transformation of *Picea glauca* by particle acceleration. *Bio/Technology* 11:84–89; 1993.
- Feinberg, A. P.; Volgestein, B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Ann. Biochem.* 132:6–13; 1983.
- Finer, J. J. Apical proliferation of embryogenic tissue of soybean [*Glycine max* (L.) Merrill]. *Plant Cell Rep.* 7:238–241; 1988.
- Finer, J. J.; McMullen, M. D. Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. *Plant Cell Rep.* 8:586–589; 1990.
- Finer, J. J.; McMullen, M. D. Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. *In Vitro Cell. Dev. Biol.* 27P:175–182; 1991.
- Finer, J. J.; Nagasawa, A. Development of an embryogenic suspension culture of [*Glycine max* (L.) Merrill]. *Plant Cell Tissue Organ Cult.* 15:125–136; 1988.
- Finer, J. J.; Vain, P.; Jones, M. W.; McMullen, M. D. Development of the Particle Inflow Gun for DNA delivery to plant cells. *Plant Cell Rep.* 11:323–328; 1992.
- Gamborg, O. L.; Miller, R. A.; Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50:151–158; 1968.
- Hadi, M. Z. Isolation and characterization of a RecA-like gene from soybean (*Glycine max* L.) Ph.D. dissertation; The Ohio State University; 1996:182.
- Hadi, M. Z.; McMullen, M. D.; Finer, J. J. Transformation of 12 different plasmids into soybean via particle bombardment. *Plant Cell Rep.* 15:500–505; 1996.
- Hazel, C. D.; Klein, T. M.; Anis, M.; Wilde, H. D.; Parrott, W. A. Growth characteristics and transformability of soybean embryogenic cultures. *Plant Cell Rep.* 17:765–772; 1998.
- Jefferson, R. A. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* 5:387–405; 1987.
- Klein, T. M.; Gradziel, T.; Fromm, M. E.; Sanford, J. C. Factors influencing gene delivery into *Zea mays* cells by high-velocity microprojectiles. *Bio/Technology* 6:559–563; 1988.
- Klein, T. M.; Kornstein, L.; Sanford, J. C.; Fromm, M. E. Genetic transformation of maize cells by particle bombardment. *Plant Physiol.* 91:440–444; 1989.
- Lonsdale, D.; Onde, S.; Cumming, A. Transient expression of exogenous DNA in intact, viable wheat embryos following particle bombardment. *J. Exp. Bot.* 41:1161–1165; 1990.
- McCabe, D. E.; Martinelli, B. J. Transformation of elite cotton cultivars by particle bombardment of meristems. *Bio/Technology* 11:596–598; 1993.
- McCabe, D. E.; Swain, W. F.; Martinelli, B. J.; Christou, P. Stable transformation of soybean (*Glycine max*) by particle acceleration. *Bio/Technology* 6:923–926; 1988.
- Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473–497; 1962.
- Pawlowski, W. P.; Somers, D. A. Transgene inheritance in plants genetically engineered by microprojectile bombardment. *Mol. Biotechnol.* 6:17–30; 1996.
- Saghei-Marouf, M. A.; Saliman, K. M.; Jorgensen, R. A.; Wallard, R. Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. *Proc. Natl. Acad. Sci. USA* 81:8014–8018; 1984.
- Santarém, E. R.; Pelissier, B.; Finer, J. J. Effect of explant orientation, pH, solidifying agent and wounding on initiation of soybean somatic embryos. *In Vitro Cell. Dev. Biol.* 32P:13–19; 1997.
- Stewart, C. N., Jr.; Adang, M. J.; All, J. N.; Boerma, H. R.; Cardineau, G.; Tucker, D.; Parrott, W. A. Genetic transformation, recovery, and characterization of soybean [*Glycine max* (L.) Merrill] transgenic for a synthetic *Bacillus thuringiensis* CRY IA (c) gene. *Plant Physiol.* 12:121–129; 1996.
- Vain, P.; McMullen, M. D.; Finer, J. J. Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Rep.* 12:84–88; 1993a.
- Vain, P.; Keen, N.; Murillo, J.; Rathus, C.; Nemes, C.; Finer, J. J. Development of the Particle Inflow Gun. *Plant Cell Tissue Organ Cult.* 33:237–246; 1993b.