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Stable transformation via particle bombardment in two different soybean regeneration systems

Shirley Sato [1], Christine Newell [2], Kathryn Kolacz [1], Loreley Tredo [1], John Finer [3], and Maud Hinchee [1]

[1] Monsanto, 700 Chesterfield Parkway North, Chesterfield, MO 63198, USA
[2] Agricultural Genetics Company Ltd., AFRC Institute of Plant Science Research, Research and Development Division, Babraham, Cambridge CB2 4AZ, UK
[3] Department of Agronomy, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, OH 44691, USA

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ABSTRACT

The Biolistics® particle delivery system for the transformation of soybean (*Glycine max* L. (Merr.) was evaluated in two different regeneration systems. The first system was multiple shoot proliferation from shoot tips obtained from immature zygotic embryos of the cultivar Williams 82, and the second was somatic embryogenesis from a long term proliferative suspension culture of the cultivar Fayette. Bombardment of shoot tips with tungsten particles, coated with precipitated DNA containing the gene for ß-glucuronidase (GUS), produced GUS-positive sectors in 30% of the regenerated shoots. However, none of the regenerants which developed into plants continued to produce GUS positive tissue. Bombardment of embryogenic suspension cultures produced GUS positive globular somatic embryos which proliferated into GUS positive somatic embryos and plants. An average of 4 independent transgenic lines were generated per bombarded flask of an embryogenic suspension. Particle bombardment delivered particles into the first two cell layers of either shoot tips or somatic embryos. Histological analysis indicated that shoot organogenesis appeared to involve more than the first two superficial cell layers of a shoot tip, while somatic embryo proliferation occurred from the first cell layer of existing somatic embryos. The different transformation results obtained with these two systems appeared to be directly related to differences in the cell types which were responsible for regeneration and their accessibility to particle penetration.

INTRODUCTION

Transgenic soybean plants have been produced using *Agrobacterium* vectors for gene transfer (Hinchee et al. 1988, Chee et al. 1989, Parrott et al. 1989, Zhou and Atherly 1990). Recently, it has been shown that DNA can be introduced into soybean via particle bombardment. Production of transgenic soybean callus using particle bombardment technology has been reported (Wang et al.1988, Christou et al. 1988). Transgenic soybean plants have been obtained by using particle bombardment of two different regeneration systems.

One system used isolated soybean shoot tips and a proprietary bombardment technology (Christou 1990a, Christou 1990b, McCabe et al. 1988); the other used embryogenic suspensions and the Biolistics® technology (Finer and McMullen 1991, Finer and Nagasawa 1988). In our study, two different soybean regeneration explants, shoot tips and embryogenic suspensions, were bombarded using a Biolistics® particle accelerator. The frequency of transgenic tissue sectors and transgenic plant production were subsequently evaluated. The location of particles after bombardment, as well the number and the location of cells responsible for regeneration, influenced the frequency of transgenic plant production in the two systems.

MATERIALS AND METHODS

Regeneration

Shoot tips-Soybean shoot tips were obtained from immature green seeds (0.5-1.0cm long) from greenhouse grown soybean plants (cv. Williams 82). After surface-sterilization of the seeds with 20% Chlorox® followed with 3 sterile water rinses, the immature embryos were extracted and the shoot tips were isolated. Primary leaves and stipules were removed, and the shoot tips were cultured on agar-solidified MS medium containing 3 mg/l benzylamino purine (BAP), 0.037mg/l naphthaleneacetic acid (NAA), 1.68 mg/l thiamine-HCl, 1.38g/l proline and 3% sucrose (McCabe et al. 1988). After bombardment, the shoot tips were moved to fresh medium and cultured in the dark for 2 weeks. The shoot tips were then transferred to agar-solidified MS medium containing 0.38mg/l BAP and 3% sucrose with 0, 25, 50, or 75mg/l kanamycin and cultured in the light (16:8) for 4 weeks. Shoot tips were subcultured every 4 weeks onto the same medium until regenerating shoots were approximately 1.5cm tall. They were then removed from the original explant and placed on B5 with no hormones (Gamborg et al. 1968) for rooting.

Embryogenic suspensions- Embryogenic suspensions of the soybean cultivar Fayette (Finer and Nagasawa 1988) were maintained by subculturing every 4 weeks into fresh liquid medium consisting of MS salts modified to contain 10mM NH_4NO_3 and 30mM KNO_3, B5 vitamins, 6% sucrose, 5mg/l 2,4-D and 5mM asparagine. At each subculture, 2 embryogenic clumps of tissue were placed into 35 mls of fresh medium in a 125 ml DeLong flask. After bombardment, the suspensions were grown without selection in liquid maintenance medium for two weeks, and then in the same medium but with 50mg/l G418 (Geneticin, Sigma) for 8 weeks, with the medium being replaced with fresh medium every 3-4 days. After 8 weeks,

Correspondence to: M. Hinchee

green embryos or clumps were separated from the brown, presumably dead, embryos and reproliferated in liquid medium without a selective agent. Further embryo development occurred on Phytagar (Gibco)-solidified MS medium containing B5 vitamins and 3% maltose. After 4 weeks, the embryos were placed on the same medium with 3% sucrose instead of maltose. Embryo germination was initiated by replating onto the previous medium after a desiccation period of 2-7 days. Desiccation was achieved by placing embryos in empty, parafilm-wrapped, plastic 100mm x 25mm petri plates.

DNA Bombardment

Shoot tips- Shoot apices were bombarded with a 1988 model (PDS 1000) of the Biolistics® particle gun. Plasmid DNA of pMON 10026 or pMON 13671 was precipitated onto M17 (DuPont) tungsten particles using the calcium chloride/spermidine method (Klein et al. 1988). The plasmids pMON 10026 or pMON 13671 contained the neomycin phosphotransferase II (NPT II) gene fused to the cauliflower mosaic virus (CaMV) 35s promoter and the ß-glucuronidase (GUS) gene fused to mannopine synthase promoter and the 7S transcription termination signal. The DNA/particle suspension was bombarded twice into apices positioned at shelf second from the bottom.

Embryogenic Suspensions-The Biolistics® particle gun designated above was used for embryogenic suspension bombardment. pMON10026 was precipitated onto M10 (DuPont) particles using the calcium chloride/spermidine method. Prior to bombardment, an entire flask of an embryogenic suspension (approximately 1 gram of tissue) was poured into a petri plate and the medium was removed. The clumpy suspension was gently smashed with a spatula prior to bombarding two times at shelf position second from the bottom.

Transformation Analysis

Regenerating shoot tips were analyzed for transformation based on their ability to express GUS 6-8 weeks post bombardment. The shoot proliferating region was free-hand sectioned and incubated with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (Jefferson et al. 1986) overnight at 37°C. The number of explants which had produced shoots containing GUS positive sectors was counted. When regenerated shoots were placed on the rooting medium, a stem cross-section was taken and evaluated for GUS expression. The number of shoots with stem cross-sections containing a GUS positive sector was counted. Rooted plantlets were evaluated for GUS expression by taking samples of each leaf from 5 different trifoliates and incubating the leaf pieces in X-Gluc overnight.

Embryogenic suspensions were evaluated for GUS expression 10 weeks after bombardment. The contents of an entire flask, containing both green and brown embryogenic clumps, were placed into X-Gluc and incubated overnight. Three subsequent stages in embryo development (green proliferating embryogenic clumps, elongated somatic embryos, and resulting plantlets) were also analyzed for GUS expression.

Histology

In order to determine the cell populations involved in regeneration, non-bombarded regenerating shoot tips were fixed in formalin-acetic acid-ethanol (FAA, Johansen 1940) at 0, 2, 7, 14, and 21 days in culture. Non-bombarded embryogenic clumps were fixed in FAA at the 4 week time point after subculture. In order to determine the distribution of particles after bombardment, shoot tips and embryogenic clumps were fixed in FAA immediately after bombardment. All FAA-fixed material was dehydrated in an ethanol-tertiary butyl alcohol series, embedded in Paraplast Plus® (Sherwood Medical), and serially sectioned at 10µm. The sections were stained with safranin-fast green (modified from Johansen 1940).

Particle Distribution

Histological sections of bombarded tissue, prepared as described above, were used for particle counting. Particle counts in different cell layers were done on median longitudinal sections from 4 different embryo clumps or shoot tips. Color photographs with a magnification factor of approximately 100x were made of these sections. Particles were easily detected by their size (approximately 2µm), shape (angular), and opaque appearance. The total number of particles was counted in each of the first two cell layers and in the remaining cell layers in the apical meristem of shoot tip explants and the surface of globular somatic embryos.

DNA Isolation and Southern Hybridization Analysis

DNA Isolation -DNA was extracted from young leaf tissue using a modification of the Dellaporta et. al (1983) procedure.

Southern Blot Analysis-To determine if the GUS gene was present in DNA isolated from leaf tissue, DNA was digested with either Bam HI(New England Biolabs); used for pMON 10026 transformants or Eco RI(New England Biolabs); used for pMON 13671 transformants. When pMON 10026 is cut with Bam HI, a 2375 bp fragment is generated which includes pMAS and the GUS gene. When pMON 13671 is cut with Eco RI, a 2503 bp fragment is generated which contains pMAS and the GUS gene. The Southern blot (Southern, 1975) analysis was performed using Church and Gilbert (1984) modifications.

RESULTS

Shoot Tip Transformation

Twenty four hours post bombardment, GUS was expressed transiently in bombarded shoot tips (Figure 1A). Thirty percent of the regenerating shoot tip explants sacrificed for GUS analysis had produced *de novo* shoot primordia and shoots which contained GUS positive sectors (Table 1; Figure 1B). However only 0.4% of nearly 3,000 regenerated plantlets had GUS positive sectors in their stems at the time of rooting (Figure 1B insert) and none of the rooted plants had GUS positive leaves. Kanamycin selection did not have a significant effect on sector formation. GUS positive sectors in regenerated shoot primordia were primarily superficial (epidermis and outer cortex), but sometimes included deeper tissues (inner cortex and leaf mesophyll cells). GUS sectors in the plantlets were only superficial (epidermis and outer cortex). These results indicated that regenerated shoots were chimeric for GUS expression, and that the transformed cells were not in the part of the primary apical meristem responsible for production of the inner cortex and vascular tissue in developing shoots.

Table 1. Histochemical GUS analysis at Three Different Stages of Regeneration Following Particle Bombardment of Soybean Shoot Tips

Regeneration Stage*	Total #	# Gus Positive
Shoot Primordia	1,295	394 (30%)
Plantlet at Rooting	2,784	10 (0.4%)
Five-Trifoliate Plant	10	0

*Shoot primordia stage regenerates were sacrificed for GUS analysis and were not part of the evaluation for the other two stages. The 10 five-trifoliate plants were the GUS positive plants identified in the plantlet rooting stage. The number of explants includes both kanamycin selected and non-selected explants.

In an examination of median longitudinal sections of bombarded shoot tips, the majority of particles appeared to be lodged in the surface cells. A count of the particles revealed that approximately 78% of all particles were found in the first two cell layers of the axillary and primary meristems (Table 2; Figure 1C).

Table 2. Particle distribution within surface cell layers in median longitudinal sections of bombarded globular somatic embryos and shoot tip apical and axillary meristems. "1" represents the most superficial cell layer.

Tissue	Sample #	Total # part.	particle #'s in cell layers 1	2	3+	% particles in cell layers 1	1+2	3+
Somatic Embryos	1	116	83	20	13	73	89	11
	2	48	24	20	4	50	92	8
	3	117	52	50	15	44	87	13
	4	148	88	43	17	59	89	11
ave:						**57**	**89**	**11**
Shoot Tip	1	30	14	10	6	47	80	20
	2	65	22	20	23	34	65	35
	3	80	46	25	19	58	76	24
	4	18	8	8	2	44	89	11
ave:						**46**	**78**	**22**

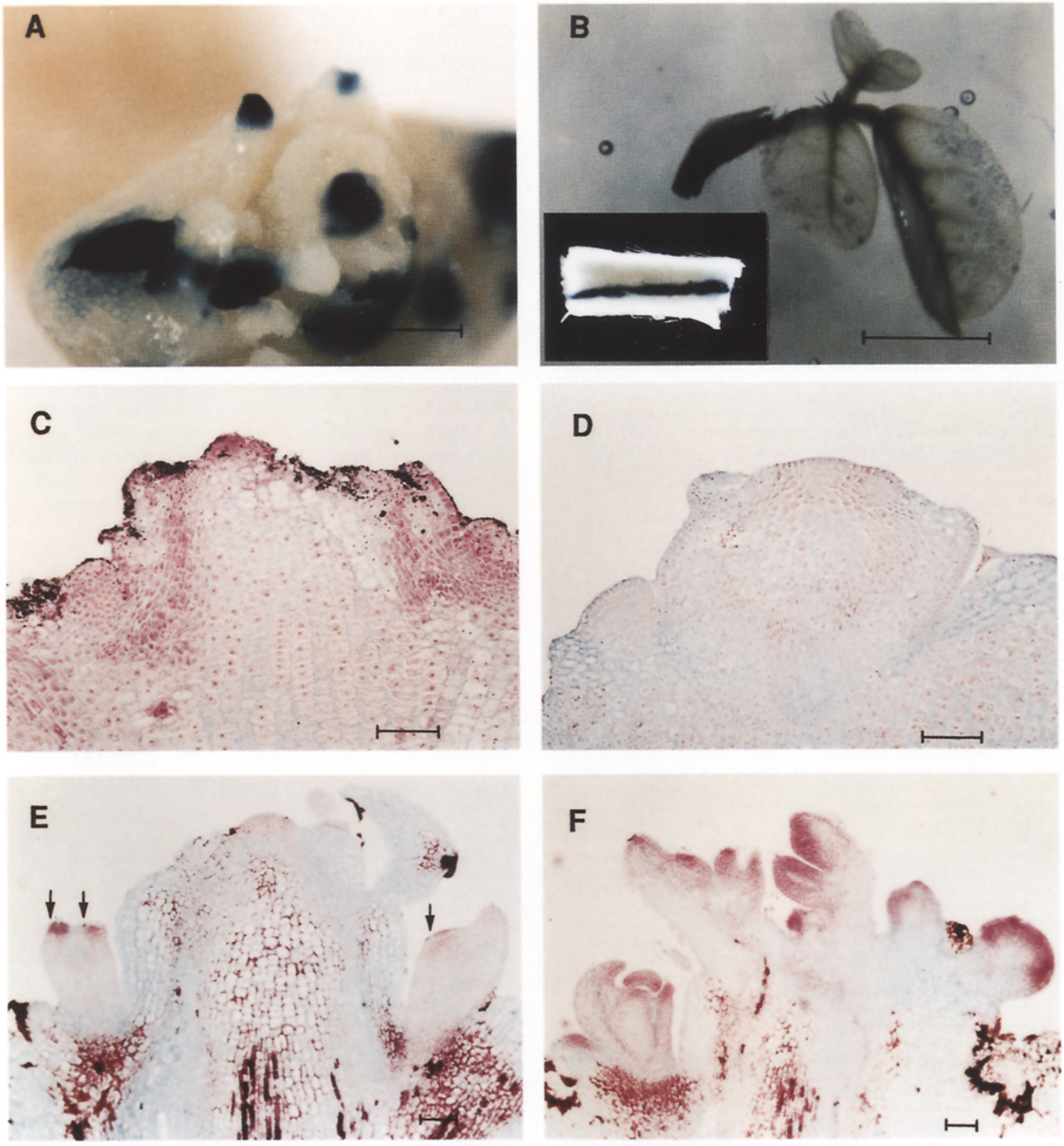


Figure 1. Shoot apice transformation and regeneration. A) Transient GUS expression in isolated shoot 24 hours post bombardment. Scale bar=0.5mm. B) GUS positive sectors in a regenerating shoot from a shoot tip at 8 weeks post bombardment. Insert: GUS positive sector in stem of regenerated shoot at time of rooting. Scale bar=1mm. C) Median longitudinal section of bombarded shoot tip with tungsten particles lodged primarily in surface cells. D-F) Stages in shoot organogenesis. D) Median longitudinal section of a shoot tip post isolation showing primary and axillary meristems. E) Median longitudinal section of an isolated shoot tip 14 days after culture. Initiation of *de novo* primordia in axillary meristems (arrows). F) Median longitudinal section of an isolated shoot tip 21 days after culture. *De novo* shoot primordia in primary meristem and axillary meristems. Figures 1C-F, scale bar=100μm.

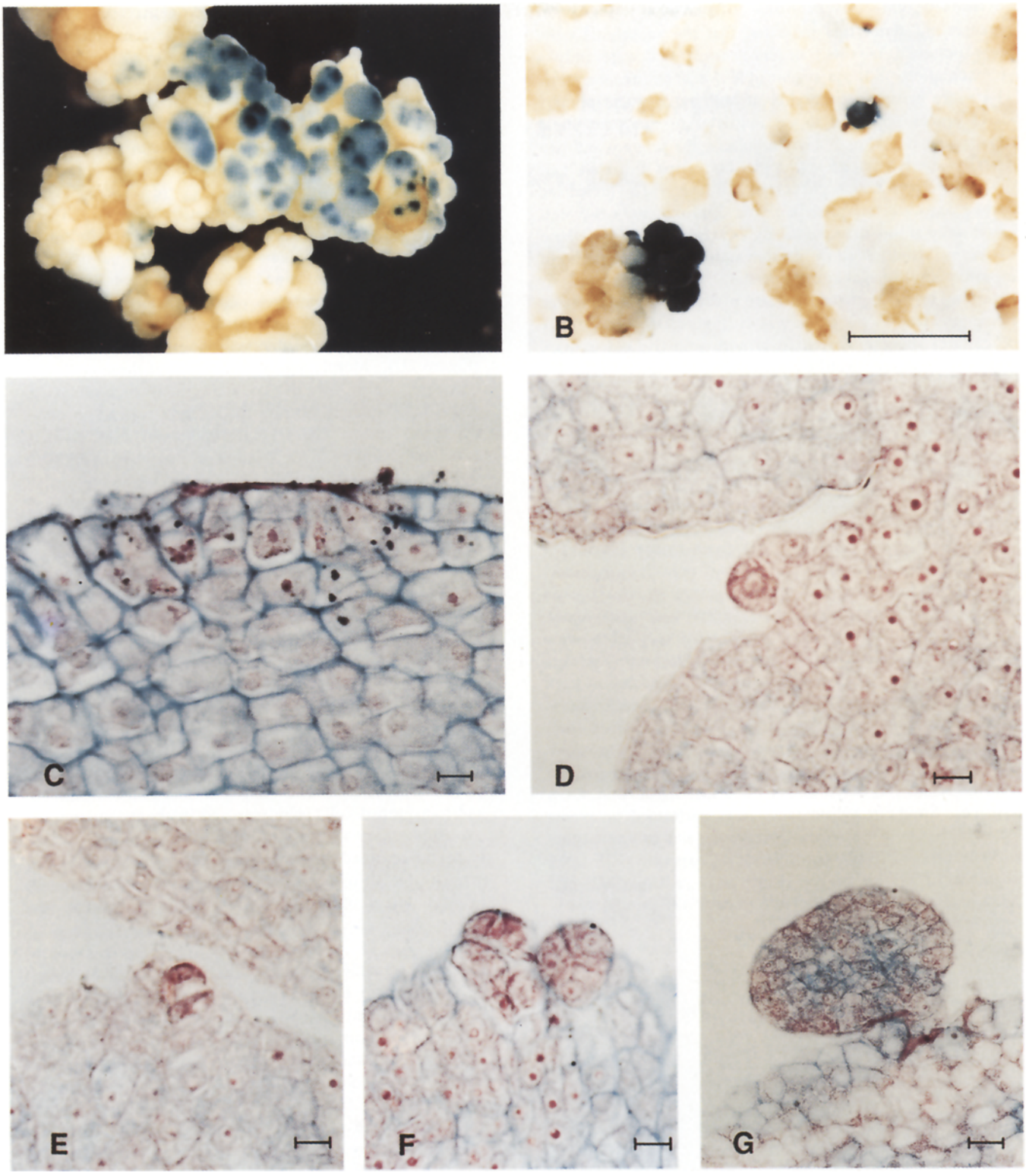
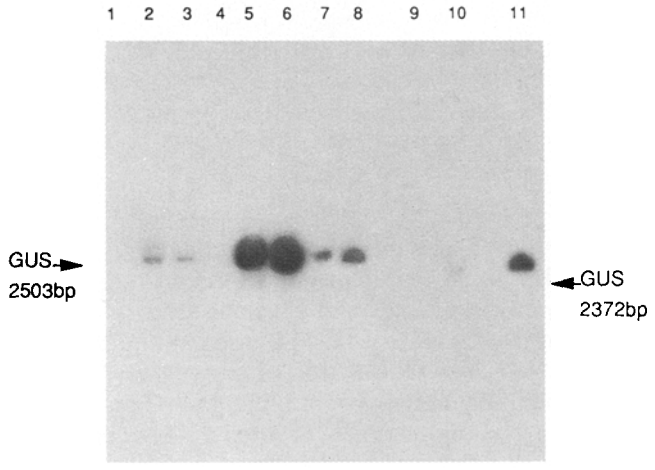


Figure 2. Embryogenic suspension transformation and regeneration. A) Transient GUS expression in suspension culture 24 hours post bombardment. Scale bar=1mm. B) Stable GUS expression in embryos after 8 weeks of selection with 50mg/l G418. Scale bar=1mm. C) Transverse section of a bombarded embryogenic clump with tungsten particles lodged primarily in first two cell layers. D-F) Stages in somatic embryo development. D) Morphologically distinct single epidermal cell prior to initiation of somatic embryo. E) Epidermal cell initiating somatic embryogenesis by dividing anticlinally instead of in typical periclinal fashion. F) Early stage in somatic embryo development. G) Globular somatic embryo. Figures 2E-F, scale bar=10μm; Figure G, scale bar=20μm.

Histological observations of several stages of regenerating shoot tips indicated that the superficial cells were involved in shoot organogenesis, but were only a subpopulation of those responsible for shoot regeneration. The entire meristematic region of both axillary and primary shoot apices appeared to be involved in *de novo* shoot organogenesis. After 7 days in culture, the primary and two axillary meristems of cultured shoot tips initiated cell divisions in both tunica and corpus cell layers. The tunica and corpus together comprise a tissue approximately 4-5 cell layers deep at this time point. By 14 days after culture, these divisions resulted in a meristematic tissue capable of initiating multiple shoot primordia. Shoot organogenesis appeared to be initiated first from the shoot organogenic tissue produced by the axillary buds (Figure 1E). By 21 days of culture, shoot initiation from both primary and axillary meristems was very prolific (Figure 1F).

Embryogenic suspension transformation

Embryogenic suspensions, twenty four hours after bombardment, contained approximately 1,500 spots of transient GUS activity. DNA bombardment of embryogenic cultures followed by selection for 8 weeks with 50mg/l G418 produced a suspension containing brown, non-growing embryos and green embryos. All green embryos analyzed were GUS positive (Figure 2B). Fifty six percent of the flasks of bombarded embryogenic suspensions had produced green embryos or embryo clusters by 10 weeks post bombardment. An average of 4 independent GUS positive clumps were produced per flask. The G418-selected green embryos were proliferated and GUS analysis of a subsample of each proliferated line showed that all the proliferated embryos in all the lines remained GUS positive. Plants produced from GUS positive embryogenic lines were also GUS positive based on histochemical analysis of their leaves. The plants were not chimeric, as all embryo and leaf tissue were GUS positive. Southern hybridization was performed on plants from five separate transformation events, and confirmed that all five of the tested plants contained the expected GUS DNA fragments (2,503 bp for Eco R1 digested pMON 13671 and 2,372 bp for Bam H1 digested pMON10026) (Figure 3).



Histology of bombarded embryogenic suspensions indicated that particles were delivered to cells responsible for the initiation of somatic embryos. An examination of median longitudinal sections of bombarded globular somatic embryos revealed that 89% of all particles were found in the first two cell layers (Table 2; Figure 2C), and approximately 57% of these particles were in the epidermal layer. Histological analysis indicated that the epidermal cells of globular somatic embryos were responsible for initiation of secondary somatic embryogenesis. Embryogenic suspensions at the time of bombardment (4 weeks post subculture) showed all stages of embryogenic development and contained a mixture of various sized globular somatic embryos and occasional elongated somatic embryos. New somatic embryos were initiated from morphologically distinct, single epidermal cells (Figure 2D). Morphological distinction of these cells was based on their slightly increased size, more rounded shape, and greater staining affinity for safranin relative to other epidermal cells. These single epidermal cells divided anticlinally (Figure 2E) instead of in the typical periclinal fashion, and subsequent divisions produced new globular somatic embryos (Figure 2F, 2G). The epidermal surface of a large globular embryo was observed to initiate many new somatic embryos.

DISCUSSION

Transformation of soybean shoot tips using the Biolistics® particle gun produced chimeric, transgenic shoot primordia and plantlets. No non-chimeric, transgenic plantlets were produced. None of the chimeric plants tested at the five-trifoliate leaf stage produced GUS positive leaves. The GUS positive sectors which were detected in regenerated shoot primordia and plantlets were primarily superficial, indicating that both non-transformed and transformed cells were involved in shoot initiation. It is possible that only the epidermal and subepidermal cells were transformed in bombarded shoot tips since the majority (78%) of particles were found in the outermost two cell layers of the apex. This corresponds with our

Figure 3 Southern hybridization analysis of soybean plants produced from GUS positive embryogenic lines. DNA was isolated from young leaves, digested with either Eco R1, if transformed with pMON 13671, or with Bam HI, if transformed with pMON 10026, and hybridized with labelled GUS coding region. Lane 1, nontransformed soybean DNA; lane 2-3, soybean DNA from plants transformed with pMON 13671; lane 4, blank; lane 5-6 soybean DNA from plants transformed with pMON 13671; lane 7-8, pMON 13671 digested with Eco RI; lane 9, nontransformed soybean DNA; lane 10, soybean DNA from a plant transformed with pMON 10026; lane 11, pMON 10026 digested with Bam HI. The arrow on the left indicates the position of a 2,503 bp fragment generated from cutting pMON 13671 that contains the GUS coding sequence. The arrow on the right indicates the position of a 2,372 bp fragment generated from cutting pMON 10026 that contains the GUS coding sequence.

observation that GUS positive sectors in chimeric plants were located primarily in the epidermis and outer cortex. Our histological studies indicated that shoot organogenesis in soybean apices involved multiple cells. Recently it was shown that shoots produced from regenerated soybean shoot tips were derived from 3 cell layers in the original apical meristem (Christou, 1990a, Christou and McCabe, 1992). Shoot apical meristems consist primarily of 3 superimposed cellular layers (L1, L2, and L3, where L1 is the outermost layer; Sussex, 1989). According to this description of apical organization, all three layers are involved in the production of a whole shoot, with L1 being responsible for the epidermis, and L2 and L3 being responsible for the production of more internal tissues. In this investigation, it appeared that the transformed cells existed primarily in the L1 and L2 layers, but not in the L3 layer, of the apical meristems of regenerated shoots. This indicates that the regenerated shoots are likely to be of multicellular origin, and that several layers of cells were involved in shoot organogenesis.

Unlike the Biolistic® shoot-tip bombardments, bombardments of embryogenic suspensions readily produced non-chimeric, GUS-positive regenerated plants. This was due to the targeting of nearly 60% of the DNA coated particles to epidermal cells. Others have reported that GUS transient expression and particles were concentrated in epidermal cells of a target tissue after bombardment (Taylor and Vasil 1991). Our histological studies indicated that single epidermal cells were responsible for initiating secondary somatic embryos. Transformed single epidermal cells could initiate secondary somatic embryogenesis which resulted in either embryo proliferation or embryo conversion into non-chimeric transformed plants. Single cell origin of somatic embryos from epidermal cells is common in several species (Maataoui et al. 1990, Maheswaran and Williams 1985, Dos Santos et al. 1983).

Work with the two different regeneration systems and the Biolistic® gun has shown that the successful production of transformed plants is dependent on which cells regenerate and the appropriate targeting of DNA-coated particles. The Biolistics® PDS 1000 gun, in our hands, did not frequently accelerate particles which penetrated more than 2 cell layers of a soybean shoot tip. Cells deeper than the first two cell layers appeared to be necessary for the development of regenerated plants. On the other hand, the shallow penetration resulting from the PDS 1000 bombardment ensured efficient targeting of cells responsible for somatic embryogenesis in soybean embryogenic suspension cultures. However, chimeric, as well as non-chimeric, transgenic soybeans have been produced from regenerated shoot tips using an "electric discharge" particle acceleration device (Christou, 1990b). This indicates that the "electric discharge" accelerator may have different performance characteristics relative to the Biolistic® PDS 1000 device. Matching regeneration potential with particle gun capabilities will be a key to developing transformation protocols which use particle-mediated transformation technology.

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