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Sonication-assisted *Agrobacterium*-mediated transformation of soybean [*Glycine max* (L.) Merrill] embryogenic suspension culture tissue

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Abstract Successful transformation of plant tissue using *Agrobacterium* relies on several factors including bacterial infection, host recognition, and transformation competency of the target tissue. Although soybean [*Glycine max* (L.) Merrill] embryogenic suspension cultures have been transformed via particle bombardment, *Agrobacterium*-mediated transformation of this tissue has not been demonstrated. We report here transformation of embryogenic suspension cultures of soybean using “Sonication-Assisted *Agrobacterium*-mediated Transformation” (SAAT). For SAAT of suspension culture tissue, 10–20 embryogenic clumps (2–4 mm in diameter) were inoculated with 1 ml of diluted (OD_{600nm} 0.1–0.5) log phase *Agrobacterium* and sonicated for 0–300 s. After 2 days of co-culture in a maintenance medium containing 100 μM acetosyringone, the medium was removed and replaced with fresh maintenance medium containing 400 mg/l Timentin[®]. Two weeks after SAAT, the tissue was placed in maintenance medium containing 20 mg/l hygromycin and 400 mg/l Timentin[®], and the medium was replenished every week thereafter. Transgenic clones were observed and isolated 6–8 weeks following SAAT. When SAAT was not used, hygromycin-resistant clones were not obtained. Southern hybridization analyses of transformed embryogenic tissue confirmed T-DNA integration.

Key words *Agrobacterium* · Embryogenic suspension · SAAT · Sonication · Soybean · Transformation

Introduction

Transgenic soybean [*Glycine max* (L.) Merrill] plants have been obtained using both *Agrobacterium* (Hinchee et al.

1988; Townsend and Thomas 1993) and particle bombardment (McCabe et al. 1988; Finer and McMullen 1991). DNA integration patterns in transformed plant tissue obtained via particle bombardment tend to be highly variable, and multiple or fragmented copies of the introduced DNAs are common, especially when older cultures are targeted (Hadi et al. 1996). The use of *Agrobacterium*, on the other hand, can result in lower copy numbers of introduced DNA with defined borders (Tinland and Hohn 1995).

Although some success has been reported with *Agrobacterium*-mediated transformation of soybean (Hinchee et al. 1988; Chee et al. 1989; Parrott et al. 1989; Townsend and Thomas 1993; Di et al. 1996), this procedure has been limited by the host- and tissue-specificity associated with the use of this biological vector. *Agrobacterium* clearly infects soybean (Pederson et al. 1983), but some cultivars are more responsive than others (Owens and Cress 1985; Hinchee et al. 1988; Parrott et al. 1989; McKenzie and Cress 1992; Bailey et al. 1994; Droste et al. 1994; Mauro et al. 1995). In addition, the efficiencies for *Agrobacterium*-mediated transformation of regenerable tissue such as the shoot meristem have been very low. Transformation inefficiencies can be partially overcome by the addition of acetosyringone to induce expression of the *vir* genes (Stachel et al. 1985; Delzer et al. 1990) or by the use of highly virulent *Agrobacterium* strains which constitutively express the *vir* genes (Hansen et al. 1994).

Agrobacterium-mediated transformation of soybean embryogenic suspension cultures has not been reported, although this tissue has certain advantages over other target tissues for transformation. These advantages include transformation of pre-determined embryogenic tissue (transformed clones will be embryogenic) and the ability to apply continual selection to eliminate chimeric clones (Finer and McMullen 1991).

Recently, a new and potentially more efficient method was developed for delivery of *Agrobacterium* to plant target tissues. This new technique, called “Sonication-Assisted *Agrobacterium*-mediated Transformation” (SAAT), involves subjecting the plant tissue to brief periods of ultrasound in the presence of *Agrobacterium* (Trick and Finer

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1997). Here, we demonstrate that SAAT can be efficiently used to transform soybean embryogenic suspension culture tissue.

Materials and methods

Plant tissue preparation

Embryogenic suspension culture tissue of soybean [*Glycine max* (L.) Merrill cv 'Chapman'] was initiated and maintained as described previously (Finer and Nagasawa 1988).

Agrobacterium and plasmid DNA

Agrobacterium tumefaciens EHA105 (Hood et al. 1993) containing the binary plasmid Vec035 was used. Vec035 (Trick and Finer 1997) was provided by Tim Hall and David Frisch (Texas A&M University). Within the T-DNA borders, Vec035 contains the coding region for hygromycin resistance under regulatory control of the NOS promoter and an intron-containing β -glucuronidase (GUS) gene driven by the CaMV35S promoter. The intron was derived from the second intron of the ST-LS1 gene from potato (Vancanneyt et al. 1990) and was used to prevent expression of the GUS gene in *Agrobacterium*.

Log-phase *Agrobacterium* cells were grown overnight in modified LB medium containing 5 g/l NaCl, 5 g/l sucrose, and 50 μ g/ml kanamycin. Bacteria were centrifuged at 1500 g for 10 min, resuspended in FN medium (Finer and Nagasawa 1988), re-centrifuged as above, and finally resuspended in FN medium at OD_{600nm} between 0.1 and 0.5.

Transient expression studies

Ten to twenty clumps of highly embryogenic soybean suspension culture tissue (2–4 mm diameter), maintained in FN medium, were transferred to sterile 13×100-mm borosilicate glass tubes. One milliliter of *Agrobacterium* suspension, diluted to OD_{600nm} 0.5 in FN medium, was added to each sample. The glass tubes were individually placed in the center of a bath sonicator (Model PC5, L&R Manufacturing Co, Kearny, N. J.) and sonicated for 0–300 s. SAAT durations were controlled with an electronic photographic timer. After SAAT treatment, the embryogenic suspension tissue was blotted on filter paper to remove excess bacteria and transferred to 30 ml FN liquid medium containing 100 μ M acetosyringone in a baffled 125-ml DeLong flask. After 1–4 days of co-culture, the medium was removed from the flasks and replaced with fresh FN medium containing 400 mg/l Timentin[®]. With co-culture periods of more than 2 days, the co-culture medium was replenished daily to reduce bacterial overgrowth. Transient GUS expression was assayed 2 days after the end of the co-culture period.

The effects of the sonication treatment on tissue growth rates and recovery were assessed by sonicating the tissues for 0, 10 or 60 s, and fresh weights of embryogenic tissue were taken after 1, 2, and 3 weeks. Sonicated tissues were grown in FN medium which was replenished weekly.

Stable transformation studies

For stable transformation, suspension culture tissues were SAAT-treated for 30 or 60 s. After a 2-day co-culture in FN medium containing 100 μ M acetosyringone, suspension culture tissues were subcultured to FN medium containing 400 mg/l Timentin[®] every week for 2 weeks. Tissues were then transferred to FN medium containing 400 mg/l Timentin[®] and 20 mg/l hygromycin. The culture medium was replaced with fresh hygromycin- and Timentin[®]-containing FN medium every week thereafter. Transgenic clones were ob-

served and isolated 6–8 weeks following SAAT treatment. Stably transformed soybean plants were regenerated from suspension culture material according to Finer and McMullen (1991).

β -glucuronidase assays

β -glucuronidase (GUS) expression was assayed by placing tissues in a GUS assay mix (Jefferson 1987) which consists of 10 mM Na₂EDTA-H₂O, 0.1% Triton X-100, 0.1 M NaH₂PO₄, 0.5 M K₃Fe(CN)₆, and 250 μ g/ml 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-GLU), and incubating overnight with gentle agitation at 37°C. The cells and tissues containing and expressing the introduced DNA (the GUS gene) were then scored by counting the number of blue foci.

Microscopy

For microscopic analyses, samples of soybean embryogenic suspension tissue were fixed in 3% glutaraldehyde, 2% paraformaldehyde, 1.5% acrolein, and 0.1 M phosphate buffer, pH 7.0, for a minimum of 2 h and then dehydrated in a graded ethanol series. For scanning electron microscopy, tissues were critical point-dried, sputter-coated, and viewed with an ISI40 Scanning Electron Microscope. For light microscopy, specimens were fixed and dehydrated as above, transferred to 100% acetone, and finally embedded in Spurr's resin (Spurr 1969). Sections for light microscopy were sectioned at 0.75- μ m thickness and stained with 0.1% toluidine blue O (Finer and McMullen 1991).

Molecular analyses

DNA from transformed embryogenic soybean clones was isolated using the CTAB procedure (Saghai-Marooof et al. 1984), digested with *Hind*III or *Eco*RI, and then electrophoresed through a 0.8% agarose gel. The gel was denatured and neutralized and the DNA was transferred to nylon membranes (Gene Screen Plus, NEN Life Science, Boston, Mass.) and hybridized as described previously (Finer and McMullen 1991). When multiple probes were used, the filter was stripped with 0.4 M NaOH for 30 min and neutralized with 0.1×SSC, 0.1% SDS, 0.6 M Tris-HCl pH 7.0. The *Bam*HI/*Sac*I fragment containing the GUS coding region of pUCGUS (Finer and McMullen 1990) and the *Bam*HI fragment containing the hygromycin phosphotransferase (HPT) coding region of pHygr (Finer and McMullen 1992) were random-prime labeled and used for hybridization. Membranes were placed on Kodak XAR-5 film with intensifying screens at –70°C for 2 days to visualize the hybridization patterns.

Results and discussion

The use of *Agrobacterium* without sonication resulted in very low transient expression of GUS in suspension tissue (Table 1). However, when *Agrobacterium* was combined with sonication, transient transformation was tremendously enhanced (Fig. 1, Table 1). Longer sonication treatments resulted in an increase in transient expression up to approximately 100 foci per clump of tissue (Table 1). This level of transient expression is roughly equivalent to levels obtained using particle bombardment of the same target tissue (Vain et al. 1993a). Using SAAT with immature cotyledons of soybean, Trick and Finer (1997) also observed an increase in transient expression with longer duration sonication.

Table 1 Effect of sonication duration on transient GUS expression in soybean somatic embryogenic cultures

Sonication duration (s)	GUS foci per clump (\pm SE) ^a
0	0.18 \pm 0.11
1	23.6 \pm 18.4
3	62.3 \pm 13.6
10	77.9 \pm 4.3
30	102.2 \pm 16.2
100	86.3 \pm 9.5
300	85.1 \pm 30.1

^a Values represent the mean (\pm standard error) of three replicate samples, each replicate consisting of 20 clumps of embryogenic suspension cultures

Table 2 Effect of sonication on the growth of soybean suspension cultures. Fresh weights of samples were determined weekly

Sonication duration (s)	Weekly determination of sample fresh weight (mg \pm SE) ^a			
	0	1	2	3
0	100 \pm 0	320 \pm 13	760 \pm 22	1602 \pm 48
10	100 \pm 0	230 \pm 11	597 \pm 45	1415 \pm 80
60	100 \pm 0	233 \pm 13	467 \pm 39	1180 \pm 58

^a Three replicates were performed for each treatment (\pm standard error)

Table 3 Effect of co-culture time on GUS expression. Tissues were SAAT-treated for 60 s with EHA 105 (Vec035) OD_{600nm} 0.24. GUS assays were performed 2 days after the end of the co-culture period

Co-culture period (days)	GUS foci per clump (\pm SE) ^a
1	0.8 \pm 0.2
2	202 \pm 59.2
3	150 \pm 51.7
4	194 \pm 46.4

^a Values represent the mean (\pm standard error) of three replicate samples, each replicate consisting of 10 clumps (2–4 mm diameter) of embryogenic suspension cultures

Unlike particle bombardment where DNA-coated particles are delivered to only one side of the target tissue and penetration is limited, use of SAAT results in efficient transformation of the total tissue surface. In addition, dissection of the clumps of embryogenic tissue revealed GUS foci buried deep within the tissue, indicating transformation of subsurface tissue. Although the soybean somatic embryos used in this study are surface-derived (Finer 1988; Finer and McMullen 1991), the transformation of subsurface tissue is critical when attempting to transform other tissues, such as meristems, that are buried several cell layers deep.

To determine whether sonication impacts the growth of embryogenic soybean suspension culture tissue, we performed a growth kinetic study. Fresh weights of sonicated embryogenic tissue that were not treated with *Agrobacterium* revealed that sonication appeared to be somewhat detrimental to the tissue directly following treatment (Table 2). Non-sonicated samples had a 3.2-fold increase

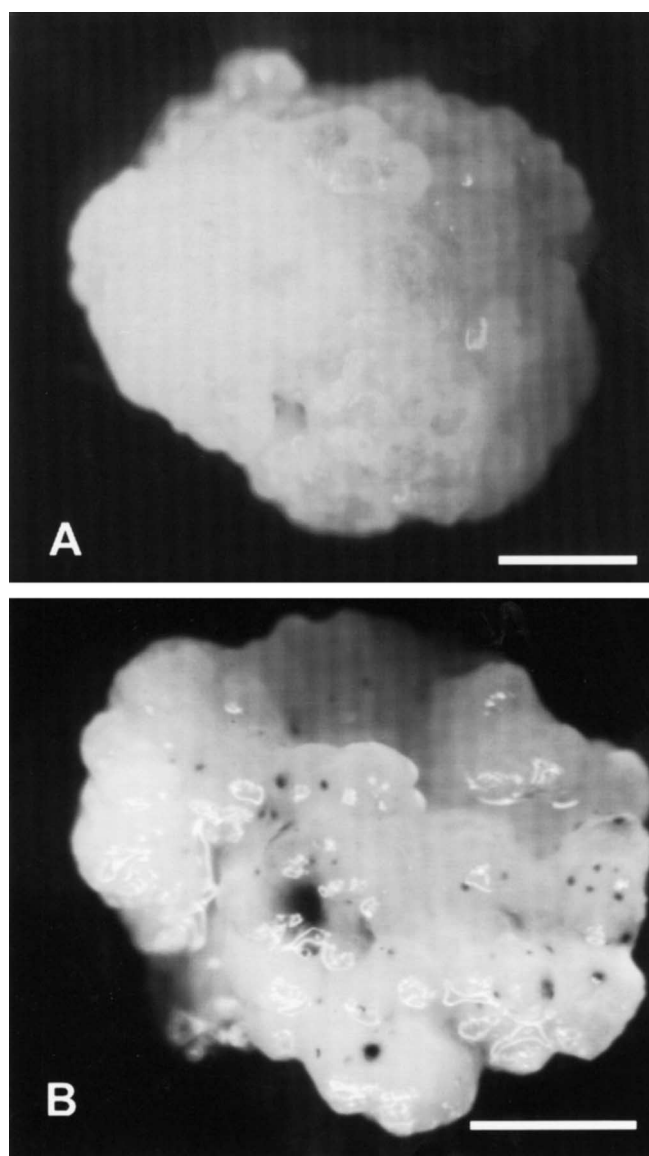


Fig. 1A, B Transient GUS expression in embryogenic suspension tissue with and without SAAT. Tissues were cleared with 70% ethanol prior to photography. **A** Soybean with *Agrobacterium* alone (non-SAAT), **B** 30-s SAAT. Bar: 1 mm

in biomass after 1 week in culture, whereas the samples sonicated for 10 s and 60 s had a biomass increase of only 2.3- and 2.1-fold, respectively. However, after 1–2 weeks, all tissues displayed approximately the same growth rate. These observations indicated that, although the treatment may have been originally damaging, the sonicated tissue survived and eventually proliferated at rates similar to that of the control.

Evaluation of co-culture durations from 1–4 days revealed that a 2-day co-culture was optimal for transient transformation of soybean embryogenic cultures using SAAT (Table 3). A 1-day co-culture period was apparently not sufficient time for the transformation process to occur using the experimental parameters reported here. A 2-day

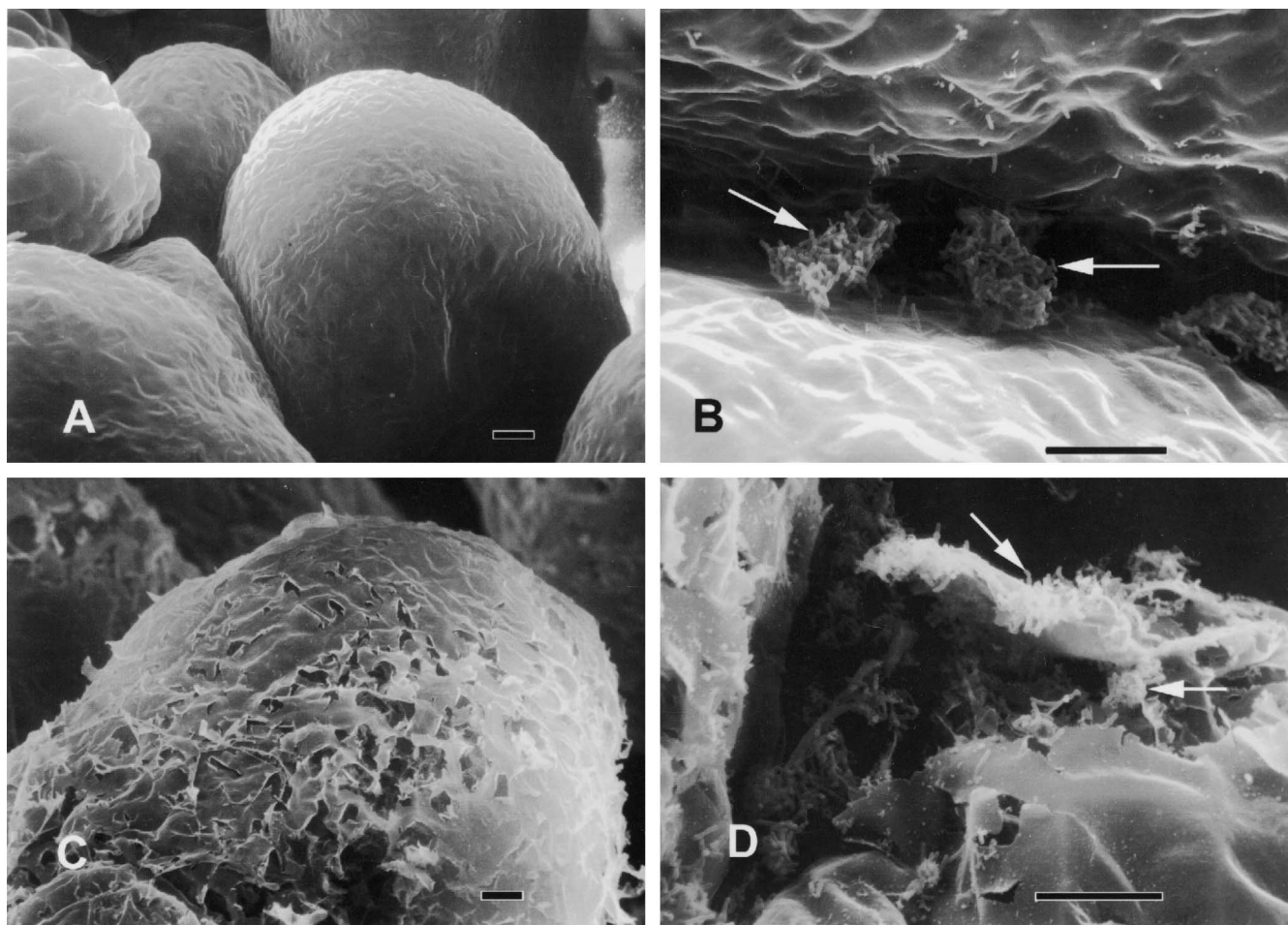


Fig. 2A–D Scanning electron micrographs of non-sonicated embryonic suspension tissue (**A** and **B**) and 30-s SAAT-treated samples with microwounds caused by sonication visible on the surface (**C** and **D**). Bacteria (arrows **B** and **D**) are clearly evident after 2 days of co-culture and can also be seen colonizing the surface and within the microwounds of the SAAT-treated tissue (**D**). Bar: 10 μ m

co-culture is commonly used for transformation of a number of different plants (Horsch et al. 1985; Villemont et al. 1997; Sangwan et al. 1992). Longer co-culture times also resulted in high levels of transient expression (Table 3), but possible negative effects due to the overgrowth of the bacteria should be avoided. A 2-day co-culture duration was therefore selected as optimum for transformation of suspension cultures.

SAAT of embryonic soybean tissues, performed at various times following subculture, showed an effect of tissue age on receptivity of the target tissue to *Agrobacterium*-mediated transformation (Table 4). Tissues that were SAAT-treated 11–14 days following subculture showed the highest levels of transient expression. This time corresponds to the late log to linear phase of growth (data not shown). Pre-treatments and selection of rapidly growing target tissue have been used for optimizing of both *Agrobacterium*- (Sangwan et al. 1992; Stachel 1985; McHughen et al. 1989) and particle bombardment-mediated (Vain et al. 1993 a,b) transformations.

Table 4 Effect of age of soybean embryonic suspension tissue on transient GUS expression. Tissues were SAAT-treated for 60 s with EHA 105 (Vec035) OD_{600nm} 0.2, co-cultured for 2 days in the presence of acetosyringone, replenished with FN medium containing Timentin[®] for 2 days, and then assayed for GUS activity

Days after subculture	GUS foci per clump (\pm SE) ^a
4	9.2 \pm 6.0
7	32.6 \pm 4.6
11	81.3 \pm 20.9
14	65.0 \pm 11.5
18	56.9 \pm 9.3
21	36.7 \pm 10.3
25	6.2 \pm 4.5
28	28.8 \pm 5.3

^a Values represent the mean (\pm standard error) of three replicate samples, each replicate consisting of 10 clumps (2–4 mm diameter) of embryonic suspension cultures

The basis for the increase in transient GUS expression from SAAT is believed to be caused by cavitation-induced microwounds which facilitate the infection by *Agrobacterium* of the target plant tissue (Trick and Finer 1997). Scanning electron microscopy revealed extensive microwounding of SAAT-treated embryonic suspension tissue (Fig. 2C,D) similar to that reported earlier for SAAT-treated immature cotyledons (Trick and Finer 1997).

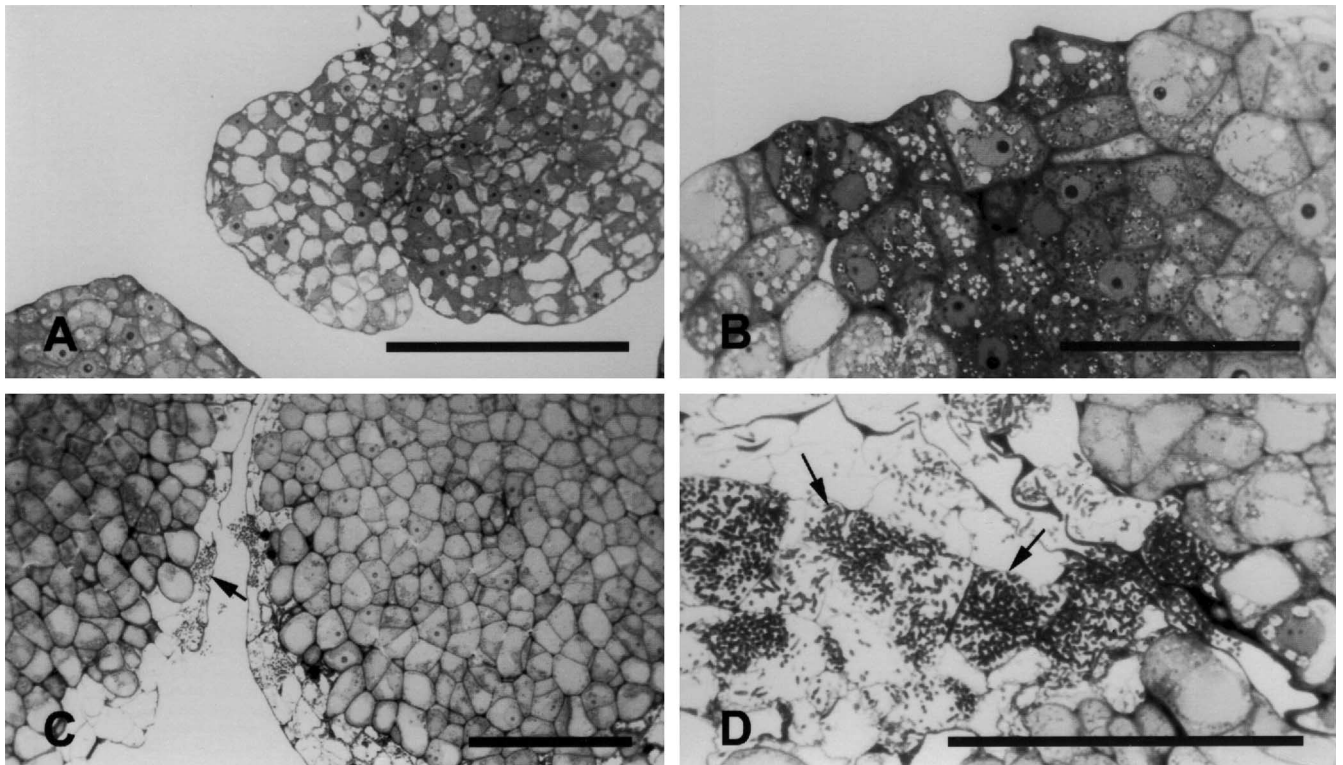


Fig. 3A–D Cross section of control (**A** and **B**) and SAAT-treated (**C** and **D**) embryogenic suspension tissue. Bacteria are indicated by the *arrows*. The apical cells of the control (non-SAAT-treated) tissues (**A** and **B**) are structurally intact. In SAAT-treated samples, bacteria were clearly evident in apical cells (**C**) and also deeper within the tissue (**D**), indicating the wounding of both surface and subsurface tissue. **A** and **C**, bar: 100 μ m; **B** and **D**, bar: 50 μ m

Wounding was evident throughout all SAAT-treated tissue (Fig. 2C,D), whereas the tissue surface was intact in the control specimens (*Agrobacterium* treatment alone) (Fig. 2A,B). The size of SAAT-induced microwounds ranged from less than 1 μ m to well over 1 mm, which is large enough to permit *Agrobacterium* to infect the embryogenic tissue. As the duration of sonication was increased, microwounds became larger and more numerous (data not shown). Bacterial colonization of control samples was restricted to areas between the lobes of the embryogenic suspension tissue (Fig. 2B). Presumably, bacteria in these regions were protected from the constant agitation of the medium and were not washed away during the co-cultivation period. In SAAT-treated samples, bacterial colonization was observed almost everywhere the surface integrity was compromised. In addition to the surface colonization, bacteria were also observed within the microwounds (Fig. 2D).

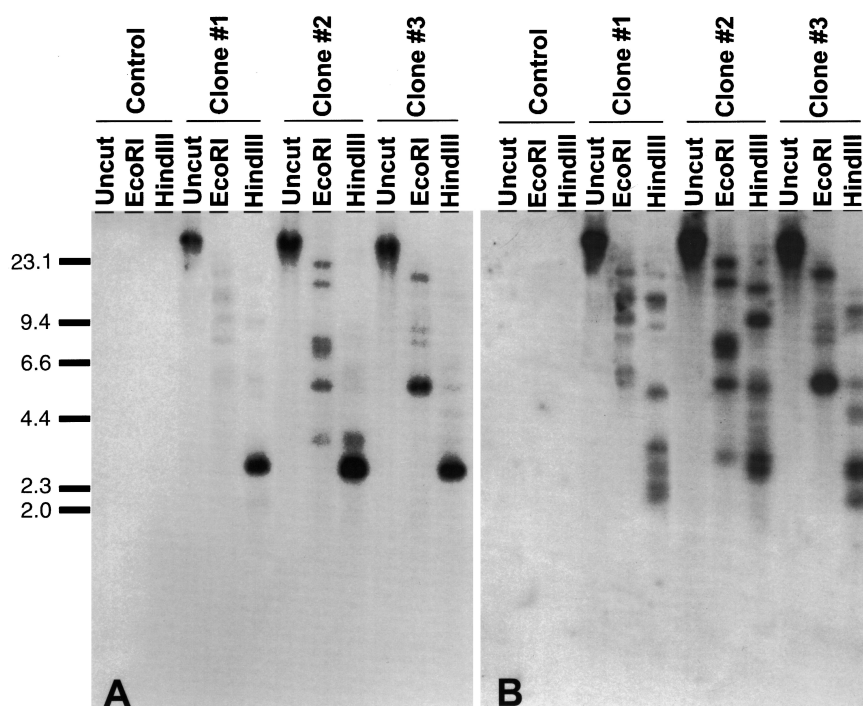
To assess the extent of *Agrobacterium* penetration of both control and SAAT-treated embryogenic suspension culture tissue, tissues were examined histologically. Cross sections of control (non-SAAT-treated) samples indicated that plant cell walls were intact and that *Agrobacterium* did not invade the tissue (Fig. 3A,B). Although bacterial col-

onization was evident between the lobes of the control samples, the internal plant tissue was intact and uninfected. However, in SAAT-treated samples, sonication caused surface and subsurface cellular disruptions within the embryogenic tissue (Fig. 3C,D), and bacterial colonization of the plant cells was observed both at and below the surface (Fig. 3C,D; arrows). Some cells were heavily colonized by the *Agrobacterium*, whereas neighboring cells were uninfected and appeared to have intact cytoplasm. This was also observed in SAAT-treated soybean immature cotyledons where intact plant cells were adjacent to infected, heavily colonized cells (Trick and Finer 1997). *Agrobacterium* binding and subsequent T-strand transfer could occur from the cytoplasmic side of these colonized cells or from various wound points in the cell wall within the tissue.

Stable transformation of soybean suspension culture tissue was obtained only with SAAT-treated cultures. A total of 121 clones were isolated from 12 separate experiments. All of the clones were selected for hygromycin resistance and tested positive for GUS expression, although there was variability in the intensity of GUS expression. No clones were recovered from cultures treated with *Agrobacterium* alone.

Southern hybridization analyses confirmed transformation of embryogenic soybean tissue (Fig. 4). Hybridization of undigested plant genomic DNAs with fragments from the coding region of either the GUS gene (Fig. 4A) or the HPT gene (Fig. 4B) yielded hybridization signals above 23 kb, indicating integration of the T-DNA into high-molecular-weight plant genomic DNA. These large bands were not the result of bacterial contamination as the binary plasmid Vec035 is only 13.6 kb, and this particular size of

Fig. 4A,B Southern hybridization analysis of DNA from stably transformed soybean. Genomic DNA was digested with either *Hind*III, which recognizes two sites within Vec035, one inside the left border and the other between the GUS and hygromycin resistance genes, or *Eco*RI, which recognizes one site just within the left border. The membrane was hybridized with **A** the 1.8-kb coding region for the GUS gene or **B** the 1-kb coding region for the hygromycin resistance gene



hybridization fragment was not observed. The hybridization signal resulting from DNA digested with *Eco*RI or *Hind*III also indicated integration of T-DNA. *Eco*RI cleaves one site just inside the left T-DNA border, yielding predicted hybridization signals greater than 4.2 kb (T-DNA is 4.2 kb). The number of hybridization signals in the *Eco*RI-digested lanes indicated that each clone had four to six copies of integrated T-DNA. The lack of a predominant unit length hybridization signal suggests that concatination of the T-DNA did not occur. Based on the restriction map of Vec035, digestion of integrated T-DNA with *Hind*III should result in at least two fragments, a 2.7-kb fragment containing the intact GUS gene and a fragment greater than 1.5 kb containing the HPT gene, the right border of the T-DNA, and various lengths of plant DNA depending on the integration site. Transgenic genomic DNA cut with *Hind*III and probed with the GUS gene resulted in the predicted fragment of approximately 2.7 kb (Fig. 4A). In addition, results from the *Hind*III-digested DNA probed with the HPT gene (Fig. 4B) confirmed the presence of four to six copies of the T-DNA as observed earlier with *Eco*RI-digested DNAs (Fig. 4A).

Transgenic soybean plants were regenerated, successfully transplanted to soil, and grown in the greenhouse. Unfortunately, both transformed and non-transformed plants (from the same starting culture) were fully sterile and progeny was not recovered. Loss of fertility of plants regenerated from long-term embryogenic suspension cultures of soybean has been previously reported (Hadi et al. 1996). The starting culture for this study was a long-term tissue culture line, and therefore sterility of regenerated plants was anticipated. However, the utility of SAAT was clearly demonstrated, and SAAT is currently being further evalu-

ated on newer lines of soybean embryogenic cultures for production of stably transformed fertile plants.

SAAT is an effective method for the delivery of *Agrobacterium* to somatic embryogenic soybean suspension cultures. Microwounds produced by sonication allow *Agrobacterium* to efficiently infect deep within the plant tissue and stably transform soybean embryogenic suspension tissue. Transformation of other target tissues such as meristems, which are difficult to transform by particle bombardment or *Agrobacterium* alone, may also be facilitated by the use of SAAT. With such efficient delivery of *Agrobacterium* into the plant tissue, SAAT should be a valuable tool for the study of *Agrobacterium*/plant interactions such as bacterial infection, cell-wall recognition and binding, and T-DNA transfer.

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