

SAAT: sonication-assisted *Agrobacterium*-mediated transformation

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Plant transformation via *Agrobacterium* can be limited by both host specificity and the inability of *Agrobacterium* to reach the proper cells in the target tissue. Described here is a new and efficient *Agrobacterium*-based transformation technology that overcomes these barriers and enhances DNA transfer in such diverse plant groups as dicots, monocots, and gymnosperms. This new technology, called sonication-assisted *Agrobacterium*-mediated transformation (SAAT), involves subjecting the plant tissue to brief periods of ultrasound in the presence of *Agrobacterium*. Scanning electron and light microscopy reveal that SAAT treatment produces small and uniform fissures and channels throughout the tissue allowing the *Agrobacterium* easy access to internal plant tissues. Unlike other transformation methods, this system has the potential to transform meristematic tissue buried under several cell layers. SAAT increases transient transformation efficiency in several different plant tissues including leaf tissue, immature cotyledons, somatic and zygotic embryos, roots, stems, shoot apices, embryogenic suspension cells and whole seedlings. A 100- to 1400-fold increase in transient β -glucuronidase expression has been demonstrated in various tissues of soybean, Ohio buckeye, cowpea, white spruce, wheat and maize. Stable transformation of both soybean and Ohio buckeye has been obtained using SAAT of embryogenic suspension culture tissues. For soybean, SAAT treatment was necessary to obtain stable transformation with this tissue.

Keywords: *Agrobacterium*; SAAT; sonication; transformation; wounding

Introduction

Over the past few years, there has been a resurgence of interest in the use of *Agrobacterium* to produce transgenic plants. Although plant tissue can be transformed using a variety of different direct naked DNA uptake methods (Finer *et al.*, 1996), the DNA integration patterns from direct DNA uptake methods are often random and largely unpredictable (Hadi *et al.*, 1996). With *Agrobacterium*-mediated transformations, although DNA integration patterns can be strain dependent (Tinland and Hohn, 1995), they are generally much simpler and gene expression may therefore be more consistent.

Monocots and certain dicot tissues are not very receptive to *Agrobacterium*-mediated transformation. Both the bacterium and the target tissue can be manipulated to obtain and enhance transformation with

these plants. Acetosyringone, an inducer of T-DNA transfer (Stachel *et al.*, 1985), enhances the transformation process and is now routinely added in transformation experiments. In addition, modifications to the binary vector in *Agrobacterium* have enabled fairly efficient transformation of rice (Hiei *et al.*, 1994) and maize (Ishida *et al.*, 1996).

The addition of anti-oxidants to the co-culture medium enhances transformation rates by reducing the hypersensitive response (Perl *et al.*, 1996), while different methods of wounding the target plant tissue have also been successfully employed to increase *Agrobacterium* infection. Wounding is an integral step in *Agrobacterium*-mediated transformation as it allows the bacterium to infect the target tissue. In addition, the wounded tissue often produces inducers of the T-DNA transfer process (Stachel *et al.*, 1985). The type or method of wounding ranges from simple wounds made during the normal course of explant preparation (Horsch *et al.*, 1985) to

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particle gun-mediated micro-wounding (Bidney *et al.*, 1992). Tissues can also be wounded using *Agrobacterium*-filled syringes which allows some degree of 'delivery' of the bacterium to the target tissue (Chee *et al.*, 1989).

One approach, not sufficiently explored, is the use of sonication to wound and modify the target tissue to enhance *Agrobacterium* infection. There are a few reports of transformation of plant tissue using sonication with naked DNA (Joersbo and Brunstedt, 1990, 1992; Zhang *et al.*, 1991), but sonication has never been used to effectively enhance *Agrobacterium*-mediated transformation of plant tissue.

For this work, the use of sonication with *Agrobacterium* was evaluated. The effects of sonication on the target tissue and the interaction of the introduced bacterium with the target tissue were examined using both light and scanning electron microscopy.

Materials and methods

Plant tissue preparation

Soybean (*Glycine max* Jack) and cowpea (*Vigna unguiculata*) plants were grown in the greenhouse under 16:8 h photoperiod at 27 °C. Immature soybean pods and leaflets of cowpea were surface-sterilized in a 20% commercial bleach solution containing 0.02% Tween-20, with slight agitation for 20 minutes and then rinsed 3 times with sterile distilled water. Leaf tissue of cowpea was used directly while immature cotyledons from soybean embryos, 4–6 mm in length, were excised and collected for treatment.

Maize (*Zea mays* A632) plants were grown either under standard greenhouse conditions or in the field. The immature embryos from self-pollinated plants were collected 9 to 21 days after anthesis and surface sterilized as above.

Wheat (*Triticum aestivum* Bobwhite) and white spruce (*Picea glauca*) seeds were surface-sterilized as above and plated on 0.5 × OMS medium containing half-strength Murashige and Skoog (MS) salts (Murashige and Skoog, 1962), half-strength B5 vitamins (Gamborg *et al.*, 1968), 1.5% sucrose and 0.2% Gelrite for germination.

Embryogenic suspension culture tissue of soybean (Chapman) was initiated and maintained as described previously (Finer and Nagasawa, 1988). Embryogenic cultures of the Ohio buckeye (*Aesculus glabra*) were initiated from seedling stem tissue cultured on a medium containing MS salts, B5 vitamins, kinetin (2 mg l⁻¹), 2,4-dichlorophenoxyacetic acid (2,4-D) (2 mg l⁻¹), 3% sucrose and solidified with 0.2% Gelrite (pH 5.7). One to two months following induction, embryogenic buckeye tissue was transferred to liquid FN medium (Finer and Nagasawa, 1988) for establishment of suspension cul-

tures. Embryogenic suspension cultures of Ohio buckeye were maintained using a biweekly to monthly subculture regime.

Agrobacterium and plasmid DNA

Agrobacterium tumefaciens EHA105 (Hood *et al.*, 1993) containing either pIG121Hm (Hiei *et al.*, 1994) or Vec035 was used. Vec035 was used with tissue of soybean, buckeye and cowpea while pIG121Hm was used with tissues of maize, wheat and white spruce. The *Agrobacterium* plasmid Vec035 (generously provided by Tim Hall and David Frisch, Texas A&M University) was made by replacing the GUS gene in the HPT version of pBIG (Becker, 1990) with the intron-containing GUS gene from p35SGUSINT (Vancanneyt *et al.*, 1990). Vec035 contains the coding region for hygromycin resistance under regulatory control of the NOS promoter and an intron-containing GUS gene driven by the CaMV35S promoter. pIG121Hm contains both a hygromycin resistance gene and an intron-containing GUS gene, each under regulatory control of CaMV35S promoters.

Log phase *Agrobacterium* cells were grown overnight in modified LB medium containing NaCl (5 g l⁻¹), sucrose (5 g l⁻¹) and kanamycin (50 µg ml⁻¹), centrifuged at 1500 g for 10 min, resuspended in liquid plant culture medium, recentrifuged as above, and finally resuspended in liquid plant culture medium. Different liquid culture media, based on the medium used to culture the plant tissues, were used to resuspend bacterial cells. Bacteria were then diluted to an OD_{600nm} between 0.01 and 0.5 with liquid medium.

Tissue sonication and co-culture

Soybean cotyledons were transferred to 1.5 ml microcentrifuge tubes, containing 0.5 ml *Agrobacterium* suspension in liquid D40 medium (Santarem *et al.*, 1997) which contains MS salts, B5 vitamins, 6% sucrose and 2,4-D (40 mg l⁻¹) (pH 7.0). Cotyledons were suspended by mild agitation and the microcentrifuge tubes were then placed in a Styrofoam float at the centre of a bath sonicator (Model PC5, L&R Manufacturing Co., Kearny, NJ, USA) which was controlled with an electronic photographic timer. Cotyledons were treated for various timed intervals (from 0.2 s to 10 s) using both horizontal and vertical wave generator outputs. Soybean cotyledons were removed from the microcentrifuge tube, placed on a sterile filter paper to blot off excess bacteria, and then transferred flat side (adaxial side) up to semi-solid D40 medium containing 0.2% Gelrite and 100 µM acetosyringone (AS). After two days of co-culture, cotyledons were washed in sterile distilled water, blot-dried on sterile filter paper, and placed (flat side up) onto semi-solid D40 medium containing cefotaxime (350–500 mg l⁻¹). After an additional two days on the medium containing antibiotics, the tissue was analysed for GUS activity.

Cowpea leaf pieces were placed in 50 ml conical polypropylene tubes containing *Agrobacterium*, resuspended in a medium containing MS salts, B5 vitamins, benzyl adenine (BA) (1 mg l^{-1}) and naphthalene acetic acid (NAA) (1 mg l^{-1}), and sonicated similarly to the soybean cotyledons for 0 to 60 s. Leaf pieces were then blotted with filter paper and placed on a medium containing MS salts, B5 vitamins, 3% sucrose, BA (1 mg l^{-1}), NAA (1 mg l^{-1}), $100 \mu\text{M}$ AS and 0.2% Gelrite (pH 5.7) (CM medium) for two days. Cowpea leaf tissues were transferred to CM medium containing cefotaxime (500 mg l^{-1}) for two days, and then assayed for GUS expression.

Immature zygotic embryos of maize were placed in a 1.5 ml microcentrifuge tube containing 1 ml of an *Agrobacterium* suspension diluted with 2T2S medium containing MS salts, B5 vitamins, 2% sucrose, and 2,4-D (2 mg l^{-1}) (pH 5.7) to an $\text{OD}_{600\text{nm}}$ 0.1 to 0.5. Embryos were treated for 0 to 30 s. After a 5 min incubation, the embryos were blot-dried on filter paper and co-cultured scutellum side up on 2T2S medium containing $100 \mu\text{M}$ AS and 0.2% Gelrite, pH 5.7 for three to five days. After co-culture, the embryos were placed on 2T2S medium containing Timentin[®] (400 mg l^{-1}) and 0.2% Gelrite, pH 5.7 for two to seven days before assaying for GUS expression.

Five-day-old wheat seedlings were placed in $150 \times 23 \text{ mm}$ borosilicate tubes with 10 ml of an *Agrobacterium* suspension diluted with liquid $0.5 \times \text{OMS}$ medium and sonicated for 0 or 100 s. Seedlings were co-cultured for three days on $0.5 \times \text{OMS}$ medium containing $100 \mu\text{M}$ AS and then transferred to $0.5 \times \text{OMS}$ medium containing Timentin[®] (400 mg l^{-1}). Wheat seedlings were assayed for transient GUS expression five days after sonication.

Fourteen-day-old white spruce seedlings were placed in $13 \times 100 \text{ mm}$ borosilicate tubes with 2 ml of an *Agrobacterium* suspension, diluted with $0.5 \times \text{OMS}$ medium. Tissues were sonicated for 0 or 50 s, blotted on sterile filter paper and then co-cultured for three days in a medium containing B5 salts and vitamins (Gamborg *et al.*, 1968), 2% sucrose, 10 mg l^{-1} BA (0.1 mg l^{-1}), indole-3-butyric acid (IBA) (0.1 mg l^{-1}), and $100 \mu\text{M}$ AS (pH 5.7). Samples were transferred to the same medium devoid of AS but containing cefotaxime (500 mg l^{-1}) for four days and then analysed for transient GUS expression.

Ten clumps of highly embryogenic soybean suspension culture tissue (2–4 mm in diameter), grown in FN medium, were transferred to sterile $13 \times 100 \text{ mm}$ borosilicate glass tubes. *Agrobacterium* suspension (1 ml) diluted to $\text{OD}_{600\text{nm}}$ 0.5 in FN medium, was added to the clumps and the tissue was SAAT-treated for 0 to 60 s. After SAAT treatment, the tissue was blotted on filter paper and transferred to 30 ml FN liquid medium in a baffled 125 ml DeLong flask containing $100 \mu\text{M}$ AS.

After 2 days of co-culture, the medium was removed from the flasks and replaced with fresh FN medium containing Timentin[®] (400 mg l^{-1}). Transient GUS expression was assayed two to five days after SAAT treatment. Embryogenic buckeye tissue was SAAT-treated in the same way as soybean suspension culture tissue except that the embryogenic clumps were sonicated in 1.5 ml microfuge tubes.

Stable transformation

For stable transformation of soybean and Ohio buckeye, embryogenic suspension culture tissues of soybean and Ohio buckeye were SAAT-treated for 30 and 60 s respectively. After a 2 day co-culture in FN medium containing $100 \mu\text{M}$ AS, suspension culture tissue was subcultured to FN medium containing Timentin[®] (400 mg l^{-1}) every week for two weeks. Hygromycin was then added to FN medium containing Timentin[®] (400 mg l^{-1}) at a concentration of 20 mg l^{-1} for soybean and 10 mg l^{-1} for buckeye. The culture medium was replaced every week for four weeks to prevent extensive browning of the medium. Transgenic clones were observed and isolated six to eight weeks following SAAT treatment. Stably-transformed soybean plants were regenerated from suspension culture material according to Finer and McMullen (1991).

β -glucuronidase assays

GUS expression was assayed by placing cotyledons in GUS assay mix (Jefferson, 1987) containing 10 mM $\text{Na}_2\text{EDTA} \cdot \text{H}_2\text{O}$, 0.1% Triton X-100, 0.1 M NaH_2PO_4 , 0.5 M $\text{K}_3\text{Fe}(\text{CN})_6$, 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-glu) ($250 \mu\text{g ml}^{-1}$) 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 spots or estimating the percentage of the surface area that was blue.

Microscopy

For microscopic analyses, samples of SAAT-treated soybean cotyledons were fixed in 3% glutaraldehyde, 2% paraformaldehyde, 1.5% acrolein, 0.1 M phosphate buffer, pH 7.0 for a minimum of 2 h and dehydrated in an ethanol series. For scanning electron microscopy, tissues were critical point dried, sputter coated and viewed by an ISI40 scanning electron microscope. For light microscopy, immature soybean cotyledons were embedded in Spurr's resin, sectioned to $0.75 \mu\text{m}$ and stained with 0.1% toluidine blue (Finer and McMullen, 1991).

Molecular analyses

DNA from transformed soybean and buckeye clones were isolated using the CTAB procedure (Saghai-Marouf *et al.*, 1984). DNAs were digested with *Hind*III and then

electrophoresed through a 0.8% agarose gel. The gel was treated and the DNA was transferred to nylon membranes and hybridized as described previously (Finer and McMullen, 1991). Membranes were placed on Kodak XAR-5 film with intensifying screens at -70°C to visualize the hybridization pattern.

Results and discussion

In all tissues tested, the SAAT treatment greatly enhanced the levels of transient expression (Table 1, Fig. 1). In most tissues, transient expression without sonication was very low, with no GUS expression being observed in tissues of wheat and white spruce seedlings as well as soybean and buckeye suspension cultures. With SAAT, transient expression was so high in areas of some tissues that individual foci could not be distinguished (Fig. 1) and it was necessary to estimate the percent of the surface that was GUS-positive. Although tissues responded to a wide range of SAAT durations (Table 2), the tissue was often damaged by the longer SAAT treatments and the tissue culture response was reduced (data not shown). The treatments that yielded high levels of GUS expression with a minimum SAAT duration were most desirable. As tissues differed in their response to SAAT (Table 1), the best treatment for each tissue needs to be empirically determined.

To determine the basis for the increase in transient GUS expression from SAAT, tissues were examined microscopically after SAAT treatment. Scanning electron microscopy revealed the formation of large numbers of micro-wounds on the surface of the SAAT-treated tissues, while the surface of the non-treated tissue was smooth and intact (Fig. 2). Micro-wounds were observed on all treated tissues and became larger with the longer duration treatments. These micro-wounds apparently resulted from cavitation, which is caused by sonication.

Cavitation can be both transient and stable. During

transient cavitation, small bubbles are formed, which enlarge and oscillate in size until they reach a critical size and implode. These implosions generate shock waves, which are strong enough to cause mechanical damage to both cells and macromolecules (Suslick, 1988). Stable cavitation results in the formation larger bubbles, which rapidly oscillate in size, causing a strong and rapid movement of the medium surrounding the bubbles (microstreaming). High velocities of microstreaming result in mechanical damage to plant tissue and macromolecules (Frizzel, 1988). It is unclear whether implosions or microstreaming resulting from transient or stable cavitation respectively are responsible for micro-wounding. The micro-wounds created by sonication covered the surface of the treated tissue and ranged in size from less than $1\ \mu\text{m}$ up to $1\ \text{mm}$ with longer duration treatments. The holes in the surface of the plant material are clearly large enough for *Agrobacterium* to invade the wounded cells or tissues (Fig. 2D). Sonication of the plant tissue with *Agrobacterium*, as opposed to sonication of tissue before the application of *Agrobacterium*, did not alter transient expression levels (data not shown). Here, for ease of handling, sonication was always performed with *Agrobacterium*.

Histological analysis of SAAT-treated tissues revealed a large degree of bacterial colonization of both epidermal and subepidermal cells (Fig. 3). Without SAAT, bacteria were observed only on the surface of the cotyledons (Fig. 3A). In some SAAT-treated tissues, epidermal cells were heavily colonized, with large numbers of bacteria present in the majority of cells (Fig. 3B). Large numbers of bacteria were also observed in subepidermal cells (Fig. 3C), with bacteria penetrating up to 7–8 cells layers in some cases (data not shown). Some of the colonized subepidermal cells were either surrounded by or adjacent to apparently intact cells, indicating that SAAT allows very localized, deep micro-wounding without damage to the neighbouring cells. These deeper wounds are

Table 1. Transient expression of GUS with and without SAAT in various plant tissues.

Plant/tissue type	SAAT Duration (s) ^b	GUS expression ^c	
		–SAAT	+SAAT
soybean/immature cotyledons (–AS) ^a	2	0.05% ± 0.16	18.6% ± 14.4
soybean/immature cotyledons (+AS) ^a	2	0.44% ± 0.72	44.0% ± 21.3
cowpea/leaflets	60	0.5%	42%
wheat/seedlings	100	0 foci	28 foci
maize/immature embryos	30	0.6% ± 0.06	80.3% ± 8.3
white spruce/seedlings	50	0 foci	26 foci
soybean/embryogenic suspension	60	0 foci	1807 foci
buckeye/embryogenic suspension	20	0 foci	112 foci

^aco-cultivated without (–) and with (+) acetosyringone (AS)

^bconditions are representative and are not fully optimized

^cPercentage values represent the proportion of surface area expressing GUS; foci is a count of GUS-positive foci; each value ± standard deviation represents the mean of 10–30 replications.

Table 2. Time course of SAAT on transient expression in immature cotyledons of soybean

SAAT Duration (s)	Surface area expressing GUS (%) ^a
0	0.6 ± 1.8
0.2	24.2 ± 22.6
1.0	48 ± 23
2.0	67 ± 35
5.0	62 ± 23
10.0	79.9 ± 9

^aEach value ± standard deviation represents the mean of 10–30 replications.

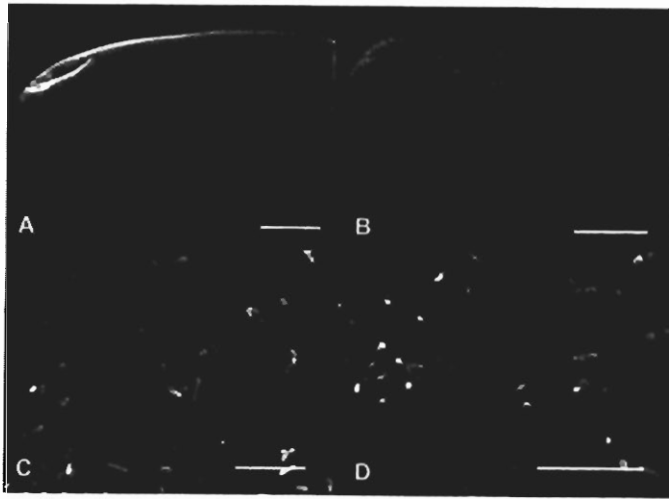


Fig. 2. Scanning electron micrographs of non-sonicated immature soybean cotyledons (A and C) and 5 s SAAT-treated samples with thousands of micro-wounds caused by sonication (B and D). A and B, bar = 500 µm; C and D, bar = 5 µm.

advantageous when subsurface targets such as cotyledonary nodes and shoot meristems are the targets for transformation. The presence of large numbers of colonized cells, adjacent to intact, apparently healthy cells, suggests that the surrounding intact cells are transformed by *Agrobacterium* from the inside of the neighbouring colonized cells. The large degree of GUS coloration within SAAT-treated tissue also indicates that large numbers of cells (those cells adjacent to colonized cells) are transiently transformed as a result of this process (Fig. 1). Because both large blue areas and small blue foci were observed together in most tissues (Fig. 1B, 1D), it is unlikely that extensive diffusion of GUS is responsible for these large GUS-positive regions. The large numbers of bacteria that were observed in many of the plant cells probably arose from introduction of one or a few bacteria into wounded cells which proliferated to give rise to the hundreds of bacteria present in these cells.

The efficiency of SAAT may be even further increased

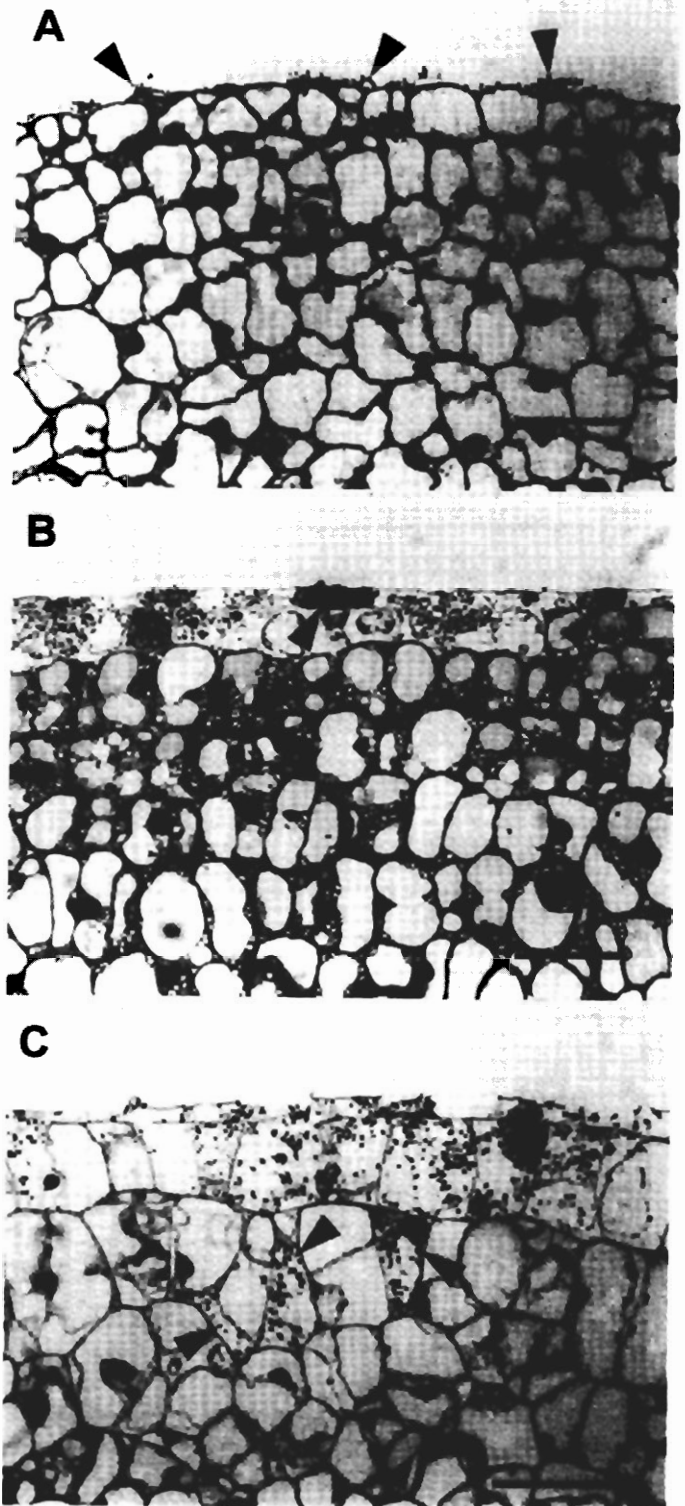


Fig. 3. Cross section of control (A) and SAAT-treated (B and C) immature soybean cotyledons. Bacteria are indicated by the arrows. The epidermal cells of the non SAAT-treated tissues (A) are structurally intact and bacteria can be seen on the surface. In SAAT-treated samples, bacteria were clearly evident in epidermal cells (B) and also deeper within the tissue (C) indicating wounding of both surface and subsurface tissue. Bar = 50 µm.

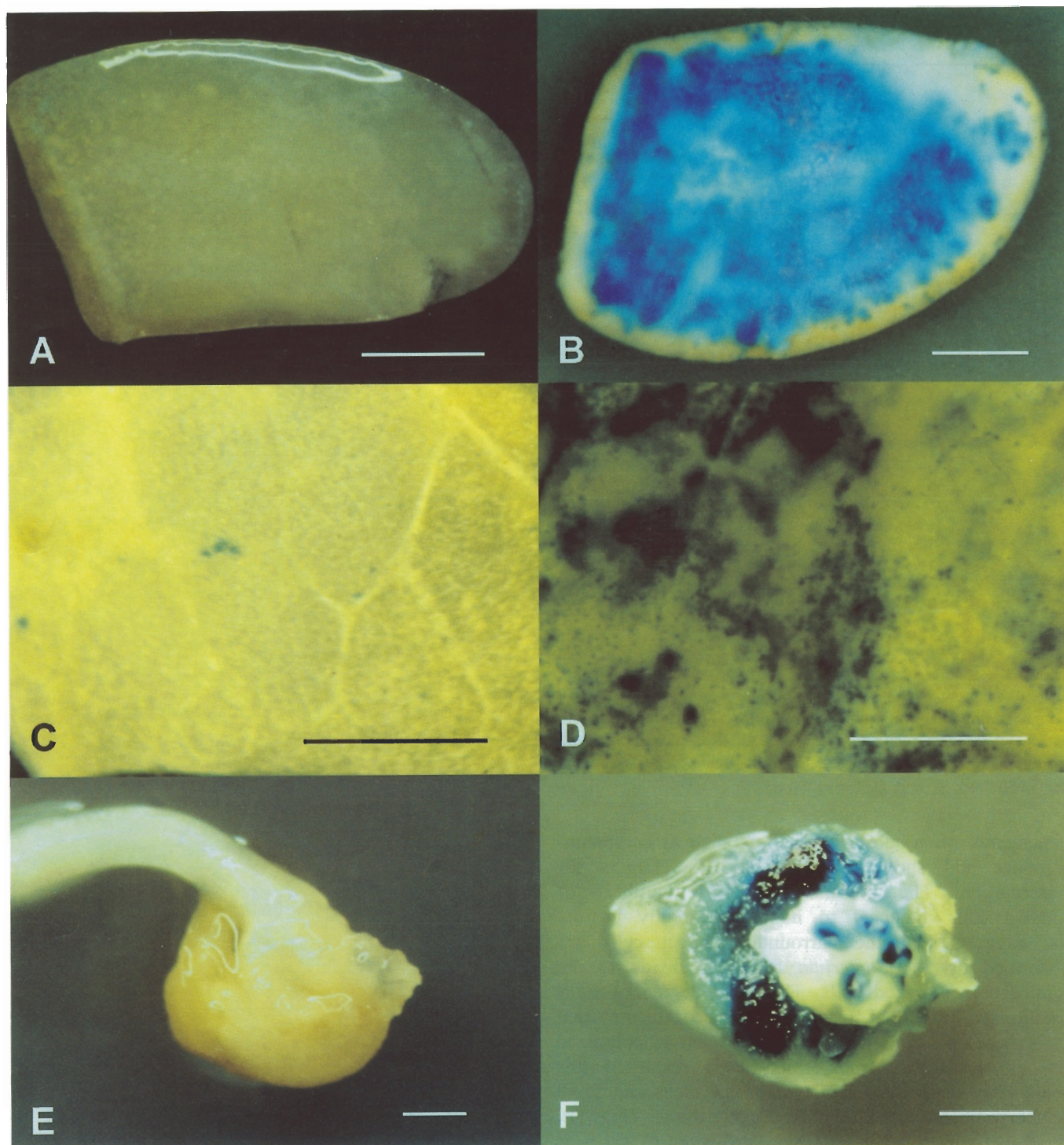


Fig. 1. Transient GUS expression in immature soybean cotyledons, cowpea leaves and immature maize embryos with and without SAAT. A. Soybean with *Agrobacterium* alone (bar = 1 mm). B. Soybean with SAAT (bar = 1 mm). C. Cowpea with *Agrobacterium* alone (bar = 500 μ m). D. Cowpea with SAAT (bar = 500 μ m). E. Maize with *Agrobacterium* alone (bar = 1 mm). F. Maize with SAAT (bar = 1 mm).

by vacuum infiltration (Bechtold *et al.*, 1993) or another method that enhances the infection process. The use of AS during co-cultivation of SAAT-treated cotyledons did increase transient expression twofold (Table 1). In addition to the practical use of SAAT to enhance transformation rates in recalcitrant plants, the efficiency of *Agrobacterium* delivery may help to elucidate factors involved in the infection and transformation processes.

Stable transformation of soybean and buckeye embryogenic suspension cultures was obtained with SAAT. Although 23 stably transformed buckeye clones were recovered without the use of SAAT, no transgenic soybean clones were recovered unless SAAT was utilized. A total of 121 soybean and 348 buckeye clones that were both hygromycin-resistant and GUS-positive were recovered from our SAAT treatments. In addition, a few putative buckeye clones were recovered which did not express GUS. It was unclear whether these GUS-negative clones were escapes or simply did not express GUS, as molecular analysis of these tissues was not pursued. However, southern hybridization analysis of 5 soybean and 12 buckeye clones that were hygromycin-resistant and GUS-positive did confirm that these clones contained the hygromycin resistance gene (data not shown).

Further analysis of a few representative soybean and buckeye clones indicated DNA integration into either one or two sites (Fig. 4). For this analysis, genomic DNA from transgenic plant tissue was digested with *Hind*III and then probed with the coding region for the hygromycin resistance gene. *Hind*III recognizes two sites within Vec035; one inside the left border and the other between the GUS gene and the hygromycin resistance gene. Therefore, digestion of DNA with *Hind*III releases the intact GUS gene and the hygromycin resistance gene along with the right border and some plant DNA (depending on the location of *Hind*III-recognition sites in the plant DNA). Hybridization of *Hind*III-digested plant DNA with the coding region for hygromycin resistance should yield various sized hybridization signals showing the different integration events. Analysis of soybean and buckeye SAAT clones showed hybridization signals indicative of low copy independent transformation events (Fig. 4). This low copy number of introduced DNA in transgenic tissue is typical of *Agrobacterium*-mediated transformation. Hybridization of undigested plant DNA with the coding region for hygromycin resistance shows a hybridization signal above 23 kb, indicating integration of the T-DNA into high molecular weight plant DNA. While transgenic Ohio buckeye plantlets remain *in vitro*, transgenic soybean plants were 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 were not recovered. Loss of fertility of plants regenerated from long-term embryo-

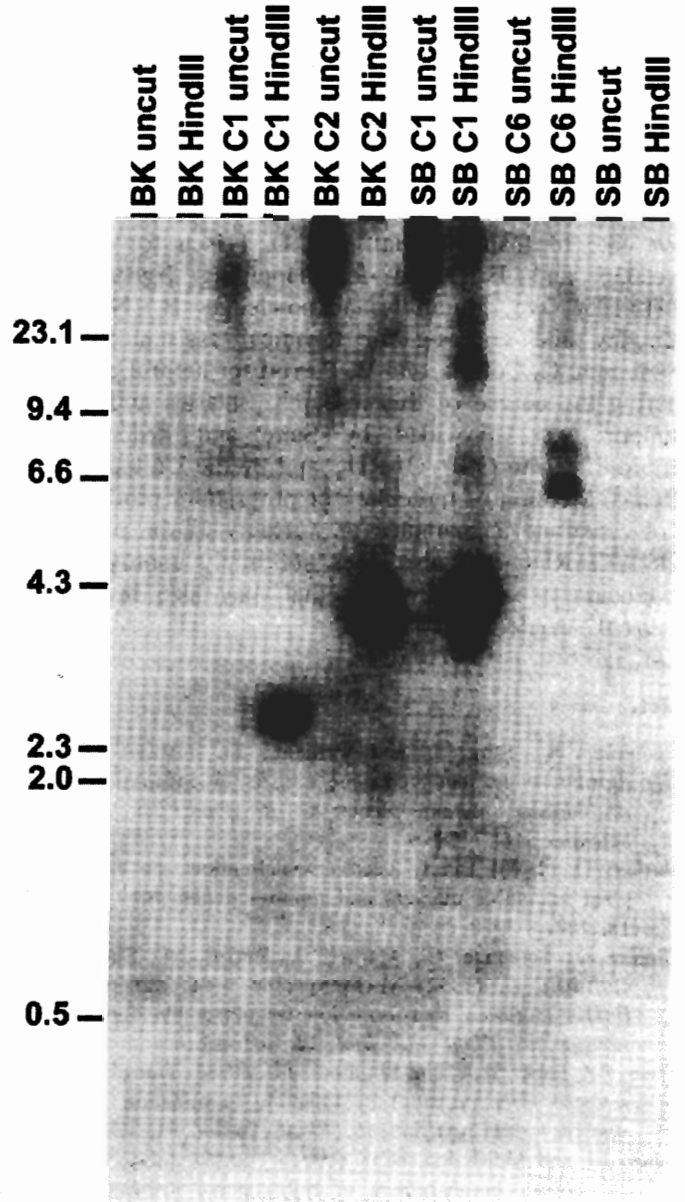


Fig. 4. Southern hybridization analysis of DNA from stably-transformed soybean and buckeye clones. Genomic DNA was digested with *Hind*III, which recognizes two sites within Vec035; one inside the left border and the other between the GUS and hygromycin resistance genes. The membrane was hybridized with the coding region for the hygromycin resistance gene.

genic suspension cultures of soybean has been previously reported (Hadi *et al.*, 1996). SAAT is currently being further evaluated on soybean as well as other plants and plant material for production of stably-transformed fertile plants.

SAAT permits the efficient delivery of *Agrobacterium* to large numbers of plant cells in a variety of different plant tissues. Using SAAT, *Agrobacterium*-plant interactions can be studied in great detail as bacterial

inoculations are consistent and controlled. This new method holds much promise for increasing transformation rates in all plants, especially those that are typically more recalcitrant to *Agrobacterium*-mediated transformation.

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