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Sonication-assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: optimization of transient expression

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Abstract Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) tremendously improves the efficiency of *Agrobacterium* infection by introducing large numbers of microwounds into the target plant tissue. Using immature cotyledons of soybean as explants, we evaluated the effects of the following parameters on transient β-glucuronidase (GUS) activity: cultivars, binary vectors, optical density of *Agrobacterium* during infection, duration of sonication treatment, co-culture conditions, length of explant preculture and addition of acetosyringone during co-culture. The extent of tissue disruption caused by sonication was also determined. The highest GUS expression was obtained when immature cotyledons were sonicated for 2 s in the presence of *Agrobacterium* (0.11 OD_{600nm}) followed by co-cultivation with the abaxial side of the explant in contact with the culture medium for 3 days at 27°C. The addition of acetosyringone to the co-culture medium enhanced transient expression. No differences were observed when different cultivars or binary vectors were used. Cotyledons sonicated for 2 s had moderate tissue disruption, while the longer treatments resulted in more extensive damage.

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Introduction

Agrobacterium provides one of the main vehicles for introducing foreign DNA into plants. A number of plant species (Wordragen and Dons, 1992; Fisk and Dandekar, 1993) and even yeast (Bundock et al. 1995) have been transformed by *Agrobacterium*. However, *Agrobacterium*-mediated transformation of many plants, including soybean, remains inefficient. *Agrobacterium*-mediated transformation of soybean has been reported using cotyledonary nodes (Hinchee et al. 1988) and immature cotyledons (Parrott et al. 1989), but the transformation frequencies were very low and the plants recovered were often chimeric (Parrott et al. 1989).

In order to enhance transformation rates, improvements have been made in the delivery of the bacterium (Bidney et al. 1992), and vectors have been modified to provide constitutive expression of *vir* genes (Hansen et al. 1994; Ishida et al. 1996). Although transformation rates have been significantly improved using these modifications, increases in efficiency are still needed.

Recently, sonication has been used to enhance *Agrobacterium*-mediated transformation of many different plant species (Trick and Finer 1997). Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) consists of subjecting the target tissue to ultrasound while immersed in an *Agrobacterium* suspension. The enhanced transformation rates using SAAT probably result from microwounding, where the energy released by cavitation (Frizzel 1988) causes small wounds both on the surface of and deep within the target tissue. Sonication enhances the delivery of naked DNA into tobacco protoplasts (Joersbo and Brunstedt 1990) and seedlings (Zhang et al. 1991) but has only recently been shown to enhance *Agrobacterium*-mediated transformation of plant tissue (Trick and Finer 1997).

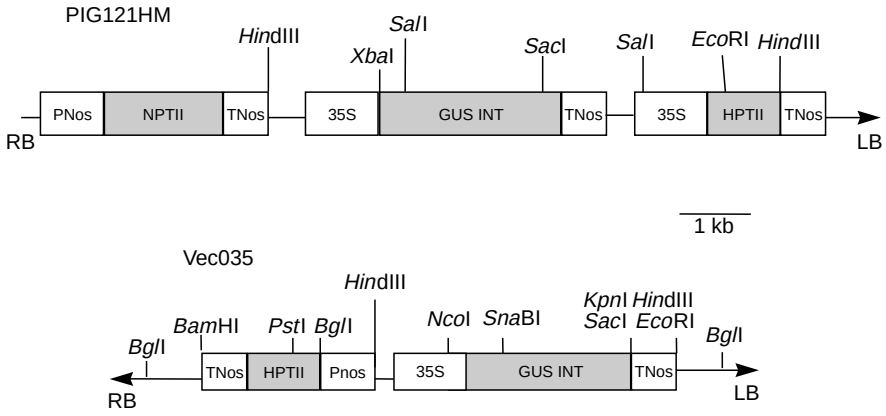

Fig. 1 T-DNA region of pIG121Hm and Vec035. Arrows indicate the direction of transcription. *RB* Right border, *LB* Left border, *HPTII* hygromycin resistance gene, *NPTII* neomycin phosphotransferase gene, *GUS INT* intron-containing GUS gene, *PNos* promoter nopaline synthase, *35S* promoter 35S of CaMV, *TNos* terminator nopaline synthase

In this report, we describe the optimization of *Agrobacterium*-mediated transient transformation of immature cotyledons of soybean using SAAT. The effects of SAAT on the structural integrity of the explants were also evaluated.

Materials and methods

Plant material

Immature pods of soybean [*Glycine max* (L.) Merrill], cvs 'Jack', 'Chapman' and 'Kunitz', were collected from plants grown in the greenhouse under a 14-h light photoperiod at 28°C. Pods were disinfested in a 20% commercial bleach solution containing 0.04% Tween-20 for 20 min and rinsed four times in sterile water. Immature seeds (4–5 mm in length) were aseptically removed from the pods and the end containing the embryonic axis was cut off and discarded. The two cotyledons were then removed from the seed coat, separated and placed on D40 medium (Santarém et al. 1997) consisting of MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 6% sucrose and 40 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), solidified with 0.2% Gelrite™ (Merck & Co, Rahway, N. J.), pH 7. Explants were maintained on D40 medium with or without 0.4 M mannitol for approximately 3 h until SAAT treatments were performed.

Bacterial strains, plasmids and culture conditions

Agrobacterium tumefaciens, strain EHA105 (Hood et al. 1993), was used as host for all the plasmids. Plasmid pIG121Hm (Hiei et al. 1994) or Vec035 were used for optimization work (Fig. 1). Plasmid Vec035 was constructed by replacing the β-glucuronidase (GUS) gene in the plasmid pBIG-Hyg (Becker 1990) with an intron-containing GUS gene from p35SGUSINT (Vancanneyt et al. 1990).

Agrobacterium was grown overnight in a modified Luria-Bertani medium (Sambrook et al. 1989) containing half the normal level of NaCl (5 g/l) and supplemented with 5 g/l sucrose and 50 mg/l kanamycin. Bacteria were centrifuged at 1500 *g* for 10 min, washed twice with equal volumes of liquid D40 medium and re-centrifuged as above. The bacterial pellet was finally resuspended in liquid D40 medium, and the OD_{600nm} was determined.

SAAT treatment

Ten cotyledons were placed in 1.5 ml microcentrifuge tubes containing 0.5 ml of the *Agrobacterium* suspension. Cotyledons were gently resuspended and placed in a float at the center of a bath sonicator (PC5 model, 55 kHz, L&R Manufacturing Company, Kearny, N. J.). The sonicator was controlled by an electronic timer. After SAAT treatment, cotyledons were removed from the tubes, placed on sterile filter paper to blot off excess bacteria and then transferred to co-culture medium.

Pre- and post-SAAT culture conditions

To evaluate the effects of sonication duration, cotyledons were sonicated for 0.1, 0.5, 1, 2, 5, 7 or 10 s using *Agrobacterium* at 0.1 OD_{600nm}, and transient expression levels were recorded. In a subsequent experiment, a combination of OD_{600nm} (0.05, 0.1 and 0.2) with different co-culture periods (2, 3 and 4 days) and temperatures of 23°C and 27° C was evaluated using a 2-s sonication treatment.

To determine the effects of acetosyringone (AS) during the co-culture period, we evaluated transient GUS expression using cotyledons co-cultured on D40 medium with or without 100 µM AS, after sonication treatments of 2 s and 5 s.

The effect of explant orientation was evaluated by placing the cotyledons adaxial side facing up or down during co-culture after a 2-s sonication treatment. Transient expression was scored on both sides of the cotyledons.

A preculture treatment period was evaluated using cotyledons that were excised and either sonicated immediately or maintained on D40 medium for 1, 2, 3 or 4 days before a sonication treatment of 2 s at 0.2 OD_{600nm}.

After co-culture in all cases, cotyledons were rinsed in sterile water, blotted dry on sterile filter paper and placed on D40 medium supplemented with 500 mg/l cefotaxime to prevent *Agrobacterium* growth.

Wounding assessment

The contribution of sonication and *Agrobacterium* to overall tissue response was determined by sonicating the cotyledons with or without *Agrobacterium*. Control treatments consisted of either no treatment or *Agrobacterium* with no sonication. Sonication was performed for 2 s and 0.2 OD_{600nm}. In an attempt to control tissue disruption from the sonication, D40 medium containing 0.4 M mannitol was used for maintaining cotyledons prior to SAAT. Mannitol (0.4 M) was also added to the D40 medium used to resuspend the bacteria. The OD_{400nm} of the plant/bacterial exudate after sonication was measured as a means of damage assessment.

Histochemical GUS assays

Histochemical GUS assays were performed 2 days after transfer of the cotyledons to the medium containing 500 mg/l cefotaxime. Cotyledons were placed in a GUS assay mix (Jefferson 1987) and incubated overnight at 37 °C with shaking at 80 rpm. The GUS assay mix was removed, and the tissue was rinsed twice with 70% ethanol. GUS activity was then determined by placing the cotyledons on a grid and estimating the percentage of the cotyledon surface that showed blue sectors under a dissecting microscope.

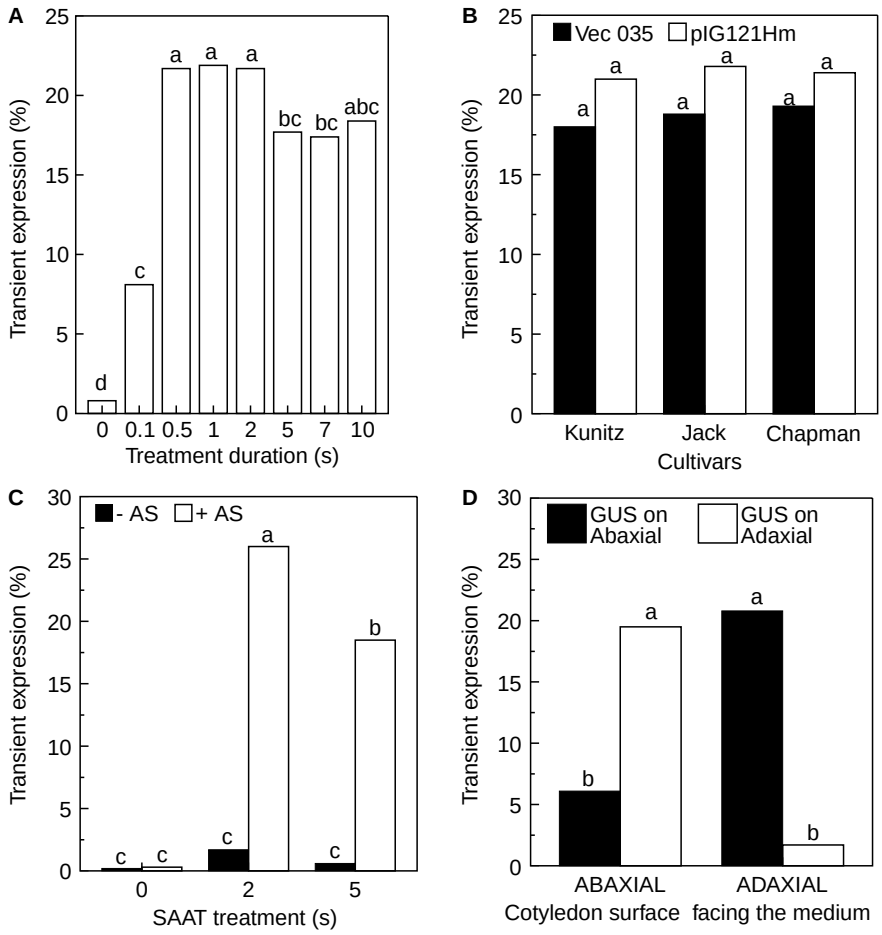

Fig. 2A–D Frequency of transient GUS expression of SAAT-treated immature soybean cotyledons using *Agrobacterium* at 0.1 OD_{600nm}. **A** Effect of duration of sonication, **B** effect of two different binary vectors and three cultivars on GUS expression using a 2-s SAAT treatment, **C** effect of the presence or absence of acetosyringone during the co-cultivation period. **D** effect of explant orientation in relation to the co-culture medium on transient GUS expression. Data represents the percentage of the cotyledon surface area showing blue sectors as an average of three replications with ten cotyledons each. *Different letters* show significant difference among treatments according to Fisher's Least Significant Difference Test (α=0.05)

Electron microscopy

For scanning electron microscopy, cotyledons were fixed in 0.2 M potassium phosphate buffer (pH 7.0) containing 3% glutaraldehyde, 2% paraformaldehyde and 1.5% acrolein for 2 h at room temperature. Samples were then dehydrated in an ethanol series (50–100% ethanol at 10 min each), critical point-dried, sputter-coated with platinum and viewed on a ISI-40 scanning electron microscope as described earlier (Trick and Finer 1997).

Data analysis

Experiments were performed with three replicates per treatment. Means of percentage of the cotyledon surface covered with blue sectors were calculated. Transformed data (sq.rt+0.5) was analyzed by ANOVA. Treatments were separated using Fisher's Least Significant Difference test (α=0.05).

Results and discussion

SAAT treatment

The duration of sonication had a dramatic effect on the transient expression of GUS in immature soybean cotyledons (Fig. 2A, Fig. 3A–D). When the cotyledons were treated with *Agrobacterium* EHA105 (Vec035) without sonication, an average of less than 1% of the surface of the cotyledons expressed GUS (Fig. 2A; Fig. 3B). A tremendous enhancement of GUS expression was observed when sonication was applied along with *Agrobacterium* (Fig. 3C–D). Treatments ranging from 0.5 s to 2 s gave the highest transient expression (Fig. 2A), although some browning of the tissue resulted from the sonication treatment.

Scanning electron microscopy revealed large amounts of microwounding of the cotyledons (Fig. 4A–H). Cotyledons treated with *Agrobacterium* without sonication showed no surface microwounding (Fig. 4A,B), while with a 2-s sonication treatment, limited microwounding was observed on the cotyledons (Fig. 4C,D). With the longer sonication treatments of 5–10 s, the entire surface of the cotyledon became covered with microwounds (Fig. 4E–H). When cotyledons were SAAT-treated for more than 10 s but less than 30 s, 30% of the cotyledons turned white a few days following culture and did not survive, while sonication treatments of more than 30 s resulted in severe tissue disruption and death of all cotyledons 5 days after sonication (data not shown).

Although high-intensity ultrasound results in immediate cell lysis (Joersbo and Brunstedt, 1992), sublethal doses result in temporary suppression of RNA and protein synthesis as well as moderate rupture of the cell walls (Joersbo

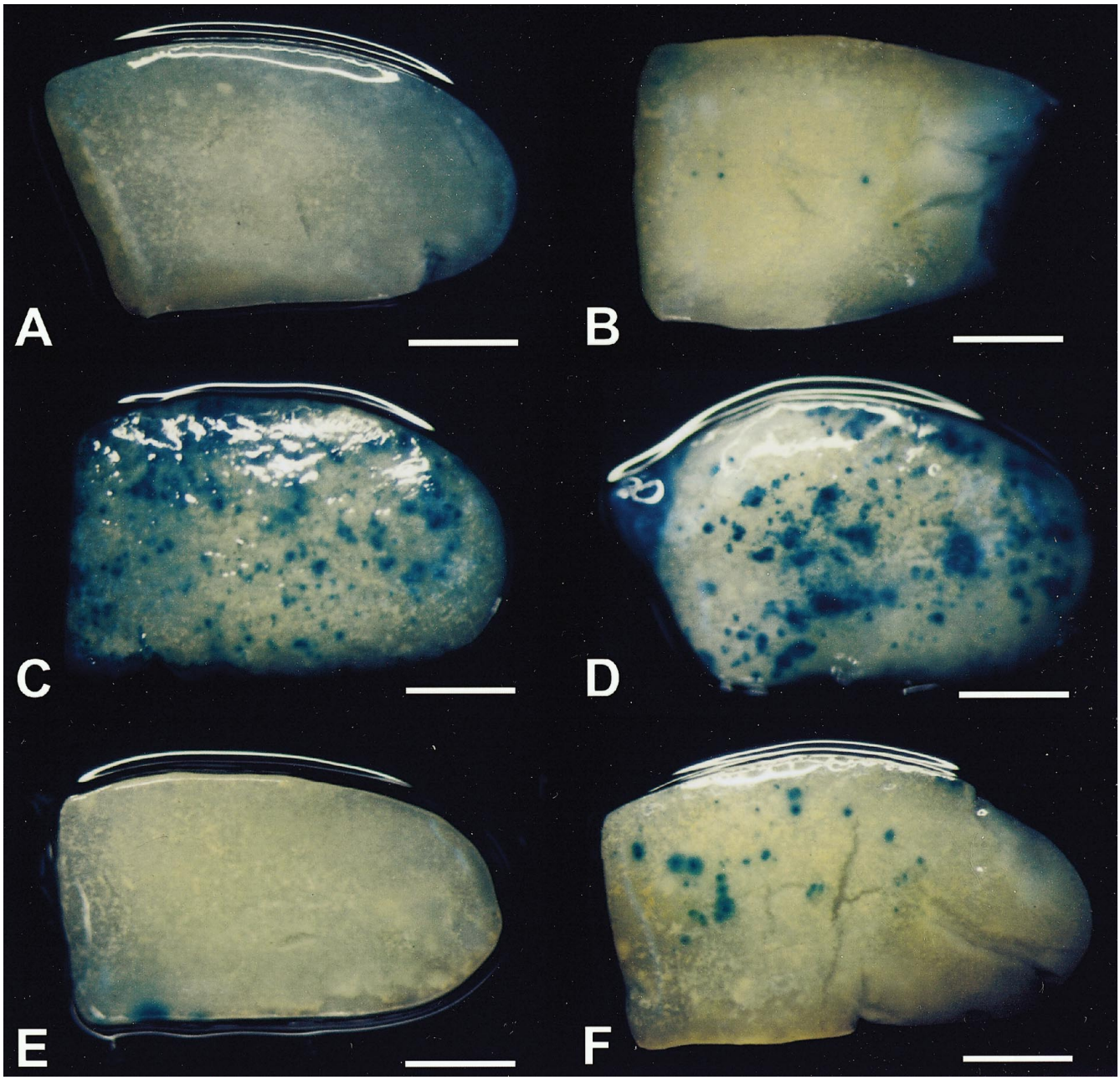

Fig. 3A–F Transient expression of GUS in the adaxial surface of SAAT-treated immature cotyledons of soybean using *Agrobacterium* (Vec035) at 0.1 OD_{600nm} with a 3-day co-culture. **A** Control (no sonication; no *Agrobacterium*). **B** *Agrobacterium*-treated cotyledons (no sonication), **C**, **D** SAAT-treated cotyledons on D40 medium (**C** 2 s, **D** 10 s), **E, F** SAAT-treated cotyledons on D40-mannitol medium (**E** 2 s, **F** 10 s). *Bars*: 1 mm

and Brunstedt, 1992). The cell-wall disruption caused by the lower energy ultrasonic frequency utilized in the present study is apparently very useful for *Agrobacterium*-mediated transformation. The wounding may aid in the production of signal phenolics (Stachel et al. 1985) and enhance the accessibility of putative cell-wall binding factors (Lippencott and Lippencott 1969) to the bacterium. Although the average GUS expression obtained with 2- and 10-s sonication treatments did not differ statistically, the large extent of microwounding observed with the longer treatment (Fig. 4C–H) indicated that the 2-s treatment was more suitable for further experiments.

The enhancement of transient GUS expression due to SAAT treatment was not genotype specific. No difference was observed among the three cultivars ('Jack', 'Chapman' and 'Kunitz') tested (Fig. 2B). Furthermore, there was no difference in transient GUS expression when either of the two different binary plasmids were used (Fig. 2B). The effect of plant genotypes has been reported for several species. Wordragen and Dons (1992) suggested that the difference in response among cultivars could be caused by differential response to the wounding stress. However, our

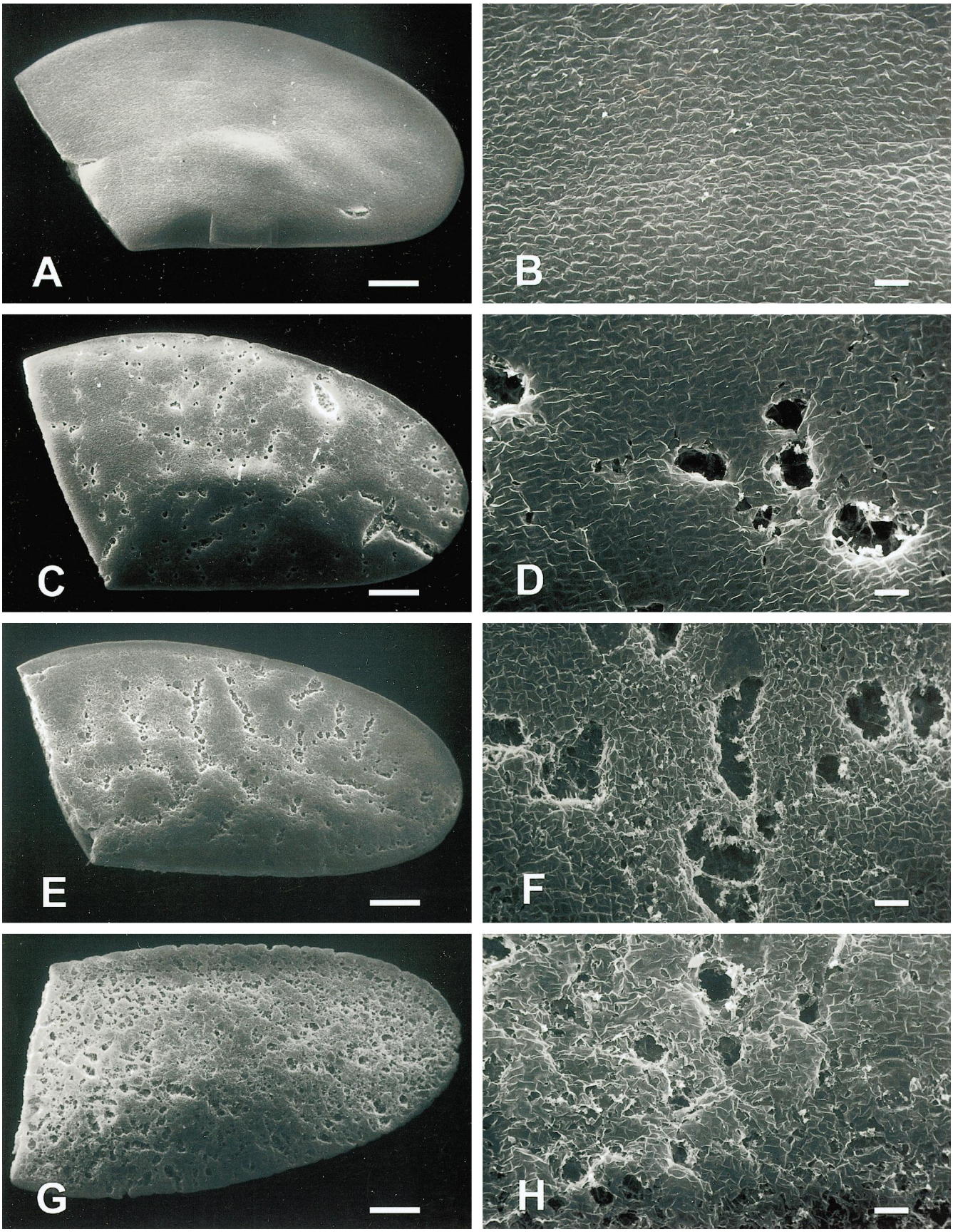

Fig. 4A–H Scanning electron microscopy of adaxial surface of sonicated immature cotyledons of soybean. **A, B** *Agrobacterium*-treated cotyledons; no sonication. **C–H** SAAT-treated cotyledons (**C, D** 2 s, **E, F** 5 s, **G, H** 10 s). *Bars* on **A**, **C**, **E**, and **G** represent 100 μm and on **B**, **D**, **F**, and **H** represent 10 μm

Table 1 Effect of OD_{600nm}, temperature and duration of the co-culture period on transient expression of immature cotyledons of soybean sonicated for 2 s

Temperature	Co-culture period (days)	Transient expression (%) [a] OD_{600nm} of *Agrobacterium*		
		0.05	0.1	0.2
23 °C	2	0.3d [b]	0.4 d	5.5 cd
	3	18.8 b	19.8 b	19.5 b
	4	20.0 b	22.6 b	21.3 b
27 °C	2	4.5 cd	8.2 c	12.8 c
	3	28.8 a	36.0 a	32.0 a
	4	29.3 a	32.3 a	36.0 a

[a] Percentage of surface area showing blue sectors as average of three replications with ten cotyledons each
[b] Means followed by the same letters are not significantly different at the 0.05 level according to Fisher's Least Significant Difference test

results indicated that the use of sonication to mediate the *Agrobacterium* infection could minimize or alleviate these differences. The cultivar 'Jack' and EHA105 containing the binary vector Vec035 were used for the remaining experiments.

Pre- and post-SAAT culturing conditions

Transient GUS expression in SAAT-treated cotyledons was affected by both temperature and length of co-culture period but was not significantly influenced by the density of the bacterial suspension (Table 1). The highest GUS expression was observed after 3- or 4-days of co-culture at 27°C, regardless of the OD_{600nm} used. At 23°C, the highest GUS expression was also observed after a 3- or 4-day co-culture period; however, transient expression was lower than at 27°C. Although a 2-day co-culture is commonly used (Holford et al 1992; Muthukumar et al 1996), a longer co-culture period of 3 or 4 days can improve transformation efficiency using *Agrobacterium* (Ducrocq et al. 1994). De Bondt et al. (1994) reported that a 4-day co-culture of *Malus* explants with *Agrobacterium* could be used but that bacterial overgrowth became problematic with this longer co-culture period. Temperature also affects the efficiency of T-DNA transfer (Fullner and Nester 1996). In stem segments of soybean infected with *Agrobacterium*, co-culture at 25°C enhanced transient expression while higher temperatures suppressed the transfer of T-DNA (Kudirka et al. 1986). Temperature can also affect pilus formation in *Agrobacterium*, which may be involved in T-DNA transfer (Fullner et al. 1996). Pilus formation was observed at 19°C but was only rarely observed at 28°C. The results obtained with soybean immature cotyledons indicated that the best conditions for co-culture after SAAT treatment were 3 days at 27°C.

Co-culture of soybean cotyledons with acetosyringone greatly increased transient expression following SAAT treatment (Fig. 2C). Although some blue foci were observed in control cotyledons (no AS), with or without sonication, a significant increase in transient expression resulted from SAAT treatments followed by co-culture with AS. Acetosyringone has been shown to enhance the transient expression of GUS in different species (Atkinson and Gardner 1991; James et al. 1993) due to activation of the *vir* genes (Stachel et al. 1985). Although the pH used in the plant medium in the present study has been reported to inhibit *vir* gene induction (Stachel et al. 1986), a microenvironment favorable for *vir* gene induction could be established on the surface of the soybean cotyledons which was not in contact with the pH 7 medium. Our results conclusively show that the addition of acetosyringone after wounding enhances transient expression regardless of the length of the sonication treatment.

The location of GUS activity was strongly influenced by the orientation of the explants during the co-cultivation period. The highest transient expression was always observed on the side of the cotyledon that was not in contact with the culture medium (Fig. 2D). It is unclear if transient expression was reduced on the side of the cotyledon in contact with the culture medium or enhanced on the other side of the cotyledon as a result of the rapid divisions in this tissue (Santarém et al., 1997). As high pH is inhibitory to *vir* gene induction (Stachel et al. 1986), it is possible that the low GUS expression on the side of the cotyledon in contact with the medium resulted from a localized high pH inhibition of *vir* gene induction. Restricted culture aeration, on the side of the tissue in contact with the medium could also have resulted in reduced *vir* gene induction (Stachel et al. 1986). Sonicated explants were therefore cultured abaxial side facing the co-cultivation medium so that the highest expression would be in the adaxial surface tissue, which typically gives rise to large numbers of somatic embryos (Santarém et al. 1997).

Since DNA synthesis appears to be required in host cells for the incorporation of T-DNA (Villemont et al. 1997), the effects of induction of cell division in the target tissue were evaluated prior to the SAAT treatment by performing a preculture timecourse using D40 medium. The highest transient expression was observed using explants that were sonicated immediately after explant excision (29.5%). Cotyledons that were precultured for 1, 2, 3 or 4 days and then SAAT-treated had 12.8%, 11.2%, 4.2% and 4.3% respectively of their surface showing GUS expression. In *Datura innoxia* and *Vigna ungiculata* the highest transformation efficiency was obtained after a 1-day preculture and decreased 50% after 8 day (Ducrocq et al. 1994; Muthukumar et al. 1996). In A*rabidopsis thaliana*, the highest transient GUS expression was observed after 4 days of preculture (Sangwan et al. 1991). Kudirka et al. (1986) did not observe an enhancement of transformation efficiency of the stem segments after the preculture period. Our results indicated no beneficial effect on transient transformation efficiency from the preculture of soybean cotyledons on induction medium. The effects of preculture may need to be re-evaluated for stable transformation work as the mitotic state of the target tissue may have different effects on transient and stable transformation.

Table 2 Comparison between immature cotyledons excised and sonicated in D40 medium or D40 0.4 *M* mannitol. Sonication treatment was 2 s with the *Agrobacterium* diluted to 0.2 OD_{600nm}

Treatment duration (s)	OD_{400} of exudate [a]		Transient expression (%) [b]	
	D40	D40-mannitol	D40	D40-mannitol
2	0.582	0.151	25.2 a [c]	1.8 b
5	0.764	0.357	24.4 a	1.4 b
10	0.995	0.433	25.2 a	1.4 b

[a] Blank consisted of sonicated *Agrobacterium* alone
[b] Percentage of surface area showing blue sectors as average of three replications with ten cotyledons each
[c] Means followed by the same letters are not significantly different at the 0.05 level according to Fisher's Least Significant Difference test

Damage assessment

Although SAAT of soybean cotyledons resulted in high levels of transient expression, browning and callus formation was apparent in many cases. To determine what was involved in the browning, we sonicated the explants in the presence or absence of *Agrobacterium*. Based on observations of embryo induction and browning, it appeared that *Agrobacterium* alone was not detrimental to tissue growth compared to the minus-*Agrobacterium* control. On the other hand, sonication of the cotyledons either with or without *Agrobacterium* resulted in callus formation with some browning of that tissue. When sonication treatments longer than 5 s were used, the primary response of the immature cotyledons to form somatic embryos was diminished or eliminated. In order to obtain stable transformation of this target tissue, the intensity of the sonication treatment should be carefully monitored to control microwounding and cell disruption.

To estimate the extent of wounding caused by sonication, we measured the OD_{400nm} of the bacterial suspension after various sonication treatments (Table 2). Light absorbance of a plant/bacterial exudate at 400 nm gives an approximation of tissue disruption. Longer sonication treatments resulted in higher plant exudate concentrations and consequently higher optical density readings (Table 2). When cotyledons were excised and SAAT-treated in D40 medium containing 0.4 M mannitol, a decrease in OD_{400nm} was observed in this exudate solution indicating less tissue wounding (Table 2). Scanning electron microscopy of cotyledons excised and SAAT-treated in either D40 medium or D40 medium with 0.4 M mannitol revealed protection of the mannitol-treated tissue from wounding (data not shown). However, the transient expression of the cotyledons treated with mannitol was also reduced (Table 2, Fig. 3E, F). Mannitol could act to protect the target tissue by causing cell plasmolysis, reducing turgidity and diminishing sonication-induced microwounding. Although osmotic treatment enhanced particle bombardment-mediated transformation of soybean and maize suspension culture material (Vain et al. 1993a, b), it was clearly not effective in enhancing transient transformation of soybean cotyledons using SAAT. The effectiveness of the SAAT treatment was apparently influenced by the water concentration or turgidity of the target tissue.

We report here a new technique which enhances the efficiency of *Agrobacterium* infection of plant cells. Immature cotyledons of soybean were very responsive for transient expression studies and were used for optimization of SAAT. While stable transformation of embryogenic suspension cultures of soybean has already been obtained using SAAT (Trick and Finer 1997, 1998), the stable transformation of soybean cotyledons as well as other plants and target tissues are still being evaluated.

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References

Atkinson RG, Gardner R (1991) *Agrobacterium*-mediated transformation of pepino and regeneration of transgenic plants. Plant Cell Rep 10:208–212

Becker D (1990) Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucleic Acids Res 18:203

Bidney D, Scelonge C, Martich J, Burrus M, Sims L, Huffman G (1992) Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. Plant Mol Biol 18:301–13

Bundock P, Dulk-Ras A den, Beijersbergen A, Hooykaas PJJ (1995) Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisae*. EMBO J 14:3206–3214.

De Bondt A, Eggermont K, Druart P, De Vil M, Goderis I, Vanderleyden J, Broekaert W (1994) *Agrobacterium*-mediated transformation of apple (*Malus* × *domestica* Borkh): an assessment of factors affecting gene transfer efficiency during early transformation steps. Plant Cell Rep 13:587–593

Ducrocq C, Sangwan RS, Sangwan-Norrell B (1994) Production of *Agrobacterium*-mediated transgenic fertile plants by direct somatic embryogenesis from immature zygotic embryos of *Datura innoxia*. Plant Mol Biol 25:995–1009

Fisk HJ, Dandekar AM (1993) The introduction and expression of transgenes in plants. Sci Hortic 55:5–36

Frizzel LA (1988) Biological effects of acoustic cavitation. In: Suslick K (ed) Ultrasound, its chemical, physical and biological effects. VCH Publ, Weinheim, pp 287–303

Fullner KJ, Nester EW (1996) Temperature effects the T-DNA transfer machinery of *Agrobacterium tumefaciens*. J Bacteriol 178: 1498–1504

Fullner KJ, Lara JC, Nester EW (1996) Pilus assembly by *Agrobacterium* T-DNA transfer genes. Science 273:1107–1109

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151–158

Hansen G, Das A, Chilton MD (1994) Constitutive expression of the virulence genes improves the efficiency of plant transformation by *Agrobacterium*. Proc Natl Acad Sci 16:7603–7607

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oriza sativa*) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6: 271–282

Hinchee MAW, Connor-Ward DV, Newell CA, McDonnell RE, Sato SJ, Gasser CS, Fischhoff DA, Re DB, Fraley RT, Horsch RB (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. Bio/Technology 6:915–922

Holford P, Hernandez N, Newbury HJ (1992) Factors influencing the efficiency of T-DNA transfer during co-cultivation of *Antirrhinum majus* with *Agrobacterium tumefaciens*. Plant Cell Rep 11:196–199

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper strains for gene transfer to plants. Transgenic Res 2:208–218.

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat Biotechnol 14, 745–50

James DJ, Uratsu S, Cheng J, Negri P, Viss, P, Dandekar AM (1993) Acetosyringone and osmoprotectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of apple. Plant Cell Rep 12:559–563

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387–405

Joersbo M, Brunstedt J (1990) Direct gene transfer to plant protoplast by mild sonication. Plant Cell Rep 9:207–210

Joersbo M, Brunstedt J (1992) Sonication: a new method for gene transfer to plants. Physiol Plant 85:230–234

Kudirka DT, Colburn MA, Hinchee MA, Wright MS (1986) Interactions of *Agrobacterium tumefaciens* with soybean (*Glycine max* (L.) Merrill) leaf explants in tissue culture. Can J Genet Cytol 28:808–817

Lippencott BB, Lippencott JA (1969) Bacterial attachment to a specific wound site as an essential stage in tumor induction by *Agrobacterium tumefaciens*. J Bacterial 97:620–628

Murashige T, Skoog FA (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol 15: 473–497

Muthukumar B, Mariamma M, Veluthambi K, Gnanam A (1996) Genetic transformation of cotyledon explants of cowpea (*Vigna unguiculata* L. Walp) using *Agrobacterium tumefaciens*. Plant Cell Rep 15:980–985

Parrott WA, Hoffman LM, Hildebrand P, Williams EG, Collins GB (1989) Recovery of primary transformants of soybean. Plant Cell Rep 7:615–617

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Sangwan RS, Bourgeois Y, Sangwan-Norreel, BS (1991) Genetic transformation of *Arabdopsis thaliana* zygotic embryos and identification of critical parameters influencing transformation efficiency. Mol Gen Genet 230:475–485

Santarém ER, Pelissier B, Finer JJ (1997) Effect of explant orientation, pH, solidifying agent and wounding on initiation of soybean somatic embryos. In Vitro Cell Dev Biol Plant 33:13–19

Stachel SE, Messens E, Van Montagu M, Zambryski P (1985) Identification of signal molecules produced by wounded plant cells which activate the T-DNA transfer process in *Agrobacterium tumefaciens*. Nature 318:624–629

Stachel SE, Nester EW, Zambryski P (1986) A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. Proc Natl Acad Sci USA 83:379–383

Trick HN, Finer JJ (1997) SAAT: Sonication-assisted *Agrobacterium*-mediated transformation. Transgenic Res 6:329–337

Trick HN, Finer JJ (1998) Sonication-assisted *Agrobacterium*-mediated transformation of soybean (*Glycine max* [L.] Merrill) embryogenic suspension culture tissue. Plant Cell Rep 17:482–488

Vain P, Keen N, Murillo J, Rathus C, Nemes C, Finer JJ (1993a) Development of the Particle Inflow Gun. Plant Cell Tissue Organ Cult 33:237–246

Vain P, McMullen MD, Finer JJ (1993b) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep 12:84–88

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol Gen Genet 220:245–250

Villemont E, Dubois F, Sangwan-Norreel BS (1997) Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of Petunia: evidence of an S-phase control mechanism for T-DNA transfer. Planta 201:160–172

Wordragen MF, Dons HJM (1992) *Agrobacterium tumefaciens*-mediated transformation of recalcitrant crops. Plant Mol Biol Rep 10:12–36

Zhang L-J, Cheng L-M, Xu N, Zhao N-M, Li, C-G, Jing Y, Jia S-R (1991) Efficient transformation of tobacco by ultrasonication. Bio/Technology 9:996–997