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A simple method for isolation, liquid culture, transformation and regeneration of *Arabidopsis thaliana* protoplasts

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Summary. An efficient technique was developed for the isolation, culture, transformation and regeneration of protoplasts derived from auxin conditioned *Arabidopsis* root cultures. On an average 30% of root protoplasts underwent cell division in liquid culture and formed somatic embryo-like structures which regenerated to plants without embedding in Ca^{2+}-alginate. The protoplast protocol was applicable to different landraces of *Arabidopsis thaliana* (L.) Heynh., such as RLD, Columbia or C24. PEG-mediated DNA uptake into protoplasts using different *uidA* reporter gene constructs yielded transient gene expression in over 25% of treated cells indicating that root-derived protoplasts are suitable recipients for transformation.

Key words: *Arabidopsis thaliana* - Root derived protoplasts - Somatic embryogenesis - DNA transformation - Transient *uidA* gene expression

Abbreviations: BA, 6-benzylaminopurine; 2,4D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; IPAR, 6-(γ,γ-dimethylallylamino)purine riboside; NAA, α-naphthaleneacetic acid; *uidA*, ß-glucuronidase gene; GUS, ß-glucuronidase enzyme; CaMV, Cauliflower Mosaic Virus; nos, nopaline synthase; MES, 2[N-morpholino]ethane-sulfonicacid; PEG, polyethylene glycol; X-gluc, 5-bromo-4-chloro-3-indolyl glucuronide; MUG, 4-methyl umbelliferyl glucuronide; MU, 4-methylumbelliferone.

Introduction

A lack of techniques allowing efficient manipulation of single plant cells hampers the application of powerful molecular approaches, such as the complementation of mutations by transformation with cDNA expression libraries, or the induction of gene mutations by homologous recombination, in *Arabidopsis thaliana*. Despite numerous reports on the isolation of protoplasts from cell suspensions (Axelos et al. 1992, Doelling and Pikaard 1993, Ford 1990, Gleba et al. 1978, Xuan and Menczel 1980) and leaf mesophyll tissues (Damm and Willmitzer 1988, Karesch et al. 1991a, Masson and Paszkowski 1992, Park and Wernicke 1993), the application of protoplast techniques for regeneration and transformation of *Arabidopsis* often yields variable results. A practical bottleneck of available methods is that the induction of cell division is achieved by embedding the protoplasts into Ca^{2+}-alginate gel matrix. Although immobilization of cells simplifies the plating (Altmann et al. 1992, Damm et al. 1989), the embedding hinders the removal of contaminating bacteria during co-cultivation with *Agrobacterium*. Current techniques are also confronted with low regeneration capability and unstable ploidy of callus cultures (Negrutiu et al. 1975, Negrutiu and Jacobs 1978, Ford 1990) that is partly caused by a systemic endopolyploidy of *Arabidopsis* tissues (Galbraith et al. 1991).

A protocol given below for isolation of protoplasts from proliferating root tissues offers an improved single cell system for PEG-mediated DNA transformation, assay of transient gene expression (Altmann et al. 1992, Axelos et al. 1992, Damm et al. 1989, Doelling and Pikaard 1993) and development of *Agrobacterium* co-cultivation technology.

Materials and methods

Culture media and solutions. **0.5MS**: MS medium (Murashige and Skoog 1962) containing half concentration of macrosalts and microelements, and 3% sucrose. **Protoplast medium**: 0.5 MS medium containing 0.4 M glucose. **Enzyme solution** for protoplast isolation: protoplast medium containing cellulase "Onozuka R-10" (Serva), with or without macerozyme R-10 (Serva), as outlined in Table 1. **Ca-agar**: 20 mM $CaCl_2$ and 0.4 M glucose solidified by 1.0% agar. **Alginate solution**: 1% sodium alginate (Sigma) and 0.4 M glucose (pH 5.8). **Gelrite solution**: 0.2% gelrite (Phytagel, Sigma) and 0.4M glucose (pH 5.8). **Glucose-Magnesium** solution: 5 mM MES (pH 5.8), 15 mM $MgCl_2$ and 0.4 M glucose. **PEG** solution: 40% PEG 4000 dissolved in a solution of 0.1 M $Ca(NO_3)_2$ (pH 5.8) and 0.4 M glucose (Negrutiu et al. 1975). **MSAR1**, callus inducing

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medium: 0.5MS medium containing mg/l: 2.0 IAA, 0.5 2,4-D, and 0.5 IPAR. **RIM**, root inducing medium: 0.5MS medium supplemented with mg/l: 1.0 NAA and 3.0 IBA. **MSAR2**, regeneration medium: 0.5MS medium containing mg/l: 2.0 IPAR and 0.05 NAA (Koncz et al. 1992a). **MSAR3**, shoot elongation medium: 0.5MS medium containing mg/l: 1.0 IAA, 0.2 IBA and 0.2 BAP (Koncz et al. 1992a). All media were adjusted to pH 5.8, and supplemented with filter sterilized growth factors after autoclaving. MSAR1 and 2 media were solidified by 0.2% gelrite, whereas MSAR3 medium contained 0.8% agar.

Root culture. Seeds of *Arabidopsis thaliana* (L.) Heynh. landraces Co (Columbia), C24 and RLD were surface sterilized in a solution of 70% ethanol, 5% calcium hypochlorite and 0.02% Triton X-100, and germinated in 0.2MS medium, as described (Koncz et al. 1992a). One-week old sterile plantlets were transferred into 250 ml Erlenmeyer flasks containing 50 ml liquid 0.5MS medium, and grown by weekly subculture with continuous shaking at 100 rpm for 3 weeks.

To adapt the root tissue culture protocol (Koncz et al. 1992a, Márton and Browse 1991, Valvekens et al. 1988) to protoplast technology, proliferating root cultures were initiated by two types of growth factor treatments. To induce differentiation and growth of secondary roots, root explants excised from plantlets grown in liquid 0.5MS medium were cut into small pieces, transferred into Petri dish (5 cm) and cultured in RIM medium by continuous shaking (80 rpm). After 4-5 days of culture, new division centers for lateral roots appeared. Elongation of secondary roots and callus formation at the cut ends of root explants became advanced following 10-15 days of auxin induction. From these explants continuously proliferating root cultures were established and maintained by weekly subculture in 0.5MS medium containing 3 mg/l IBA. Chromosome counting in acetocarmine stained root tips (Altmann et al. 1992) after one year culture period revealed no alteration in the diploid (2n=10) chromosome complement (data not shown). A high frequency regeneration of normal, fertile plants obtained by plating of such root cultures on MSAR2 medium indicated, that roots growing in the presence of IBA maintained their regeneration potential for a long time period.

A second type of root culture was established in callus inducing MSAR1 medium. An intense cell division starting from the central vascular cortex of roots was apparent after 3-4 days that resulted in development of globular meristematic structures (Figure 1A and B). Root cultures showing cell proliferation and adventitious root formation were harvested after 7 to 10 days for protoplast isolation. When these root cultures were plated on MSAR2 regeneration medium, shoot differentiation from such globular structures occured within a period of 2-3 weeks.

Protoplast isolation and culture. After removing the culture medium, proliferating root cultures were suspended in protoplast enzyme solution (5 ml per Petri dish), and incubated for different durations at 24°C either with, or without shaking (80 rpm). A preliminary assay of different cell wall digesting enzymes (e.g. Onozuka cellulase types RS, R-10, SS, Driselase, and various macerozymes) indicated that cellulase Onozuka R-10 provides the best yield of protoplasts from root explants (data not shown). Optimal conditions for protoplast isolation with Onozuka R-10 were determined as outlined in Table 1. Protoplasts were purified by sequential filtering through 50 μm and 25 μm meshes, pelleted by centrifugation (50-100 g, 5 min), and washed twice with protoplast medium. The sedimented protoplasts were suspended in MSAR1 medium containing 0.4 M glucose at a density of 3-7 x 10^5 cell/ml. The protoplast suspension was distributed in 1 ml aliquotes into Petri dishes (5 cm) that were incubated at 24°C in dim light. Gradual dilution of protoplasts was performed by addition of 1 ml MSAR1 medium containing 0.4M glucose at days 5, 10 and 15 after protoplast isolation, then 2 ml MSAR1 medium was added at days 21, 27, 33 and 40 of culture.

For comparison of culture conditions, root protoplasts were also embedded in Ca-alginate or Ca-gelrite gels. After the last wash in protoplast medium (see above), the protoplasts were suspended in either alginate, or gelrite solution at a density of 3-7 x 10^5 cell/ml, and layered onto Ca-agar plates. After 45 min, solidified Ca-alginate or Ca-gelrite gel discs were released from the Ca-agar in 10ml 0.5MS medium containing 0.4M glucose by a scalpel, transferred to new Petri dish and cultured in 7 ml liquid MSAR1 medium containing 0.4 M glucose. Subculture of gel-embedded protoplasts was as described for protoplast liquid culture above.

Plant regeneration. Proliferating colonies derived from protoplasts were plated on solid MSAR1 medium when their diameter reached approximately 1 mm, and cultured at 24°C using 15 hrs light period. Regenerating shoots were individually transferred to MSAR3 medium to induce rooting. After 4-6 days plants were transferred to 0.5MS medium containing 0.5% sucrose in glass jars (700 ml) sealed by loose cotton plugs. The plants were either allowed to flower and set seed in jars, or when rooted, they were planted in soil and transferred to greenhouse.

PEG-mediated DNA uptake and transient gene expression in root protoplasts. Plasmids pGE1 and pROK2275 were used to adjust the conditions for assaying transient gene expression in root protoplasts. To construct plasmid pGE1, the promoter of the nopaline synthase gene (pnos) was isolated as a SalI-SmaI fragment from plasmid pPCV720 (Koncz et al. 1994) and inserted into SalI-SmaI sites of a polylinker sequence located upstream of a promoterless *uidA* reporter gene in plant promoter test vector pPCV812 (Walden et al. 1990). pROK2275 is a derivative of plasmid pBI121 (Jefferson et al. 1987) that carries the coding region of *uidA* gene from plasmid pRAJ275 (Jefferson 1987) linked to the promoter of CaMV 35S RNA (p35S) and the polyadenylation signal sequence of the nopaline synthase gene (pAnos)(Jefferson, unpublished). Plasmid pGE1 and pROK2275 DNAs were transformed into root protoplasts by PEG-mediated DNA uptake (Damm et al. 1989, Altmann et al. 1992). Protoplasts, isolated as described above, were incubated in protoplast medium on ice for 60 min, pelleted by centrifugation, and resuspended in Glucose-Magnesium solution at a density of 10^6 cells/ml. 300 μl aliquots of protoplasts were distributed in 12 ml glass centrifuge tubes and mixed with 20 to 50 μg linearized plasmid DNA, added in 20 to 50 μl sterile water. After 5 min incubation at room temperature, the protoplasts were gently mixed with 300 μl PEG solution. Following 30 min incubation, the protoplasts were slowly diluted by 8 ml protoplast medium and pelleted by centrifugation. After repeated washing with protoplast medium, the protoplasts were resuspended and cultured in MSAR1 medium containing 0.4 M glucose as described above.

To estimate the efficiency of DNA uptake, the transient expression of *uidA* reporter gene constructs was assayed by histochemical staining and fluorimetric determination of GUS enzyme activity (Jefferson 1987). Two days after DNA uptake, protoplasts were collected by centrifugation, and resuspended at a density of 3 x 10^5 cells/ml in GUS-staining solution containing 1 mg/ml X-gluc in 50mM Tris.HCl (pH7.0), 125mM $CaCl_2$ and 0.4M mannitol. After 24 hr incubation, the proportion of cells showing blue staining was determined by counting 1000 to 2000 protoplasts using a hemocytometer and inverted microscope. Fluorimetric GUS assays were carried out using MUG as substrate according to Jefferson (1987). After termination of the enzyme reaction by addition of 20 μl of reaction mixture to 980 μl of 0.2M Na_2CO_3, the fluorescence of samples was measured by excitation at 365nm and emission at 455nm using a Perkin Elmer LS-2B fluorimeter. The protein concentration of extracts was determined according to Bradford (1976), and the fluorescence values were converted to GUS activity units expressed in nmol MU min^{-1} mg $protein^{-1}$.

RESULTS

Protoplast isolation

Roots of plants grown in 0.5MS liquid medium, and two types of auxin conditioned root cultures were used as starting material for protoplast isolation. Protoplast isolation from roots cultured without auxin treatment proved to be practically impossible due to a very low yield. Digestion by

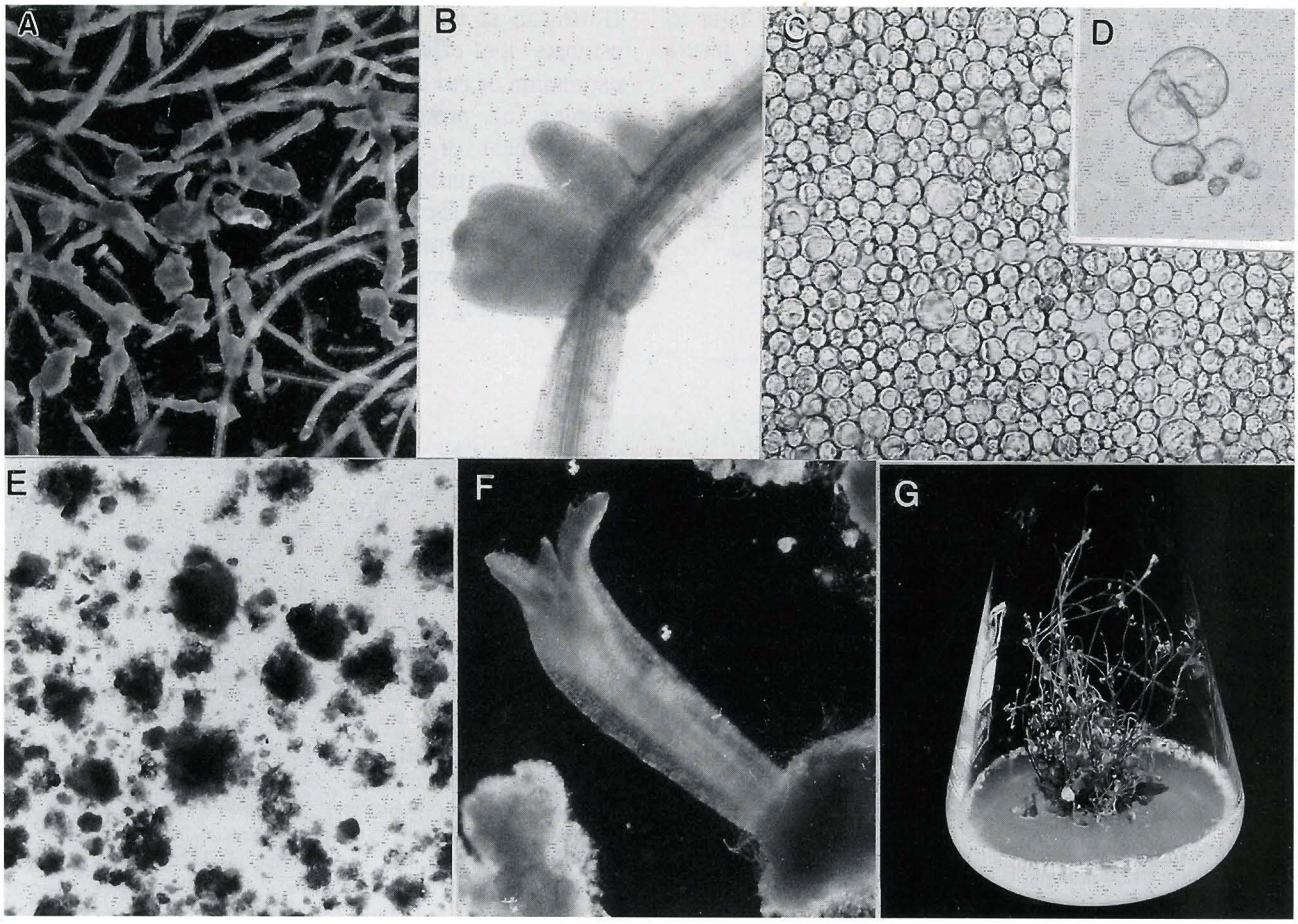

Figure 1. Isolation, culture and regeneration of *Arabidopsis* root protoplasts. A: 10-day old root culture in MASR1 liquid medium. B: Globular, meristematic structures on auxin treated roots. C: Protoplasts isolated from 7-10 day old MSAR1 root cultures. D: Root derived protoplasts after the first division. E: Colony formation in 4 week old liquid culture. F: Shoot differentiation from colonies plated on MSAR2 regeneration medium. G: Root protoplast derived, regenerated *Arabidopsis* plant.

various combinations of cellulases and macerozymes resulted in protoplast formation from cells constituting the apex of secondary roots grown in RIM medium. However, the yield of protoplasts remained below an average of 5-8 x 10^4 protoplasts/g root. In contrast, overnight digestion of roots cultured in MSAR1 medium for 7-10 days (Fig.1A and B) by 1% cellulase R-10 and 0.25% macerozyme R-10 yielded on average 1.5-3x10^7 protoplasts/g fresh weight root. Titration of enzyme concentration and duration of enzyme treatment showed that digestion of roots with 2.0-2.5% cellulase Onozuka R-10 for 3-5 hrs was optimal for protoplast release (Table 1). Over 50% of protoplasts were small, did not possess vacuoles and starch grains, thus appeared to derive from meristematic cells (Fig. 1C and D).

Protoplast culture and regeneration

Regeneration, division, colony formation and plating efficiency of protoplasts were examined using K3, MS and MSAR media, with or without embedding the protoplasts into Ca-alginate or Ca-gelrite matrices. It was found that root derived protoplasts regenerated their cell wall with or without embedding, but divided earlier and reached higher division frequencies in liquid MSAR1 medium containing 0.4 M glucose. The rate of protoplast survival and division correlated with the duration of enzyme digestion (Table 1).

A short period of digestion provided good quality of protoplasts that displayed 75% survival and 20-40% division during the first 3-5 days of culture. The density of cultured protoplasts and the schedule of dilution procedure influenced the efficiency of colony formation (Table 2). The initial high protoplast density (1x10^6 cells/ml), required for the induction of cell division, had to be gradually reduced, because undiluted protoplasts tended to aggregate and collapse. A proper dilution schedule (see Materials and methods) resulted in colonies of 20-60 cells within 4-5 weeks of culture (Fig. 1E). Plating of colonies on MSAR2 medium induced the development of embryo-like structures, over 80% of which regenerated rosette leaves in light within 2-3 weeks of culture (Fig. 1F). Because the high cytokinin concentration of MSAR2 medium inhibited the root development, rosettes of 2-5 mm were first transferred to

MSAR3 medium to induce rooting, and 4-6 days later to 0.5MS medium with 0.5% sucrose to regenerate fertile plants (Fig. 1G).

Table 1. The effect of different enzyme treatments used for protoplast isolation from roots of *Arabidopsis thaliana* (landrace RLD) cultured in MSAR1 medium for 7-10 days.

Enzyme concentration [%]		Time required for protoplast release [hr]	Protoplast survival [%]	Protoplast division [%]
Cellulase	1.0	12-16	10-25	7-10
	1.5	10-14	15-30	7-10
	2.0	6 - 8	30-75	10-25
	2.5	3 - 5	>75	20-40
Cellulase	1.0			
Macerozyme	0.25	12-16	10-25	7-10
Cellulase	1.0			
Macerozyme	0.5	12-16	10-25	7-10

Protoplast survival and division rates were scored after 5 days of culture in MSAR1 medium containing 0.4 M glucose as described (Altmann et al. 1992). The table shows a summary of data from 5 independent experiments.

Table 2. Division rate and colony formation of root derived *Arabidopsis thaliana* (landrace RLD) protoplasts in different culture conditions.

Density [10^6 cells/ml]	Dividing protoplasts [%]			Colony formation [%]		
	Liquid	Alginate	Gelrite	Liquid	Alginate	Gelrite
1.0	10-15	3 - 4	3 - 5	15-25	7 -10	7 -15
0.7	30-40	3 - 4	3 - 5	35-50	7 -10	7 -15
0.5	30-40	7 -10	7 -10	35-50	7 -15	7 -15
0.3	30-40	7 -10	7 -10	35-50	7 -15	7 -15
0.1	13-20	3 - 4	3 - 5	15-30	3 - 5	3 - 5

The rate of protoplast division was determined by inspecting 25 Petri dishes and counting 500 cells on each after 5 days of culture in 3 independent experiments. The efficiency of colony formation, that represents the proportion of protoplasts developing to microcalli, was determined in a similar fashion after 45 days of culture.

Data described above were obtained with protoplasts from *Arabidopsis* landrace RLD. The same protocol was found to be equally applicable to Columbia and C24 landraces (data not shown).

PEG-mediated DNA uptake and transient gene expression assay with root protoplasts

To assay the competence of root protoplasts in DNA transformation, plasmids pGE1 and pROK2275 were used in PEG-mediated DNA uptake experiments (Fig.2). pGE1 and pROK2275 DNAs were transformed to protoplasts as described by Axelos et al. (1990), Altmann et al. (1992), Damm et al. (1989), Doelling and Pikaard (1993), Hoffmann et al. (1993) and Karesch et al. (1991a). To estimate the efficiency of DNA uptake, the transient expression of *gus* reporter genes was assayed by histological staining of protoplasts with X-gluc and fluorimetric measurement of GUS enzyme activity using MUG as substrate. Counting of X-gluc stained cells 24 hrs after DNA uptake showed that transient expression of both reporter gene constructs resulted in detectable GUS activity in an average proportion of 28.5 ± 5.2% of transformed protoplasts (Fig. 3).

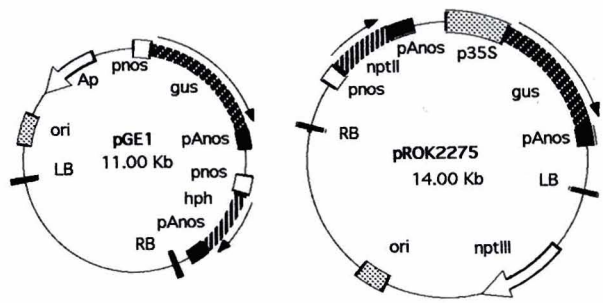

Figure 2. Schematic map of T-DNA-based plant transformation vectors pGE1 and pROK2275. pGE1: ori, ColE1 replication origin; Ap, ampicillin resistance gene; pnos, promoter of the nopaline synthase gene; gus, *uidA* (ß-glucuronidase) reporter gene; pAnos, polyadenylation sequence of the nopaline synthase gene; hph, hygromycin phosphotransferase gene; LB and RB, left and right 25 bp border repeats of the T-DNA. Other abbreviations in the schematic map of pROK2275: nptII, neomycin phosphotransferase (*aph*(3')II) gene of transposon Tn*5*; p35S, the promoter of Cauliflower Mosaic Virus 35S RNA gene; nptIII, bacterial kanamycin resistance gene of pBI121; ori, *oriV* of plasmid RK2.

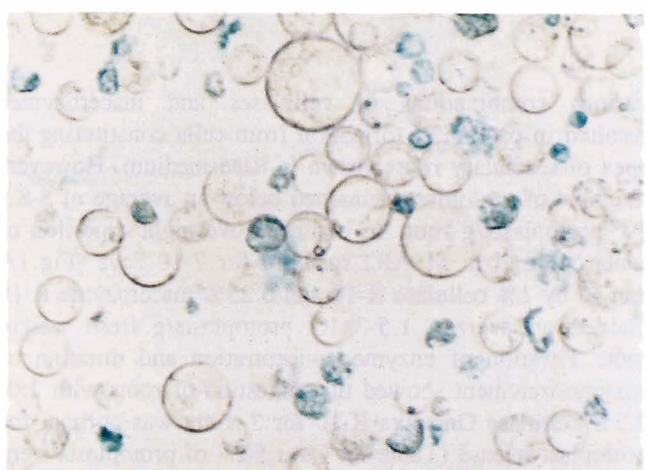

Figure 3. Assay of transient ß-glucuronidase gene expression in root derived protoplasts by histochemical staining with X-gluc. Due to precipitation and toxicity of X-gluc dye, GUS expressing protoplasts collapse during the staining procedure.

Fluorimetric measurement of GUS activity at different time periods after PEG-mediated DNA uptake (Table 3) showed a considerable variation between absolute values of transient GUS expression obtained in different experiments. The

CaMV35S promoter driven *gus* reporter gene resulted in 2 to 4-fold higher levels of GUS activity in comparison to that obtained by the pnos-*gus* construct in nondividing protoplasts harvested after 24 and 48 hrs of DNA transformation. In dividing protoplasts assayed 72 hrs after DNA uptake, the difference between GUS activities of pROK2275 and pGE1 transformed cells increased dramatically.

Table 3. Factors affecting the reproducibility of transient *gus* gene expression assay in root protoplasts.

Sample	Experiment	GUS activity pGE1	GUS activity pROK2275	pROK2275/pGE1 GUS ratio
	I.	36.2	114.0	3.15
24 hr	II.	29.6	88.8	3.0
	III.	75.6	101.2	1.34
	I.	86.8	339.6	3.91
48 hr	II.	65.3	156.8	2.40
	III.	166.2	204.0	1.22
	I.	87.1	3312.5	38.0
72 hr	II.	72.8	1180.0	16.2
	III.	654.3	2956.0	4.52

GUS activities are given in nmol MU min^{-1} mg $protein^{-1}$. In experiment III. 25 μg/ml single-stranded salmon sperm DNA was used as carrier. In all transformation experiments 1.5 x 10^6 protoplasts were transformed by 40 μg plasmid pGE1, or pROK2275 DNA.

In support of earlier observations (Damm et al. 1989, Doelling and Pikaard 1993, Karesch et al. 1991b), the data in Table 3. suggests that the use of carrier DNA during PEG mediated DNA uptake may differentially influence the levels of transient expression and integration of transforming DNAs. The difference noted between GUS activities before and after the onset of cell division also supports the results of Rasmussen and Rasmussen (1993) indicating that a proper determination of the time course of transient gene expression is necessary to reduce the standard deviation of data derived from protoplast transformation assays.

DISCUSSION

Efficient use of the *Arabidopsis* protoplast system is hindered by several technical problems. In spite of careful preculture of donor plants (Masson and Paszkowski 1992), density fractionation of protoplasts (Park and Wernicke 1993), and optimization of protoplast plating by embedding in Ca-alginate gels (Damm and Willmitzer 1988, Karesch et al. 1991a), the best efficiencies of colony formation obtained with leaf mesophyll protoplasts are between 0.5 and 10%. From a heterogeneous population of leaf cells (Park and Wernickle 1993) only protoplasts from elongating, young mesophyll cells give rise to cell division when embedded in Ca-alginate (Masson and Paszkowski 1992, Damm and Willmitzer 1988, Karesch et al. 1991a, Masson and Paszkowski 1992). In contrast, plating efficiencies ranging from 40% to 60% (Doelling and Pikaard 1993, Ford 1990, Xuan and Menczel 1980) were reported for protoplasts derived from cell suspensions.

A major problem with cell suspensions is that a long duration of cultures in callus media containing high levels of 2,4D drastically reduces the efficiency of shoot differentiation and yields regenerated plants with aberrant chromosome complements. The leaf protoplast system is also biased due to systemic endopolyploidy of mesophyll tissues.

A need for an improved protoplast system is underlined by the success of T-DNA insertional mutagenesis (see for review: Bechtold et al. 1993, Feldmann 1991, Koncz et al. 1989, 1992ab; Márton and Browse 1991, Valvekens et al. 1988) that would be potentially applicable to e.g. isolation of gain of function mutations (Hayashi et al. 1992) using different selection schemes in Petri dishes. To approach this goal, we established a novel protoplast system using auxin conditioned root cultures. Auxin induced proliferation of root vascular meristem in liquid culture results in globular, meristematic structures which, when induced by cytokinin, differentiate to rosette leaves at near 100% frequency. A brief cellulase treatment preferentialy releases protoplasts from such meristematic centers. Root protoplasts derived from meristematic cells undergo cell division within 3-5 days in MSAR1 liquid culture yielding an average division rate of 30%. Following gradual dilution through a period of 4-6 weeks, protoplast derived cell suspensions can be plated on MSAR2 medium and show high frequency regeneration. By starting from 1g root culture, this protocol yields reproducibly 3 x 10^6 protoplasts, from which after 6 weeks over 3 x 10^5 regenerating colonies can be obtained. Transient ß-glucuronidase gene expression assays indicate that root protoplasts are suitable recipients for DNA uptake. Although the auxin sensitivity of *Arabidopsis* landraces varies considerably (Koncz et al. 1992a, Márton and Browse 1991, Valvekens et al. 1988), the protocol described above was found to be applicable to different *Arabidopsis* landraces, such as RLD, Columbia and C24. In conclusion, the root protoplast protocol offers a novel liquid culture and regeneration system that appears to be suitable for development of an *Agrobacterium* co-cultivation method.

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