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 glucuronide; MU, 2[N-morpholino]ethanesulfonic acid; enzyme; embedding in glucuronide; MUG, 4-methylumbelliferone.

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, β-glucuronidase gene; GUS, β-glucuronidase enzyme; CaMV, Cauliflower Mosaic Virus; nos, nopaline synthase; MES, 2[N-morpholino]ethane-sulfonic acid; PEG, polyethylene glycol; X-gluc, 5-bromo-4-chloro-3-indolyl glucuronide; MUG, 4-methylumbelliferyl 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 Ca2+-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 cocultivation 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).


Materials and methods

Culture media and solutions. 0.5MS: MS medium (Murashige and Skoog 1962) containing half concentration of macromals 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 CaCl2 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 MgCl2 and 0.4 M glucose. **PEG solution**: 40% PEG 4000 dissolved in a solution of 0.1 M Ca(NO3)2 (pH 5.8) and 0.4 M glucose (Negrutiu et al. 1975). **MSAR1**, callus inducing...
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 as outlined in Table 1.

Protoplast isolation and culture. Root cultures were initiated by two types of growth factor treatments. To construct plasmid pGEL1, the promoter of the nopaline synthase gene (pnos) was isolated as a SalI-Smal fragment from plasmid pCV720 (Koncz et al. 1994) and inserted into SalI-Smal 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 3SS RNA (p3SS) and the polyadenylation signal sequence of the nopaline synthase gene (pAnos)(Jefferson, unpublished). Plasmid pGEL1 and pROK2275 DNAs were transferred 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 fluorometric 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 10^6 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. Fluorometric 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 NaCO_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
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 $5 \times 8 \times 10^4$ 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 $1.5 \times 3 \times 10^7$ 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 ($1 \times 10^6$ 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 MSARl medium for 7-10 days.

<table>
<thead>
<tr>
<th>Enzyme concentration</th>
<th>Time required for protoplast release [hr]</th>
<th>Protoplast survival [%]</th>
<th>Protoplast division [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulase 1.0</td>
<td>12-16</td>
<td>10-25</td>
<td>7-10</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>10-14</td>
<td>15-30</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>6 - 8</td>
<td>30-75</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>3 - 5</td>
<td>&gt;75</td>
</tr>
<tr>
<td>Cellulase 1.0</td>
<td>Macerozyme 0.25</td>
<td>12-16</td>
<td>10-25</td>
</tr>
<tr>
<td></td>
<td>Cellulase 1.0</td>
<td>1.0</td>
<td>12-16</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>12-16</td>
<td>10-25</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>12-16</td>
<td>10-25</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>12-16</td>
<td>10-25</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>12-16</td>
<td>10-25</td>
</tr>
</tbody>
</table>

Protoplast survival and division rates were scored after 5 days of culture in MSARl 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.

<table>
<thead>
<tr>
<th>Density [10^6 cells/ml]</th>
<th>Dividing protoplasts [%]</th>
<th>Colony formation [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liquid Alginate Gelrite</td>
<td>Liquid Alginate Gelrite</td>
</tr>
<tr>
<td>1.0</td>
<td>10-15</td>
<td>3 - 4</td>
</tr>
<tr>
<td>0.7</td>
<td>30-40</td>
<td>3 - 4</td>
</tr>
<tr>
<td>0.5</td>
<td>30-40</td>
<td>7 -10</td>
</tr>
<tr>
<td>0.3</td>
<td>30-40</td>
<td>7 -10</td>
</tr>
<tr>
<td>0.1</td>
<td>13-20</td>
<td>3 - 4</td>
</tr>
</tbody>
</table>

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 pGEl and pROK2275 were used in PEG-mediated DNA uptake experiments (Fig. 2). pGEl 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 pGEl and pROK2275. pGEl: ori, ColEl replication origin; Ap, ampicillin resistance gene; pnos, promoter of the nopaline synthase gene; gus, nldA (b-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 Tn5; p35S, the promoter of Cauliflower Mosaic Virus 35S RNA gene; nptIII, bacterial kanamycin resistance gene of pBlI21; ori, oriV of plasmid RK2.

Figure 3. Assay of transient b-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.

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

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 10⁶ protoplasts were transformed by 40 µg plasmid pGE1, or pROK2275 DNA.

In support of earlier observations (Damm et al. 1989, Doelling and Pikaard 1993, Kareesch 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, Kareesch 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 Wernicke 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, Kareesch 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 preferentially 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 10⁶ protoplasts, from which after 6 weeks over 3 x 10⁶ regenerating colonies can be obtained. Transient β-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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