Transformation of *Physcomitrella* protoplasts

Overview

For the transformation of *Physcomitrella*, polyethylene glycol (PEG) mediated transformation of protoplasts is almost exclusively applied (Schaefer et al., 1991) even though partical bombardment is as well applicable to transform *Physcomitrella* (Sawahel et al., 1992; Cho et al., 1999; Bezanilla et al., 2003). Compared to the PEG-mediated transformation procedure the application of the biolistic method requires less DNA. Furthermore, it was shown that the transformation efficiency obtained by particle bombardment seems to be ten times higher when compared to PEG-mediated transformation of protoplasts (Cho et al., 1999). The transformation using particle bombardment is carried out by shooting the DNA onto protonemal tissue growing on cellophane. After the transformation transgenic cells can be isolated by blending the tissue and subsequent plating onto selective medium (Cho et al., 1999). For PEG-mediated transformation protoplasts are obtained by using the enzyme mixture Driselase for cell wall digestion of *Physcomitrella* protonema tissue (Grimsley et al., 1977). Protocols have been described starting with plant material grown on solidified medium using ammonium tartrate as a medium supplement (Ashton and Cove, 1977). The addition of ammonium tartrate to liquid culture medium results in predominant growth of the moss protonema in the chloronema stage, which is thought to be the best source for protoplast isolation (Jenkins and Cove, 1983). The cell walls of subsequent developmental stages seem not to be sufficiently susceptible to cell wall degrading enzymes. The reduction of the calcium concentration or lowering the pH of the medium also result in increased protoplast yields (Rother et al., 1994; Hohe and Reski, 2002). In general, for protoplast preparation *Physcomitrella* liquid cell cultures are commonly used.

The DNA constructs used for the generation of transgenic *Physcomitrella* lines should contain a selection marker cassette to allow an easy screening procedure to be used for the identification of plants that have undergone an integration of the DNA construct. The most commonly used selection marker comprises the *nptII* gene encoding the enzyme neomycin phosphotransferase which confers resistance to geneticin (G418). Alternatively, the *aph* (aminoglycoside phosphotransferase) gene conferring resistance to kanamycin, the *hph* (hygromycin phosphotransferase) gene mediating hygromycin resistance or the *ble* (phleomycin-binding protein) gene conferring resistance to zeomycin may be used (Schaefer et al., 1991; Imaizumi et al., 2002; Kasahara et al., 2004; Mittmann et al., 2004). To drive expression, the resistance genes are usually flanked by the promoter and terminator sequences of the cauliflower mosaic virus (CaMV) *35S RNA* or the *nopalin synthase* (*nos*) gene from *Agrobacterium tumefaciens*. The 35S-driven selection marker cassette can be subcloned from the pRT99 plasmid vector (Topfer et al., 1988). Besides these viral and

bacterial promoters, plant-derived promoter regions were shown to be suitable driving expression of transgenes in *Physcomitrella*. These include heterologous promoters of the rice actin 1 and maize ubiquitin genes as well as homologous promoters derived from *Physcomitrella* alpha-1,3-fucosyltransferase, beta-1,2-xylosyltransferase and beta-tubulin genes (Bezanilla et al., 2003; Horstmann et al., 2004; Jost et al., 2005). Before transformation of *Physcomitrella* protoplasts it is recommended to linearize the DNA construct using appropriate restriction enzymes. The transformation of circular plasmids may result in extrachromosomal replication and the generation of unstable transformants (Ashton et al., 2000).

For large-scale protoplast isolation, the use of pH-controlled bioreactor cultivation is most suitable (Hohe and Reski, 2002). Alternatively, plant material grown under standard growth conditions in Erlenmeyer flasks containing Knop medium with reduced calcium concentrations (1/10 $Ca(NO_3)_2$) or reduced pH (4.5) can be used for protoplast isolation. When starting with approximately 200 ml of these cultures, protoplast yield will be sufficient for at least ten independent transformations. The moss material is harvested by filtration through a 100 µm protoplast sieve. Digestion of the cell walls is carried out using 2% Driselase enzyme mixture dissolved in 0.5 M mannitol. The digested moss material is successively passed through sieves with a mesh size of 100 and 45 µm and carefully washed in 0.5 M mannitol once. The transformation procedure can be performed as described by Strepp et al. (1998). After transformation the protoplasts are cultivated in Knop medium supplemented with 3% mannitol and 5% glucose and grown under reduced light conditions in the dark for 12-16 h followed by cultivation in the same medium for ten days under normal growth conditions. During this time regeneration of protoplasts is initiated. Afterwards, the regenerating protoplasts are plated onto Petri dishes containing solidified Knop medium. The medium should be covered with a sterile cellophane sheet which facilitates the transfer of the regenerating plants at subsequent stages. To select stably transformed plants the cellophane with the cultures is transferred onto solidified medium containing the appropriate antibiotic for two week, followed by a two weeks release period on medium without antibiotic and a second selection period of two weeks. Plants surviving the second round of selection are considered to be stable transformants. Detailed analysis of those plants by PCR revealed that more than 98% had stably integrated the transgene into the genome (Schween et al., 2002).

Reagents and material

1. Knop medium with 1/10 $Ca(NO_3)_2$ [alternatively medium with a reduced pH value of 4.5 can be used]: Prepare 1 l Knop medium as described above, but add 1 ml of the

$Ca(NO_3)_2$ stock solution instead of 10 ml. This modified medium is used to set up suspension cultures for small-scale transformation.

2. For large-scale protoplast isolation use of pH-controlled bioreactor cultivation is recommended. The bioreactor cultures are grown at pH 4.5 with HCl.
3. Mannitol solution (0.5 M): Dissolve 91.1 g mannitol in 800 ml H_20, adjust to pH 5.8 with KOH or HCl, add H_20 to 1 l. The osmolarity of the solution should be approximately 560 mOs. Sterilize by autoclaving.
4. 4% (w/v) Driselase solution: Dissolve 0.4 g Driselase (Sigma) in 10 ml 0.5 M mannitol in a 15 ml Falcon tube and vortex briefly. Keep the solution light protected by covering the tube with aluminum foil and incubate for complete dissolving of the enzyme for 1 h at room temperature on a rotating table. Centrifuge at 2300 x g for 10 min and filter sterilize the supernatant using a 0.22 µm filter.
5. 3M medium: Dissolve 3.05 g $MgCl_2$, 1 g MES (2-(N-morpholino)-ethanesulfonic acid), 87.4 g mannitol in 800 ml H_20, adjust pH to 5.6 with KOH. The osmolarity of the solution should be approximately 580 mOs. Sterilize by autoclaving.
6. PEG solution (40%): Weigh out 10 g PEG 4000, add 3M medium to 25 ml, sterilize by filtration through a 0.22 µm filter.
7. DNA solution: Digest the DNA construct with a suitable restriction enzyme producing a linearized DNA fragment containing the gene of interest and the *nptII* selection marker cassette. Purify the DNA by standard ethanol precipitation and dissolve the construct at a final concentration of 0.25 µg/µl in 100 µl 0.1 M $Ca(NO_3)_2$.
8. Regeneration medium: For 1 l use 10 ml of each stock solution described for preparation of Knop medium (KH_2PO_4, KCl, $MgSO_4$ x 7 H_2O, $Ca(NO_3)_2$), add 12.5 mg $FeSO_4$ x 7 H_2O, add 50 g glucose, add 30 g mannitol, adjust pH to 5.8 with KOH. Adjust the osmolarity to approximately 540 mOs using mannitol. Filter sterilize through a 0.22 µm filter.
9. Selection medium: Prepare standard solid Knop medium and autoclave. Let cool down to 60°C and add 500 µl G418 sulfate solution (50 mg/ml; Promega) to reach a final G418 concentration of 25 µg/ml. Pour the medium into petri dishes. The plates can be stored up to four weeks at 4°C.
10. Counting chamber, glass tubes with screw cap, protoplast sieves (100 µm, 45 µm), petri dishes (9 cm diameter), 6 well cell culture plates, pipettete tips with cut tips, forceps, pipetteboy, rotating table, sterile cellophane sheets (Schütt, Hamburg, Germany).

Method

1. Small-scale preparation of moss material: Starting from a standard *Physcomitrella* liquid culture grown in Erlenmeyer flasks inoculate 200 ml 1/10 $Ca(NO_3)_2$ Knop medium with moss material corresponding to 10 mg dry weight. Grow the culture in a 500 ml

Erlenmeyer flask under standard conditions. After four days exchange the medium and cultivate for another three days. The complete culture is used for protoplast isolation.

2. Large scale preparation of moss material: *Physcomitrella* plants are grown in bioreactors with reduced pH (pH 4.5). For protoplast isolation 100-200 ml of these cultures are used.
3. The respective moss material is harvested by filtration through a 100 µm protoplast sieve. Transfer the material to a petri dish (9 cm diameter) and add 8 ml 0.5 M mannitol solution. Add 8 ml of the Driselase stock solution. The final concentration of Driselase is 2% (w/v). Seal the petri dish with Parafilm and cover it with aluminum foil. Incubate for 45 min at room temperature on a rotary shaker.
4. Pass the moss material successively through sieves with a mesh size of 100 and 45 µm (Wilson, UK) and divide the filtrate into two glass tubes. Centrifuge the filtrate for 10 min at 45 x g in the glass tube. Discard the supernatant and wash the protoplasts by resuspending each pellet in 10 ml of 0.5 M mannitol by gentle rolling of the glass tubes between your hands. Centrifuge again for 10 min at 45 x g, discard the supernatant and resuspend each pellet in 5 ml 0.5 M mannitol. Combine both samples.
5. Take a 100 µl aliquot with a cut pipettete tip and determine the protoplast number using a counting chamber. Meanwhile centrifuge the combined protoplasts again for 10 min at 45 x g. Discard the supernatant and re-suspend the pellet in 3M medium adjusting a density of 1.2×10^6 protoplasts/ml.
6. Transfer the DNA solution into a glass tube and carefully add 250 µl of the protoplast solution using a cut pipettete tip. Add 350 µl of the PEG solution and mix gently by rolling the tube. Incubate the mixture for 30 min at room temperature and mix again every 5 min by gentle rolling. Dilute the mixture with 3M medium every 5 min adding 1, 2, 3, and 4 ml, respectively, and carefully mix the solution after each step by rolling the tube.
7. Centrifuge for 10 min at 45 x g, discard the supernatant and re-suspend the protoplasts in 3 ml regeneration medium. Transfer two times 1.5 ml of the protoplast solution into two wells of a 6-well culture plate. Seal the plate with Parafilm and incubate overnight in the dark at 25°C followed by incubation under normal growth conditions for 10 d. During this time regeneration of protoplasts is initiated.
8. Plate 1 ml of the regenerating protoplasts onto a 9 cm petri dish containing solidified Knop medium. The medium should be covered with a sterile cellophane sheet which facilitates the transfer of the regenerating plants at subsequent stages. Grow the cultures under standard conditions for three days.
9. To select stable transformed plants transfer the cellophane with the cultures onto solidified Knop medium containing 25 µg/ml G418 for two weeks, followed by a two weeks release period on medium without G418 and a second selection period of two weeks. Plants surviving the second round of selection are considered to be stable

transformants. Detailed analysis of those plants by PCR have shown that more than 98% had stably integrated the transgene into the genome (Schween et al., 2002).

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