Fate of a Mutant Macrochloroplast in Somatic Hybrids

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Summary

Protoplast isolation from a moss, Physcomitrella patens (Hedw.)B.S.G., was optimized regarding preculture of plants, digestion procedure and isolation protocol. PEG-mediated protoplast fusion in batch experiments resulted in at least 29% fusion products. All of these appeared to be one-to-one fusions. In about 50% of the fusions nuclei fused within 90 minutes as well. Protoplast fusion with wild-type and chloroplast mutant PC22 was performed. Fusion products harbouring wild-type plastids as well as a giant mutant chloroplast were transferred to feeder-cultures in millicells and fate of the macrochloroplast was observed. Analysis of regenerants revealed that deficiencies in plastid division and in cellular differentiation are two uncoupled genetic traits in moss mutant PC22.

Key words: Physcomitrella patens (Hedw.)B.S.G.; cytokinin; moss; plant regeneration; plastid division; protoplast.

Abbreviations: DAPI = 4',6-diamidino-2-phenylindole; MES = 2(N-morpholino)ethanesulfonic acid; PEG = polyethylenglycol; PVP = polyvinylpyrrolidone.
Introduction

The haploid protonemata of mosses are well established plant systems for studying the morphogenetic potential of different stimuli such as cytokinin (Hahn and Bopp, 1968) and light (Bopp, 1959, 1981). Furthermore, the availability of differentiation mutants and protoplast fusion techniques facilitate the genetic analysis of morphogenesis (Grimsley et al., 1977a, b; Watts et al., 1985; Bhatla and Bopp, 1985; Mejia et al., 1988; Hansen et al., 1988; Ye et al., 1989). In order to identify genes involved in chloroplast division, we analyse Physcomitrella patens (Hedw.)B.S.G. (Reski and Abel, 1985) with special emphasis on the mutant PC22, which is defective in chloroplast division. Thus, the majority of cells possess only one giant chloroplast (Reski et al., 1986). The mutant macrochloroplast is a mechanical obstacle which is divided during cell division in a hitherto unknown manner: It is severed by the enlarging cell plate, resulting in abnormal cross walls, less orderly branching of the protonema, and retarded growth (Abel et al., 1989; Reski et al., 1992). The defect in plastid division can be partially compensated for by cytokinin (Reski et al., 1986) and by blue light (Reski and Abel, 1992). Cytokininduced chloroplast division is accompanied by a transient accumulation of several plastid polypeptides and a differential regulation of plastid gene expression (Reski et al., 1991; Kasten et al., 1992). However, we do not know, whether the lack in chloroplast division is due to a nuclear or to a plastid mutation. As mosses are self-fertile haplonts and, moreover, PC22 is not only a chloroplast mutant, but also a sterile differentiation mutant, conventional genetic analysis is impossible. We report here on our approach to this problem via somatic hybridization techniques.

Materials and Methods

Plant material and culture conditions

The wild-type strain of Physcomitrella patens (Hedw.)B.S.G. and mutant PC22 have been characterized previously (Reski et al., 1986; Abel et al., 1989; Reski et al., 1991). Protonemata were grown under controlled conditions (growth chamber at 25 ± 1°C; light pro-
vided from above by two Philips TL 65W/25; light flux of 32 μmol m⁻² s⁻¹; light-dark regime 16:8 h; Reski et al., 1991) in modified liquid Knop medium (0.25 g/L KH₂PO₄; 0.25 g/L KCl; 0.25 g/L MgSO₄·7H₂O; 1.0 g/L Ca(NO₃)₂·4H₂O; 12.5 mg/L FeSO₄·7H₂O; pH 5.8; Reski and Abel, 1985).

**Protoplast isolation, fusion and regeneration**

1 g of 6–10 days old protonemata were digested by agitated incubation for 15 hours in the dark in 0.5% Driselase (Sigma) in 0.5 M mannitol. The suspension was passed through sieves (Wilson) of 100 μm and 45 μm/40 μm pore size and centrifuged for 5 minutes at 70 g. The pellet was solubilized in 10 mL of 20% sucrose in CPW salts (1.48 g/L CaCl₂·2H₂O; 27.2 mg/L KH₂PO₄; 101 mg/L KNO₃; 312 mg/L MgSO₄·7H₂O; 976 mg/L MES; 10 g/L PVP-10 (Sigma P-6755); pH 5.6; according to Banks and Evans, 1976), topped with 0.5 mL W5 solution (18.4 g/L CaCl₂·2H₂O; 9 g/L NaCl; 1 g/L glucose; 0.8 g/L KCl; pH 5.6; according to Menzel et al., 1981) and centrifuged for 10 minutes at 150 g. Protoplasts from the interface were washed twice by centrifugation in W5, and their concentration was adjusted. PEG 1500, 4000 and 6000 (Merck), respectively, in 50 mM CaCl₂ was adjusted to pH 8.0 and was sterilized by filtration (Sartorius minisart; 0.2 μm). Fusions were performed in petri dishes with 500 μL PEG and 250 μL protoplast suspension. After 15 minutes 1 mL regeneration medium (Knop medium, 3% mannitol, 5% glucose, pH 5.8; Batra and Abel, 1981) was added, followed by 2 mL and 6 mL after 10 minutes each. Fusion products were selected individually and transferred to Millicells (Millipore) in 3 cm petri dishes containing 3 mL regeneration medium. 0.1 g vigorously growing protonema served as feeder. After 24 h incubation in the dark, protoplasts were transferred to normal growth conditions. DAPI-staining was carried out according to Ye et al. (1989).

**Results**

**Protoplast isolation**

Based on the protocols of Grimsley et al. (1977 a, b) and Hansen et al. (1988) we optimized the following parameters: 1. Digestion conditions, 2. purification, and 3. preculture of the plant material.

1. Preplasmolysis in 0.5 M mannitol and incubation for 3 hours in 2% Driselase/0.4 M mannitol resulted in a high number of protoplasts which were contaminated with cell debris. An incubation for 15 hours in 0.5% Driselase/0.5 M Mannitol gave the same results (data not shown). Furtheron, we used this protocol as it is less expensive. Moreover, it is more convenient as protoplast purification, fusion and selection of fusion products can be performed in one day. 2. Protoplasts could be purified through sterile sieves with 45 μm (WT) or 40 μm (PC22) pore size, respectively, and floated in 20% sucrose/CPW salts. As cells appeared to be stressed by the sucrose, we topped it with W5 wash medium. Thus, pure and viable protoplasts (Fig. 1 a, b) could be collected from the interface. Nevertheless, the yield was small, as the majority of protoplasts pelleted or stuck in the sucrose (Fig. 2), glueing to one another. 3. When plants were precultured in modification of Saxena and Rashid (1981) for 7 days with 10% of the Ca(NO₃)₂ amount, cell wall digestion was complete, and protoplasts lost their sticky behaviour, resulting in significant more pure protoplasts (Fig. 2 b). Thus, we obtained routinely 2 × 10⁶ protoplasts per gram protonemata and even obtained them from leafy gametophores (Fig. 3).

As a control we substituted Ca(NO₃)₂ by equimolar amounts of NH₄NO₃ or KNO₃, respectively. In the first case, digestion was inefficient, but protoplasts did not glue

Fig. 1: Isolated protoplasts of (a) wild-type and (b) mutant PC22 of *Physcomitrella patens*. Bar represents 50 μm.

Fig. 2: Centrifuge tubes with floated protoplasts of protonemata grown on modified Knop-medium (a), and of protonemata precultured on medium with reduced (10%) Ca(NO₃)₂ content (b).
Fig. 3: Protoplasts from a leafy gametophore. Plants were precultured in reduced Ca\(^{2+}\) concentrations.

Together, whereas in the latter no differences to protoplast isolations from plants precultured on reduced Ca(NO\(_3\))\(_2\) were discernible. Therefore, culture in NH\(_4\)\(^{+}\) does not only alter cell shape (Ashton and Cove, 1977) but hampers Driselase-mediated cell wall digestion, whereas high Ca\(^{2+}\) concentrations result in incomplete cell wall digestion and gluing of protoplasts in P. chemonema.

Protoplast fusion

In order to obtain the highest yield of one-to-one fusions even during mass fusion, we optimized the following parameters, based on the protocol of Grimsley et al., 1977 b: 1. Molecular weight of the PEG, 2. final concentration of the PEG, and 3. protoplast density during fusion.

1. From all tested kinds of PEG only PEG 4000 yielded numerous fusions, which remained single after dilution. Therefore, this type of PEG was used in subsequent experiments. 2. PEG 4000 in final concentrations of 25%, 27.5%, 33.5%, 37.5%, and 40% with 1–2 × 10^6 protoplasts/mL gave no differences during fusion. For mere practical reasons we furtheron used PEG 4000 in f.c. of 33.5%. 3. We added 250 µL protoplast in concentrations of 1 × 10^7, 5 × 10^7, 1 × 10^8, 5 × 10^8, and 1 × 10^9 protoplasts/mL, respectively, to 500 µL PEG. With 1 × 10^7 and 5 × 10^8 protoplasts/mL fusions and cell aggregates could be detected rarely (Fig. 4 a), while their number significantly increased with 1 × 10^8 and 5 × 10^9 protoplasts/mL (Fig. 4 b). Upon dilution the aggregates crumbled and gave rise to numerous fusions. With 1 × 10^9 protoplasts/mL we predominantly obtained large cell aggregates (Fig. 4 c) which did not crumble after dilution. Therefore, subsequent fusion experiments were performed with 2–3 × 10^9 protoplasts/mL.

Analysis of somatic hybrids

Having optimized the protocols for protoplast isolation and fusion, we fused protoplasts from wild-type and the chloroplast mutant in a 1:1 ratio. In order to analyse the fusion process, we took samples at different times, starting five minutes after transfer of the protoplasts to PEG. Cells were fixed and stained with DAPI. We randomly analysed 500 protoplasts in each sample according to the number of nuclei per cell. While in controls no protoplast with more than one nucleus was discernible, 29% of them had two nuclei 5 minutes after PEG treatment. None had three or more nuclei. Thus, our optimized protocol for batch experiments yielded 29% one-to-one fusions. The number of binucleate protoplasts decreased over time to 14% after 90 minutes (Fig. 5), indicating nuclei fusion in more than 50% of the fusions within 1.5 hours.

We transferred 70 cells harbouring numerous wild-type plastids as well as one mutant macrochloroplast (Fig. 6 a) individually to semi-single culture. 80% of these fusions remained viable for more than two days. During this time, all plastids were concentrated around the nucleus, and individual chloroplasts could hardly be detected (Fig. 6 b). Some cells showed budding with at least part of the macrochloroplast in the constriction (Fig. 6 c). Protoplasts became asym-
Fig. 5: Plot of the percentage of binucleate protoplasts against the time in minutes after protoplast fusion by PEG-treatment. Five minutes after PEG treatment 29% of all protoplasts were binucleate. This figure was taken as 100%. 500 protoplasts per time point were randomly analysed.

metric and four days after fusion, first divisions occurred, resulting in a new outgrowth. Several fusants divided less than five times, exhibiting massive budding and necrosis. Thus, 20% of the fusants (= 14 fusion products) could be regenerated to whole plants. As judged by DAPI-fluorescence, all of them contained one single nucleus with a DNA content significantly higher than wild-type or mutant alone. Eleven of the somatic hybrids exhibit normal chloroplasts and protonema differentiation. Nevertheless, they remain unfertile. One somatic hybrid possesses normal chloroplasts but is impeded in bud formation. Two somatic hybrids exhibit macronuclear chloroplasts. These plants form buds and even leafy gametophores (Fig. 7).

Discussion

Somatic hybridization of moss protoplasts in batch experiments has been repeatedly reported (Grimsley et al., 1977 a, b; Watts et al., 1985; Bhatla and Bopp, 1985). Even microtechniques such as one-to-one electrotusion of selected mutant protoplasts have been established for Funaria hygrometrica (Mejia et al., 1988) and Physcomitrella patens (Hansen et al., 1988). In our current effort we aimed to work out a protocol which does not need expensive equipment, but nevertheless frequently results in numerous one-to-one fusions, thus combining the advantages of the «mass-approach» with those of the «micro-approach».

One of the problems when working with mosses is their rigid cell wall, which impedes digestion with any other enzyme combination than Driselase. Even then protoplasts are not pure enough to be fused without problems. Therefore, several authors purified moss protoplasts by sedimentation only (Grimsley et al., 1977 a, b; Batra and Abel, 1981; Burgess and Linstead, 1981) and even digested them with protease K prior to fusion (Mejia et al., 1988; Hansen et al., 1988). To circumvent these rigid methods, we optimized protocols for Physcomitrella and gained three significant advantages: 1. For the first time protoplasts could be obtained even from leafy gametophores and protoplast yields were significantly higher than hitherto reported. 2. Protoplasts could be purified by floatation in sucrose, as they were pure enough to diminish gluing. 3. With these pure and viable protoplasts we obtained at least 29% one-to-one fusions in batch experiments. No multi-fusion event could be detected, while in former reports 40% of the fusants were more than diploid (Grimsley et al., 1977 b); a result that significantly hampers genetic analyses.

Fig. 7: Leaflet from a WT(+)PC22 somatic hybrid with mutant macrochloroplasts. Bar represents 50 μm.

Fig. 6: WT(+)PC22 fusants in Millicell culture a) immediately after transfer, b) and c) two days after transfer. Note that the giant chloroplast is sorted out in a constriction in the fusant depicted in c). Bar represents 25 μm.
We intended to allocate the mutation which impedes plastid division either to nuclear or to plastid DNA and, therefore, analysed the fate of a mutant macrochloroplast in WTI(+)/PC22 somatic hybrids. Our anticipations were the following: 1. All somatic hybrids would have wild-type plastids, if the mutant macrochloroplast is due to a recessive nuclear mutation as described for some chloroplast mutants of Arabidopsis thaliana (Mourad and White, 1992). 2. A mixture of plastids would occur in the fusants with a subsequent sorting out of the macrochloroplast, if the mutant plastid is due to a plastome mutation.

Nevertheless, our results did not exactly fulfill one of these expectations: First of all, we could not identify individual plastids in the original fusion product, as all plastids were concentrated around the nucleus. Surprisingly, every new outgrowth contained only one type of plastid, the vast majority of them small wild-type chloroplasts. As 12 regulators harbour wild type plastids and only 2 have mutant chloroplasts, our experiments seem to favour a nuclear mutation as cause for the lack in plastid division. According to this assumption both variant plants occur because parts of the dominant wild-type genome had been inactivated. Plant tissue culture generally leads to somaclonal variations (Wang et al., 1992) and somatic hybrids may exhibit chromosomal losses (Gléba and Sytnik, 1984; Kumar and Cocking, 1987; Templar et al., 1991). Nevertheless, nothing is known about chromosome behaviour in moss somatic hybrids and chromosomal losses in these cases appear to be unlikely as moss protoplasts regenerate to differentiated plants without passing an unstable callus phase. Furthermore, chromosome losses are rare in intraspecific hybrids of higher plants.

Our results may also be interpreted in favour of a plastid mutation that impedes plastid division: Fusants show massive budding and several constrictions contained at least parts of the mutant macrochloroplast, indicating its persistence after introduction of the wild-type nucleus. The high percentage of regenerants with wild-type plastids in the first outgrowth may then be due to a sorting out of the giant chloroplast in the very first cell. This could have happened in two ways: 1. Several fusants showed massive budding with the giant chloroplast in these dying constrictions. 2. It is well documented that the PC22 chloroplast is a mechanical obstacle during cytokinesis (Abel et al., 1989; Reski et al., 1992) and it is easy to imagine that it is an obstacle during asymmetric cell formation and subsequent division as well.

Thus, we were unable to unequivocally allocate the mutation which hampers plastid division utilizing somatic hybridization techniques. As there are no equivalent mutants described so far, we got no clue from comparable experiments with other plant species. Therefore, we will perform mutant curing experiments with wild-type organellar DNA to address this problem. However, two WTI(+)/PC22 plants harbouring mutant plastids produce wild-type like leaflets. Additionally, one somatic hybrid with wild-type plastids is impeded in bud formation. This is evidence for the deficiencies in plastid division and in cellular differentiation being two uncoupled genetic traits in Physcomitrella mutant PC22.

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References


