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Short communication

Production of somatic hybrids of moss by electrofusion

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Summary. Mixtures of protoplasts of two auxotrophic mutants of *Physcomitrella patens,* one requiring thiamine (aneurine), the other *p*-aminobenzoic acid, have been subjected to electrofusion. The protoplasts were aligned in an alternating electric field (500 KHz, 20 V RMS/cm) and induced to fuse by a brief DC pulse (800 V/cm, time constant lms). After culture, first on complete medium and then on selective medium, hybrid plants were obtained at a frequency of 3%. One hybrid with a morphology typical of polyploidy was also observed.

The use of electric pulses to fuse animal cells and plant and microbial protoplasts appears to offer marked advantages over the chemical methods of fusion now in common use (Keller and Melchers 1973; Kao and Michayluk 1974): fusion of dikaryons is completed within 10 min and the method avoids the use of potentially toxic chemicals. The most elegant of the electrofusion procedures is that due to Zimmermann and his collaborators (Zimmermann and Scheurich 1981) in which the cells or protoplasts are first aggregated by a high frequency alternating electric field. A brief high voltage DC pulse then causes the cells to fuse. Despite its attractions the method has inherent defects, not least because of the tiny volumes that can be handled, and has not lived up to its initial promise. Kohn and Schieder (1985) and Kohn et al. (1985) have successfully used the method to obtain complementation hybrids of nitrate reductase deficient strains of tobacco but workers using animal cells have not been so successful and have found it better to aggregate the cells first by chemical methods before applying the high voltage DC pulse (Lo et al. 1984). Watts and King (1984) described modifications to the method which removed the principal disadvantages of the procedure of Zimmermann and Scheurich (1981) so that relatively large volumes of culture (up to 2 ml per batch) could be uniformly subjected to electrofusion with no tendency for the protoplasts to attach to the electrodes and fusion products could be recovered aseptically and in high yield. We have now demonstrated the effectiveness of this procedure by using it to obtain hybrids from complementary auxotrophic strains of the moss, *Physcomitrella patens.*

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Dedicated to Professor Georg Melchers to celebrate his 50-year association with the journal

The methods for the culture of *P. patens* and the isolation and culture of protoplasts were as described by Ashton and Cove (1977) and Grimsley et al. (1977a). The auxotrophic strains of *P. patens* used were *pab-3*, which requires *p*-aminobenzoic acid (Ashton and Cove 1977), and BAR 2 *thi-1,* which requires thiamine (Grimsley et al. 1980). The BAR 2 *thi-1* strain was used solely because of its auxotrophic allele, the BAR 2 phenotype being irrelevant to the present investigation.

Electrofusion was carried out in 10 cm square Petri dishes internally divided in 2 cm square chambers (Sterilin). The electrode was the transferrable type described by Watts and King (1984). A mixture of protoplasts (1.5 ml) at a concentration of 2×10^5/ml in 0.44 M mannitol containing 0.1 mM $CaCl_2$ was pipetted into the chamber, the electrode was pressed into place and 10V RMS, 500 KHz applied for about 30 sec. A single 400V DC pulse was applied by discharging an 8 μF capacitor through a 160 ohm resistor in parallel with the fusion electrodes (pulse length lms) (Watts and King 1984). The electrode was then removed and the electrofused protoplasts were diluted with 3 volumes of culture medium. After standing for 1 h to complete fusion the protoplasts were suspended in cooled molten soft agar (Grimsley et al. 1977a) and plated out on complete medium to regenerate walls and grow. After 2–3 weeks individual plants were transferred to minimal medium and allowed to grow for 3 weeks; auxotrophic plants failed to develop at this stage, diploid complementation hybrids grew normally and polyploid complementation hybrids produced atypical attenuated micro plants.

Freshly prepared moss protoplasts behaved like protoplasts of higher plants in the alternating field, aligning in rows along the field lines of force (Fig. 1a) but a DC pulse caused them to burst rather than fuse. Even pulses as low as 300 V/cm, which would not be expected to induce fusion, made over 50% protoplasts burst. This type of instability in higher plant protoplasts is usually associated with ionic deficiencies (Watts and King 1984). The moss protoplasts were therefore incubated for 30 min before electrofusion in Knop's medium in 0.44 M mannitol. After washing and resuspending in 0.44 M mannitol containing 0.1 mM $CaCl_2$, the instability of the protoplasts had largely disappeared but protoplasts were reluctant to fuse even with DC pulses as high as 800 V/cm.

The low efficiency of fusion may be due to the presence of residual wall material, the rapidity of wall regeneration (Burgess and Linstead 1981) or an inherent difference be-

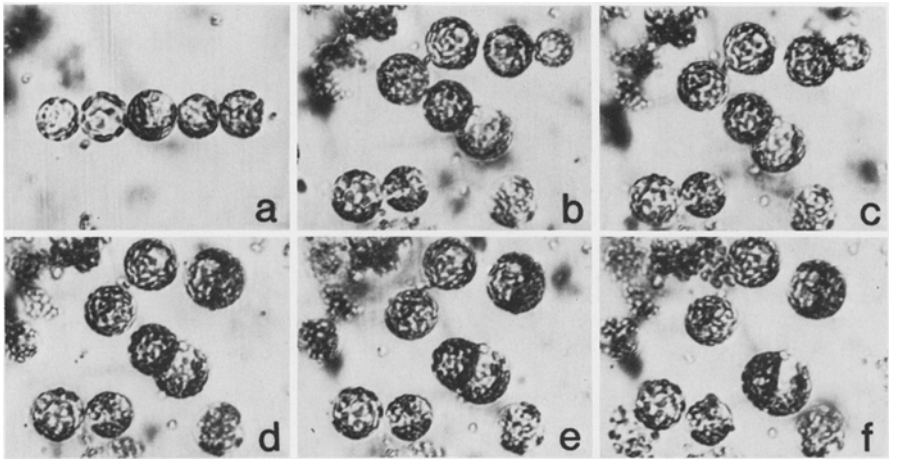

Fig. 1a–f. Electrofusion of moss protoplasts. Protoplasts were subjected to a radiofrequency field (10V RMS, 500 KHz) for 30 s followed by a DC pulse (400 V). The inter-electrode distance was 5 mm. **a** Protoplasts aligned in the RF field; **b** 30 s after the DC pulse; **c** 1 min; **d** 2 min; **e** 5 min; **f** 8 min after the DC pulse. Two pairs of protoplasts can be seen fusing, one pair much faster than the other (*upper right*)

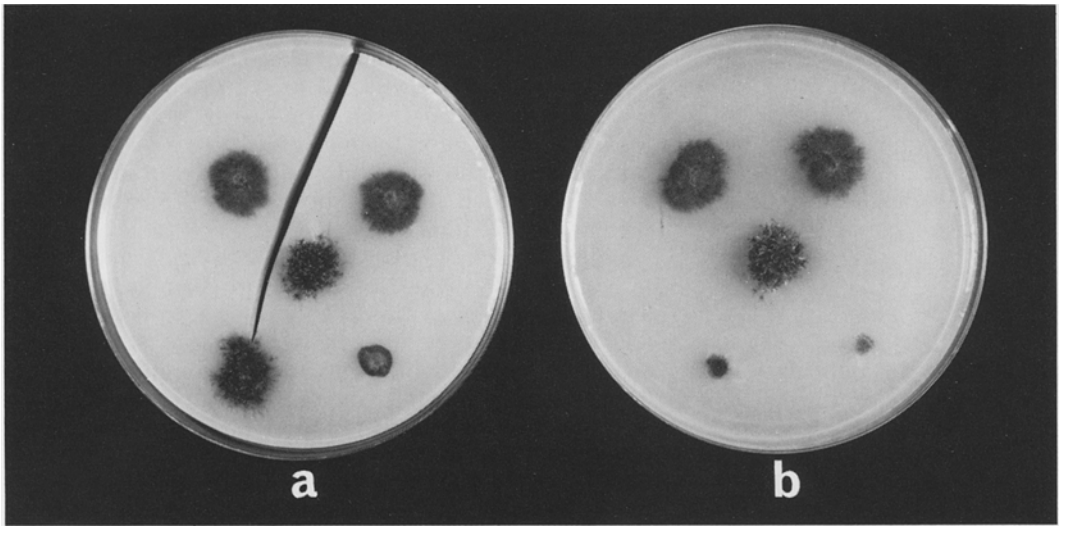

Fig. 2a, b. Hybrids produced by electrofusion. **a** Supplemented medium, **b** minimal medium. On each part: *top left* and *right,* hybrids; *centre, wt; bottom left,* BAR 2 *thi-1; bottom right, pab 3*

tween moss and higher plant protoplasts. Protoplasts were therefore given two additional treatments before fusion: pronase (1 mg/ml) to remove surface proteins and a cellulase (Onozuka R10, 0.1%) to assist in wall solution, were present during preparation and preincubation of protoplasts.

After these modifications fusion occurred in 10–25% protoplasts and was best with the highest voltage used (800 V/cm) (Fig. 1b). The progress of fusion was often rather different from that observed, for example, with mesophyll protoplasts of *Nicotiana tabacum*. Immediately after the DC pulse there was no apparent change in the appearance of the protoplasts but after 1–2 min the first signs of active fusion appeared and then proceeded rapidly so that pairs of protoplasts were rounded up after a further 5 min (Fig. 1b–f).

Electrofusion of protoplasts of individual mutant strains gave no plants able to grow on minimal medium. In contrast, despite the disappointingly low percentage of fusions, electrofusion of a mixture of the mutant strains readily gave hybrids able to grow on minimal medium. Out of some 200 plants screened 8 grew on minimal medium; none of the 8 had the typical haploid morphology, 7 showed growth characteristic of diploids and one appeared to be a polyploid hybrid (referred to as Class I, II and III respectively by Grimsley et al. 1977b) (Fig. 2).

A genetic analysis of the hybrids will be necessary to establish that these are true somatic hybrids, but there is no reason to doubt that they are. The incidence of back mutation is several orders of magnitude less than the frequency of autotrophic plants observed here. The encouraging feature of the experiments is the efficiency with which hybrids were recovered: fusion induced by polyethylene glycol gives at best a recovery of 0.5% hybrids (Grimsley et al. 1977a, b) compared with the present 3–4%. The electrofusion method of Watts and King (1984) thus offers the possibility of a simple, more efficient somatic hybridization.

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