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The Production of Somatic Hybrids by Protoplast Fusion in the Moss, *Physcomitrella patens*

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Summary. A technique has been developed for the isolation of large numbers of protoplasts from protonemal tissue of *Physcomitrella patens,* and for their regeneration to give whole plants. Somatic hybrids have been selected following treatment of mixtures of protoplasts from complementary auxotrophic strains with 50 mM $CaCl_2$ at high pH. The hybrids have a morphology different from that of normal haploid strains, but similar to that of aposporously produced diploids. The progeny resulting from self-fertilisation of the hybrids show a segregation which is consistent with their being the products of meioses in an autotetraploid.

Introduction

Mutant strains showing a variety of abnormal patterns of development are easily obtainable in the moss, *Physcomitrella patens* (Engel, 1968; Ashton, 1974; Ashton and Cove, unpublished data) and we are using these mutant strains to study the regulation of development. However, many such strains have developmental abnormalities which lead to sterility, and therefore it is not possible to use conventional methods of genetic analysis. We have tried several ways to circumvent this problem, and have attempted, for example, to obtain developmentally abnormal mutants which are temperature sensitive. We report here a more radical approach, which achieves hybridisation by protoplast fusion, and is therefore independent of the sexual process.

Protoplast fusion has been induced in a number of systems, and in a few cases it has been possible to select the hybrid product of fusion between two strains, and to culture this further (Melchers and Labib, 1974; Schieder, 1974; Ferenczy, Kevei and Szegedin, 1975; Anne and Peberdy, 1976). We have devised a method for the isolation and regeneration of protoplasts in *Physcomitrella patens,* and have attempted to obtain protoplast fusion using a number of techniques which have been described for other systems. Of these, the method of Keller and Melchers (1973) was the most successful, and routinely gives a high yield of somatic hybrids.

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Materials and Methods

a) P. patens Strains

Details of the strains used in these studies are given in Table 1.

b) Sterilisation Procedure

Except where stated otherwise media, and reagents were sterilised by autoclaving for 40 min at 115°. Petri dishes were plastic, and were purchased presterilised.

c) Culture Conditions

In these studies. *P. patens* was grown sterilely in petri dishes using a Knop's medium solidified with 1.5% (w/v) agar (Ashton and Cove, 1977). Petri-dishes were incubated at 25° in continuous white light (5000–8000 lux) on a horizontal laminar flow work station to aid sterility. Protonemal tissue, free of agar, was obtained for protoplast preparation by inoculating 90 mm petri-dishes containing appropriately supplemented (Ashton and Cove, 1977) medium overlaid with sterile cellophane (Bopp, Jahn and Klein, 1964) with 2 ml of a dilute homogenate of protonemata, obtained by blending protonemata in sterile distilled water for 2 min at 12,000 r.p.m., using a homogeniser (M.S.E.). After 12–14 days incubation, this technique yields 0.5–1.0 g wet weight of protonemata per petri-dish.

d) Isolation of Protoplasts

Protoplasts were obtained by incubating protonemal tissue with Driselase (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan). Drise-

Table 1. Origins and phenotypes of *P. patens* strains used

Genotype	Phenotype	Origin	Reference
wild-type	wild-type	from a single spore from a plant in Gransden Wood, Huntingdonshire, England	H.L.K. Whitehouse (personal communication)
thi-1	requires thiamine (aneurine)	by UV treatment of wild-type spores	Engel (1968)
thi-1 *nic*-10	requires thiamine and nicotinic acid	by N-methyl-N′-nitro-N-nitrosoguanidine treatment of *thi*-1 spores	Ashton and Cove (1977)
pab-3	requires p-amino benzoic acid	by N-methyl-N′-nitro-N-nitrosoguanidine treatment of wild-type spores	Ashton and Cove (1977)
thi-1 *gam*-26	requires thiamine, does not produce gametophores	by N-methyl-N′-nitro-N-nitrosoguanidine treatment of thi-1 spores	Ashton and Cove (unpublished data)

lase was first dissolved (2% w/v) in 8% (w/v) mannitol solution and then centrifuged at 2500 × g for 5 min. The supernatant was sterilised by passage through a Millipore filter (pore size 0.45 μm). Protonemal tissue was harvested from cellophane overlay plates with a sterile spatula, soaked for 30 min in sterile 8% (w/v) mannitol solution, and added to the sterile enzyme preparation at about 1 g wet weight of protonemata per 10 ml enzyme solution, in sterile 25 ml glass vials. The vials were incubated at 27° in a water bath with gentle agitation (80 strokes/min) for 1 h, after which time most of the protonema was reduced to protoplasts. The protoplasts were separated from large cell debris by filtration through a stainless steel gauze (pore size ca. 100 μm × 100 μm), harvested by low speed centrifugation (100 to 200 × g for 3 min) and washed twice in sterile 8% mannitol solution. The final preparation consisted almost entirely of protoplasts, but contained a few single cells still enclosed in their cell walls. 1 g wet weight of protonemata yields about 10^6 viable protoplasts.

e) Regeneration of Protoplasts

Isolated protoplasts are capable of regeneration into whole moss plants in the absence of any exogenous hormone supply. In the initial wall-formation stage of regeneration, which takes 2 to 5 days, the protoplasts must be kept in osmotically buffered medium to prevent them from bursting. After a cell wall has formed, however, the regenerants grow much faster if they are transferred to normal growth medium, as high osmolarity inhibits their growth. The following technique for protoplast regeneration was therefore used. 1 ml of a protoplast suspension, diluted appropriately, was added to 9 ml of molten medium containing 6% (w/v) mannitol, but only 0.8% (w/v) agar, kept at 35° in a water bath. This mixture was pipetted gently at once onto petri-dishes (2 ml to each 90 mm petri-dish) containing normal medium to which 6% (w/v) mannitol and appropriate supplements had been added, and which had been overlaid with sterile cellophane. After 1 week's incubation, the cellophane, together with the top layer of medium containing the regenerating protoplasts, was removed and placed onto fresh normal medium. Individual moss plants could be counted with the naked eye after several days further incubation. Using this technique it was possible to achieve regeneration of up to 80% of the protoplasts plated.

An alternative method for obtaining and regenerating protoplasts from *P. patens* has been described by Stumm, Meyer and Abel (1975).

f) Protoplast Fusion Procedure

Protoplasts were isolated from two complementary auxotrophic strains using the standard procedure described above. The two preparations were mixed, centrifuged at 100 to 200 × g for 3 min and resuspended gently in the fusion solution, which had been previously incubated at 35°, to give a protoplast concentration for each strain of 10^5–10^6 protoplasts/ml. The fusion solution contained 50 mM glycine/NaOH buffer (pH 10.5) 4% (w/v) mannitol and 50 mM $CaCl_2$. The mixed protoplasts were centrifuged at 100–200 × g for 3 min and the pellet was incubated in the fusion solution at 35° for 40 min. The mixture was then washed twice by centrifugation and resuspended in a solution containing 50 mM $CaCl_2$ and 8% (w/v) mannitol. The survival of this treatment by the component strains was estimated by plating on media supplemented so that only one or other strain could grow. Survivals in the range 10 to 40% were obtained routinely. The fusion mixture was also plated on unsupplemented media to select for somatic hybrids which might arise following protoplast fusion. Control treatments on each component strain were carried out in parallel.

Results

1. Selection of Somatic Hybrids Following Protoplast Fusion Treatments

Although we have tried sodium nitrate (Carlson, Smith and Dearing, 1974) and polyethyleneglycol (Kao and Michayluk, 1974) to induce fusion, we have been unable to select hybrids following these treatments. This may not be because these treatments do not induce fusion as survival from these treatments is very low (< 1%) and sufficient protoplasts may not therefore have been screened. However, we have been successful in selecting hybrids using a modification of the method of Keller and Melchers (1973) as described above. Table 2 summarises the results of six experiments using this technique, using the *pab*-3 strain as one component, and either *thi*-1 *nic*-10 or *thi*-1 *gam*-26 as the other. Hybrids were selected on unsupplemented medium, and when *thi*-1 *gam*-26 was used as one of the strains, there was initially considerable background growth from this strain. However, after about 14 days' incubation, the *thi*-1 *gam*-26 strain began to yellow and the vigorous green hybrids were obvious. None of the parallel control treatments with the individual parental strains yielded

Table 2. The selection of somatic hybrids after treatment of protoplast mixtures

Strains used		Control treatments				Mixed treatment		
1	2	Strain 1		Strain 2		No of viable protoplasts plated		No. of plants growing on minimal medium
		No. of viable protoplasts plated	No. of plants growing on minimal medium	No. of viable protoplasts plated	No. of plants growing on minimal medium	Strain 1	Strain 2	
pab-3	*thi*-1 *nic*-10	1.0×10^5	0	2.2×10^4	0	4.1×10^5	8.9×10^4	10
pab-3	*thi*-1 *gam*-26	1.1×10^4	0	1.2×10^4	0	4.5×10^4	4.8×10^4	17
pab-3	*thi*-1 *gam*-26	4.0×10^3	0	5×10^2	0	4.0×10^3	5×10^2	16
pab-3	*thi*-1 *nic*-10	3.7×10^4	0	6.1×10^3	0	3.0×10^4	5.1×10^3	16
pab-3	*thi*-1 *nic*-10	3.4×10^4	0	1.5×10^5	0	1.3×10^5	1.5×10^5	24
pab-3	*thi*-1 *nic*-10	5.8×10^4	0	4.5×10^4	0	9.6×10^4	1.1×10^5	13

Each line of the table represents an independent experiment. All protoplasts were subjected to the fusion treatment described in the methods section. Protoplast survival was estimated by plating on appropriately supplemented media

Table 3. Classification of progeny obtained following self-fertilisation of *thi*-1 *nic*-10/*pab*-3 somatic hybrid

Phenotype	Sporophyte			Total A+B+C	Total as %	Expected ratios as %	
	A	B	C			1	2
Prototrophic	162	176	77	415	53.5	57.9	48.6
Requiring thiamine	34	33	11	78	10.1	11.6	13.2
Requiring nicotinic acid	46	39	18	103	13.3	11.6	13.2
Requiring p-amino benzoic acid	43	41	20	104	13.4	11.6	13.2
Requiring thiamine+nicotinic acid	14	7	4	25	3.2	2.3	3.6
Requiring, thiamine+p-amino benzoic acid	4	7	6	17	2.2	2.3	3.6
Requiring nicotinic acid+p-amino benzoic acid	15	10	5	30	3.9	2.3	3.6
Requiring thiamine+nicotinic acid +p-amino benzoic acid	1	1	1	3	0.4	0.5	1.0
'Hybrid-like'	32	27	142	201	25.9	–	–
'Haploid-like'	287	287	0	574	74.1	–	–
Total all classes	319	314	142	775			

The expected ratios are calculated assuming no linkage between the *thi*-1, *nic*-10 and *pab*-3 genes (no linkage between those genes is detected in normal sexual crosses (Ashton and Cove, 1977)). Ratio 1 assumes "random chromosome segregation" while ratio 2 assumes "random chromatid segregation" during meiosis of an autotetraploid

any plants growing on minimal medium indicating that the strains used did not revert at a high frequency.

2. *Phenotype of Somatic Hybrids*

All hybrid strains selected in this series of experiments have a phenotype different from that either of the normal hyploid wild-type grown on minimal medium, or of the haploid component strains grown on supplemented minimal medium. The hybrids have more caulonema and produce fewer gametophores. This phenotype is stable, being retained after repeated subculturing, and is not altered by supplementation. The hybrids are self-fertile, but take about six months to complete their life cycles, in contrast to the haploid wild-type which takes only 2–3 months (Engel, 1968; Ashton and Cove, 1977). The hybrids produce fewer sporophytes than the haploid wild-type.

3. *Analysis of Progeny Obtained by Self-fertilisation of Hybrids*

Spores from three different sporophytes obtained from a hybrid between *thi*-1 *nic*-10 and *pab*-3 have been cultured and classified for Table 3. These progeny were of two morphological types, resembling either the parental hybrid, or a typical haploid strain. The frequency of these two types varied from sphorophyte to sporophyte (see Table 3). There was no correlation between those morphological types and the segregation of the auxotrophies.

Discussion

It is clear from the results presented here that protoplast fusion is a satisfactory technique for the somatic hybridisation of *P. patens*, and we are now proceeding to use it as a method of genetical analysis of developmentally abnormal strains.

Although we cannot be certain that the hybrids we have produced are diploid until cytological studies of their karyotype have been carried out, there are a number of indications that the hybrids are indeed diploid. Firstly they resemble in their morphological differences from normal haploid strains, diploids which have been obtained by aposporous regeneration of sporophyte tissue (Engel, 1968; Ashton and Cove, 1977). Secondly the ratio of progeny classes obtained following self-fertilisation of the *thi*-1 *nic*-10/*pab*-3 somatic hybrid is consistent with the spores being produced by meiosis from an autotetraploid. (The expected proportion of diploid cells of genotype *aa*, arising by meiosis of an autotetraploid *AAaa*, falls within the range 16.7% to 21.4%. These are the limits defined respectively by "chromosome segregation", which occurs when gene A is closely linked to the centromere, and by "random chromatid segregation" which is dependent upon gene A being distant from the centromere, and upon quadrivalents being formed during the first division of meiosis.) The proportion of thiamine – requiring progeny in the samples examined was 17.5%, of nicotinic acid – requiring progeny 19.1% and of p-amino benzoic acid – requiring progeny 19.9%.

It is not clear why the somatic hybrids obtained by protoplast fusion have a different morphology from haploid strains. The similarity of *thi*-1 *nic*-10/*pab*-3 hybrids to *thi*-1 *gam*-26/*pab*-3 hybrids indicates that the *nic*-10 and *gam*-26 mutations are recessive, and the failure of the hybrids to respond to supplementation with thiamine, nicotinic acid or p-amino benzoic acid either singly or in combination suggests incomplete dominance of one of the component mutations is unlikely to be the cause of the changed phenotype. Since aposporous diploids also have this phenotype, it is probable that the morphological differences are a consequence of the change in ploidy. However, it is difficult to see why the progeny obtained following self-fertilisation of the *thi*-1 *nic*-10/*pab*-3 hybrid should be of two morphological types, nor why their proportions should vary between sporophytes. Further studies will show how general this phenomenon is.

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