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Selection of hybrid plants obtained by electrofusion of vacuolated × evacuolated plant protoplasts in hypo-osmolar solution

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Vacuolated and evacuolated tabacco mesophyll protoplasts were electrically fused in hypo-osmolar media by using an alternating field of modulated amplitude for alignment. The vacuolated fusion partner was isolated from *Nicotiana tabaccum* L. cv Xanthi and the evacuolated one from the streptomycin-resistant strain *Nicotiana tabaccum* L. cv Petit Havana SR1. The field and osmolarity conditions used ensured relatively high yields of heterologous fusion products despite the differences in density and size of the parental cells. After removal of the evacuolated, streptomycin-resistant fused and unfused protoplasts by flotation of vacuole-containing cells on iso-osmolar sucrose medium, the cybrids and hybrids were cultured in 25 μl drops of agarose. During the first 5 weeks the non-fused Xanthi-protoplasts were used as a nurse culture. After addition of streptomycin to the growth media, cybrids and hybrids were successfully selected whereas fused and unfused vacuole-containing protoplasts died within 6 days. Only the streptomycin-resistant cybrids and hybrids developed into whole plants. On average a yield of 0.025% of streptomycin-resistant plants (referred to the total number of parental cells) was obtained. Polyacrylamide gel electrophoresis of leaf extracts of these plants showed that at least 50% of the streptomycin-resistant plants had a hybrid-esterase isoenzyme pattern. The protocol can be generalised by fusion of iodoacetamide-inactivated vacuolated protoplasts with meristematic (or evacuolized) protoplasts carrying no genetic marker. Use of evacolated protoplasts for electrofusion with vacuole-containing protoplasts therefore offers a way of overcoming the lack of suitable genetic markers for hybrid selection.

Introduction

Electrofusion has been extensively utilized in recent years for somatic hybridization of plant protoplasts [1–8]. However electrofusion as well as conventional fusion techniques are faced with the problem of separation of the generated hybrids from unfused parental cells. In contrast to the situation with bacteria, yeast and mammalian cells, somatic hybridization of higher plants suffers from the lack of genetic markers suitable for selection of hybrids [9]. An alternative is the use of mutants as fusion partners [2,10,11]. However, this is disadvantageous if specific genotypes are required.

Micromanipulation of single fused cells, combined with optical control of the fusion process [3] and separation of fluorescent labelled hybrids from parental cells by sophisticated techniques such as FACS [12], are the most common techniques to mitigate the inherent problems of plant regeneration from somatic hybrids. However, for routine large-scale production of plant hybrids these techniques are too time-consuming and/or expensive.

An elegant separation technique for plant heterokaryon protoplasts was recently suggested by Naton et al. [13]. These authors electrofused evacuolated with vacuole-containing protoplasts which have a lower density. Heterologous fusion products exhibited an intermediate density and could therefore be separated by density gradient centrifugation, at least in principle. However, the selected heterokaryon fractions were strongly contaminated by parental cells due to multiple homogeneous fusion events and due to the very low yield of heterologous fusion products. The low yield resulted from the difference in density of the parental cells leading to differential cell sedimentation and therefore to separation during the alignment process. The latter problem has been overcome – at least for evacuolated and vacuolated oat protoplasts – by using an alternating field of modulated field strength for cell alignment and by fusion in hypo-osmolar solutions [14].

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The object of this research was to improve the selection procedure for evacuolated-vacuolated heterokaryons and to demonstrate the regeneration of whole hybrid plants. To this end we fused vacuolated tobacco protoplasts of one strain with evacuolated protoplasts of another strain, which exhibited a cytoplasmatically coded resistance against streptomycin [15]. The results showed that enrichment by flotation in sucrose-containing solution followed by culture in streptomycin-containing growth media represents an efficient way to select viable cybrid- and heterokaryon protoplasts which could be used for plant regeneration.

Materials and Methods

Plants

Nicotiana tabacum L. cv Xanthi (Xanthi-tobacco) was grown in potting compost in a greenhouse under natural light. The streptomycin-resistant strain *Nicotiana tabaccum* L. cv Petit Havana SR1 (SR1-tobacco; kindly provided by Prof. Czygan, University of Würzburg, F.R.G.) was grown in sterile shoot culture as described elsewhere [16]. SR1-tobacco carries a maternally-inherited (plastid-coded) streptomycin-resistance mutation [15].

Protoplast preparation

Leaf mesophyll-protoplasts from SR1- and Xanthi-tobacco were prepared as described by Potrykus and Shillito [16] after abraiding the lower epidermis of 4–6-week-old plants with Carborundum powder [17]. The leaves of Xanthi tobacco were surface pre-sterilized by immersion in 70% ethanol for 5 s, followed by treatment with 7.5% Domestos (Lever, F.R.G.) for 20 min at 22°C.

Evacuolization of SR1-protoplasts

Freshly prepared SR1-protoplasts were mixed with 45 ml Percoll, containing 0.5 M sorbitol, 14 mM $CaCl_2$, 5 mM 2-morpholinoethanesulfonic acid (pH 6.8). After centrifugation for 20 min at $85000 \times g$ in a vertical rotor (VTI 50, Beckmann, München) at 22°C, the evacuolated SR1-protoplasts were collected and washed twice with a solution containing 0.5 M mannitol, 0.1 mM $CaCl_2$ and 0.1% BSA (iso-osmolar fusion medium). The viability of the evacuolated (and vacuolated) protoplasts was determined by dye exclusion using Erythrosin-B (0.1%, [14]). The vacuolated protoplasts were counted with an haemocytometer and were stored at a cell density of $1 \cdot 10^6$/ml in iso-osmolar fusion medium for 18 h at 4°C. The vacuolated Xanthi-protoplasts were prepared just before the fusion experiments.

Electrofusion

Vacuolated Xanthi-protoplasts and evacuolated SR1-protoplasts were sedimented and resuspended separately in the fusion medium at a cell density of $1 \cdot 10^6$/ml. If not otherwise stated this solution contained 0.4 M mannitol, 0.1 mM $CaCl_2$ and 0.1% BSA (hypo-osmolar fusion medium). The two suspensions of SR1- and Xanthi protoplasts were mixed at a ratio of 2:1 and the total cell density was adjusted to $5 \cdot 10^5$/ml with hypo-osmolar fusion medium. 360 µl of this protoplast solution were pipetted into a helical fusion chamber [8,14]. Electrofusion was performed with a Biojet CF (manufactured by Biomed, Theres, F.R.G.). Celis were aligned in an alternating field of 500 V/cm (peak to peak) at 1.5 MHz for 30 s, followed by an alternating field of 100 V/cm (peak to peak) at 1.5 MHz for 5 s. This field strength profile prevented separation of the parental cells of different densities and sizes during alignment, without causing deterioration of the cells [14]. Then a single rectangular breakdown pulse of 30 µs duration and of pre-determined strength was applied. 30 min after the breakdown pulse the chambers were opened and the electrode assembly was rinsed with 1 ml hypo-osmolar fusion medium in order to remove cells from between the electrodes.

Separation of evacuolated from vacuole-containing protoplasts after electrofusion

For cybrid and heterokaryon selection the field exposed protoplasts were allowed to sediment for 15 min at $1 \times g$. The supernatant was discarded and the cells were resuspended in 1 ml K3-medium [16]. Centrifugation at $1000 \times g$ for 10 min caused all protoplasts and fusion products that contained an intact vacuole to rise to the top, whereas unfused evacuolated SR1-protoplasts and their homologous fusion products as well as debris sedimented to the bottom of the centrifuge tube. After removal of the upper layer the vacuole-containing cells were diluted by a factor of 4 with iso-osmolar fusion medium. Subsequently the protoplasts were sedimented at $300 \times g$ for 20 min.

Selection of streptomycin-resistant cybrids and hybrids

The pellet, containing protoplasts enriched with heterologous fusion products, was resuspended in 200–300 µl K3A medium, containing 1% molten Sea-Plaque agarose at 35°C. The protoplasts were plated in 25 µl droplets on the bottom of a petri-dish (diameter 3.5 cm, Nunc, Denmark). After 1 to 5 min the agarose had solidified and 1–1.5 ml K3A medium [16] without agarose was added. The dishes were sealed with parafilm and incubated for 5 weeks in the dark at 22°C. Every 10th day the osmolarity of the K3A-medium was reduced by 20%. Cell wall regeneration (detected with 0.02% Calcofluor-white St. [18]) and cell division were observed after approximately 3 and 5 days, respectively. After 5 weeks the K3A-medium was replaced by LS medium [16], containing 500 mg/l streptomycin, 0.2

mg/l kinetin, 1 mg/l naphthylacetate and 0.1 mg/l 6-benzylaminopurine. The calli were cultivated in the light (1500 lux, 12/12 h light/dark regime). Control experiments showed that calli originating from vacuole-containing unfused protoplasts or vacuole-containing homologous fusion products died after 6 days in the selection medium. After a further 5 weeks the green, streptomycin-resistant calli were transferred to LS medium [16] with 0.5 mg/l 6-benzylaminopurine as the only phytohormone. When the plants had formed their first small leaves, they were transferred to hormone-free shoot-culture medium [16]. The plants formed roots within 3 weeks under these conditions.

For identification of the hybrid status of these plants the leaf-isoenzyme pattern of esterases was investigated by gel electrophoresis [19,20]. To this end, young leaves of the parental and hybrid plants (0.3–0.5 g fresh weight) were ground in a mortar at 4°C with 100 µl 1 M 2-hydroxyethanepiperazinesulfonic acid, 1% Polyklar AT (Serva, Heidelberg, F.R.G.), 0.1% bovine serum albumin, 50% glycerol, 0.1% 2-mercaptoethanol at pH 7.5. After centrifugation for 20 min at 15000 × *g* in an Eppendorf-tube at 4°C, 20 µl of the given leaf extract were subjected to 7.5% polyacrylamide gel electrophoresis (200 V, [19]) in a Protean™ slab gel apparatus (Bio-Rad, München, F.R.G.). After 3 hours the esterase isoenzyme patterns were analysed according to Stegemann and Schnick [20] using 0.17% α-naphthylacetate and 0.045% Fast Blue RR.

Chemicals were of the highest purity available from Merck (Darmstadt, F.R.G.) or Sigma (Taufkirchen, F.R.G.). Enzymes used for protoplast preparation were from Serva (Heidelberg, F.R.G.).

Results

The yield of intact protoplasts isolated from leaves of Xanthi and SR1 plants was $(2–4) \cdot 10^5$ and $(1–2) \cdot 10^6$ protoplasts/g fresh weight. Evacuolation of the SR1-protoplasts resulted in a 50–70% yield of viable (dye-excluding) protoplasts. Microscopy showed that the suspension of evacuolated SR1-protoplasts was free from vacuolated protoplasts.

Successful separation of hybrids from unfused parental cells, from homologous cell-to-cell fusion events and from multiple heterologous fusion products required a fusion protocol which ensured high fusion efficiency. As shown for electrofusion of evacuolated with vacuolated oat protoplasts [14] the most critical parameters are the alignment field and, in particular, the osmolarity of the fusion medium and consequently the field strength of the fusion (breakdown) pulse. Screening experiments demonstrated that protoplast alignment in an alternating field of high strength (500 V/cm, peak-to-peak) and of 30 seconds duration preferentially induced pairing and subsequent fusion of vacuolated Xanthi with evacuolated SR1 protoplasts, provided that the aligned cells were kept in position by an alternating field of weak strength (100 V/cm, peak-to-peak) for 5 s before the fusion pulse. In general, 30% of the aligned protoplast pairs consisted of SR1 × Xanthi pairs. By contrast, conventional alignment with an alternating field of constant but moderate field strength (180 V/cm, peak-to-peak) resulted in the formation of cell chains which were (with few exceptions) almost homologous [13,14]. Obviously, initial application of an alternating field of high strength prevented separation of cells of different densities and sizes due to rapid migration of the cells towards the electrodes. Viability tests showed that the high field strength was not lethal to the aligned protoplasts. Investigations of the fusion yield also showed that a field strength of 100 V/cm in the post-alignment phase was sufficient to keep the protoplasts in fusion position.

In another experimental series the fusion frequency was studied as a function of osmolarity of the medium by application of single fusion pulses of 500 to 2500 V/cm strength.

The yield of heterologous fusion events was determined by microscopic examination of at least 100 aligned pairs of SR1 × Xanthi protoplasts about 20 min after application of the breakdown pulse. In order to view the fusion process under the microscope and to count the fusion products an alternating field of weak strength (100 V/cm, peak-to-peak value) was applied for this period of time. After fusion in hypo-osmolar media, most of the products had assumed a nearly spherical shape after 20 min. The fusion yield (expressed in percentage of fused SR1 × Xanthi pairs in relation to the total number of aligned heterologous pairs) is given in Fig.1. It is evident that optimum fusion yields were obtained in 0.4 M mannitol solutions over a broad range of the fusion pulse strengths. Maximum yield at this osmolarity (about 68%) was obtained with a field pulse of 2000 V/cm. Towards higher and lower osmolarities the fusion yield decreased significantly independently of the field strength used.

Since hypo-osmolar conditions might reduce the ability for cell wall regeneration of fused plant protoplasts [31] cells of the individual strains were subjected to homologous electrofusion in media of different osmolarity. Cell wall regeneration was determined after 5 days. The field strength of the fusion pulse in these experiments was varied between 500 V/cm and 2500 V/cm. Fig. 2 shows the results for a fusion pulse of 1500 V/cm strength. It is evident that the regeneration rate of both types of protoplasts was reduced by 25–30%, but independent of the mannitol concentration in the range of 0.4 M to 0.5 M. This value is comparable to the regeneration rate of 83% which was observed for control cells which were not subjected to the electrofusion treatment. Below 0.4 M mannitol the regeneration rate decreased

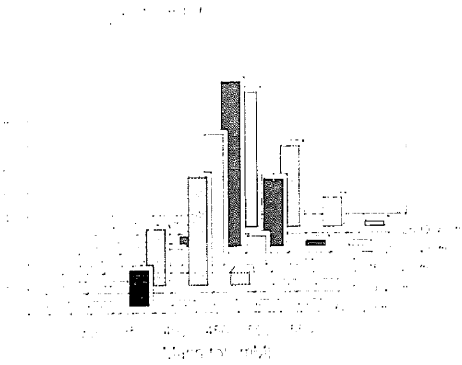

Fig. 1. Yield of heterologous fusion products after electrofusion of evacuolated, streptomycin-resistant SR1 protoplasts with vacuole-containing Xanthi protoplasts in media containing different mannitol concentrations. Protoplasts were aligned in an alternating field of modulated strength (500 V/cm for 30 s followed by 100 V/cm for 5 s, peak- to-peak values). After application of the fusion pulse the 100 V/cm alternating field was applied for 20 min in order to keep the fusing protoplasts in position for counting. Fusion was initiated by injection of a single breakdown pulse of 30 μs duration and of variable strength. Black columns: 500 V/cm, white columns: 1000 V/cm, lightly speckled columns 1500 V/cm, dashed columns: 2000 V/cm and heavily speckled columns 2500 V/cm. The yield of heterologous fusion products at a given osmolarity and field strength of the fusion pulse was determined microscopically from 100 aligned pairs of SR1 × Xanthi-protoplasts after 20 min. The data are taken from a typical experiment.

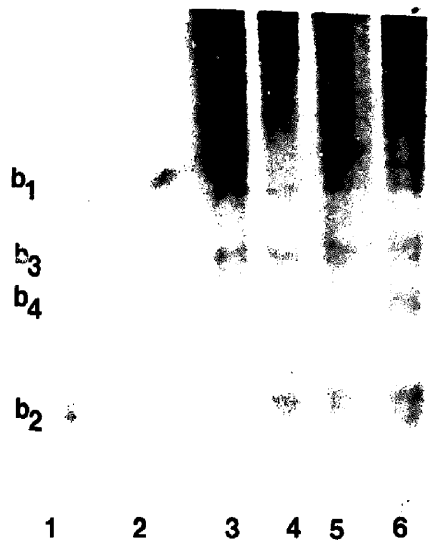

Fig. 3. Esterase-Isoenzyme profile after polyacrylamide gel electrophoresis of leaf extracts from untreated SR1-plants (track 1), untreated Xanthi-plants (track 2) and four streptomycin-resistant hybrid plants (tracks 3–6). b_1: Major isoenzyme from N. tabacum cv Xanthi. b_2: The 2 major isoenzymes from *N. tabacum* cv Petit Havana SR1. b_3 and b_4: Additional isoenzymes occurring in somatic hybrid plants (tracks 3–6) regenerated from electrofused protoplasts.

dramatically. According to Fig.2 the evacuolated SR1 protoplasts were apparently more tolerant of osmotic stress than the vacuolated Xanthi protoplasts. In 0.35 M mannitol the protoplasts of this species either burst or showed no cell wall regeneration whereas about 30% of the evacuolated SR1-protoplasts survived. The mannitol concentration had to be reduced to 0.3 M before these protoplasts burst or lost regeneration ability.

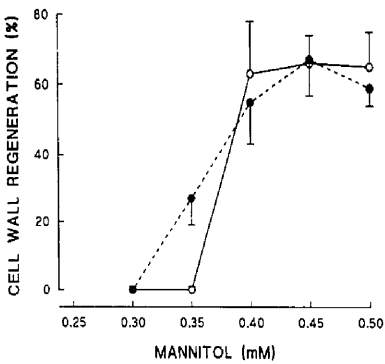

Fig. 2. Cell wall regeneration of SR1-protoplasts (filled circles) and Xanthi-protoplasts (open circles) after electrofusion at different concentrations of mannitol in the fusion medium. SR1-protoplasts or Xanthi-protoplasts were fused and cultured as described under Method, except that the protoplasts solutions were not mixed, but were fused separately. After 4 days, cell wall regeneration rate was determined from at least 300 protoplasts with Calcofluor white. The data are from a single representative experiment.

After demonstration of the absence of adverse side effects of 0.4 M mannitol solutions on cell wall regeneration of both parental fusion partners, plants were grown from electrofused cybrids and heterokaryons in the selection medium after isolation of the vacuole-containing fused and unfused protoplasts by sucrose flotation. On average 100 to 150 streptomycin-resistant calli could be regenerated from 10^6 input cells fused by a single breakdown pulse of strength 2000 V/cm and of 30 μs duration (data from 20 experiments). This corresponded to a yield of 0.025%. Control experiments with protoplast mixtures which were not exposed to the alignment field and fusion pulse, but otherwise to the same treatment, did not lead to any regenerated plant. Spontaneous fusion could therefore be excluded. It should be noted that only three plants could be regenerated on average when fusion was performed in isoosmolar media using the same field conditions.

At least 50% of plants regenerated from the streptomycin-resistant calli were hybrids, as judged by the esterase-isoenzyme pattern (Fig. 3). Gel electrophoresis of extracts of leaves of field-untreated SR1 plants showed the presence of two mayor esterase bands of high electrophoretic mobility, whereas the leaf-extracts

of control Xanthi plants exhibited only one mayor esterase band of low electrophoretic mobility. In contrast, the leaf extract of hybrid plants showed the bands of the parental fusion partners (Fig. 3). In addition one or two bands of intermediate electrophoretic mobility occurred, presumably because of new combinations of the subunits of the parental esterases.

Discussion

The results reported here show that evacuolization of one fusion partner is an efficient method to improve the yield of heterokaryons from plant protoplast fusion. The enrichment by flotation on iso-osmolar sucrose-media could be further improved if the evacuolated fusion partner contained a cytoplasmatically coded antibiotic resistance. The yield of hybrid plants obtained by this method was relatively high compared to heterokaryon yield from electrofused *vacuolated* protoplasts even though it is somewhat difficult to compare our results with data published in the literature (see, for example, Refs. 2,4,10,11,22–29) and literature quoted in Refs. 30 and 31). In the past the use of *evacuolated* fusion partners was hindered by the low yield of heterologous fusion products. The large differences in density and size between vacuolated and evacuolated plant protoplasts caused difficulties in heterologous pairing of the protoplasts in the alternating field. Due to sedimentation and to the volume-dependence of the alignment force, segregation of the two fusion partners occurred during cell pairing when a field of constant strength was used.

We have shown that similar yields of heterologous fusion products to those under microgravity conditions [32] can be achieved if pairing is conducted in an alternating field of modulated strength. However, in order to obtain high fusion frequencies between heterologous cell pairs, hypo-osmolar conditions are also required. The reasons for facilitation of electrofusion under hypo-osmolar conditions are not understood. However, it is likely that due to water uptake and concomitant swelling of the cells, tensions are created in the membrane which lead to the dissolution of cell- and membrane-skeleton proteins [33]. Such processes would increase the mobility of membrane components, but also – as shown for mammalian cells [34] – the permeability of the attached membranes. This, of course, should facilitate the intermingling process of the membranes after the fusion pulse has been applied. The high and reproducible yield of hybrid plants shows that this osmotic treatment apparently did not affect cell viability. This is also indicated by the regeneration rate of the cell walls of field-treated cells which was comparable to that of the control cells electrofused in iso-osmolar solutions.

Therefore hypo-osmolar electrofusion seems to be a useful technique not only for fusion of mammalian cells [35] but also for fusion of plant protoplasts of different sizes and densities. Efforts to elucidate the underlying mechanism of hypo-osmolar electrofusion will certainly help in future to further increase the yield of heterokaryons and of hybrid plants. The present protocol allows large-scale selection of hybrids which is an advantage compared to single cell techniques for isolation of heterokaryon protoplasts [3]. The procedure is simple and does not need sophisticated equipment such as FACS. The method can be generalised in future by fusing small meristematic protoplasts with large mesophyll protoplasts. If meristematic or evacuolized protoplasts from metabolic defective mutants are available, they can be electrofused with wild-type (metabolic complementary) mesophyll protoplasts and vice versa. Inactivation of the large fusion partner by iodoacetamide [36] can be utilized, if no genetic marker is available. Thus, universal application of this selection protocol is conceivable. A further application of the technique presented in this communication is the controlled transfer of plastids from one plant species to another in high yield.

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