# Viable somatic hybrids are obtained by direct current electrofusion of chemically aggregated plant protoplasts

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Using low levels of polyethylene glycol as aggregating agent, protoplasts of *Nicotiana glauca* and *N. langsdorffii* were electropulsed (square wave direct current electric pulses) and on subsequent culture on a selective medium (without phytohormones), calli developed. The hybrid character of some of the calli was demonstrated by direct comparison of the isoelectrophoretic pattern of the ribulose-1, 5-bisphosphate carboxylase subunits with those of the parent plants. In optimum conditions, the electrofusion technique described is about 30-fold more efficient for hybrid production than the conventional polyethylene glycol method when applied to the same plant systems.

*Electrofusion Protoplast (Nicotiana) Ribulose-1,5-bisphosphate carboxylase*

## 1. INTRODUCTION

Somatic hybridization is a means of by-passing interspecific or intergeneric sexual incompatibility in plants. Since the hybrids can be propagated by vegetative multiplication, the method is of great interest in plant breeding [1].

Protoplast fusion is the most useful technique for obtaining somatic hybrids [2]. During the past 10 years, fusion of protoplasts has been routinely performed by treatment with PEG [3,4] and numerous viable hybrids have been obtained [1]. However, in spite of many improvements, the yields remain low and the technique has proved to be difficult to use.

A physical approach to protoplast fusion has recently been proposed: electrofusion [5–7]. Even though the basic process in the different laboratories involved remained the same (intercellular closing of the electric field-induced pores), the approaches to bring the protoplasts in close contact were different. The largely advertised technique of Zimmermann [5] is efficient and easy to use in routine experiments. It involves two steps: dielectrophoresis-induced contact between the cells followed by electrical pulse-triggered fusion [5,8]. However, it also suffers some limitations: small number of protoplasts treated per assay and difficulties encountered in the regeneration of viable hybrids.

Several versions of the initial process of Zimmermann have been proposed to overcome these limitations [9–11] and indeed some of them have led to the recovery of hybrids. However, in most cases, no absolute proof of the hybrid character of the calli or plants obtained (by determination of the number of chromosomes or of isoenzyme patterns) has been given [12–14]. Bates and Hasenkampf [15] recently obtained viable hybrids of *Nicotiana* characterized by their esterase isoenzyme patterns. However, the yield of the process compared to the classical PEG method was not presented.

Indeed, procedures where the protoplasts are ag-

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*Abbreviations:* PEG, polyethylene glycol; d.c. electrofusion, direct current electrofusion; IEF, isoelectric focusing; RuBPCase, ribulose-1,5-bisphosphate carboxylase

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gregated by other means and then fused by d.c. electric pulses are now known to be highly efficient [14–16]. With this method somatic hybrids have been produced with high yields for animal [16] and yeast cells [17] and genetic evidence of the hybrid character of the fusion products has been given [18]. Adapted to plants, this method involves firstly the aggregation of the plant protoplasts by a chemical agent (spermine) and then fusion by means of short d.c. electric pulses [19]. This technique is very efficient (routinely 50% of the protoplasts are fused) and of general application (interspecific or intergeneric fusions were obtained with a comparable efficiency). d.c. electrofusion of chemically aggregated protoplasts gives rise to viable hybrids as is clearly demonstrated in this paper.

The well-known model *Nicotiana glauca* + *N. langsdorffii* was retained for this study because: (i) protoplasts of the two parents can be cultured easily; (ii) parasexual hybrids have already been obtained in a number of cases by different processes [20,22]; (iii) hybrids of tumorous nature grow on a medium devoid of plant hormones (in contrast to the parents) [23] and this property provides a simple selection test.

Using this sytem we were able to obtain large numbers of viable hybrids which were further characterized at the molecular level by isoelectric focusing polypeptide analyses of the enzyme RuBPCase.

## 2. MATERIALS AND METHODS

### 2.1. *Media*

Medium A – 340 mM KCl, 18 mM $CaCl_2$, pH 5.7; medium B – 0.5 M mannitol, 1 mM $MgSO_4$, 50 mM NaCl, 0.01% Tween 80 and 750 $\mu$M spermine or 1–10% PEG 6000, the pH being adjusted to 5.9 with 0.7 M 2-(*N*-morpholino)ethanesulfonic acid (Mes); $T_0$ medium – the total culture medium of Chupeau et al. [20]; selective medium – $T_0$ medium minus plant hormones; $SG_3$ medium – selective medium without mannitol but containing twice the concentration of macroelements and 30 $g \cdot l^{-1}$ glucose.

### 2.2. *Plant material*

Seeds of *N. glauca* and *N. langsdorffii* were a generous gift from Dr Chupeau and Mr Goujaud (INRA, Versailles, France). The plants were grown in a growth chamber under the following conditions: photoperiod, 14 h; light, 21 $\mu E \cdot m^{-2} \cdot s^{-1}$; temperature, 25°C in the light, 20°C in the dark. Young but fully expanded leaves from at least 8-week-old plants were used.

### 2.3. *Protoplast preparation*

All the manipulations were performed in sterile conditions under a horizontal laminar flow hood. Leaf mesophyll protoplasts were obtained essentially as described by Chupeau et al. [20]. After washing the protoplasts twice in medium A and centrifugation ($50 \times g$, 3 min), the pellet was resuspended in medium B and the concentration adjusted to $75 \times 10^4$ protoplasts/ml.

### 2.4. *Electrofusion*

Electrofusion was performed under a horizontal laminar flow hood. The protoplasts were left in contact with the aggregating agent (spermine or PEG) for 60 min. Then 0.3 ml of the protoplast suspension was transferred to a petri dish (55 mm diameter). After 10 min settling, the protoplasts were submitted to electropulsation. Two flat stainless-steel electrodes (5 mm wide, 20 mm long, sterilized in ethanol) were dipped into the protoplast suspension and one to three square wave electric pulses of 1 kV/cm were applied for various durations. The overall process was controlled and monitored with an oscilloscope (Electrofuser, project ATEIM-CNRS, France). After the pulses, the electrodes were removed and the suspension allowed to stay under the laminar flow hood for 1.5 h at 21°C and then diluted 11-fold with 3 ml $T_0$ medium. After 4 days in the dark at 25°C, the protoplasts were transferred to light ($17.5 \mu E \cdot m^{-2} \cdot s^{-1}$, temperature 25°C). It should be emphasized that in our technique the cellular suspension is not pipetted out of the chamber after pulsation and that the duration of contact with the electrodes is very short (less than 1 min). Furthermore, the ratio of the surface of the electrodes in contact with the suspension to the volume of the suspension is very small, thus the percentage of protoplasts susceptible to sticking to the electrodes is also very small.

After 1 week on culture, the plating efficiency (number of dividing protoplasts) was determined either by direct counting under an inverted microscope or with a hemocytometer after careful re-

covery of the colonies in $T_0$ medium and adjustment to a given volume (this second method gives more accurate estimation of the dividing protoplasts, however it is time-consuming and not very easy to perform in sterile conditions).

### 2.5. *Selection of hybrid colonies*

After 3 weeks, the colonies were washed twice in selective medium ($T_0$ liquid medium lacking phytohormones) and plated on the same medium solidified with 0.6% agar. 3–4 weeks later, the surviving colonies were individually subcultured on $SG_3$ medium.

### 2.6. *RuBPCase isolation and analysis*

Hybrid calli (2 cm diameter) were cut in half, one half transferred to $SG_3$ medium but without glucose to stimulate the synthesis of the RuBPCase and cultivated for at least 1 week, the second half being subcultured on $SG_3$ medium.

RuBPCase of *Nicotiana* species and of hybrid calli was isolated by immunoprecipitation [24] and analyzed by isoelectric focusing in the presence of 8 M urea [25]. 1 g leaves or calli were homogenized at 4°C in a mortar, with sand, and 2 ml of 0.2 M Tris-HCl (pH 7.5) containing 2.5% (Polyclar) polyvinyl-polypyrrolidone, 0.5% PEG 6000, 10 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 $\mu$M leupeptin. The homogenates were centrifuged at 25 000 × $g$ for 15 min at 4°C and the supernatants loaded onto a Sephadex G-25 column equilibrated with 50 mM Tris-HCl (pH 7.5) and 5 mM DTT. The desalted extracts from the calli were concentrated by dialysis against PEG 20000 and clarified by filtration through a 0.8 $\mu$m filter unit.

RuBPCase from all extracts was precipitated by heterologous antiserum raised against sunflower RuBPCase. The specificity of the immunoserum was demonstrated by electrophoresis [26]. The cross-reactivity of RuBPCase of different origins (sunflower and different *Nicotiana*) was checked by Ouchterlony double diffusion [27]. Aliquots of plant extracts were incubated at 4°C for 24 h with an excess of immunoserum and the pellet washed twice with 25 mM Tris-HCl (pH 7.5) containing 1% Triton X-100 and 1 M NaCl and twice with 25 mM Tris-HCl (pH 7.5). The precipitate was dissolved in 20 $\mu$l of 8 M urea, 2% Nonidet P-40, 5% $\beta$-mercaptoethanol and 5% ampholines, pH 5–8 (Pharmacia) and submitted to electrofocusing.

Isoelectrofocusing was performed at 20 W for 4000 $V \cdot h^{-1}$ in a 4.5% polyacrylamide slab gel containing 8 M urea, 2% Nonidet P-40 and ampholines (Pharmacia, pH range 5–8, dilution 1:15) with 1 M NaOH and 0.04 M L-glutamic acid as catholyte and anolyte, respectively. Polypeptide bands were stained with fast-green [28] and gels were scanned with a laser densitometer (Ultroscan LKB 2202).

## 3. RESULTS

### 3.1. *Optimization of the pulsation medium*

Using the results of Chapel et al. [19], the spermine concentration was set at 750 $\mu$M for the aggregation of the protoplasts. However, on subsequent culture, division of the protoplasts was greatly reduced and stopped after 1 week. This shortcoming was not present when we used PEG as an aggregating agent. PEG was kept at a low enough concentration so that it could not induce fusion by itself.

Table 1 shows the percentage of surviving protoplasts of *N. glauca* and *N. langsdorffii* upon culture with different concentrations of PEG added to the fusion (medium B) in the absence of electric pulsations. Compared to the assay without PEG, no significant influence of this molecule was noticed on the survival of the protoplasts of *N.*

Table 1

Effect of different concentrations of PEG added to the fusion medium on the plating efficiency of protoplasts

| % PEG in the fusion medium | % dividing protoplasts of | | | |
|---|---|---|---|---|
| | *N. glauca* | | *N. langsdorffii* | |
| | A | B | A | B |
| 0 | 15 | nd | 11 | 5 |
| 2.5 | 13 | 15 | 12 | 5 |
| 5 | 32 | 50 | 10 | 5 |
| 10 | 20 | 20 | 11 | 5 |

(A) The number of dividing protoplasts was estimated by direct counting on the petri dish with an inverted microscope or (B) using a hematocytometer after the recovery of the protoplasts in culture medium. Values are means of 3 separate determinations. nd, not determined

*langsdorffii* while it shows a slight beneficial effect in the case of *N. glauca.*

### 3.2. *Electrofusion of mixed populations of* N. glauca *and* N. langsdorffii *and selection of the hybrids*

*N. glauca* and *N. langsdorffii* protoplasts were mixed in the presence of different concentrations of PEG. A 1:2 ratio was retained in all the experiments because of the better plating efficiency of *N. glauca* compared to *N. langsdorffii* (see table 1) (assays involving other ratios are in progress and will be published later). The protoplast suspension was electropulsed and the heterocaryons cultured and selected as described in section 2. The results are presented in table 2.

The best yield was obtained using 2.5% PEG as aggregating agent and a 1 $kV \cdot cm^{-1}$ electric pulse of 100 $\mu s$ duration. No callus was recovered if the mixed population was not electrofocused or when one species alone was submitted to electrofusion and cultured on the selective medium, whatever the conditions retained. Fig. 1 shows photographs taken at different steps after hybrid selection.

Table 2

Effect of different concentrations of PEG and of changes in the electrofusion parameters on hybrid production

| PEG concentration (%) | Number of colonies recovered per $10^5$ cultured protoplasts[a] | | | | |
|---|---|---|---|---|---|
| | Electrofused | | | | Not electrofused |
| | Duration of pulses ($\mu s$) | Number of pulses | | | |
| | | 1 | 2 | 3 | |
| 1 | 50 | 0 | 0 | nd | 0 |
| | 100 | 0 | 0 | 0.8 | 0 |
| 2.5 | 50 | 4 | 6.8 | 7.2 | 0 |
| | 100 | 14 | 6 | 5.2 | 0 |
| 10 | 50 | 2.5 | 2.6 | 1.4 | 0 |
| | 100 | 5.1 | 1.1 | 0.4 | 0 |

[a] Determination of the number of surviving colonies was performed after 8 weeks of culture on the selective medium. nd, not determined

### 3.3. *Biochemical characterization of the hybrids*

IEF analysis of RuBPCase is presented in fig.2. The large subunit of the enzyme was dissociated into 3 polypeptides (a–c and b–d), two of which migrate similarly (b,c) in *N. glauca* and *N. langsdorffii*, respectively. However, on the gel slab presented in fig.2, a faint band was obtained for polypeptide b in the case of *N. langsdorffii*. Such a result was not obtained with the extract of

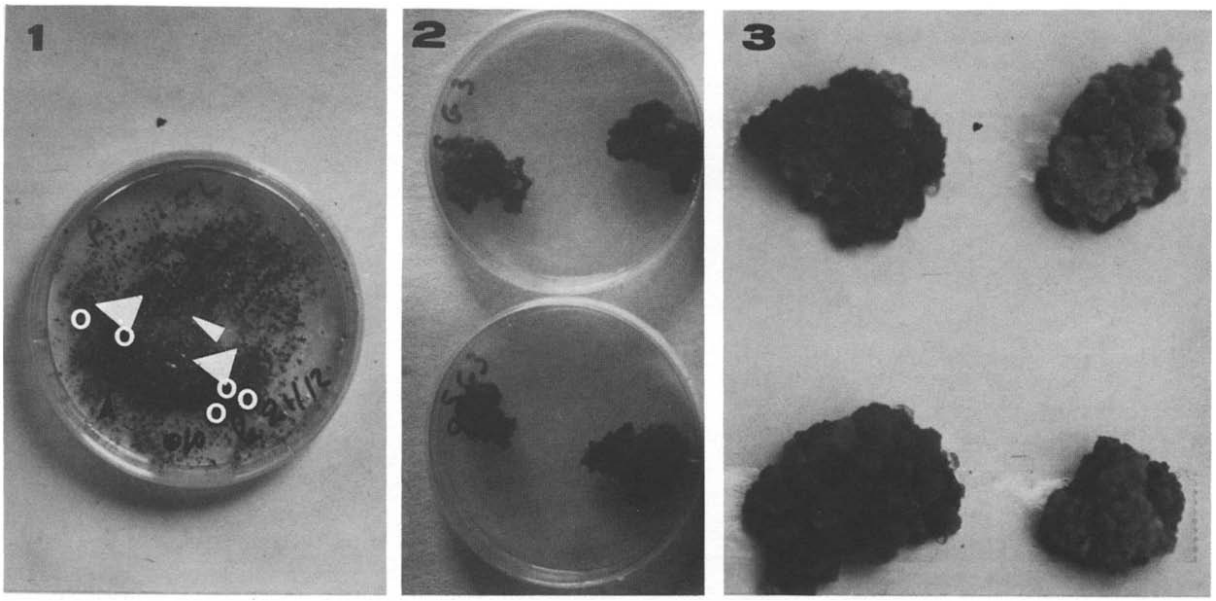


Fig.1. Photographs of the hybrid calli. (1) After 14 days on the selective medium: (▽) dead protoplasts, (○,▽) developing colonies. (2) After the first transfer to $SG_3$ medium. (3) 3-week-old hybrid calli.

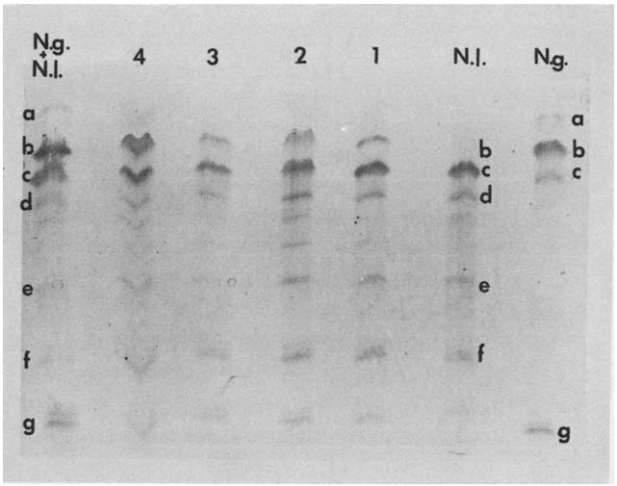


Fig.2. Isoelectric focusing of RuBPCase. Nl, *N.langsdorffii*; Ng, *N.glauca* (100 μg protein extracts each); Nl + Ng, mixture of protein extracts from *N.langsdorffii* and *N.glauca*; (1–4) hybrid calli (500 μg protein extracts each); (a–d) large subunit polypeptides; (e–g) small subunit polypeptides.

separate experiments which show that the level of b was similar in *N. glauca* and *N. langsdorffii*. This unexpected result may be due to specific breakdown of this polypeptide by proteinases during the extraction process [29], occurring neither for *N. glauca* nor for the hybrid extracts (however, because direct comparison of the migration of the polypeptides of the parents and of the hybrids was obtained only on this gel slab, we present this photograph instead of an arrangement of two different slabs).

However, polypeptide a (having the lowest mobility) appeared to be specific to *N. glauca* while polypeptide d (of highest mobility) seemed specific to *N. langsdorffii*. The polypeptide composition of the small subunit of RuBPCase was also different in the two species: *N. glauca* exhibited only one band (g) but *N. langsdorffii* showed two (e,f) on the gel. When extracts of the two species were mixed, a polypeptide pattern showing the presence of all the polypeptides of the two *Nicotiana* was observed.

A different pattern was obtained from the hybrid calli. The polypeptides of both parents (e–g) were observed in the small subunit and a relative estimation of their proportions by densitometry scanning (table 3) indicated that they were present in similar amounts among the hybrids. In contrast, polypeptide a, characteristic of *N. glauca*, was absent from

Table 3

Relative polypeptide estimation of the large and the small subunits of the RuPBCase from the parents *Nicotiana* and the hybrid calli after IEF analysis

| Polypeptides | | % of peak area obtained by scanning densitometry of the gel | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | N.g. | N.l. | Hybrid calli | | | | N.g. + N.l. |
| | | | | 1 | 2 | 3 | 4 | |
| Large subunit | a | 10.7 | – | t | t | t | t | 8.7 |
| | b | 37.4 | 1[a] | 16 | 18.7 | 19.5 | 7 | 24.8 |
| | c | 11 | 37.6 | 37.7 | 26.6 | 37.5 | 33 | 37.6 |
| | d | – | 10 | 9 | 8.8 | 8.2 | 6.9 | 4.5 |
| Small subunit | e | – | 14.6 | 6.8 | 7.9 | 4.2 | 9.5 | 4.6 |
| | f | – | 17.6 | 15.9 | 14.4 | 15.4 | 15.6 | 17.6 |
| | g | 23 | – | 8.6 | 9.3 | 5.6 | 10 | 7.9 |
| % of total peak area recovered | | 82.1 | 80.8 | 94 | 86.4 | 90.4 | 82 | 78.4 |

[a] Underestimated, see comments in section 3

For legend, see fig.2. t, trace; N.g., *N.glauca*; N.l., *N.langsdorfii*

the large subunit and the quantitative analysis (table 3) shows clearly that this subunit is similar to the RuBPCase large subunit of *N. langsdorffii.*

## 4. DISCUSSION

Definite advances in the production of viable interspecific or intergeneric plant somatic hybrids require, firstly, the utilization of a highly efficient and simple technique of protoplast fusion. The electrofusion method used here is particularly well-suited for large-scale fusion of protoplasts. In contrast to other techniques using electric fields as fusogenic agent [10,30] where a fusion chamber with a special design is required, the present experiment was performed directly in the culture dish. As in the case of animal cell electrofusion [16,18] the electrodes are dipped directly into a petri dish containing the sample, and the protoplasts electropulsed. The electrodes are withdrawn from the dish, rinsed with alcohol, dried under the sterile flow hood and are ready to be used again. Throughout the experiment the protoplasts are kept in the same petri dish thus avoiding disruption of the fragile newly formed heterocaryons.

In [19], we achieved the electrofusion of protoplasts after a step of aggregation with spermine. Efforts were made first to improve the method of fusion and the subsequent viability of the protoplasts isolated from *N. glauca* and *N. langsdorffii.* Thus it appeared that spermine, added to the fusion medium at 750 $\mu$M to induce the aggregation of the protoplasts before fusion, delayed and finally stopped the division of the protoplasts whether electrofused or not. Even though polyamines are now considered to possess plant hormone-like properties [31], the possibility remains that, at the optimal concentration used for aggregating the protoplasts, this compound is toxic (toxicity of spermine toward protoplast division was clearly demonstrated by Huhitinen et al. [32] for *Alnus glutinosa* and *A. incana*). We changed spermine for PEG 6000 at low concentrations in the fusion medium. At the concentration retained, PEG has no effect on protoplast fusion and slightly stimulates protoplast division and growth.

Using the above-defined medium, fusion products which grew on the selective medium were obtained. Under optimal conditions (2.5% PEG, 1 pulse of kV/cm for 100 $\mu$s) a mean yield of 14 calli per $10^5$ cultured protoplasts was obtained. It is misleading to compare the yield of hybrids obtained with our technique to those reported for other electrofusion methods since, in addition to the specific yield of the fusion process, total hybrid recovery is strongly dependent upon the mode of selection retained (manual isolation, genetic complementation, resistance to antibiotics, etc. [33]). However, direct comparison with the results of Chupeau et al. [20] on the same system *(N. langsdorffii + N. glauca),* but using PEG as the fusogenic agent, can be performed: under optimal conditions 14 calli per $10^5$ protoplasts were obtained. If we consider the results in table 1 a mean of 10 protoplasts per 100 divide in 2.5% PEG, for the two parents. Thus taking this value as a basis for calculation, instead of the total protoplasts involved in the fusion process, the yield will be 10-fold higher: $14 \times 10 = 140 \cdot 10^{-5}$. This is 28-times more efficient than the values obtained by Chupeau et al. with 30% PEG.

Considering that, in the experiments of Pelletier and Chupeau [2], the PEG fusion efficiency is about 5–30%, and that our electrofusion method gives routinely about 40–60% fusion, the higher yield in hybrids obtained using our technique can only be explained by electrofusion being more innocuous. In fact, it is now recognized that at the concentrations used to induce fusion (30–40% PEG), PEG is toxic for protoplasts [34,35].

As the culture of the protoplasts on a selection medium is not an irrefutable proof of the hybrid character of the recovered calli [36], IEF analysis of the enzyme RuBPCase was performed.

The results demonstrate unequivocally that all the analysed calli contain the small subunits of RuBPCase polypeptide patterns of both parents. It is now well established that the small subunit of RuBPCase is encoded by the nuclear genome [37]. By contrast, all the hybrids analysed contain the polypeptide pattern of the large subunit of RuBPCase of *N. langsdorffii,* encoded by the chloroplastic genome [37]: this result indicates the presence in the cells of the calli of only one type of plastid. Taken together these data favour the idea of an amphiploid nature of the cells and exclude the possibility of chimerism [36].

The segregation phenomenon, at the plastid level, demonstrated in this work has already been shown in numerous experiments involving PEG-

mediated fusions of either *N. glauca* + *N. langsdorffii* [38], other *Nicotiana* species [39,40] or other genera [36]. However, recent studies [41,42] have shown that in the early stages after fusion, both parental types of plastid coexist inside the hybrids; thus the sorting out of one kind of chloroplast takes place later after an undetermined number of mitoses. In accordance with these findings, it should be noted that the analysis of the calli obtained by electrofusion was performed after at least 2 subculture steps explaining the recovering of the plastids of only one parent [41]. In this way, electrofusion, when compared to PEG-induced fusion, seems to have no particular effects on the recombination processes that follow the fusion events.

As presented here, electrofusion of chemically aggregated plant protoplasts is a very promising technique owing to the high yield of viable hybrids produced in comparison with the PEG technique. The next step in the routine production of plant somatic hybrids of commercial importance requires the ability to distinguish easily the parental protoplasts from the fused ones without the constraints of the use of mutants and selective media. In this way, sorting out the hybrids using either micromanipulation methods or a cell sorter [43] in association with electrofusion should be the most efficient combination in the production of hybrids from any cultivated plant protoplasts.

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