[29] Plant Protoplast Fusion and Somatic Hybridization

By P. T. LYNCH, M. R. DAVEY, and J. B. POWER

Introduction

Conventional plant breeding is often limited by pre- and/or postzygotic incompatibility barriers, and fusion of somatic cells to generate somatic hybrid plants has been considered as a method of overcoming such limitations.[1] The lack of constraints to interspecific or intergeneric protoplast fusion permits hitherto reproducibly isolated plant genomes to be combined at the protoplast (heterokaryon) level, thus providing the basis for the generation of novel hybrids. Protoplast fusion also enables the genetic manipulation of vegetatively propagated crops, such as sterile or subfertile individuals, and those plants, including woody species, with naturally long life cycles.[2] Somatic hybridization of highly heterozygous species also provides an element of predictability in relation to the hybrid, because meiotic recombination is avoided. Cytoplasmic factors, such as mitochondrial-based cytoplasmic male sterility, may also be transferred from one species to another by protoplast fusion.[3]

Somatic hybridization involves four discrete, yet interrelated, stages: (1) protoplast isolation and culture with efficient plant regeneration in at least one of the fusion partners; (2) induced protoplast fusion, preferably at high frequency, without loss of viability; (3) the development of a selection strategy incorporating somatic hybrid plant regeneration; and (4) the confirmation of hybridity or cybridity. Such confirmation utilizes cytological and morphological markers, and a range of biochemical-based techniques.

Protoplast Isolation and Culture

The ability to isolate protoplasts that, when cultured under defined conditions, divide mitotically and regenerate plants has now been established for many species,[4-7] including woody plants.[8]

[1] G. Pelletier and Y. Chupeau, *Physiol. Veg.* **22**, 377 (1984).

[2] Y. Y. Gleba and K. M. Sytnik, "Protoplast Fusion—Genetic Engineering in Higher Plants," Monogr. Theor. Appl. Genet., No. 8. Springer-Verlag, Berlin, 1984.

[3] A. Kumar and E. C. Cocking, *Am. J. Bot.* **74**, 1289 (1987).

[4] S. C. Maheshwari, R. Gill, N. Maheshwari, and P. K. Gharyal, *Results Probl. Cell Differ.* **12**, 3 (1986).

[5] M. R. Davey and J. B. Power, *in* "Progress in Plant Protoplast Research" (K. J. Puite, J. J. M. Dons, H. J. Huizing, A. J. Kool, M. Koornneef, and F. A. Krens, eds.), p. 15. Kluwer Academic Publishers, Dordrecht, Boston, London, 1987.

Protoplast Fusion

Induced protoplast fusion can be achieved using chemical[9] and electrical treatments.[10] In both cases, fusion is a two-stage process. First, protoplasts are brought into close membrane contact, the degree of plasma membrane adhesion depending on the parental protoplasts. Tight contact may occur only in localized regions between adhering protoplasts.[11] Subsequently, the plasma membranes are stimulated to interact, for example, by modification of the electrical charges on the membranes,[12] resulting in protoplast fusion.

Fusion generates products (heterokaryons) with two or more nuclei within a mixed cytoplasm containing organelles from the parental protoplasts. The cytoplasms derived from the respective parental protoplasts mix at different rates within the heterokaryons, according to the protoplast types.[9] Cell wall formation and nuclear fusion to produce hybrid cells occur early in culture. Nuclear fusion takes place either during interphase by the formation of nuclear bridges, or at the first mitosis.[11] The fate of plastids in hybrid cells varies, and includes loss of one parental type or recombination between plastids of the two parents.[13] Vacuoles in heterokaryons may fuse,[14] and microtubules integrate.[15] However, the fate of other cell organelles is unclear.

The extent of protoplast fusion, heterokaryon formation, and survival of fusion products can be monitored using naturally occurring visual markers. Thus heterokaryons can be readily identified following the fusion of chlorophyll-containing leaf mesophyll protoplasts with suspension cell

---

[6] Y. P. S. Bajaj, *in* "Biotechnology in Agriculture and Forestry. 8. Plant Protoplasts and Genetic Engineering I" (Y. P. S. Bajaj, ed.), p. 3. Springer-Verlag, Berlin, 1989.

[7] R. P. Finch, P. T. Lynch, J. P. Jotham, and E. C. Cocking, *in* "Biotechnology in Agriculture and Forestry. 14. Rice" (Y. P. S. Bajaj, ed.), p. 251. Springer-Verlag, Berlin, 1991.

[8] S. J. Ochatt and J. B. Power, *in* "Comprehensive Biotechnology 2" (M. Moo-Young, G. S. Warren, and M. W. Fowler, eds.), p. 99. Pergamon, New York, 1992.

[9] J. A. Saunders and G. W. Bates, *in* "Cell Fusion" (A. E. Sowers, ed.), p. 497. Plenum, New York, 1987.

[10] J. A. Saunders, B. F. Matthews, and P. D. Miller, *in* "Electroporation and Electrofusion in Cell Biology" (E. Neumann, A. E. Sowers, and C. A. Jordan, eds.), p. 343. Plenum, New York, 1989.

[11] L. C. Fowke, *in* "Biotechnology in Agriculture and Forestry. 18. Plant Protoplasts and Genetic Engineering I" (Y. P. S. Bajaj, ed.), p. 289. Springer-Verlag, Berlin, 1989.

[12] B. Hahn-Häqerdal, K. Hosono, A. Zachrisson, and C. H. Bornman, *Physiol. Plant.* **67,** 359 (1986).

[13] H. Lörz, *in* "Plant Genetic Engineering" (J. H. Dodds, ed.), p. 27. Cambridge Univ. Press, Cambridge, 1985.

[14] F. Constabel, H. Koblitz, J. W. Kirkpatrick, and S. Rambold, *Can. J. Bot.* **58,** 1032 (1980).

[15] B. Hahne and F. Hoffmann, *Plant Sci.* **47,** 199 (1986).

protoplasts lacking this pigment.[16] Fluorescent dyes have also been used as visual markers to label protoplasts.[17]

Chemically Induced Protoplast Fusion

The plasma membranes of isolated plant protoplasts have a net negative electrical charge of approximately 10–35 mV,[18] as a consequence of which adjacent protoplasts naturally repel each other. To induce the close membrane contact required for membrane fusion, the charges on the surfaces of protoplasts must be neutralized by exposure, for example, to polycations such as polyethylene glycol (PEG), or by the use of a high-pH solution. A number of protocols have been described for chemically induced protoplast fusion.[9,19] The use of PEG coupled with solutions buffered at high pH in the presence of Ca^{2+} (high pH/Ca^{2+}) is the most commonly used method to induce protoplast fusion. Carbonyl-free PEG has been shown to improve protoplast fusion, to diminish the formation of large protoplast aggregates, and to retain protoplast viability.[20]

General Protocols for Chemically Induced Fusion of Plant Protoplasts

Polyethylene Glycol Treatment. The following steps are required.

1. Protoplasts are suspended in CPW13M solution (Table I), typically at a density of 2.0×10^5 ml^{-1}, and 4.0-ml aliquots of each of the respective protoplast suspensions are mixed in 16-ml capacity screw-capped centrifuge tubes (Corning, Ltd., Stone, Staffordshire, England). The protoplasts are pelleted by centrifugation (100 *g*; 10 min, 22°C) and the supernatant removed.

2. Aliquots (2.0 ml) of PEG solution (Table I) are added to the pellets and the protoplasts gently resuspended prior to incubation at 22° for 10 min.

3. The PEG solution is diluted, at 5-min intervals, by the addition of 0.5, 1.0-, 2.0-, 2.0-, 3.0-, and 4.0-ml aliquots of CPW9M solution (Table I). Protoplasts are gently resuspended after each dilution.

4. Protoplasts are centrifuged (100 *g*; 10 min, 22°) and the supernatant removed. Subsequently, they are resuspended in an appropriate culture

[16] R. P. Finch, I. H. Slamet, and E. C. Cocking, *J. Plant Physiol.* **136,** 592 (1990).
[17] K. P. Pauls and P. V. Chuong, *Can. J. Bot.* **65,** 834 (1987).
[18] T. Nagata and G. Melchers, *Planta* **142,** 235 (1978).
[19] J. B. Power, M. R. Davey, M. McLellan, and D. Wilson, "Laboratory Manual: Plant Tissue Culture." University of Nottingham, 1989.
[20] P. K. Chand, M. R. Davey, J. B. Power, and E. C. Cocking, *J. Plant Physiol.* **133,** 480 (1988).

TABLE I
COMPOSITION OF FUSION AND WASHING SOLUTIONS

Solution	Composition[a]
CPW13M	KH_2PO_4 (27.2), KNO_3 (101.0), $Ca_2Cl_2 \cdot 2H_2O$ (1480.0), KI (0.16), $MgSO_4 \cdot 7H_2O$ (246.0), $CuSO_4 \cdot 5H_2O$ (0.025), 13% (w/v) mannitol, pH 5.8, autoclaved
CPW13M/Ca^{2+}	As above, but supplemented with 7.4 g $CaCl_2 \cdot 2H_2O$ per liter
CPW9M	As CPW13M, but with 9% (w/v) mannitol
PEG	30% (w/v) Polyethylene glycol 6000 (Koch-Light, Ltd., Haverhill, England), 4% (w/v) sucrose, 0.01 *M* $CaCl_2 \cdot 2H_2O$, autoclaved
High pH/Ca^{2+}	0.05 *M* Glycine–NaOH buffer, 1.1% (w/v) $CaCl_2 \cdot 2H_2O$, 10% (w/v) mannitol, pH 10.4, filter sterilized
Purified PEG	PEG 1540 (Boehringer-Mannheim, Indianapolis, IN) in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 8.0, filter sterilized
Electrofusion solution	0.05 m*M* $CaCl_2 \cdot 2H_2O$, 11% (w/v) mannitol, filter sterilized

[a] Data in milligrams per liter unless indicated otherwise.

medium before plating at a density of 5.0×10^4 ml^{-1} (this plating density depends on the protoplast partners used for fusion).

High-pH/Ca^{2+} Treatment. Three steps are required.

1. Protoplasts are suspended in CPW13M solution, spun down as in step 1 of the previous section, and 8.0 ml of a high-pH/Ca^{2+} fusion solution (Table I) added. The protoplasts are gently resuspended, immediately centrifuged (60 *g*; 3 min, 22°) and maintained at 30° for 15 min.

2. Sterile distilled water (2.0 ml/tube) is added and gently mixed with the fusion solution, leaving the protoplast pellet intact. Incubation is continued for a further 10 min (30°).

3. The supernatant is removed, the protoplasts washed once in CPW13M/Ca^{2+} solution (Table I), and resuspended in the appropriate culture medium.

Polyethylene Glycol with High-pH/Ca^{2+} Treatment. Fusion frequencies have been enhanced by the use of PEG in combination with high pH/Ca^{2+}.[21] The success of this latter modification probably relates to a combined effect of the two fusogens, which have separate modes of action. Primarily, PEG acts as a protoplast agglutinator, whereas high pH/Ca^{2+} modifies the surface charges of the plasma membrane.[9]

[21] K. N. Kao and M. Saleem, *J. Plant Physiol.* **122,** 217 (1986).

1. Protoplasts are treated with PEG as described in steps 1 and 2 for the section on polyethylene glycol treatment (above), but are diluted with 8.0-ml volumes of high-pH/Ca^{2+} solution per tube. Protoplasts are incubated at 22° for 10 min.

2. The protoplasts are centrifuged (60 *g*; 3 min) and treated as in step 3 of the previous section.

Purified Polyethylene Glycol Fusion Treatment. Polyethylene glycol is known to reduce the viability of fusion products and this cytotoxic effect has been attributed to membrane dehydration[12] and impurities in the polymer, such as α-tocopherol and phenolic derivatives.[22,23] An improved procedure, using PEG preparations (MW 1540) with a low carbonyl content,[20] has been developed for plant protoplasts. This method is applicable to a wide range of plant protoplast systems and results in a high frequency of heterokaryon survival compared with treatments using unpurified PEG.

1. Protoplasts of the species to be fused are suspended separately in 13% (w/v) mannitol solution at a density of 1.0×10^5 ml^{-1} and are allowed to stand for 5–10 min.

2. Equal volumes of the protoplasts suspensions are mixed and 1.0- to 1.5-ml aliquots dispensed into the wells of a 25-compartment 120-mm^2 grid dish (Sterilin, Ltd., Hounslow, Middlesex, England).

3. An aliquot (0.5 ml) of the purified, low-carbonyl PEG solution (Table I) is added and the mixture left for 15–20 min at 22°.

4. One milliliter of 5% (w/v) mannitol solution is added and the fused protoplasts are left for approximately 5 min to become spherical.

5. The mixture of PEG and mannitol solution is removed and the protoplasts are washed in 13% (w/v) mannitol solution. Protoplasts are left in this concentration of mannitol solution for 30 min before a final wash in 13% (w/v) mannitol solution and transfer to culture medium.

Other Methods of Chemical Fusion. Several other methods have been developed for the chemical fusion of plant protoplasts, but none has been used as extensively as the four procedures already described. Three examples of other compounds used to fuse protoplasts are given in the following three sections.

Dextran and dextran sulfate: Both high molecular weight dextran and dextran sulfate have been used to induce protoplast aggregation, although dextran sulfate was found to be toxic to protoplasts. Protoplast fusion has

[22] K. Honda, Y. Maeda, S. Sasakawa, H. Ohno, and E. Tsuchida, *Biochem. Biophys. Res. Commun.* **100,** 442 (1981).

[23] K. Honda, Y. Maeda, S. Sasakawa, H. Ohno, and E. Tsuchida, *Biochem. Biophys. Res. Commun.* **101,** 165 (1981).

also been achieved by using dextran in the presence of organic salts but, to date, somatic hybrid plants have not been reported using this method.[24]

Polyvinyl alcohol: Protoplast adhesion and fusion have been induced by polyvinyl alcohol (PVA) in the presence of $CaCl_2$ and mannitol, with little loss of protoplast viability.[25] Again, this technique has not resulted in the production of somatic hybrid plants.

Agarose and calcium nitrate: Protoplasts plated at high density (1.0×10^5 ml^{-1}) in 2.0% (w/v) agarose in 0.2 *M* $Ca(NO_3)_2$ can be kept in close contact during subsequent treatment with a high-pH/$Ca(NO_3)_2$ solution, which induces the protoplasts to fuse. After a 20-min incubation period the fusion solution is replaced by culture medium. Somatic hybrid and cybrid plants have been produced between *Solanum tuberosum* and *Solanum nigrum* by using this method.[26]

Electrofusion of Protoplasts

Using electrofusion to fuse plant protoplasts can have several advantages when compared with chemically induced fusion.[27] For example, electrofusion eliminates the need for toxic chemical fusogens and extensive washing procedures. The areas of membrane disturbance are restricted to zones of membrane contact, thus maintaining protoplast viability. Most important, electrofusion usually results in a higher frequency of heterokaryon formation.[28]

Electrofusion has several inherent shortcomings. Having to suspend protoplasts in an essentially electrolyte-free solution may adversely affect protoplast viability due to a loss of membrane stability and leakage of cellular electrolytes. Generally, the electronics required for electrofusion are sophisticated and, as a consequence, expensive. Additionally, only relatively small volumes of material can be fused at one time because of the restricted volume of the fusion chamber. Thus, flat chambers of 7-μl capacity, and helical fusion chambers of 200-μl volume, have been explored.[29,30] A convenient electrode system, consisting of a series of parallel

[24] I. Kishinami and J. M. Widholm, *Plant Cell Physiol.* **28,** 211 (1987).
[25] T. Nagata, *Naturwissenschaften* **65,** 263 (1978).
[26] H. Binding, M. Zuba, J. Rudnick, and G. Mordhorst, *J. Plant Physiol.* **133,** 409 (1988).
[27] G. W. Bates, J. A. Saunders, and A. E. Sowers, *in* "Cell Fusion" (A. E. Sowers, ed.), p. 367. Plenum, New York, 1987.
[28] A. Zachrisson and C. H. Bornman, *Physiol. Plant.* **67,** 507 (1986).
[29] G. Pilwat, U. Zimmermann, and H. P. Richter, *FEBS Lett.* **133,** 169 (1981).
[30] U. Zimmermann and J. Vienken, *in* "Hybridoma Technology in Agricultural and Veterinary Research" (N. J. Stern and H. R. Gamble, eds.), p. 173. Rowman & Allanheld, Totowa, NJ, 1984.

brass plates that fit the square wells of a Sterilin 25-compartment dish, enabling volumes of 1.0 ml or more to be handled, has been constructed.[31]

When subjected to a nonuniform alternating electric (AC) field, protoplasts suspended in an electrolyte-free solution move together to form "pearl chains" in which point-to-point membrane contact develops between adjacent protoplasts. Such pearl chains form because the polarized protoplasts move toward the region of higher field strength (dielectrophoresis), and become attracted to each other (mutual dielectrophoresis). Fusion is stimulated by short pulses of direct current (DC), which causes breakdown of the closely aligned membranes. During this process, membrane lipids become randomly oriented and pores develop in the plasma membranes of the protoplasts. Membrane bridges result, leading to the actual fusion process,[32] with the cytoplasms of adjacent protoplasts becoming continuous. Protoplasts can also be brought together and fused by using microelectrodes, avoiding the necessity for a potentially damaging AC field.[33] This technique, if combined with single-cell culture, can permit hybrid cell formation from a defined pair of protoplasts.[34]

Chemical treatments of protoplasts prior to electrofusion have been reported to improve both protoplast stability and fusion frequency. Proteases, polyamines, and dimethyl sulfoxide have been used. These compounds probably decrease membrane fluidity and increase membrane lipid domains.[35-37]

To maximize heterokaryon formation, it is important to optimize the conditions under which short pearl chains (preferably consisting of pairs of protoplasts) are formed. This can be achieved by minimizing the alignment time and AC field strength. Generally, smaller protoplasts less than 25 μm in diameter must be maintained at high densities, usually in excess of 5.0×10 ml^{-1}, in order to maximize heterokaryon formation.

Example of An Electrofusion Protocol. The electrofusion of protoplasts of *Rudbeckia hirta* and *Rudbeckia laciniata* illustrates a typical electrofusion protocol[38] using the plate electrode system.[31]

[31] J. W. Watts and J. M. King, *Biosci. Rep.* **4**, 335 (1984).
[32] U. Zimmermann and H. B. Urnovitz, this series, Vol. 151, p. 194.
[33] H. Morikawa, Y. Hayashi, Y. Hirabayashi, M. Asada, and Y. Yamada, *Plant Cell Physiol.* **29**, 189 (1988).
[34] H. G. Schweiger, J. Dirk, H.-U. Koop, E. Kranz, G. Neuhaus, G. Spangenberg, and D. Wolff, *Theor. Appl. Genet.* **73**, 769 (1987).
[35] P. T. Lynch, S. Isaac, and H. A. Collin, *Planta* **178**, 207 (1989).
[36] L. J. Nea, G. W. Bates, and P. J. Gilmer, *Biochim. Biophys. Acta* **897**, 293 (1987).
[37] L. J. Nea and G. W. Bates, *Plant Cell Rep.* **6**, 337 (1987).
[38] J. S. Al-Atabee, B. J. Mulligan, and J. B. Power, *Plant Cell Rep.* **8**, 517 (1990).

1. Leaf mesophyll protoplasts of *R. hirta* and callus protoplasts of *R. laciniata* are isolated as described.[39] Protoplasts of *R. laciniata* are stained with fluorescein diacetate (25 μg ml^{-1}) during enzyme incubation.

2. Protoplasts of the two species are washed twice in electrofusion medium (Table I) and mixed (1:1) to give a final protoplast density at 2.0×10 ml^{-1}. Aliquots (1.0 ml) of the protoplast mixture are transferred into the 9 central wells of a 25-compartment square-grid dish. The dish is gently agitated to distribute the protoplasts evenly throughout each well, and the protoplasts are allowed to settle for 5 min.

3. The dish is placed on the stage of an inverted microscope, which is situated in a laminar air-flow hood. The parallel-plate electrode assembly is sterilized by immersion in ethanol (for 30 sec) and allowed to dry in the sterile air flow.

4. The electrode is inserted into one of the wells of the dish and the protoplasts are subjected to an AC field of 0.5 MHz, 54 V cm^{-1} for approximately 45 sec. Subsequently, two DC pulses of 810 V cm^{-1}, and 2000 μsec duration, are applied to induce protoplast fusion. The AC field is reduced to zero over a 15-sec period.

5. Twenty-microliter aliquots of CPW13M solution (Table I) are added to each well of the dish. After 15 min the protoplasts are gently transferred to 8.0-ml capacity screw-capped centrifuge tubes. The protoplasts are allowed to settle for 15–20 min, after which the electrofusion-CPW13M solution is withdrawn. The protoplasts are resuspended in culture medium. Examples of the electrical parameters used to fuse plant protoplasts are given in Table II.[35,38,39a–d]

Selection of Somatic Hybrids

Despite efforts to increase protoplast fusion frequencies, the formation of viable, binucleate heterokaryons is typically restricted to less than 5% of the protoplast population. Therefore, it is necessary to select these fusion products against a background of homokaryons, unfused parental protoplasts, and/or multiple fusion bodies. Several selection methods have been described, but a universally applicable system has not yet been developed. Some commonly used selection systems are described below.

Genetic Complementation

Complementation methods depend on fusion of two protoplast systems, each of which carries different recessive selectable markers. The resulting somatic hybrid cells are functionally restored. A range of comple-

[39] J. S. Al-Atabee and J. B. Power, *Plant Cell Rep.* **6**, 414 (1987).

TABLE II
ELECTROFUSION PARAMETERS

Plant species	Alignment		Fusion		Ref.
	Field strength ($V\ cm^{-1}$)	Frequency (MHz)	Field strength ($V\ cm^{-1}$)	Pulse period (μsec)	
Apium graveolens	200	1.5	150	99	35
Nicotiana tabacum, N. plumbaginifolia	300	0.5	500	2000	39a
Oryza sativa	100	2	1000	50	39b
Picea abies, Pinus sylvestris	150	0.35	2000–4000	50–1000	39c
Rudbeckia hirta, R. laciniata	54	0.5	810	2000	38
Solanum tuberosum, S. brevidens	100	1	1250–1500	10	39d

mentation systems has been used to recover somatic hybrid tissues and somatic hybrid plants.

Use of Chlorophyll-Deficient Mutants (Albinos). Fusion of protoplasts from two nonallelic chlorophyll-deficient lines results in somatic hybrid cells that are chlorophyll proficient, as in the case of the fusion of protoplasts from albino cell lines of *Medicago sativa* and *Medicago borealis*.[40] Selection can also be based on complementation between wild-type and albino lines. Thus somatic hybrid cells between wild-type mesophyll protoplasts of *Petunia parodii* and protoplasts from an albino line of *Petunia inflata* exhibit chlorophyll synthesis and sustained growth.[41]

Use of Light-Sensitive Mutants. The fusion of mesophyll protoplasts from a light-sensitive mutant of *Nicotiana plumbaginifolia* with wild-type *mesophyll* protoplasts of *Nicotiana gossei,* irradiated with 200 J Kg^{-1} of ^{60}Co γ rays (0.066 J kg^{-1} sec^{-1} dose rate) prior to fusion, has been used to select heterokaryon-derived green hybrid cell colonies.[42] Regenerated plants have the morphology of *N. plumbaginifolia,* and normal green coloration.

[39a] J. D. Hamill, J. W. Watts, and J. M. King, *J. Plant Physiol.* **129,** 111 (1987).
[39b] K. Toriyama and K. Hinata, *Theor. Appl. Genet.* **76,** 665 (1988).
[39c] U. Kirsten, H. E. Jacob, M. Tesche, and S. Kluge, *Stud. Biophys.* **119,** 85 (1987).
[39d] N. Fish, A. Karp, and M. G. K. Jones, *Theor. Appl. Genet.* **76,** 260 (1988).
[40] D. M. Gilmour, M. R. Davey, and E. C. Cocking, *Plant Cell Rep.* **8,** 29 (1989).
[41] L. S. Schnabelrauch, F. Kloc-Bauchan, and K. C. Sink, *Theor. Appl. Genet.* **70,** 57 (1985).
[42] P. Medgyesy, R. Golling, and F. Nagy, *Theor. Appl. Genet.* **70,** 590 (1985).

Use of Nitrate Reductase-Deficient Lines. Following fusion, parental protoplasts from nitrate reductase-deficient (NR^-) cell lines are eliminated by their inability to utilize nitrate in the culture medium. This deficiency can be overcome in hybrid tissues through complementation by the other fusion partner. Thus, for hybrid cell/tissue selection, two selectable markers are required, with the result that protoplasts from NR^- lines are combined with those carrying other selectable markers. For example, protoplasts from NR^- *Nicotiana tabacum* fused with wild-type *Nicotiana glutinosa* pollen tetrad protoplasts (which do not undergo sustained cell division) produce hybrid cells that utilize nitrate. Such cells regenerate to form green plants.[43] Other types of autotrophic plant mutants can be employed in somatic hybridization selection schemes, including amino acid autotrophic lines for the intraspecific fusion of *Datura innoxia* protoplasts.[44]

Use of Resistance Markers. Dominant characteristics for traits such as resistance to herbicides[45] and amino acid analogs[46] are employed in selection. When protoplasts from two separate and mutually exclusive resistant lines are fused, the tolerance of each parental species is acquired by the somatic hybrid cells and the latter exhibit dual resistance. Unfused parental protoplasts and homokaryons are eliminated during selection. Intraspecific somatic hybrids are produced between parental lines of *S. tuberosum* that have resistance to different amino acid analogs, including *S*-aminoethylcysteine and *S*-methyltryptophan.[47]

Use of Double Mutants. Protoplasts of many potential fusion partners are of the wild type and, as a result, do not possess any markers suitable for selection. One method of overcoming this limitation is to construct a parental line carrying both negative and positive selectable markers, that is, an auxotrophic trait and a resistant trait. Only the heterologous fusion products with complemented auxotrophic–resistant traits will survive selection. Somatic hybrids between *Sinapis turgida* and *Brassica oleracea,* using protoplasts from a double mutant (NR^- and an *S*-methyltryptophan resistant) of *S. turgida,* have been produced by this approach.[48]

Use of Transformed Cell Lines. Resistance markers used in somatic hybrid selection schemes can be introduced by transformation. Protoplasts from transformed lines of *S. tuberosum,* carrying kanamycin or hygromy-

[43] A. Pirrie and J. B. Power, *Theor. Appl. Genet.* **72,** 48 (1986).
[44] P. K. Saxena and J. King, *Plant Cell, Tissue Organ Cult.* **9,** 61 (1987).
[45] J. Gressel, N. Cohen, and H. Binding, *Theor. Appl. Genet.* **67,** 131 (1984).
[46] M. E. Horn, T. Kameya, J. E. Brotherton, and J. M. Widholm, *Mol. Gen. Genet.* **192,** 235 (1983).
[47] S. E. de Vries, E. Jacobsen, M. G. K. Jones, A. E. H. M. Loonen, M. J. Tempelaar, J. Wijbrondi, and W. J. Feenstra, *Theor. Appl. Genet.* **73,** 451 (1987).
[48] K. Toriyama, T. Kameya, and K. Hinata, *Planta* **170,** 308 (1987).

cin B resistance genes, are fused, resulting in hybrid tissue that is resistant to both antibiotics. In forage legumes, kanamycin resistance combined with the use of the metabolic inhibitor sodium iodoacetate (see the next section), are used to select somatic hybrids between *Lotus corniculatus* and *Lotus tenuis*.[49]

Use of Antimetabolites. Complementation selection systems can also be based on the use of irreversible biochemical inhibition, which blocks metabolic pathways when the parental protoplasts are treated prior to fusion.[50] Inactivated parental lines cannot undergo cell division in their own right, but hybrid cells exhibit metabolic complementation and undergo sustained growth. The metabolic inhibitor sodium iodoacetate is used in combination with other markers, including lack of sustained cell division in one of the parental protoplast lines, to select somatic hybrid plants.[51] An example of this selection system involves the fusion of sodium iodoacetate-inactivated *Oryza sativa* protoplasts with protoplasts from a range of wild *Oryza* species. Protoplasts of the wild species fail to divide in culture. Iodoacetate usage requires a careful determination of treatment levels, so as to minimize cross-toxicity from parental protoplasts.[50]

Use of Tumorous Growth of F_1 Hybrids

To permit continued development of regenerated shoots from calli derived from the fusion of protoplasts of *Nicotiana langsdorffii* and *Nicotiana glauca,* the tissues are grafted onto plants of *Nicotiana glauca.*[52] Tumor formation, a characteristic of the sexual F_1 hybrid between these two *Nicotiana* species, is observed on the scion, thus providing a method for somatic hybrid selection.

Use of Differential Growth and Plant Regeneration

The differential response of parental protoplasts to culture conditions provides a method for selecting somatic hybrid tissues. Following the fusion of iodoacetamide-inactivated *O. sativa* protoplasts with those of *Echinochloa oryzicola,* the treated protoplasts are cultured in a medium that supports the growth of rice protoplasts and somatic hybrid cells, but not protoplasts of *E. oryzicola.*[53]

[49] M. A. Aziz, P. K. Chand, M. R. Davey, and J. B. Power, *J. Exp. Bot.* **41,** 471 (1991).
[50] C. T. Harms, *in* "Plant Protoplasts" (L. C. Fowke and F. Constabel, eds.), p. 169. CRC Press, Boca Raton, FL, 1985.
[51] R. Nehls, *Mol. Gen. Genet.* **166,** 117 (1978).
[52] P. S. Carlson, H. Smith, and R. D. Dearing, *Proc. Natl. Acad. Sci., U.S.A.* **69,** 2292 (1972).
[53] R. Terada, J. Kyozaka, S. Nishibayashi, and K. Shimamoto, *Mol. Gen. Genet.* **210,** 39 (1987).

The different mechanisms of plant regeneration also provide a method for somatic hybrid selection. Thus plant regeneration in *R. hirta* occurs through shoot formation, whereas shoot production in *R. laciniata* is via rhizogenesis. Somatic hybrids and plants of *R. laciniata* are regenerated through rhizogenesis. The somatic hybrids are identified by the presence of pigmented roots, a feature of *R. hirta*.[38]

Use of Electrical Stimulation

Electrical pulse treatments have been shown to enhance the division of plant protoplast-derived cells,[54] and to stimulate shoot formation from protoplast-derived cells of several plants, including woody species such as *Prunus avium* × *pseudocerasus*.[55] This technology is applied successfully in somatic hybridization. Thus electroporation of parental protoplasts prior to electrofusion promotes the division of heterokaryons and facilitates the recovery of somatic hybrids between the two woody species *Pyrus communis* var. *pyraster* and *Prunus avium* × *pseudocerasus*.[56] Somatic hybrid tissues are not produced when parental protoplasts are not electrostimulated prior to fusion. Electrostimulation of protoplast division and plant regeneration may prove particularly useful in cases in which parental protoplasts respond to this treatment with increased growth and plant regeneration, especially if used in combination with other selection techniques.

Physical Isolation of Heterokaryons

Biochemical complementation/selection systems usually lead to preferential recovery of amphidiploid somatic hybrids.[57] Asymmetric hybrids, such as those possessing one complete genome but only a few chromosomes of the other parent, are likely to be lost during selection due to an inability of the cells to survive the strong selection pressure, through incomplete complementation to growth proficiency.[57] This, combined with the lack of suitable selectable markers for many parental species, makes physical identification, isolation, and culture of fusion products an important alternative. Heterokaryons can be identified by a dual-labeling system, such as red chlorophyll autofluorescence used in combination with the

[54] E. L. Rech, S. J. Ochatt, P. K. Chand, J. B. Power, and M. R. Davey, *Protoplasma* **141**, 169 (1987).

[55] S. J. Ochatt, P. K. Chand, E. L. Rech, M. R. Davey, and J. B. Power, *Plant Sci.* **54**, 165 (1988).

[56] S. J. Ochatt, E. M. Patat-Ochatt, E. L. Rech, M. R. Davey, and J. B. Power, *Theor. Appl. Genet.* **78**, 35 (1989).

[57] E. C. Cocking, M. R. Davey, D. Pental, and J. B. Power, *Nature (London)* **293**, 265 (1981).

yellow-green fluorescence of fluorescein diacetate.[58] Fluorescein diacetate labeling combined with the use of red fluorochromes such as rhodamine isothiocyanate has also been employed.[59]

Somatic hybrid tissues of *Medicago* species[60] and somatic hybrid plants of *Solanum* species[61] have been recovered from dual-labeled heterokaryons by using micromanipulation. However, micromanipulation is a laborious technique and the number of heterokaryons that can be selected with ease is limited.

Flow cytometry is another procedure that permits the selection of larger numbers (usually several thousand) of labeled heterokaryons.[62] Until recently, the range of somatic hybrid plants recovered from flow-sorted heterokaryons was limited to the genera *Nicotiana* and *Brassica*.[63,64] However, sorting has been extended to fused protoplasts from a wide combination of plant species, in some cases with somatic hybrid plant production.[65]

Confirmation of Hybridity

The first indication of the hybridity of cell lines/callus is their ability to survive the selection procedure. To eliminate potential problems such as reversion, cross-feeding, and residual leakiness from the selection system, additional confirmation is required at both the callus and plant levels.[66] Verification of hybridity requires demonstration of the presence and expression of genetic traits from both parents.

Morphological Characteristics of Regenerated Plants

Intermediate morphologies can be used to identify somatic hybrid material. Leaf shape and size[56] and floral characteristics, including flower size, color, and number of ray florets, can be evaluated.[38] Ideally, several independent characteristics should be considered. The more distant the

[58] G. Patnaik, E. C. Cocking, J. Hamill, and D. Pental, *Plant Sci. Lett.* **24**, 105 (1982).
[59] T. L. Barsby, J. F. Shepard, R. J. Kemble, and R. Wong, *Plant Cell Rep.* **3**, 165 (1984).
[60] D. M. Gilmour, M. R. Davey, and E. C. Cocking, *Plant Sci.* **53**, 267 (1987).
[61] K. J. Puite, S. Roest, and L. P. Pijnacker, *Plant Cell Rep.* **5**, 262 (1986).
[62] D. W. Galbraith, *in* "Cell Culture and Somatic Cell Genetics of Plants" (I. K. Vasil, ed.), Vol. 1, p. 433. Academic Press, London, 1984.
[63] C. L. Afonso, K. R. Harkins, M. A. Thomas-Compton, A. E. Krejci, and D. W. Galbraith, *Bio/Technology* **3**, 811 (1985).
[64] N. Hammatt, A. Lister, N. W. Blackhall, J. Gartland, T. K. Ghose, D. M. Gilmour, J. B. Power, M. R. Davey, and E. C. Cocking, *Protoplasma* **194**, 34 (1990).
[65] C. Sjodin and K. Glimelius, *Theor. Appl. Genet.* **77**, 651 (1989).
[66] R. Nehls, G. Krumbiegel-Schroeren, and H. Binding, *Results Prob. Cell Differ.* **12**, 67 (1986).

taxonomic relationship between the parental species, the greater the number of morphological characteristics that are available for assessment. Morphological features, such as pigmentation[67] and relative growth rates,[68] can be used to identify hybridity, even in protoplast-derived callus. In some cases, morphological analysis may be complicated by abnormalities arising from aneuploidy, somatic incompatibility, or somaclonal variation from the effects of the tissue culture procedure.[50]

Chromosomal Complement of Hybrids

The chromosome complements from actively dividing somatic cells, such as those from root tips, provide further evidence of hybridity and of ploidy levels. Hybrid plants are identified by their chromosome numbers,[43] and the structure and size of somatic cell chromosomes when compared with the karyotypes of parental species.[69] In some cases, chromosome counts may be inaccurate due to doubling or elimination of chromosomes.[70]

Isoenzyme Analysis

The different electrophoretic mobilities of isoenzymes that catalyze basic cell functions can be used to identify hybrid tissues/plants, as is the case of somatic hybrids between wild pear and colt cherry,[56] *Rudbeckia* species,[38] and *Oryza* species.[71] Hybrid tissue may possess isoenzyme band profiles characteristic of each parent, as well as additional bands. These additional bands may be regarded as possible artifacts, or as hybrid molecules or genes present in parent cells that are expressed within the new genetic background.[72]

Molecular Analysis

The development of molecular techniques, such as restriction fragment analysis and DNA hybridization of nuclear and organelle DNAs,[1] has permitted detailed analysis of the genetic constitution of somatic hybrids. Specific patterns of restricted DNA of both mitochondria and chloroplasts

[67] K. Klimaszewska and W. A. Keller, *Plant Sci.* **58,** 211 (1988).
[68] M. Niizeki, *in* "Biotechnology in Agriculture and Forestry. 8. Plant Protoplasts and Genetic Engineering" (Y. P. S. Bajaj, ed.), p. 410. Springer-Verlag, Berlin, 1989.
[69] L. R. Wetter and K. N. Kao, *Theor. Appl. Genet.* **576,** 272 (1980).
[70] F. D'Amato, *CRC Crit. Rev. Plant Sci.* **3,** 73 (1985).
[71] Y. Hayashi, J. Kyozuka, and K. Shimamoto, *Mol. Gen. Genet.* **214,** 6 (1988).
[72] H. Binding, G. Krumbiegel-Schroeren, and R. Nehls, *Results Probl. Cell Differ.* **12,** 37 (1986).

confirm hybridity, and elucidate organelle segregation and DNA recombination patterns.[73] Species-specific DNA fragments are used to determine the relative parental contributions to somatic hybrids.[74] Restriction fragment length polymorphism (RFLP) mapping[75] permits a more detailed examination of the inheritance of nuclear and organelle genomes in somatic hybrids. Thus a variety of established methods are available that permit accurate determination of the presence of genetic material from both parents in somatic hybrids.

Future Prospects for Plant Protoplast Fusion and Somatic Hybridization

Although plant protoplast fusion is now a routine procedure, methods are still being refined and new techniques developed, including radio-frequency electric field-induced fusion (electroacoustic fusion)[76] and laser-induced cell fusion.[77] Electroacoustic fusion may prove particularly useful for small protoplasts, which often require extreme treatment, such as high fusogen concentrations or longer DC pulses. However, in general, the culture of protoplasts postfusion and hybrid cell selection present more problems than the actual process of fusion.

Although conventional methods of plant breeding will continue to play a major role in crop improvement, somatic hybridization will offer a unique opportunity for achieving gene flow in plants, particularly for the transfer of reproductively isolated multigenic traits. The application of protoplast fusion to plant breeding depends on continued extension of the range of crop plants that can be regenerated from protoplasts, together with refinement of the procedures for the selection of somatic hybrid tissues and plants.

[73] A. Morgan and P. Maliga, *Mol. Gen. Genet.* **209**, 240 (1987).
[74] M. W. Saul and I. Potrykus, *Plant Cell Rep.* **3**, 65 (1984).
[75] G. Kochert, "Introduction to RFLP Mapping and Plant Breeding Applications," *in* Rockefeller Found. Int. Program Rice Biotechnol., New York, 1989.
[76] D. C. Chang, *in* "Electroporation and Electrofusion in Cell Biology" (E. Neumann, A. E. Sowers, and C. A. Jordan, eds.), p. 215. Plenum, New York, 1989.
[77] E. Schierenberg, *in* "Cell Fusion" (A. E. Sowers, ed.), p. 409. Plenum, New York, 1987.