Chapter 13

Somatic Hybridization and Cybridization

13.1. INTRODUCTION

Plant protoplasts represent the finest single cell system and offer exciting possibilities in the fields of somatic cell genetics and crop improvement. In culture, isolated protoplasts often perform better than single, whole cells (Nagata and Takebe, 1971; Kao and Michayluk, 1975) and should, therefore, serve as an excellent starting material for cell cloning and development of mutant lines. They also provide experimental material for many other fundamental and applied studies. Freshly isolated protoplasts have been employed in studies related to cell wall synthesis, membrane properties and virus infection. However, the feature of isolated protoplasts that has brought them into the limelight is the ability of these naked cells to fuse with each other irrespective of their origin.

Protoplast fusion has opened up a novel approach to raising new hybrids. This technique of hybrid production through the fusion of body cells, bypassing sex altogether, is called somatic hybridization.

Unlike sexual reproduction in which organelle genomes are generally contributed by the maternal parent, somatic hybridization also combines cytoplasmic organelles from both the parents. In somatic hybrids recombination of mitochondrial genome occurs frequently. Chloroplast genome recombination is rare but segregation of chloroplasts of the two sources in hybrids causes selective elimination of chloroplasts of one or the other parent, forming novel nuclear-cytoplasmic combinations. Fusion products with the nucleus of one parent and extra-nuclear genome/s of the other parent are referred to as cybrid and the process to obtain cells or plants with such genetic combination/s is called cybridization. Somatic cell fusion, thus, offers new ground to achieve novel genetic changes in plants.

This chapter deals with the techniques of somatic hybridization and cybridization which involve a series of interdependent steps, shown in Fig. 13.1. Isolation and culture of protoplasts have been dealt with in Chapter 12. Regeneration of plants from hybrid cells does not warrant further discussion after having dealt with plant regeneration from unfused protoplasts in Section 12.3.3. Other aspects of the technique are discussed in the following pages. Application of the protoplast system in genetic transformation is dealt with in Chapter 14.

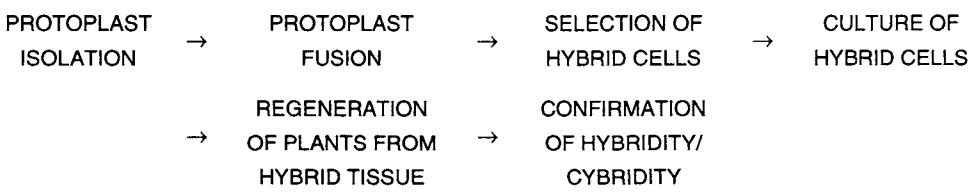

Fig. 13.1. Steps involved in somatic hybridization/cybridization.

13.2. PROTOPLAST FUSION

During enzymatic degradation of cell walls some of the adjacent protoplasts fuse together forming homokaryons (also referred to as homokaryocytes, each with two to several nuclei (Miller et al., 1971; Motoyoshi, 1971; Woodcock, 1973). This type of protoplast fusion, called 'spontaneous fusion', has been ascribed to the expansion and subsequent coalescence of the plasmodesmatal connections between the cells (Withers and Cocking, 1972). The occurrence of multinucleate fusion bodies is more frequent when protoplasts are prepared from actively dividing cultured cells. About 50% of the protoplasts prepared from callus cells of maize endosperm (Motoyoshi, 1971) and suspension cultures of maize embryos (Brar et al., 1979) were multinucleate. A sequential method of protoplast isolation, or exposing the cells to strong plasmolyticum solution before treating them with mixed enzyme solution would sever the plasmodesmatal connection and, consequently, reduce the frequency of spontaneous fusion.

So far as somatic hybridization and cybridization are concerned spontaneous fusion is of no value; these require the fusion of protoplasts of different origin. To achieve induced fusion a suitable chemical agent (fusogen) or electric stimulus is generally necessary. Since 1970 a variety of fusogens have been tried to fuse plant protoplasts of which $NaNO_3$, high pH and high Ca^{2+}, and polyethylene glycol treatments have been successfully used to produce somatic hybrid/cybrid plants. During the last decade fusion of protoplasts by electric stimulus (electrofusion) has gained increasing popularity.

13.2.1. Chemical fusion

(i) *$NaNO_3$ treatment*. As early as 1909, Kuster demonstrated that a hypotonic solution of $NaNO_3$ induces the fusion of sub-protoplasts within a plasmolysed epidermal cell. However, a reproducible, and controlled fusion of isolated protoplasts by $NaNO_3$ was reported by Power et al.

(1970). The technique is fully described by Evans and Cocking (1975). Although this fusogen was used by Carlson et al. (1972) to produce the first somatic hybrid in plants, the technique suffers from a low frequency of heterokaryon formation, especially when highly vacuolated mesophyll protoplasts are involved (Power and Cocking, 1971; Keller and Melchers, 1973; Burgess and Fleming, 1974; Melchers and Labib, 1974). This led to the search for more efficient fusion techniques.

(ii) *High pH and high Ca^{2+} treatment.* In 1973 Keller and Melchers reported that mesophyll protoplasts of two lines of tobacco could be readily fused by treating them in a highly alkaline (pH 10.5) solution of high Ca^{2+} ions (50 mM $CaCl_2 \cdot 2H_2O$) at 37°C for about 30 min. Using this technique Melchers and Labib (1974) and Melchers (1977) produced intraspecific and interspecific somatic hybrids, respectively, in the genus *Nicotiana*. For somatic hybridization in petunias this method of protoplast fusion was regarded as superior to the other two common chemical methods in terms of the throughput of hybrids (Power et al., 1980). However, for some protoplast systems such a high pH may be toxic (Kao and Wetter, 1977). For practical details of the technique, see Appendix 13.I.1.

(iii) *Polyethylene glycol (PEG) treatment.* Since 1974 (Kao and Michayluk, 1974; Wallin et al., 1974) PEG has achieved widespread acceptance as a fusogen of plant protoplasts because of the reproducible high frequency heterokaryon formation and comparatively low cytotoxicity to most cell types. Another merit of PEG-induced fusion, over the other two methods of chemical fusion of protoplasts, is the formation of a high proportion of binucleate heterokaryons (Wallin et al., 1974; Kao, 1977). Burgess and Fleming (1974) reported that treatment with a highly alkaline solution containing Ca^{2+} ions, at 37°C, produced large clumps comprising many protoplasts whereas with PEG the aggregation occurred mostly between two to three protoplasts. PEG-induced fusion is non-specific. In addition to fusing soybean-maize and soybean-barley (Kao et al., 1974), PEG brings about effective fusion between animal cells (Ahkong et al., 1975a; Pontecorvo, 1975), animal cells with yeast protoplasts (Ahkong et al., 1975b), and animal cells with higher plant protoplasts (Dudits et al., 1976b).

The steps involved in fusing protoplasts by PEG are given in Appendix 13.I.2. Briefly, the freshly isolated protoplasts from the two selected parents are mixed in appropriate proportions and treated with 15–45% PEG (1500–6000 MW) solution for 15–30 min followed by gradual washing of the protoplasts with the culture medium. Kao et al. (1974) observed that eluting PEG with a highly alkaline solution (pH 9–10) containing a high

Ca^{2+} ion concentration (50 mM $CaCl_2 \cdot 2H_2O$) led to a higher frequency of fusion than washing with the culture medium (see also Kao and Wetter, 1977). This method, which is essentially a combination of the original PEG method described by Kao and Michayluk (1974) and the high pH high Ca^{2+} ions method of Keller and Melchers (1973), is currently the most widely used method for plant protoplast fusion.

Several factors affect protoplast fusion by PEG:

(a) PEG of molecular weight (MW) higher than 1000 induces tight adhesion and high frequency fusion of protoplasts. Generally, PEG of MW 1500–6000 has been used at concentrations ranging from 15 to 45%.

(b) PEG-induced fusion is enhanced by enriching the PEG solution with Ca^{2+} ions.

(c) The dilution of PEG should be gradual. Drastic elution would result in the formation of very few heterokaryons.

(d) Prolonged incubation in PEG solution reduces heterokaryon formation.

(e) Protoplasts from young leaves and fast growing calli give better fusion.

(f) Whereas the protoplasts from cultured cells can tolerate enzyme, PEG, and high pH high Ca^{2+} treatments fairly well, those from the mesophyll cells have proved sensitive to these conditions. Preculturing the leaves for a few days improves the tolerance of mesophyll protoplasts to these treatments.

(g) Excessive dilution of the enzyme solution leads to poor fusion, probably because of rapid wall synthesis by the protoplasts.

(h) The types of enzymes and their concentrations used for protoplast isolation is another factor influencing protoplast fusion. Driselase yields highly fusable protoplasts but it may also adversely affect the viability of the protoplasts (Kao, 1978).

(i) Protoplast density also influences the fusion frequency. A 4–5% protoplast suspension (protoplast volume/liquid volume) usually gives the highest frequency of heterokaryon formation.

(j) High temperature (35–37°C) promotes fusion frequency while low temperature (15°C) promotes protoplast adhesion. According to Burgess and Fleming (1974) high temperature is especially promotive for the fusion of highly vacuolated protoplasts. In practice, however, the entire fusion experiment is performed at around 24°C.

(k) Repeated centrifugation after the fusion treatments, as is necessary when the fusion experiment is performed in centrifuge tubes, adversely affects the yield and viability of fused protoplasts.

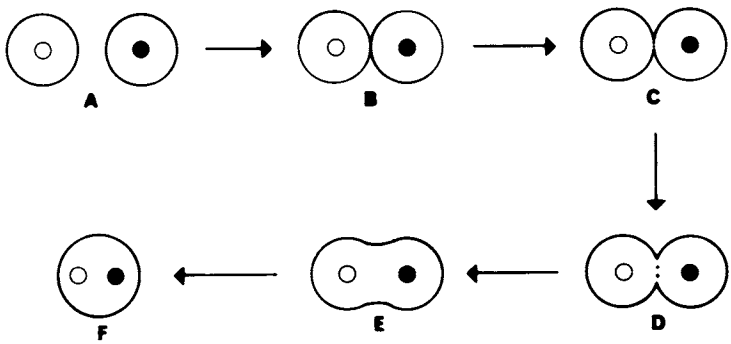

Fig. 13.2. Diagrams showing the sequential stages in protoplast fusion. (A) Two separate protoplasts. (B) Agglutination of two protoplasts. (C,D) Membrane fusion at localized sites. (E,F) Formation of a spherical heterokaryon.

Therefore, the technique of fusing the protoplasts on a coverslip, in 150 μl droplets, as described by Kao and Michayluk (1974), is preferred (for details see Appendix 13.I.2).

(iv) *Mechanism of fusion.* Protoplast fusion consists of three main phases: (a) agglutination, during which the plasma membrane of two or more protoplasts are brought into close proximity (see Fig. 13.2A,B), (b) membrane fusion at small localized regions of close adhesion resulting in the formation of cytoplasmic continuities or bridges between protoplasts (see Fig. 13.2C,D) and, (c) rounding-off of the fused protoplast due to the expansion of the cytoplasmic bridges forming spherical hetero- or homokaryons (see Fig. 13.2E,F).

Protoplast adhesion, which is temperature independent, can be induced by a variety of treatments but this does not necessarily lead to membrane fusion. Plant protoplasts carry a negative surface charge (Grout et al., 1972; Nagata and Melchers, 1978). Depending on the species this charge may vary from –10 to –30 mV. Due to the common charge the plasma membranes of agglutinated protoplasts do not come close enough to fuse. Membrane fusion requires that the membranes must be first brought into apposition at molecular distances of 10 Å or less (Cocking, 1976). The high pH-high Ca^{2+} ions treatment has been shown to neutralize the normal surface charges thus allowing the membranes of agglutinated protoplasts to come in intimate contact (Melchers, 1977). Ten millimolar $CaCl_2 \cdot 2H_2O$ completely removes the charge from tobacco protoplasts. High temperature, which promotes membrane fusion in plants as well as in animals, has been shown to cause perturbance of the lipid molecules in the plasma membranes, and the fusion occurs due to

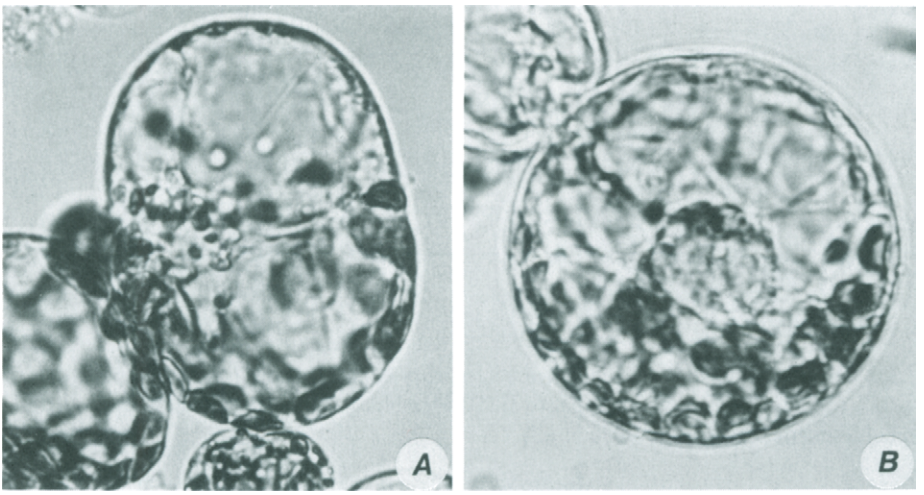

Fig. 13.3. Two stages in the fusion of a non-chlorophyllous protoplast from a suspension culture of *Petunia hybrida* with a green mesophyll protoplast of *P. parodii*. The cytoplasms are becoming progressively mixed together and a heterokaryon has been formed (B) (courtesy of J.B. Power, UK).

the interaction and intermingling of the lipid molecules in the intimately adhering plasma membrane (Ahkong et al., 1975a).

The protoplasts treated with PEG instantaneously agglutinate to form clumps of two or more protoplasts. Tight adhesion of the plasma membranes may occur over a large surface area (Wallin et al., 1974) or it may be restricted to small localized sites in the region of agglutination (Fowke et al., 1975) or both (Burgess and Fleming, 1974). Localized fusion of closely apposed plasma membranes occurs in the regions of tight adhesion, and small cytoplasmic channels are established. These channels gradually expand and the fusing protoplasts, passing through the dumbell-shaped stage (Fig. 13.3A), becomes spherical (see Fig. 13.3B). As the PEG is eluted the fusion bodies are deplasmolyzed and active streaming of the cytoplasm is re-established. This facilitates the rounding of the fusion bodies and mixing up of the cytoplasm which is completed in 3–10 h (Fowke et al., 1975; Gosch and Reinert, 1978).

The actual mechanism of PEG-induced fusion is not clear. Kao and Wetter (1977) have suggested that the PEG molecule, which is slightly negative in polarity, can form hydrogen bonds with water, protein, carbohydrate, etc., which possess positively polarized groups. When the PEG molecule chain is large enough it acts as a molecular bridge between the surface of adjacent protoplasts and adhesion occurs. PEG can bind Ca^{2+}

as well as other cations. The Ca^{2+} may form a bridge between the negatively polarized groups of protein (or phospholipids) and PEG, thus, enhancing adhesion. During the washing process the PEG molecules bound to the membrane, either directly or through Ca^{2+}, are eluted, resulting in disturbance and redistribution of the electric charge. Such a redistribution of charge in the regions of intimate contact of the membranes can link some of the positively charged groups of one protoplast to the negatively charged groups of the other protoplast and vice versa, resulting in protoplast fusion.

In animal cells PEG is reported to cause alterations in membrane structure, such as aggregation of intramembranous protein/glycoprotein particles. As a result, protein-free lipid bilayer regions appear in the plasma membrane. It has been suggested that membrane fusion during PEG treatment occurs in these regions (Ahkong et al., 1975a).

13.2.2. Electrofusion

Chemical fusion of plant protoplasts has many disadvantages: (1) The fusogens are toxic to some cell systems. Benbadis and de Virville (1982) observed destruction of mitochondria following PEG treatment at fusogenic level. (2) It produces random, multiple cell aggregates. (3) The fusogen must be removed before culture. In contrast, electrofusion is rapid (usually complete within 15 min), simple, synchronous, and more easily controlled (Walton and Brown, 1988; Jones, 1991). For somatic hybridization of *Solanum tuberosum* and *S. brevidens*, without selection, electrical fusion (12.3% hybrid shoots) was more effective than PEG-induced fusion (2.6% hybrid shoots; Jones et al., 1990). The somatic hybrids produced by electrofusion of protoplasts often show much higher fertility than those produced by PEG-induced fusion (Han San et al., 1990; Hossain et al., 1994; Asao et al., 1994).

Electrofusion of protoplasts was first demonstrated by Senda et al. (1979). Zimmermann and his co-workers (see Zimmermann and Vienken, 1982) developed this method further, and their work led to the production of an automatic 'Zimmermann Electrofusion System', by GCA Corp., Precision Scientific Group, USA, which is claimed to be 10 000 times more effective than any other method for protoplast fusion.

Since then electrofusion of protoplasts has been applied with great success to a range of systems, and a number of different electrofusion systems with fixed or movable electrodes have been tested (Koop et al., 1983; Zhakrisson and Bornman, 1984; Watts and King, 1984; Gaynor, 1986; Buckley et al., 1990; Hidaka et al., 1995). During the last 5 years several hybrids have been produced by electrofusion of protoplasts

(Mattheij et al., 1992; Hossain et al., 1994; Ling and Iwamasa, 1994; Asao et al., 1994; Motomura et al., 1995). Electrically fused egg and sperm protoplasts of maize also regenerated full plants (see Section 10.4).

The fusion systems generally consist of a DC pulse generator and a sine wave generator connected in parallel to a fusion chamber fitted with two electrodes about 200 μm apart. The fusion chamber, mounted on a glass microscope slide, is connected to two syringe pumps, one containing the protoplasts to be fused and the other fresh sterile osmoticum for washing and flushing the chamber. The chamber, connecting tubing and associated valving can be sterilized by first pumping through 70% ethanol for ca. 10 min followed by a large volume of sterile water. Before fusion the sterilized chamber is flushed with a large volume of fusion mixture. The protoplasts, suspended in the fusion medium of low conductivity (e.g. mannitol solution of appropriate osmolarity) are introduced into the chamber and placed between the two electrodes. A non-uniform high frequency (0.5–1.5 MHz) AC field (10–200 V cm^{-1}) is applied across the protoplasts. As the surface charge on the protoplasts becomes polarized they act as dipoles and migrate along the electric field lines to a region of highest field intensity (Fig. 13.4). If the field intensity is high (ca. 200 V cm^{-1}) the protoplasts may migrate to the electrode with higher electric field but with lower field intensity the aggregation occurs between the electrodes. As the protoplasts have been aligned in chain, one or two short (10–20 μs) DC pulses of high voltage (0.125–1 kV cm^{-1}) are applied which causes reversible membrane breakdown (pore formation) in the contact area of the adjacent protoplasts (Fig. 13.5). The AC field is briefly reapplied to maintain close protoplast contact as fusion begins and then reduced to zero. The fusion process (Fig. 13.6) takes about 10 min. Pretreatment of protoplasts with spermine and the presence of 1 mM $CaCl_2$ in the fusion mixture increases the fusion frequency. With both the treatments up to 60% fusion can be achieved (Lindsey and Jones, 1990). To achieve high frequency one-to-one fusion the protoplast density should be low (ca. 1×10^4 protoplasts ml^{-1}). Fusion in pairs of two protoplasts is also enhanced if the divergence of the electric field and the field strength are not too large (Zimmermann and Greyson, 1983).

In the microdroplet method of electrofusion (Fig. 13.7), developed by Koop et al. (1983), the desired pair of protoplasts is transferred, as described in the microdroplet method of protoplast culture (Section 12.3.2), to a droplet containing low ionic strength fusion medium. In one experiment several fusions are performed using several microdroplets on one coverglass overlaid with a common layer of mineral oil. Fusion is performed by introducing into the droplet a pair of platinum wire electrodes (0.5 μm diameter, 10 mm long). The distance between the electrodes,

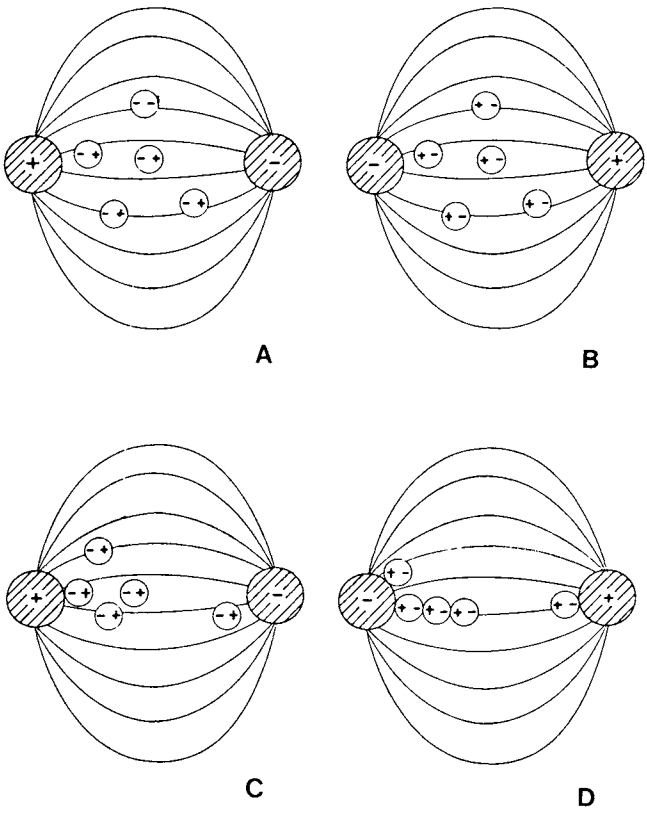

Fig. 13.4. Dielectropole collection of protoplasts (blank circles) in a non-homogeneous AC field. (A,B) The electric field-induced transient dipole in the protoplast membrane (represented by + and −). (C,D) Due to non-homogeneous electric field, the protoplasts move in the direction of higher field strength and attach to the nearest surface of the electrode (hatched circles). Since the bathing medium is of relatively low conductivity (in comparison to the cells) the protoplasts attached to the electrode surface act as local high field-strength region and attract other protoplasts. This leads to the formation of pearl-chain of protoplasts (after J.J. Gaynor, 1986, Handbook of Plant Cell Culture, Vol. 4, with permission of McGraw-Hill Co.).

mounted under the condenser, can be adjusted. After alignment of the protoplasts on one of the electrodes by AC current (1 MHz, 66 V cm^{-1}) fusion is induced by a single negative DC pulse (0.9 kV cm^{-1} for 50 μs). By this method, 50 one-to-one fusions could be performed in 1 h. Full plants have been regenerated after electrofusion of defined pairs of leaf protoplasts (Koop and Schweiger, 1985b).

Electrofusion is more suitable for the fusion of mesophyll protoplasts than root or callus protoplasts (Pelletier, 1993). The presence of large vacuole or amyloplasts is detrimental for the protoplasts during the fu-

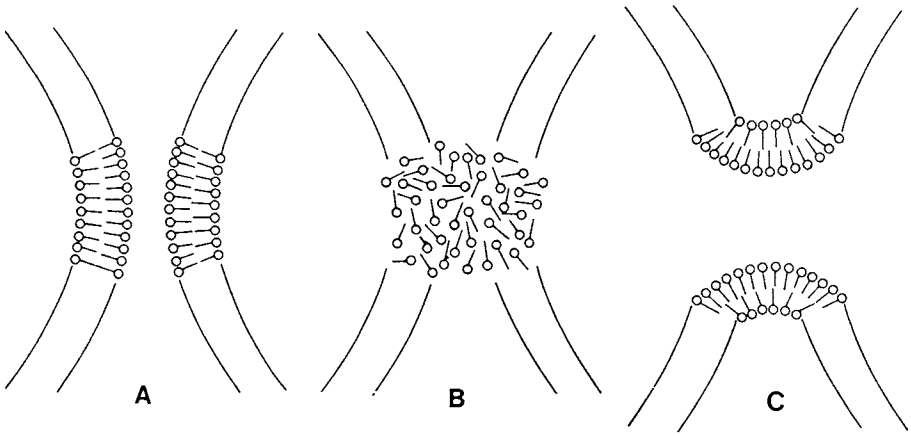

Fig. 13.5. Model of the molecular events occurring during electrofusion. (A) Membranes of neighbouring protoplasts are brought together and held in intimate contact by the process of dielectrophoresis. (B) Application of a single, high strength DC pulse leads to a breakdown of the plasma membrane at the poles of the cells. (C) Reannealing of membranes following fusion pulse. If the two neighbouring protoplasts were in close proximity at the time of local membrane disruption, then lipids reassemble into a single bilayer, fusing the protoplasts ((after J.J. Gaynor, 1986, Handbook of Plant Cell Culture, Vol. 4, with permission of McGraw-Hill Co.).

sion process. Despite the several advantages of the electrofusion method over PEG-induced fusion, the latter continues to be more popular probably because of high technical accuracy and, to some extent, high initial investment associated with the former.

13.3. SELECTION OF FUSION PRODUCTS

In somatic hybridization by electrofusion of protoplasts it may not be difficult to follow the fate of the fusion products because the fusion frequency is very high and sometimes it is possible to achieve one-to-one fusion of desired pairs of protoplasts. However, a chemical fusion treatment results in a heterogeneous mixture of the parental type protoplasts, heterokaryons and a variety of other nuclear-cytoplasmic combinations. The heterokaryons which are the potential source of future hybrids constitute a very small (0.5–10%) proportion of the mixture. Only a fraction of these heterokaryons show nuclear fusion (Pelletier, 1993). Moreover, being novel genetic combinations, several things may happen following fusion treatment which further reduce the number of potential hybrid cell lines to a very low level. It is, therefore, of key importance in somatic hybridization to be able to select the hybrid cells or their products. Numerous different ways of selecting hybrids have been proposed and practised, including morphological basis, complementation of biochemical and

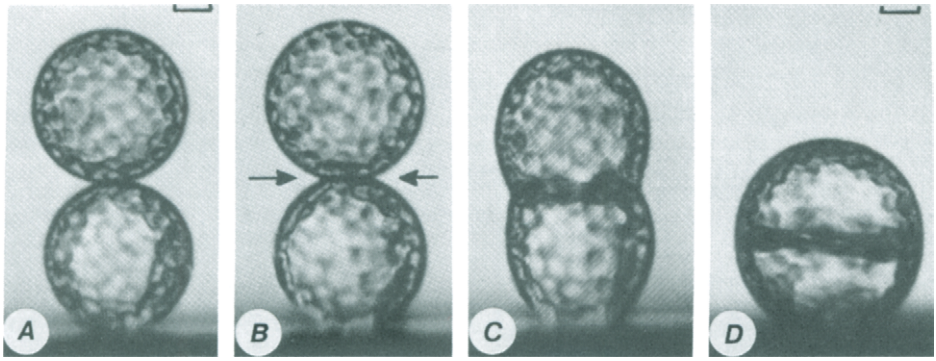

Fig. 13.6. Stages in the electrofusion of *Avena sativa* protoplasts. (A) Point-to-point contact between adjacent protoplasts suspended in 0.5 M mannitol solution and exposed to electric field (frequency 500 kHz and strength 200 V cm^{-1}). (B) Flattening of the protoplasts in the area of membrane contact. (C,D) Fusion of the protoplasts 10 and 30 s after a 15 μs high voltage (600 V cm^{-1}) DC pulse was applied. Bar = 10 μM (reprinted by permission from: U. Zimmermann and J. Vienken, 1982, J. Membr. Biol., 67: 165–182; © Springer-Verlag).

genetic traits of the fusing partners, and manual or electronic sorting of heterokaryons/hybrid cells. The last method is by far the most reliable and of wide application for somatic hybrid production.

13.3.1. Morpho-physiological basis

Some workers have cultured the whole mixture of protoplasts after fusion treatment and screened the calli or regenerated plants for their hybrid characteristics. However, it is a labour intensive method and may require considerable glasshouse space. Occasionally, the hybrid calli may exhibit heterosis and outgrow the parental cell colonies. Selection of putative hybrids based on callus morphology has been used in intra- (Deimling et al., 1988) and interspecific (Guri and Sink, 1988; Mattheij et al., 1992) somatic hybridization in the genus *Solanum*. In the cross *S. tuberosum* + *S. ceraceifolium* one of the parents produces bright green callus (*S. tuberosum*) and the other forms brown-yellow callus with purple coloured cells (*S. ciraceifolium*). The putative hybrid calli were identified by their intermediate morphology, i.e. green with purple coloured cells (Mattheij et al., 1992).

Nakano and Mii (1993a) obtained interspecific somatic hybrids between *Dianthus chinensis* and *D. barbatus* without an artificial selection system. PEG-fused protoplasts were cultured in the absence of a selection pressure and 30 calli exhibiting vigorous growth were selected. Two of the calli differentiated shoots, one more profusely than the other. The

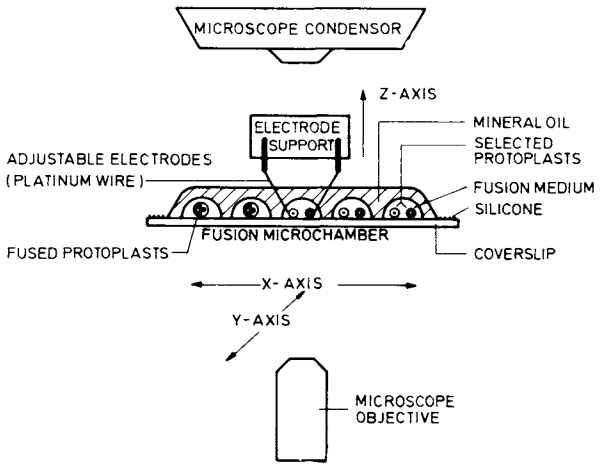

Fig. 13.7. Set-up for the microdroplet method of electrofusion of individually selected protoplasts (reprinted by permission from: H.G. Schweiger et al., 1987, Theor. Appl. Genet., 73: 769–783; © Springer-Verlag)

shoots were grown to the flowering stage in vitro. The flower colour, chromosome number and esterase isozyme pattern confirmed the hybrid nature of the plants. Similar hybrid vigour for callus growth was reported in an intraspecific combination involving *S. tuberosum* (Austin et al., 1985; Waara et al., 1989), interspecific combinations in *Datura* (Schieder, 1978), and *Brassica* (Taguchi and Kameya, 1986) as well as the intergeneric combination *Lycopersicon esculentum* + *Solanum muricatum* (Sakamoto and Taguchi, 1991).

In the interspecific cross *Dianthus chinensis* + *D. barbatus*, mentioned above, the two parents differed in their morphogenic potential which could have acted as a selection force at the final stage of plant regeneration. Whereas the protoplast-derived callus of *D. chinensis* showed 30% regeneration the other parent proved non-regenerable under the prevailing culture conditions. Plant regeneration in many cases has proved to be a dominant trait.

Recently, several reports have demonstrated that somatic hybrids can be efficiently selected by combining the dominant uniparental regeneration potentiality with iodoacetamide induced inactivation of the protoplasts of regenerable parent (Terada et al., 1987; Wright et al., 1987; Takamizo et al., 1991; Nakano and Mii, 1993b; Krasnyanski and Menczel, 1995). In such crosses the protoplasts of the regenerable parent are incapable of forming a callus. Since the calli derived from the other parent lacks regeneration potential, only the hybrid calli differentiate plants. This system seems to be quite promising as it requires neither the

production of mutants or transformants with genetic markers nor special equipment and technique.

The scope of this selection method is limited to certain combinations showing differences in their regeneration potential under specific culture conditions.

13.3.2. Complementation

In this method complementation of genetic or metabolic deficiencies of the two fusion partners are utilized to select the hybrid component. When protoplasts of two parents, each carrying a non-allelic genetic or metabolic defect are fused it reconstitutes a viable hybrid cell of wild type in which both defects are mutually abolished by complementation, and the hybrid cells are able to grow on minimal medium non-permissive to the growth of the parental cells. For such a complementation it is necessary that the defects are recessive and expressed in cultures (Harms, 1983).

Melchers and Labib (1974) demonstrated complementation to wild type green calli and plants after fusion of protoplasts from two chlorophyll deficient varieties of tobacco. Cocking et al. (1977) and Power et al. (1979, 1980) produced three interspecific hybrids of *Petunia* by applying a selection scheme involving complementation of cytoplasmic albino trait of one parent (*P. hybrida, P. inflata, P. parviflora*) and sensitivity to culture medium of the other parent (*P. parodii*). In all these combinations (see Fig. 13.8) when the protoplasts were plated in MS medium after the fusion treatment *parodii* protoplasts were eliminated at the small colony stage. Only the protoplasts of the other parent and the hybrid component developed into full callus. The calli of the hybrid nature could be clearly distinguished from the parental-type tissue by their green colour. The selection procedures used by Schieder (1978a), Dudits et al. (1977) and Krumbiegel and Schieder (1979) also rely partly on the use of nuclear albinos as one of the parents.

Glimelius et al. (1978) obtained tobacco colonies capable of growing on nitrate as the sole source of nitrogen when they fused protoplasts from a *cnx*-type and a *nia*-type mutant cell lines deficient in nitrate reductase activity.

Complementation selection can also be applied to dominant characters, such as dominant resistance to antibiotics, herbicides or amino acid analogues (Harms et al., 1981). Dominant expression of resistance in one parental line is supplemented, on the other hand, with sensitivity to a second drug for which the other fusion partner is resistant. Drug sensitivity behaves as a recessive trait. On a medium containing toxic levels of both drugs the double resistance of hybrid cells enables them to grow but ei-

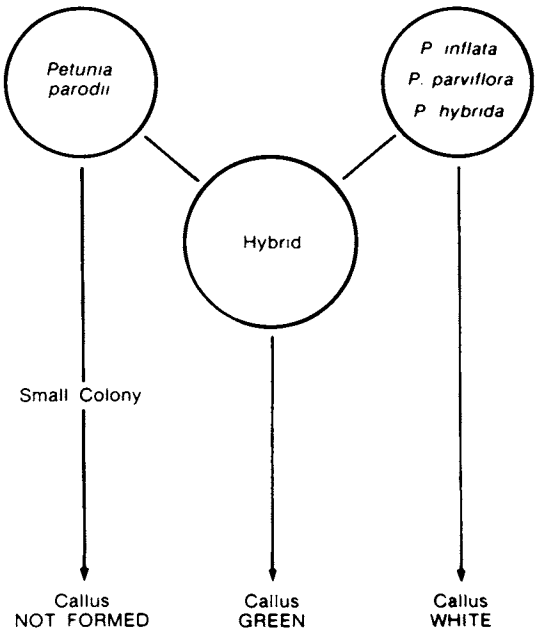

Fig. 13.8. Diagrammatized summary of the selection system used in interspecific somatic hybridization of *Petunia parodii* (wild type) with *P. hybrida*, *P. inflata* and *P. parviflora* (albinos). For details see text (after Cocking et al., 1977; Power et al., 1979, 1980).

ther parent is killed. Marker traits can be introduced into the parents either by mutagenesis or genetic engineering. Resistance to antibiotics (Hamill et al., 1984), amino acid analogues (Harms et al., 1981) and herbicides (Evola et al., 1983) have been employed in some cases using induced or spontaneously occurring mutations. Masson et al. (1989) and Thomas et al. (1990) utilized the transformation of both fusion parents, one with hygromycine and the other with kanamycin resistance genes and selected hybrids by adding both antibiotics to the culture medium.

The combination in the same parent of an auxotrophic mutation (such as nitrate reductase deficiency or albinism) and a dominant trait (such as antibiotic or herbicide resistance) result in so-called universal hybridizer. Hybrids between this and any wild type genotype belonging to the same or another species can be directly selected after fusion by culturing the fusion products in a minimal medium supplemented with the antibiotic or herbicide where both parents are unable to grow. Toriyama et al. (1987b) isolated a double mutant of *Sinapis turgida* which was deficient in nitrate reductase and was resistant to 5-methyltryptophane (5MT).

This line was used to produce intergeneric hybrids with *Brassica oleracea* and *B. nigra* by first culturing the fusion products on a medium containing NO_3^- as the sole source of nitrogen, which eliminated *S. turgida* protoplasts, and later transferred to NO_3^- medium containing 5MT to eliminate the other parent. All the plants finally regenerated were hybrids.

13.3.3. Isolation of heterokaryons or hybrid cells

The most reliable and widely applicable selection system is one which involves isolation of the heterokaryons or hybrid cells and their culture individually or at low density. This approach has gained strength from the success achieved with low density culture of protoplasts using media highly enriched with organic components, conditioned medium, feeder cell-layer technique, and microdrop technique (see Section 12.3.2). This approach not only allows definitive picking up of the hybrid components but also in purer forms. In other selection systems, especially where more than one type of cells is favoured to grow, the neighbouring cell clusters may fuse and make an adulterated tissue mass.

Manual isolation of heterokaryons requires that the two parental type protoplasts have distinct morphological markers and are easily distinguishable. Kao (1977) demonstrated that if green, vacuolated, mesophyll protoplasts of one parent were fused with richly cytoplasmic, non-green protoplasts from cultured cells of another parent the fusion products could be identified for some time in culture. Using this approach, Gleba and Hoffmann (1978, 1979) produced an intergeneric hybrid between *Arabidopsis thaliana* and *Brassica campestris*. Mesophyll protoplasts of *Brassica* were fused with cultured cell protoplasts of *Arabidopsis*. From 3 to 5-day-old cultures, the individual fusion products, mostly with four to eight cells, were mechanically isolated using a micropipette and cultured separately in Cuprak dishes. The inability of the parental protoplasts to grow well in the medium used initially served as the added selection pressure. A similar procedure was followed to obtain somatic hybrids between *Nicotiana tabacum* and *N. knightiana* (Menczel et al., 1981). A major advance in this field has been the development of a dual fluorescence labelling system for heterokaryons. Protoplasts labelled green by treatment with fluorescein diacetate (1–20 mg l^{-1}) are fused with protoplasts emitting a red fluorescence, either from chlorophyll autofluorescence or from exogenously applied rhodamine isothiocyanate (10–20 mg l^{-1}). The labelling can be achieved by adding the compound into the enzyme mixture. This system can be applied even for visual selection of heterokaryons formed by the fusion of morphologically indistinguishable protoplasts, such as mesophyll protoplasts of two parents.

Manual isolation of the heterokaryons by Pasteur pipette or an automatic pipette connected to a gentle suction has the advantage that multiple fusion products can be rejected but it is tedious and time consuming. Therefore, for physical isolation of dual-labelled heterokaryons some workers (Hammatt et al., 1990; Sundberg et al., 1991) have used fluorescence-activated cell sorter (FACS; Galbraith, 1991) which is accurate and exceptionally rapid (about 5×10^3 cells s^{-1}). After fusing the protoplasts labelled with different fluorochromes that fluoresce at different wavelengths, the mixture is fed to the FACS. In the machine the fluid stream carrying the protoplasts is passed between focused laser light and photocells that detect the fluorescence. The stream is dispersed into droplets and the droplets containing cells with single (parental) and double (heterokaryons) fluorescence are electrostatically deflected into different sterile containers.

13.4. VERIFICATION OF HYBRIDITY

Despite the numerous procedures that have been employed to select the desired hybrids/cybrids following protoplast fusion, it is evident from the available literature that no system is foolproof. Most of the selection systems have their characteristic disadvantages which preclude their widespread use. For example, the selection schemes relying on differential responses of the parental species towards the medium or culture regimes used, suffer from cross-feeding between the parental protoplasts when the fusion products are cultured. Therefore, successful passage through a selection system should be treated as first evidence for the hybridity of the selected materials. Further proof must be added from other, independent markers to finally prove or disprove the hybrid nature of the selected putative hybrids. This proof requires a demonstration of genetic contribution from both parents. Some of the commonly employed criteria for this purpose are listed below.

13.4.1. Morphology

Intermediate expression of numerous vegetative and floral characters, such as stalk height and diameter, leaf shape, type of trichomes formed, pigmentation, flower colour and morphology, have been screened for evaluation of presumed somatic hybrids to demonstrate their hybridity. This criterion is, however, not very reliable. Variations induced by tissue culture environment may alter some morphological characters or the hybrid may show entirely new traits not displayed by either of the parents.

13.4.2. Cytological analysis

One of the primary features to characterize somatic hybrids is the chromosome complement. Comparison of number and morphology of chromosomes would reveal if the putative hybrid possesses the expected chromosome complements from the two parents, it is an aneuploid or it involves intergenomic translocations. This is relatively easy when the parental species exhibit prominent differences in chromosome number and morphology. However, this approach will not be applicable to all species, particularly where fusion involves closely related species or where the chromosomes are very small (e.g. *Brassica* species).

A more generalized approach recently introduced to detect relative contributions of each parental genome and restructuring of chromosomes in somatic hybrids is in situ hybridization of 'species-specific' repetitive DNA probes to mitotic chromosomes (Piastuch and Bates, 1990; Itoh et al., 1991).

13.4.3. Isozyme analysis

Isozymes are defined as multiple molecular forms of an enzyme exhibiting similar or identical catalytic properties (Harms, 1983). If the two parents exhibit different band patterns for a specific isozyme the putative hybrid can be easily verified. The banding pattern displaying isozymes from both parents are usually sufficient proof of hybridity. The hybrid may also show isoenzyme bands derived from new combinations of enzymatic subunits. The isozymes commonly used for hybrid identification include acid phosphatase, esterase, peroxidase, phosphoglucoisomerase, phosphoglucomutase and glutamate oxaloacetate transaminase.

Isozyme banding pattern of a particular enzyme may vary considerably depending on the tissue source examined. Some enzymes, i.e. peroxidase isozymes, are particularly variable and do not provide reliable markers unless special care is taken. It is essential to use strictly comparable tissue samples when performing isozyme analysis.

13.4.4. DNA analysis

Recent developments of molecular biological techniques have greatly expanded our analytical tools which can serve to characterize somatic hybrids and cybrids. Demonstration of the presence of DNA from both parents provides the most direct proof of hybridity.

Restriction fragment length polymorphism (RFLP) analysis of nuclear (Williams et al., 1990) and organelle (Pehu, 1991) DNA has been widely

used to verify the hybrid and cybrid nature of the selected fusion products. Lately, southern blot analysis using species-specific repetitive DNA probes (Piastuch and Bates, 1990; Itoh et al., 1991; Fahleson et al., 1994a) or non-radioactive rDNA (ribosomal RNA genes) probes (Honda and Hirai, 1990; Nakano and Mii, 1993ac) have also been used to analyse nuclear genomes of somatic hybrids, and these methods are becoming increasingly popular in confirmation of hybridity of the putative somatic hybrids because of their efficiency and simplicity. These DNA-based methods of verifying hybrid nature are independent of the tissue source and can, therefore, be applied at a relatively early stage in somatic hybridization.

Randomly amplified polymorphic DNA (RAPD) is another recently introduced molecular method to screen hybrids (Xu et al., 1993; Nakano and Mii, 1993ac). Like rDNA analysis (Honda and Hirai, 1990) RAPD requires very small amount of tissue. However, it has been suggested that RAPD should be used only as a quick preliminary screening for putative somatic hybrids, and it should be followed by RFLP, species-specific DNA probes and/or chromosome counting to accurately confirm the nature of the hybrids (Xu et al., 1993).

13.5. GENETIC CONSEQUENCES OF PROTOPLAST FUSION

Fusion of protoplasts at the level of the plasmalemma is non-specific, and there is no barrier to interspecific, intergeneric, interfamily, or even interkingdom fusion of cells. Therefore, a range of wide crosses between sexually incompatible parents have been attempted through cell fusion to incorporate useful genes from wild species into present day cultivars of crop plants. Some examples of somatic hybrids with potential agronomic value are listed in Table 13.1.

Detailed cytological and biochemical analyses of somatic hybrid cell lines and the plants regenerated from them have revealed that hybrid cells can give rise to hybrid plants with full nuclear genomes from both the fusion partners (symmetric hybrids). More often, however, gradual elimination of chromosomes of one of the partners occurs during successive cell cycles, resulting in hybrids with full nuclear genome of one of the parents and only a part of the nuclear genome of the other parent (asymmetric hybrids). The third category of plants obtained from fused protoplasts are those which retain the nuclear genome of only one of the partners but with at least some alien extra-nuclear genes (Cybrids).

The fate of the nuclear genome in the course of somatic hybridization largely depends on three factors: (1) the number and type of parental cells participating in fusion; (2) genomic segregation during the first di-

TABLE 13.1

Examples of somatic hybrids with potential agronomic value

Combination	Resistance/ trait[a]	Reference
Nicotiana tabacum + N. repanda	TMV	Bates (1990b)
Solanum tuberosum + S. ciraceifolium	*Phytophthora*, *Nematode*	Mattheij et al. (1992)
S. tuberosum + S. brevidens	PLRV *Phytophthora* *Erwinia*	Austin et al. (1985), Helgeson et al. (1986), Austin et al. (1988)
S. tuberosum + S. phureja	Higher yield	Matheij and Puite (see Puite, 1992)
S. melongena + S. integrifolium	*Pseudomonas*	Kameya et al. (1990)
S. melongena + S. saintwongsei	*Pseudomonas*	Asao et al. (1994)
Oryza sativa + O. officinalis	Blast	Hayashi et al. (1989)
Brassica napus + B. carinata	*Phoma*	Sjodin and Glimelius (1989b)
B. napus + B. juncea	*Phoma*	Sjodin and Glimelius (1989b)
B. napus + B. nigra	*Phoma*	Sjodin and Glimelius (1989a,b)
B. napus + B. tournefortii	*Phoma*	Liu et al. (1995)

[a]TMV, tobacco mosaic virus; PLRV, potato leaf roll virus.

vision of the fusion product; and (3) chromosome segregation and/or rearrangement during colony formation and/or plant regeneration. Consequently, in a mass protoplast fusion experiment a wide variety of genetic recombinants may arise with different frequencies (Gleba and Shlumukov, 1990). Table 13.2 illustrates the degree of variation in chromosome number, and chloroplast segregation in somatic hybrids between different species in the tribe Brassiceae.

Somatic hybrid cells also show segregation of cytoplasmic inclusions. A heterokaryon formed after cell fusion contains two or more nuclei in a cytoplasm with plastids and mitochondria of the two parents. As a rule, the plastid population shows random segregation of the parental types in successive cell generations, so that the daughter cells, as early as small colony stage, are left with exclusively one or the other type of plastids (Kung et al., 1975; Chen et al., 1977; Melchers et al., 1978; Aviv et al., 1980; Akada and Hirai, 1986). Interparental recombination of plastid genomes (plastome) after cell fusion occurs only rarely (Medgyesy et al., 1985; Thanh and Medgyesy, 1989). In contrast, mitochondrial genomes (chondriome) very often undergo interparental recombination (Belliard et al., 1978, 1979; Rothenberg et al., 1985; Vedel et al., 1986; Menczel et al., 1987; Akagi et al., 1989; Kyozuka et al., 1989; Yang et al., 1989; Derks et al., 1991), and after segregation one of the many recombined genomes is

TABLE 13.2

Chromosome number, chloroplast segregation and fertility in somatic hybrids produced between different species within the tribe Brassiceae (after Glimelius et al., 1990)

Hybrid combination (no. of hybrid plants)	Chromosome number	% of hybrid plants	% of plants with chloroplasts from parent		Self fertility (% of rape)
A + B			A	B	
B. oleracea +	38	30			17
B. campestris	<38	9			–
(23)	>38	52	54	46	0.2
	Chimeric	9			–
B. napus +	54	67			4.6
B. nigra	<54	23			6.4
(30)	>54	7	88	12	0.4
	Chimeric	3			–
B. napus +	56	44			50
B. oleracea	<56	0			–
(18)	>56	50	68	32	2.0
	Chimeric	6			–
B. napus +	74	88			10
B. juncea	<74	0			–
(8)	>74	12	100	0	10
	Chimeric	0			–
B. napus +	60	8			2
Eruca sativa	<60	59			7
(24)	>60	29	85	15	1
	Chimeric	4			–

retained in a regenerated plant or its progeny (Pelletier, 1991). Independent assortment of chloroplasts and mitochondria results in a very large number of mitochondria-plastid combinations (Fig. 13.9). However, the most frequent cytoplasmic constitution of the cells derived after cell fusion are those where mitochondria with recombinant genomes are associated with one or the other parental plastid genomes (cases 7 and 8 in Fig. 13.9). The observed behaviour of nuclear genomes and cytoplasmic organelles in the course of somatic hybridization generates plants with novel nuclear-chloroplast-mitochondrial combinations.

Since asymmetric hybrids and cybrids are, generally, more valuable than full hybrids between completely unrelated plants, methods have been developed to promote asymmetric hybridization or cybridization.

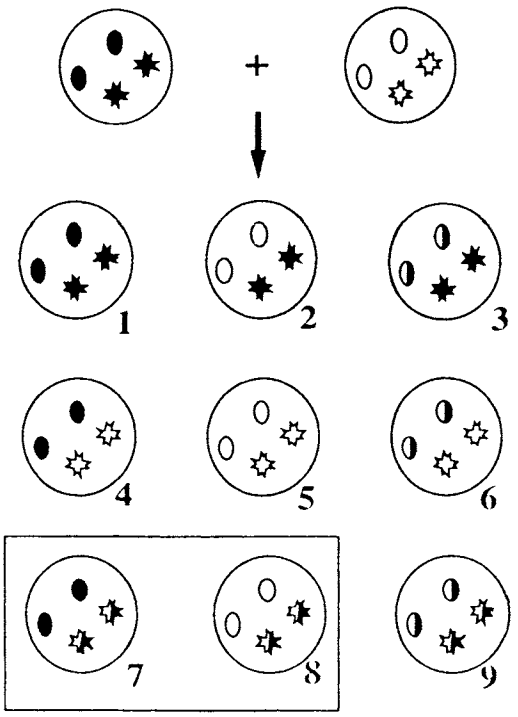

Fig. 13.9. The nine theoretical products of protoplast fusion between parents differing in plastid (○ , ●) and mitochondria (✡ , ✱), considering random elimination of one or the other parental genome and interparental recombination (◑ , ✡). Combinations No. 7 and No. 8 are most frequently observed (after Pelletier, 1993).

13.5.1. Symmetric hybridization

Despite a high incidence of chromosome elimination following fusion of protoplasts of distantly related parents, several interspecific, intergeneric, inter-tribal or even interfamily somatic hybrids have been produced, some of which are also fertile.

In the Brassicaceae several interspecific and intergeneric somatic hybrids have been produced (see Table 13.3). Sundberg and Glimelius (1986) and Sundberg et al. (1987) resynthesized *B. napus* by fusing the protoplasts of *B. oleracea* ($2n = 18$) and *B. campestris* ($2n = 20$). The heterokaryons were isolated 24 h after culture of PEG-treated protoplasts, either manually with a micropipette or by fluorescein activated cell sorter FACS-III (Glimelius et al., 1986) and cultured at low density. About 14% of the isolated fusion products formed calli of which 2% differentiated shoots. All the shoots regenerated from manually isolated heterokaryons were hybrids as against 87% shoots from flow sorted heterokaryons. As

TABLE 13.3

Examples of interspecific and intergeneric somatic hybrids produced during the past decade

Combination	Reference
Interspecific	
Brassica juncea + B. spinescens	Kirti et al. (1991)
B. napus + B. carinata	Sjodin and Glimelius (1988b)
B. napus + B. juncea	Sjodin and Glimelius (1988b)
B. napus + B. nigra	Sjodin and Glimelius (1988a,b)
B. napus + B. oleracea[a]	Jourdan et al. (1989a)
B. oleracea + B. campestris[a]	Sundberg and Glimelius (1986), Sundberg et al. (1987), Yamagishi et al. (1994)
Citrus sinensis + C. limon	Tusa et al. (1990)
C. sinensis + C. paradisi	Ohgawara et al. (1989)
C. sinensis + C. unshiu	Kobayashi et al. (1988)
Dianthus chinensis + D. barbatus	Nakano and Mii (1993a)
D. caryophyllus + D. chinensis	Nakano and Mii (1993b)
Helianthus annuus + H. giganteus[a]	Krasnyanski and Menczel (1995)
Lycopersicon peruvianum + L. pennellii	Adams and Quiros (1985)
L. esculentum + L. peruvianum[a]	Kinsara et al. (1986)
Oryza sativa + O. brachyantha	Hayashi et al. (1988a)
O. sativa + O. eichingeri[a]	Hayashi et al. (1988a)
O. sativa + O. officinalis[a]	Hayashi et al. (1988a)
O. sativa + O. perrieri[a]	Hayashi et al. (1988a)
Solanum melongena + S. integrifolium	Kameya et al. (1990)
S. melongena + S. sisymbriifolium	Gleddie et al. (1986)
S. melongena + S. khasianum[a]	Sihachakr et al. (1988)
S. melongena + S. torvum	Guri and Sink (1988a), Sihachakr et al. (1989)
S. melongena + S. nigrum	Guri and Sink (1988b)
S. melongena + S. ethiopicum	Daunay et al. (1993)
S. melongena + S. integrifolium	Kameya et al. (1990)
S. melongena + S. saintwongsei[a]	Asao et al. (1994)
S. tuberosum + S. brevidens[a]	Austin et al. (1985, 1986), Helgeson et al. (1986)
S. tuberosum + S. circaeifolium	Mattheij et al. (1992)
Intergeneric	
Brassica campestris + Barbarea vulgaris	Oikarinen and Ryoppy (1992)
B. carinata + Camelina sativa[a]	Narasimhulu et al. (1994)
B. juncea + Diplotaxis muralis[a]	Chatterjee et al. (1988)
B. juncea + Moricandia arvensis[a]	Kirti et al. (1992b)
B. juncea + Trachystoma ballii[a]	Kirti et al. (1992a)
B. napus + Arabidopsis thaliana	Forsberg et al. (1994)
B. napus + Barbarea vulgaris[a]	Fahleson et al. (1994)
B. napus + B. tournefortii[a]	Liu et al. (1995)

B. napus + *Eruca sativa*	Fahleson et al. (1988)
B. napus + *Thlaspi perfoliatum*[a]	Fahleson et al. (1994b)
B. oleracea + *Moricandia arvensis*	Toriyama et al. (1987a)
B. oleracea + *Sinapis turgida*[a]	Toriyama et al. (1987b)
Citrus aurantifolia + *Feroniella lucida*[a]	Takayanagi et al. (1992)
C. aurantifolia + *Swinglea glutinosa*	Takayanagi et al. (1992)
C. sinensis + *Atalantia ceylanica*[a]	Louzada et al. (1993)
C. reticulata + *Severinia buxifolia*[a]	Grosser et al. (1992)
C. sinensis + *Citropsis gilletiana*[a]	Grosser et al. (1990)
C. sinensis + *Murraya paniculata*[a]	Shinozaki et al. (1992)
C. sinensis + *Poncirus trifoliata*	Ohgawara et al. (1985), Grosser et al. (1988a)
C. sinensis + *Severinia disticha*[a]	Grosser et al. (1988b)
C. reticulata × *C. paradisi* + *Atalantia monophylla*[a]	Motomura et al. (1995)
C. reticulata + *Citropsis gabunensis*[a]	Ling and Iwamasa (1994)
C. reticulata × *C. paradisi* + *Severinia buxifolia*[a]	Motomura et al. (1995)
Oryza sativa + *Echinochola oryzicola*[a]	Terada et al. (1987)
Solanum lycopersicoides + *Lycopersicon esculentum*	Hossain et al. (1994)
Solanum tuberosum + *Lycopersicon pimpinellifolium*	Okamura (1988)

[a]Sexually incompatible combination, as indicated by the authors.

determined by chromosome counting and DNA content analyses, 30% of the hybrids showed 38 chromosomes. The 6 amphidiploid somatic hybrids with normal morphology showed 38–70% pollen viability (75% of the pollen viability of normal *B. napus*), and seed-set varied from 1 to 40%.

Some new species produced in the Brassicaceae by protoplast fusion are '*Brassicomoricandia* (*Brassica oleracea* + *Moricandia arvensis*; Toriyama et al., 1987a) and *B. naponigra* (*B. napus* + *B. nigra*; Sjodin and Glimelius, 1989a; Yamagishi et al., 1989). Fahleson et al. (1994a) made intertribal crosses between *B. napus* and *Barbarea vulgaris* but the somatic hybrids suffered from poor growth and differentiation of vital organs, such as roots. Even the rooted hybrids could not be established as full plants in the glasshouse. In contrast, fertile, intertribal symmetric somatic hybrids were obtained following the crosses *B. napus* + *Arabidopsis thaliana* (Forsberg et al., 1994) and *B. napus* + *Thlaspi perfoliatum* (Fahleson et al., 1994b).

A major objective of traditional plant breeding is the transfer of genes conferring disease resistance or stress tolerance from closely or distantly related wild species into modern high yielding crop cultivars, which is often thwarted because of sexual incompatibility barriers. Somatic hybridization can bypass these barriers. This is well illustrated by several

successful attempts to produce somatic hybrids involving *Solanum tuberosum* (potato) as one of the parents. Austin et al. (1985, 1986) produced somatic hybrids between *S. tuberosum* (tetraploid) and *S. brevidens* (diploid); the latter is a non-tuber producing wild species with genes for resistance against some common viral and fungal diseases of potato. The resulting tuber-bearing amphiploid (hexaploid) hybrids, with full chromosome compliments of the two parents were fertile and cross compatible with *S. tuberosum* (Ehlenfeldt and Helgeson, 1987). The wild species genes conferring resistance to potato leaf roll virus (PLRV) and *Phytophthora infestans* and tuber forming trait of *S. tuberosum* were present in the hybrids (Austin et al., 1985; Helgeson et al., 1986). Moreover, the hybrid tuber had resistance to 'tuber soft rot', a disease caused by the bacteria *Erwinia cartovora*, which was not predictable because *S. brevidens* does not form tubers.

Mattheij et al. (1992) reported another successful attempt to transfer disease resistance into potato from a wild *Solanum* species by somatic hybridization. Three out of four somatic hybrids obtained after fusing the protoplasts of diploid *S. circaeifolium* and dihaploid *S. tuberosum* were fully resistant to the pathogen *Phytophthora infestans*, and all four hybrids were highly resistant to the nematode, *Globodera pallida*. Sexual crosses between the somatic hybrids (as a female parent) and tetraploid *S. tuberosum* yielded viable seeds, demonstrating the potential of the hybrids in potato breeding. In a field trial of six tetraploid somatic hybrids between *S. tuberosum* and *S. phureja*, one hybrid gave three times higher tuber yield than the potato cultivar (Matheij and Puite; cited in Mattheij et al., 1992).

Somatic hybrids between *S. melongena* and *S. integrifolium*, possessing total chromosome number of the two parents ($2n = 48$) and showing high resistance to *Pseudomonas solanacearum* than either of the parents, were produced by Kameya et al. (1990). Fertile somatic hybrids showing resistance to *P. solanacearum* were obtained by PEG induced protoplast fusion of *S. melongena* and *S. saintwongsei* (Asao et al., 1994).

Melchers et al. (1978) had produced 'pomato' by fusing the protoplasts of potato and tomato but all the hybrids were sterile, probably because the potato protoplasts involved in the fusion were derived from aneuploid callus cells. Fertile 'pomato' plants could be obtained by fusing mesophyll protoplasts of potato and *Lycopersicon pimpinellifolium* (Okamura, 1988). Somatic and sexual hybridization in the genus *Lycopersicon* has been reviewed by Lefrancois et al. (1993).

Recently, Krasnyanski and Menczel (1995) have produced fertile somatic hybrids between *Helianthus annuus* and *H. giganteus*. Seeds from two of such hybrids produced normal fertile F_2 plants. Several inter-

specific (Grosser et al., 1989; Ohgawara et al., 1989; Tusa et al., 1990) and intergeneric (Grosser et al., 1988, 1990) somatic hybrids of *Citrus* with sexually compatible and incompatible partners have been developed during the past decade (see Table 13.3).

13.5.2. Asymmetric hybridization

Interspecific somatic hybrids, as also sexual hybrids, between a wild species and a cultivated species contain many undesirable traits of the wild species besides the desired ones. Backcrossing of the hybrid with the cultivated species, required to remove the unwanted genes of the wild parent, is hampered because the more spectacular hybrids between remote species are generally sterile. Combining alien genomes may also interfere with normal development of the hybrid, so that the hybrid callus is incapable of regenerating plants (Gupta et al., 1984), the hybrid plants are necrotic and die before attaining maturity (Terada et al., 1987) or produce abnormal flowers. Gleba and Hoffmann (1979) had suggested that the formation of asymmetric hybrids through the induction of unilateral chromosome elimination might improve hybrid morphology and fertility.

It is evident from the analyses of somatic hybrids that even if barriers preventing sexual hybridization between two remote species are bypassed by protoplast fusion, barriers may still exist at the genomic level, resulting in spontaneous elimination of chromosomes during culture of the hybrid cells. Consequently, some of the hybrid plants regenerated from the fusion products lack some or most of the chromosomes of one of the fusion partners. The nucleolar chromosomes of *Solanum phureja* were eliminated preferentially in the somatic hybrid *S. tuberosum* + *S. phureja* (Pijnacker et al., 1987). The somatic hybrids that have lost chromosomes of one of the parents and are, therefore, phenotypically closer to the other parent are called asymmetric hybrids. The mechanism that determines which lot of chromosomes is to be partially eliminated is not well understood. Generally, the chromosomes of the parent with shorter cell cycle are retained. Another factor which may cause genomic incompatibility is the difference in the state of differentiation of the two cells involved in fusion (Warren, 1991). For example, when cells from actively growing suspension cultures and non-dividing mesophyll cells are fused there are higher chances of loss of chromosomes of the latter parent.

The observation of spontaneous occurrence of asymmetric hybrids led to a search for methods to achieve directed elimination of chromosomes of the donor parent, to allow the synthesis of asymmetric but fertile hy-

brids. This is now possible by X- or γ-irradiation of the donor protoplasts before fusing them with normal protoplasts of the recipient parent, a method originally developed for the transfer of cytoplasmic genetic information (Zelcer et al., 1978; see Section 13.5.3). Irradiation causes fragmentation of the chromosomes rendering the protoplasts incapable of dividing. When such protoplasts are fused with normal protoplasts of the recipient parent, some of the fragments may get integrated into the hybrid genome. Such asymmetric hybrids can be recovered by selection for nuclear traits of the donor. Dudits et al. (1980) first described the restoration of chlorophyll synthesis in carrot albino mutants by fusing its protoplasts with irradiated parsley protoplasts. Similarly, Somers et al. (1986) restored nuclear-coded nitrate reductase (NR) activity in a NR deficient tobacco mutant by fusion with irradiated barley protoplasts. Following similar strategies several asymmetric hybrids, with one or a few traits (Dudits et al., 1980, 1987; Gupta et al., 1984; Bates et al., 1987; Wolters et al., 1991) to many chromosomes (Imamura et al., 1987; Muller-Gensert and Schieder, 1987; Gleba et al., 1988; Famelaer et al., 1989; Yamashita et al., 1989; Wijbrandi and Koornneef, 1990) of the donor genome have been obtained based on irradiation of the donor parent protoplasts.

Yamashita et al. (1989) produced asymmetric hybrids by fusing *B. oleracea* protoplasts with X-irradiated protoplasts of *B. campestris* and established their hybridity on the basis of morphology, isozymes and chromosome number. Itoh et al. (1991) confirmed the asymmetric hybrid nature of these plants by using in situ hybridization of *B. campestris*-specific, middle repetitive DNA sequences to their metaphase chromosomes. By backcrossing of the asymmetric hybrids with *B. oleracea* twice, Itoh et al. obtained fully fertile plants with all the morphological characters of *B. oleracea* but showing a disease resistance derived from *B. campestris*. Sjodin and Glimelius (1989b) achieved interspecific transfer of resistance to *Phoma lingam*, a fungus causing severe diseases in several cruciferous crops, by asymmetric hybridization. Complete resistance to the pathogen occurs in *B. nigra* and related species, *B. carinata* and *B. juncea*. By fusing *B. napus* protoplasts with irradiated protoplasts of *B. juncea* or *B. carinata* and by addition of sirodesmin (a toxin produced by *P. lingam*) in the culture medium, as a selection pressure, asymmetric hybrids were regenerated which proved fully resistant to infection by pycnospores of the fungus. Another interspecific asymmetric hybrid in the genus *Brassica* was produced by Schieder et al. (1991). Irradiated protoplasts of *B. nigra* ($2n = 16$) were fused with *B. napus* ($2n = 38$) protoplasts and hybrid colonies were regenerated on the basis of hygromycin resistance introduced in *B. nigra* by genetic engineering. Some of the hy-

brids were resistant to *Plasmodiophora brassicae*, a trait contributed by *B. nigra*. The hybrids showed a great inter- and intra-clonal variation in chromosome number. Cells with different chromosome numbers occurred in the same root, suggesting that chromosome elimination in somatic hybrids may continue even after plant regeneration.

Bates (1990a,b) recovered asymmetric hybrids following fusion of *Nicotiana tabacum* protoplasts with irradiated protoplasts of *N. repanda*. The hybrids, which appeared similar to *N. tabacum*, were obtained by selection for kanamycin resistance, introduced into *N. repanda* by *Agrobacterium*-mediated transformation. Two of the hybrids displayed *N. repanda's* hypersensitivity response when inoculated with TMV. Although largely male sterile, many of the hybrids produced viable seeds when backcrossed with *N. tabacum*. Bates (1990b) has also transferred hypersensitivity to TMV from *N. glutinosa* to *N. tabacum* through asymmetric hybridization.

In asymmetric hybridization the degree of elimination induced by irradiation varies considerably with the species involved. When normal protoplasts of *N. plumbaginifolia* were fused with *N. tabacum* protoplasts exposed to γ-irradiation as low as 50 Gy, cybrids containing *N. plumbaginifolia* nuclear DNA and *N. tabacum* chloroplasts were obtained (Menczel et al., 1982). In contrast, fusion of irradiated *Solanum tuberosum* protoplasts with the protoplasts of *Lycopersicon esculentum* did not yield even a single hybrid lacking potato DNA (Wolters et al., 1991). γ-Irradiation up to 500 Gy was insufficient for total elimination of potato chromosomes from the fusion products.

Partial donor genome transfer to raise asymmetric hybrids can also be achieved by using microprotoplasts containing one or a few chromosomes (Ramulu et al., 1992). Miniprotoplasts can be obtained after inducing micronuclei formation by treatment with antimicrotubule agents (Sree Ramulu et al., 1991).

13.5.3. Cybridization

In sexual hybridization the plastid and mitochondrial genomes are generally contributed by only the female parent whereas in somatic hybridization the extranuclear genomes from both the parents are combined. Consequently, the latter approach to crossing plants offers a unique opportunity to study the interaction of the cytoplasmic organelles. Interparental recombination of mitochondrial genomes and independent assortment of chloroplasts and mitochondria following cell fusion results in plants with novel combinations of nuclear/plastid/mitochondria genomes. A plant having nuclear genome mostly derived from one of the

fusion partners with at least some alien organelle genome, derived from the other fusion partner, is termed cybrid (Galun, 1993).

Some of the desirable traits, such as cytoplasmic male sterility (CMS), certain types of disease resistance and herbicide resistance are encoded in extranuclear genomes. Alloplasmic lines, with nucleus of one parent in the cytoplasm of another parent are conventionally obtained by crossing the two parents with the cytoplasm-donor (hereafter called 'donor') as the female parent, followed by a series of backcrossing with cytoplasm-recipient (hereafter, called 'recipient') parent as the recurrent pollinator. This process is time consuming and may require several years. Another drawback of this method is that alloplasmic transfer can only be performed between sexually compatible species. Moreover, this approach does not allow combining two cytoplasmically controlled traits occurring in different plants. By cell fusion, on the other hand, cybrids can be produced in a single manipulation, and it is an efficient method to transfer cytoplasmic characters from one parent to the other or combining cytoplasmic characters from two parents.

(i) *Methods to produce cybrids.* In an experiment involving fusion of full protoplasts of two parents, cybrids may arise through: (a) fusion of a normal protoplast with an enucleate protoplast; (b) fusion between a normal protoplast and a protoplast containing non-viable nucleus; (c) elimination of one of the nuclei after heterokaryon formation; or (d) selective elimination of chromosomes at a later stage. Although cybrids have been produced by this approach, lacking a nuclear fusion control, it is possible to improve the chances of recovery of desired cybrids by inactivating the nucleus of the donor parent by X- (Menczel et al., 1987) or γ- (Barsby et al., 1987a) irradiation (5–30 kR) of its protoplasts, before fusion (Zelcer et al., 1978; Aviv and Galun, 1980). These treatments apparently do not have any deleterious or mutagenic effect on organelle genomes, probably because these genomes are present at a high copy number in each plant cell. Consequently, when the irradiated protoplasts of the donor plant are fused with normal protoplasts of the recipient plant and fusion products are cultured, only the protoplasts of the recipient parent with cytoplasmic genome of either or both the parents are able to divide and regenerate plants. However, in cybrid production using irradiated donor protoplasts incorporation of fragmented nuclear DNA of the donor parent into the recipient genome may occur. After all this is also the most popular method to raise asymmetric hybrids (Section 13.5.2). Probably, the chances of cybrid formation by this method can be increased by additional selection pressure in favour of the nuclear genome of the recipient parent.

Contribution of nucleus by the donor parent can also be avoided by using their cytoplasts (enucleated sub-protoplasts) for fusion with normal protoplasts of the recipient parent. Highly purified preparations of cytoplasts can be obtained in a single step by high-speed centrifugation (20 000–40 000 × *g* for 45–90 min) of protoplasts in an iso-osmotic density gradient, with 5–50% Percoll (Lorz et al., 1981). A modification of this method of cytoplast production is described by Lesney et al. (1986). Maliga et al. (1982) were the first to demonstrate the transfer of streptomycin-resistant chloroplasts in *Nicotiana* by cytoplast-protoplast fusion. Sakai and Imamura (1990) produced CMS *B. napus* by fusion of cytoplasts isolated from CMS *Raphanus sativus* Kosena with iodoacetamide-inactivated protoplasts of male *B. napus*. A problem with using cytoplasts could be that often nucleate miniprotoplasts may occur with cytoplasts as contaminant (Pelletier, 1991).

When an irradiated protoplast or a cytoplast of the donor parent is fused with a normal protoplast of the donor parent, the fusion product receives the cytoplasm of both the parents, and interparental recombination of organelle genomes may occur before random segregation of the organelles. To retain only the cytoplasmic organelles of the donor parent, Medgyesy et al. (1980) recommended the treatment of the recipient protoplasts with metabolic inhibitors, such as iodoacetate (IOA) or iodoacetamide (IOAM). In an experiment involving fusion of irradiated donor protoplasts and metabolically inactivated recipient protoplasts the parental protoplasts are unable to divide but due to metabolic complementation the fusion products may divide and form tissues in which cells possess the nucleus of the recipient partner and cytoplasm of the donor parent. This donor-recipient method (Sidorov et al., 1981), which considerably enhances the chances of selecting the desired nucleus-cytoplasm combinations is now most widely used to produce cybrids. However, in this approach a careful analysis of the cybrids is required because some nuclear information of the irradiated donor may persist as individual or translocated chromosomes (Menczel et al., 1987; Sidorov et al., 1981).

(ii) *Some examples of useful cybrids produced through cell fusion.* Cybridization has been used successfully to make intergeneric and interspecific transfer of cytoplasm in tobacco, petunia, rice and *Brassica* species. In recent years most extensive work on cybrid production has been done in the genus *Brassica*.

Several CMS systems (encoded in mitochondrial genome) are available in the genus *Brassica*, including *Nap* (Thompson, 1972), *Ogu* (Bannerot et al., 1977) and *Polima* (Sernyk, 1983) types. *Ogu* type of CMS, discovered in *Raphanus sativus* by Ogura (1968), was introduced into *B. oler-*

acea and *B. napus* through intergeneric sexual crosses (Bannerot et al., 1974, 1977). However, the resulting plants, although male sterile, suffered from several deficiencies. In all the alloplasmic CMS lines produced by sexual crosses the plants exhibited yellowing at low temperatures (<15°C) and, although green at higher temperature they always maintained low level of chlorophyll. Screening among varieties of various *Brassica* species for genes to correct the chloroplast deficiency or restorer genes for CMS trait have been unsuccessful (Yarrow, 1992). Moreover, the flowers of CMS plants of *Brassica* species exhibited poor development of nectaries, resulting in reduced production of nectar (Pelletier et al., 1983). By fusing the protoplasts of chlorophyll deficient CMS lines of *B. napus* with those of male fertile *B. napus* (with normal cytoplasm) or *B. campestris* (with atrazine resistant chloroplasts), Pelletier et al. (1983) obtained CMS *B. napus* plants in which cold sensitive chloroplasts of *R. sativus* were replaced by those of *B. napus* or *B. campestris* as a result of random segregation of the plastids. Plants with a new combination of mitochondrial male sterility and fully functional plastids were identified by morphological traits after random regeneration from parental and fused protoplasts. In field tests some of the cybrids also showed correction for other deficiencies related to floral morphology (Pelletier et al., 1988). They produced enough nectar to be as attractive to bees as fertile plants and possessed female fertility. The findings of Pelletier et al. (1983) were confirmed by Menczel et al. (1987). However, these workers used X-irradiated (5.7 kR) protoplasts of the CMS donor parent which made selection of the desired genotypes easier because protoplasts of one of the parents did not divide.

A wild *B. campestris* showing resistance to the triazine herbicide atrazine, due to a single point mutation in the plastome genome, was isolated by Matais and Bouchard (1978). This cytoplasmically controlled trait had been transferred to cultivars of *B. napus* and *B. campestris* (Beversdorf et al., 1980). Yarrow et al. (1986) and Barsby et al. (1987b) combined, by cybridization, the two useful cytoplasmically controlled traits, viz. CMS (encoded in chondriome) and atrazin resistance (encoded in plastome), occurring in different plants. However, the two studies employed different selection strategies. Yarrow et al. manually picked up the heterokaryons and cultured them using *Nicotiana tabacum* nurse cell system, earlier used for the selection of novel cytoplasm-nuclear combinations in *N. tabacum* (Flick et al., 1985) and in the synthesis of *B. napus* through somatic hybridization (Sundberg and Glimelius, 1986). The atrazine resistant CMS (*nap*) *B. napus* plants produced by Yarrow et al. (1986) were morphologically normal and produced seeds on pollination with viable pollen. Barsby et al. (1987b) combined the *Polima* type CMS

and herbicide resistance of *B. campestris* in *B. napus* using the donor-recipient selection system.

Jourdan et al. (1989b) synthesized atrazine resistant CMS *B. napus* by cybridization between CMS (*Ogu*) *B. oleracea* and atrazine-resistant *B. campestris*. This group also transferred atrazine-resistant chloroplasts from *B. napus* to *B. oleracea* (Jourdan et al., 1989a).

Kameya et al. (1989) demonstrated that somatic hybridization between sexually incompatible species offers the possibility of producing male sterile plants. They fused IOAM-treated protoplasts of *B. oleracea* with normal protoplasts of *B. campestris* and cultured them on MS medium which does not favour division of *R. sativus* protoplasts. From the regenerants CMS plants with nucleus of *B. oleracea* and plastids of *R. sativus* were obtained.

13.6. CONCLUDING REMARKS

The current methods of isolated gene transfer (Chapter 14) have somewhat overshadowed the importance of somatic hybridization in crop improvement. However, the application of genetic engineering is limited to transfer of single gene traits. The characters, such as yield and stress resistance, which require transfer of cluster of genes are not amenable to improvement by the present methods of genetic engineering. In this regard somatic cell fusion acquires significance.

Somatic hybridization provides a new approach to widen the genetic base of our crop plants by facilitating gene flow between sexually incompatible species. Somatic cell fusion may not be so useful to produce spectacular hybrids such as 'pomato' as once expected but certainly has helped in producing several intergeneric and interspecific hybrids, which could not be obtained sexually and has generated some useful breeding material. The progress in asymmetric hybridization and cybridization by somatic cell fusion has considerably enhanced the importance of this asexual method of plant breeding.

Hybrid selection after protoplast fusion has been a serious bottle-neck in somatic hybridization. By electrofusion of protoplasts it has become possible to achieve very high frequency fusion. In some cases protoplasts can be fused in desired pairs and the fusion products cultured separately to obtain hybrids without an additional selection system.

APPENDIX 13.I: PROTOCOLS FOR FUSING PLANT PROTOPLASTS

13.I.1. High pH and high Ca^{2+}-induced fusion (after Keller and Melchers, 1973; Melchers and Labib, 1974)

(a) Mix freshly isolated protoplasts of the selected parents in a ratio of 1:1 with a final density of ca. 2.5×10^5 protoplasts ml^{-1}.
(b) Pellet the protoplasts by centrifuging at $50 \times g$ for 3–5 min.
(c) Remove the supernatant and add 2 ml of the fusion mixture, containing 50 mM glycine–NaOH buffer, 50 mM $CaCl_2 \cdot 2H_2O$, and 400 mM mannitol (pH 10.5).
(d) Pellet the protoplasts by centrifuging at $50 \times g$ for 3–5 min.
(e) Place the centrifuge tube in a water bath at 37°C for 10–30 min.
(f) Replace the fusion mixture by washing medium (600 mM mannitol, 50 mM $CaCl_2 \cdot 2H_2O$) and leave for 30 min.
(g) Wash twice with the washing medium.
(h) Suspend the protoplasts in culture medium and culture as small drops.

13.I.2. PEG-induced fusion (after Kao, 1976)[1]

(a) Mix freshly isolated protoplasts (while still in the enzyme solution) of the two desired parents in a ratio of 1:1. Pass the suspension through a 62-μm pore size filter and collect the filtrate in a centrifuge tube. Seal the mouth of the tube with a screw cap.
(b) Centrifuge the filtrate at $50 \times g$ for 6 min to sediment the protoplasts.
(c) Remove the supernatant with a Pasteur pipette.
(d) Wash the protoplasts with 10 ml of solution I (500 mM glucose, 0.7 mM $KH_2PO_4 \cdot H_2O$ and 3.5 mM $CaCl_2 \cdot 2H_2O$, pH 5.5).
(e) Resuspend the washed protoplasts in solution I to make a suspension with 4–5% (v/v) protoplasts ml^{-1}.
(f) Put a 2–3 ml drop of Silicon 200 fluid (100 cs) in a 60 × 15 mm petri dish.
(g) Place a 22 × 22 cm coverslip on the drop.
(h) Pipette ca. 150 μl of the protoplast suspension onto the coverslip with a Pasteur pipette.

[1] This method could be modified to fuse protoplasts in a centrifuge tube but in that case every washing would be followed by centrifugation which has been reported to adversely affect the fusion process and the viability of mesophyll protoplasts.

(i) Allow about 5 min for the protoplasts to settle on the coverslip forming a thin layer.

(j) Add drop-by-drop 450 μl of PEG solution (50% PEG 1540, 10.5 mM $CaCl_2 \cdot 2H_2O$, 0.7 mM $KH_2PO_4 \cdot H_2O$) to the protoplast suspension. Observe protoplast adhesion under an inverted microscope.

(k) Incubate the protoplasts in the PEG solution for 10–20 min at room temperature (24°C).

(l) Gently add two 0.5 ml aliquots of solution II (50 mM glycine, 50 mM $CaCl_2 \cdot 2H_2O$, 300 mM glucose, pH 9–10.5) at 10-min intervals. After another 10 min add 1 ml of protoplast culture medium.

(m) Wash the protoplasts five times at 5-min intervals with 10 ml of the fresh protoplast culture medium. At the end of each washing do not remove the entire medium from the coverslip. Leave behind a thin layer of the old medium over the protoplasts and add to it fresh medium. If the parent protoplasts are distinguishable visually it may be possible to assess the frequency of heterokaryon formation at this stage.

(n) Culture the fused products together with the unfused protoplasts on the same coverslip in a thin layer of 500 μl of culture medium. Put additional 500–1000 μl medium in the form of droplets around the coverslip to maintain the humidity inside the petri dish.