Chapter 5
Protoplast Cultures

With suitable enzymes the cell wall of plant cells can be removed through hydrolysis of its macromolecular building material, i.e., "naked" cells called protoplasts are derived. In an isotonic medium, these protoplasts are healthy and can survive. Protoplasts are used to investigate a broad range of physiological problems reaching from the significance of the cell wall for nutrient uptake to mechanisms related to the synthesis of the cell wall. In an early investigation Bush and Jacobson (1986) show for protoplasts the same kinetics, time course and pH response, e.g., of potassium uptake as the intact cells of a suspension. Besides such basic problems since the 1960s in many instances protoplasts were used to solve problems of practical plant breeding.

It is an old dream of plant breeders to produce hybrids of different plant species not to be obtained by cross pollination to have plant material with properties characteristic of both parents. The probably most prominent example is a hybrid of potatoes and tomatoes as parents with the ability to produce tomatoes as fruits and potatoes growing on subterranean stolons. As can be seen from a reproduction from Strasburger's Lehrbuch der Botanik printed at the beginning of last century (Fig. 5.1; cf. it is missing in later editions), this was also the aim of Winkler's occulation experiments using two solanaceous species. A histological inspection of the shoot apex clearly shows that the hybrids obtained are chimeras with quite interesting morphologies of fruits and leaves. The arrangement of cell layers of both "parents" is probably the result of a mixture of cells of wound callus formation on the cutting edges made for the occulation procedure.

Based on first successful fusion experiments with protoplasts of different species in the seventies of last century hybrids of tomatoes and potatoes produced by fusion of protoplasts of the two species were reported (see Fig. 5.2). Mesophyll protoplasts of *Solanum lycopersicum*, and callus protoplasts of *Solanum tuberosum* were fused by Melchers et al. (1978; 80 mM $CaCl_2$, 4.5% PRG, pH 10), but the plants produced were sterile.

Restriction analyses of chloroplast DNA, and characterization of RuBisCO of both parents as well as the hybrid by electrophoresis clearly indicated the

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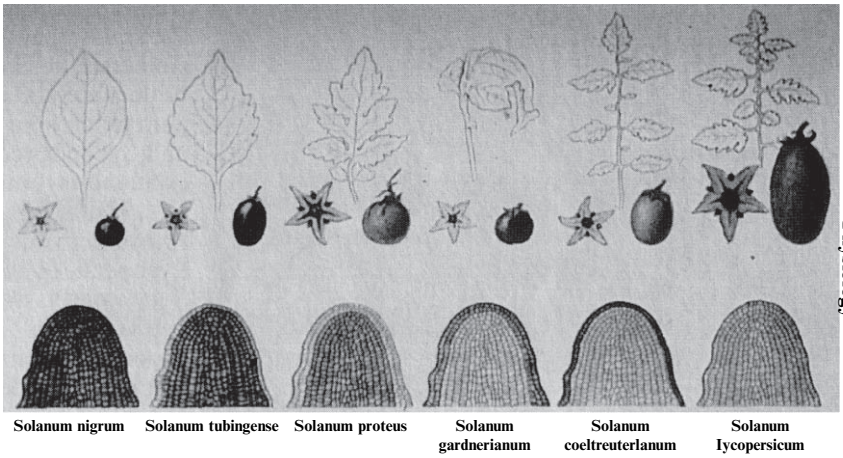

Fig. 5.1 Grafting chimeras of *Solanum nigrum* and *Solanum lycopersicum*, and parents (original H. Winkler). A leaf, a flower, the shoot apex, and a fruit are shown for each hybrid. In the apex, the cell layers stemming from *S. nigrum* are dark colored, those from *S. lycopersicum* are light (from Strasburger et al. 1913)

Fig. 5.2 A hybrid produced by protoplast fusion of *Solanum tuberosum* and *Solanum lycopersicum* (topatoes; hybrid nucleus of *S. tuberosum* and *S. lycopersicum*, plastids from *S. lycopersicum*). *Top* Fruits developed after pollination with *Solanum stenotomum*. *Bottom* Underground storage organs

occurrence of two types of hybrids, despite fusion of the nuclei. Still, the number of chromosomes was higher than those of either parent. One type of hybrid apparently contained plastids only of the potato (pomatoes), and the other those of the tomato (topatoes). Mixed cases were not found, but only a limited number of individuals were investigated, of which two thirds were pomatoes and one third were topatoes. A successful fusion was identified after microscopically detecting the fusion of color-free (pre-grown in the dark) potato protoplasts, with protoplasts of light green tomato plants containing a genetically disturbed chlorophyll system. A transfer of the potato cells from darkness to the light resulted in the formation of chlorophyll, and regenerates had leaves with normal chlorophyll concentration. Callus cultures of the tomato parent regenerated only adventitious roots. Regenerates of the fusion experiments with normal chlorophyll concentration were either of potato origin, or offspring of a protoplast fusion, i.e., a hybrid. Based on numerous morphological properties, it was possible to distinguish between potatoes and the hybrid. Interestingly, a gas chromatographic analysis of volatile components of undifferentiated callus cultures of hybrids indicated the occurrence of substances absent in those of the parents.

These early experiments proved the possibility of producing crosses between different species, though these hybrids could not be used in practical plant breeding programs. At about the same time, the fusion of *Arabidopsis thaliana* and *Brassica campestris* was reported by Gleba and Hofmann (1978), and somewhat later the production of "synthetic" rapeseed plants by in vitro fusion of protoplasts of *Brassica oleracea* and of *Brassica campestris* (Schenck 1982), which are thought to be the parents of rapeseed following a spontaneous hybridization about 1,000 years ago. In the examples given above, plant species of the same genera or family were used as hybridization partners. Later, fusions were attempted also of species of quite distant systematics, like tobacco and carrot producing so-called NICA plants (Dudit et al. 1987). This fusion was successful and callus was produced, but the regeneration of plants failed. This could be achieved after the genome of carrot protoplasts was destroyed by irradiation with X-rays. As can be seen in Fig. 5.3, these NICA plants have the habitus of tobacco plants with narrow leaves.

The original aim of protoplast cultures was to produce new genomes with properties exhibited by neither parent. This has now been replaced by many experimental systems able to insert selected foreign genes into a recipient genome—gene technology. The first to successfully use this approach were possibly Potrykus and his research group in Basel (Potrykus et al. 1987). Here, a virus was employed as a vector to transfer the genetic information for resistance to the antibiotic kanamycin to tobacco protoplasts. These transformed protoplasts could be raised to intact plants carrying this resistance. Gene technology will be dealt with in a later chapter discussing its advantages and shortcomings (Sect. 13.2).

Even nowadays, protoplasts are often employed as recipients of foreign genetic material, and to produce plants through somatic embryogenesis that can be used in plant breeding programs. As an example, some results on using protoplasts of rice in gene technology shall be mentioned. Of the several methods available to transfer foreign genetic material based on biolistics, or *Agrobactrium*-mediated transfer to

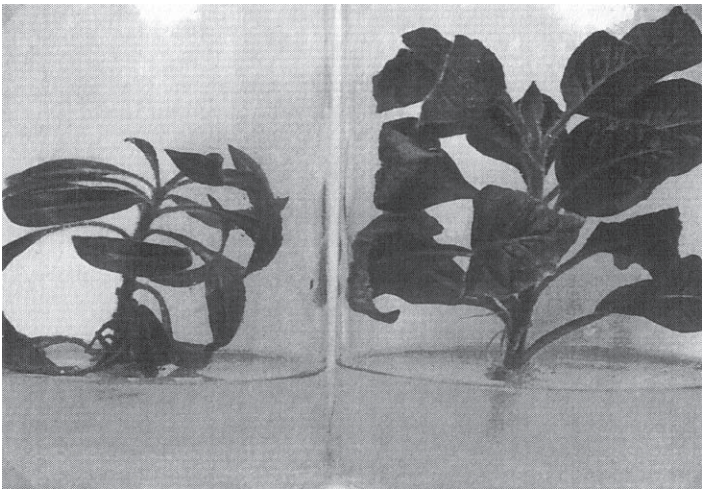

Fig. 5.3 The habitus of leaves of NICA plants (*left*), and that of the tobacco parent (*right*). NICA plants are the result of protoplast fusions of *Daucus* and *Nicotiana*

competent cells, best results and highest efficiency are achieved by a direct introduction into rice protoplasts. Whereas it is almost routine to obtain protoplasts of japonica varieties, those of indica rice are still recalcitrant to tissue culture procedures. A method to this end was published by Zhang (1995).

5.1 Production of Protoplasts

The methods described in this chapter were originally developed to obtain protoplasts from leaves of various Brassicaceae (Elmshäuser et al. 1979). Later, these could be successfully adapted to other plant species (various orchids, *Datura*, carrots, and others). For sterilization, the tissue used to obtain protoplasts is first exposed to 70% ethanol for 1 min, followed by submergence into a hypochlorite solution (0.6%) for 20 min. After this, the leaf material is washed 4 times with sterile aqua dest., and then transferred for 15 min to the nutrient medium used subsequently for the cultivation of the protoplasts (Table 5.1), without the organic components. Instead, 0.4M mannitol is supplied to detach the plasmalemma from the cell wall through plasmolysis. After this pre-incubation period, the leaves (still intact) are cut into pieces approximately 0.5 mm in length. Then, 150–200 mg fresh weight of this leaf material is incubated with 10 ml of the enzyme solution in Table 5.1, in 60 x15 mm plastic Petri dishes. Beforehand, the enzyme solution is passed through a membrane filter (45 μm) for sterilization. The Petri dishes are sealed with Parafilm, and covered with aluminum foil to prevent illumination.

Table 5.1 Culture media used for protoplast culturing (Elmshäuser et al. 1979; macro- and microelements as in B5 medium, Table 3.3)

Component	Concentration	Component	Concentration
Macro- and micronutrients (mg/l; Gamborg et al. 1968)			
$NaH_2PO_4 \times H_2O$	1,110.0	$MnSO_4 \times H_2O$	10.000
KNO_3	3,000.0	H_3BO_3	3.000
$(NH_4)_2SO_4$	134.0	$ZnSO_4 \times 7H_2O$	2.000
$MgSO_4 \times 7H_2O$	250.0	$Na_2MoO_4 \times 2H_2O$	0.250
$CaCl_2 \times 2H_2O$	1,025.0[a]	$CuSO_4$	0.025
Fe-EDTA	46.3[a]	KI	0.750
Organic components (mg/l; Kartha et al. 1974)			
Nicotinic acid	1.0	Mannitol	0.5M[a]
Thiamine	10.0	2.4D	2.3×10^{-6}M
Pyridoxine	1.0	BA	4.4×10^{-6}M
m-Inositol	100.0	NAA	1.6×10^{-5}M
Glutamine	200.0[a]		
Casein hydrolysate	250.0[a]		
Glucose	2,500.0		
Ribose	125.0		
Enzyme solution to produce protoplasts (pH 6.2)			
Cellulase Onozuka SS1 500	2.0%		
Mazerozyme[b]	1.0%		
Pectinase (Serva)	0.5%		
Potassium dextransulfate[b]	0.5%		
Mannitol	0.5M		

[a]Changed from the original
[b]Welding & Co., Hamburg

The leaf material is left for 6 h at 28°C in darkness in the enzyme solution. At the end of this incubation, the individual protoplasts are detached by gentle shaking (Fig. 5.4). By passing through a glass filter, or glass wool, the remaining leaf material is removed. After this, the protoplasts are separated from the incubation medium by gentle centrifugation at about 100 g with the help of a hand centrifuge, and the sedimented material containing the protoplasts is washed 4 times with the nutrient medium to be used for culture of the protoplasts (Table 5.1). Finally, 2 ml of the protoplast suspension is transferred to the same plastic Petri dishes as described above for incubation.

In experiments to produce protoplasts of several Brassicaceae, the highest efficiency in obtaining a healthy population was achieved by using leaves of 6-week-old plants. Important were also the growing conditions of the donor plant immediately before the experiment. An illumination period of 10 h was optimal. Possibly during this illumination period, the cells accumulate enough energy by photosynthesis to withstand the stress of being transformed into protoplasts. An optimal density at the beginning of culture is about 10^5 protoplasts per ml of suspension.

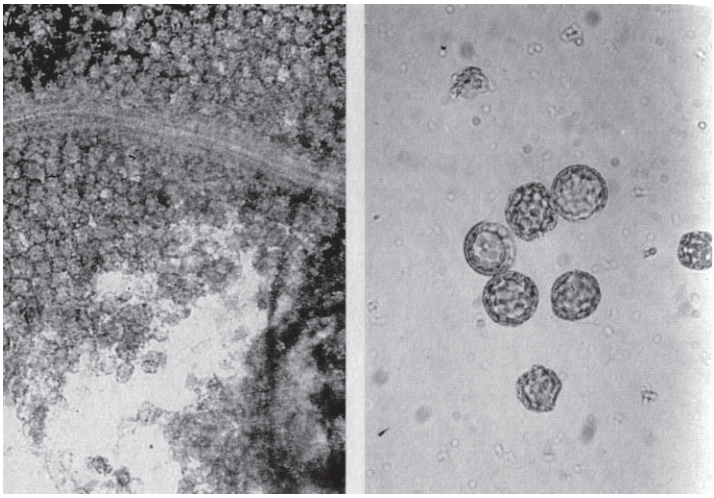

Fig. 5.4 The isolation of protoplasts (*Brassica* ssp.: *left* initiation of disintegration and first free protoplasts, *right* freshly isolated protoplasts

It was possible to successfully replace the enzymes in the solution by the culture supernatant from *Clostridium cellulovorans*, as shown for cultured cells of tobacco and *Arabidopsis thaliana*.

During the first 40 h after the protoplast production, culture is carried out at 500 lux, followed by a period of 5 days at 2,000 lux. The temperature is kept at 26–28°C, under 12/12 h light/dark illumination. Then follows the application of 0.2 ml of fresh medium of the same composition as that originally used, but in which mannitol is replaced by sucrose (2%; Table 5.2). After another 7 days, 2 ml of this nutrient medium is supplied per Petri dish, and the total volume is partitioned into two Petri dishes of the same size and volume as that of the former. Cell aggregates produced after another 10 days are transferred onto agar.

About 3 h after the start of incubation in the enzyme solution, a disintegration of tissue, and the first free-floating protoplasts can be observed (Fig. 5.4). In the spherical protoplasts, the chloroplasts initially gather at the periphery of the cell and later, i.e., about 30–35 h after the start of the experiment, an accumulation of chloroplasts occurs around the nucleus. Often these organelles exhibit a brownish color. Using calcofluoro white as a stain specific for cell wall material, the beginning of restructuring of the cell wall can be observed. Concurrently, the originally spherical protoplasts become oblong (oval; Fig. 5.5), and at about 100 h after isolation, the initiation of the first cell divisions can be observed. Apparently, the regeneration of the cell wall is a prerequisite to initiate cell division—a plausible explanation of this phenomenon is to date not possible.

An interesting, though usually negatively viewed phenomenon, is a “budding” of protoplasts (Fig. 5.6) during the regeneration of the cell wall. Apparently during

Table 5.2 Nutrient solution to culture cell aggregates developed from protoplasts (macro- and microelements following MS medium, vitamins following Gamborg et al. 1968, Table 3.3)

Component	Concentration
Sucrose	2.0%
Agar	0.6%
Casein hydrolysate	0.1%
2.4D	0.2 ppm
Kinetin	0.1 ppm

the formation of the new cell wall, parts of the cytoplasm protrude through areas of the cell wall not yet completely regenerated. In these buds, no material belonging to the cell nucleus has been detected, but occasionally some plastids can be seen. Cells with such buds can not survive, and after 2 weeks at the latest they die. If the concentrations of the components of medium in Table 5.1 are halved, then budding can be considerable reduced, but not entirely prevented.

Cell division activity of protoplast material is usually limited, accounting for about 2–3% of cells after isolation. These few cells are the origin of cell clusters consisting of 200–300 cells after 2 weeks of culture. An increase of the population of healthy cells able to divide can be observed after a supplement of 0.05% charcoal, to absorb toxic substances produced during the process of protoplast isolation. This increase can be up to tenfold. These clusters can be used to produce somatic embryos, and eventually intact plants, as shown for *Daucus* and others.

The basic principle of the many methods described in the literature is the same as that described above; the procedure adopted has to be worked out for each plant species or tissue used.

5.2 Protoplast Fusion

The major aim of protoplast fusion has been to combine the genomes of two species that can not be combined by pollination. Due to the fast development of gene technology during the last 10–15 years, through which selected genes can be transferred from a donor genome to the genome of any other species, this aim can be achieved more precisely. Still, it may sometimes be desirable to include protoplast fusion in one or the other research program, and so the topic shall be briefly discussed here.

If protoplasts of different species are mixed, then a high percentage of fusionates are autofusion products of specimens of the same origin. To distinguish these from those of fusion between any two species, a reliable marker is required. The simplest way to this end is the use of different tissues with distinct morphological or other characteristics. A good example are cells with chloroplasts from the leaves of one "parent", and cells free of chloroplasts from another part of the other parent (Fig. 5.7). Isoosmolarity of the two types of cells is a prerequisite. In an early

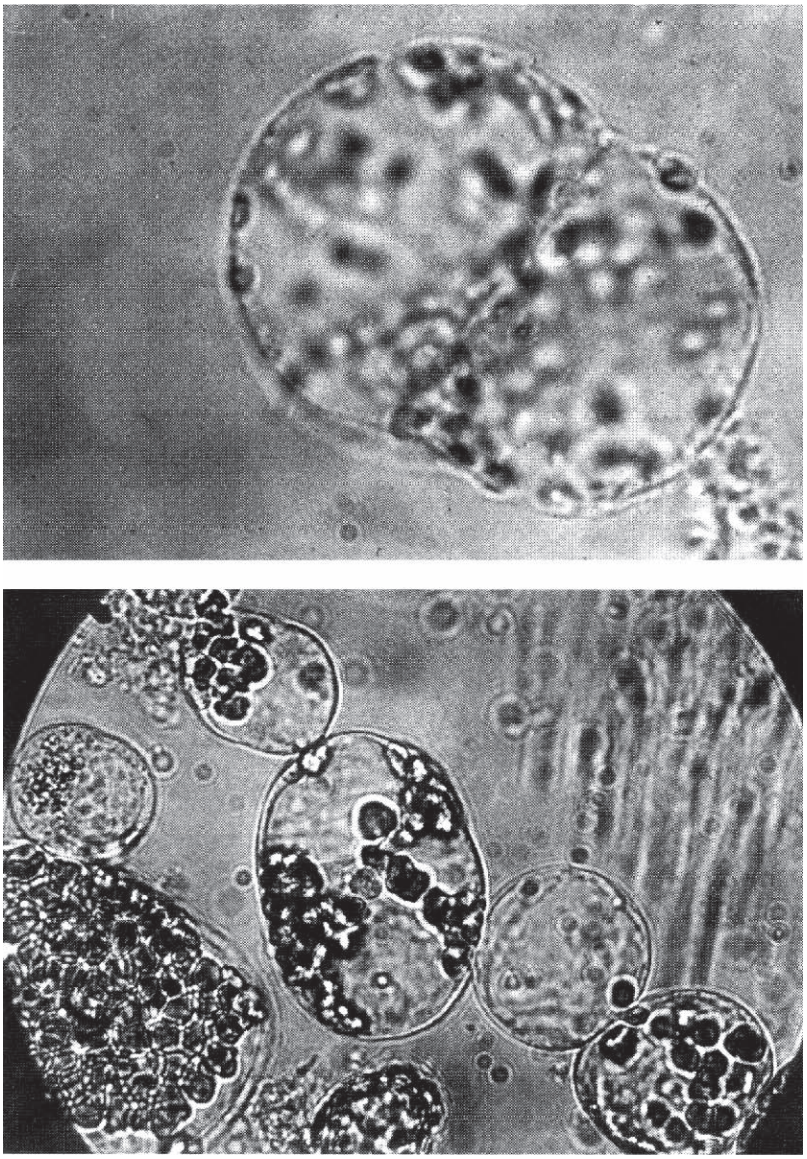

Fig. 5.5 Protoplast isolation: *top* regenerated cell wall (70 h after isolation), *bottom* first cell division (about 100 h after isolation)

experiment using protoplasts of rapeseed leaves, and protoplasts of cells from the carrot root, an "explosion" of the leaf protoplasts was observed due to the high sugar content of the carrot root cells. Still, this was an indication of a successful combination.

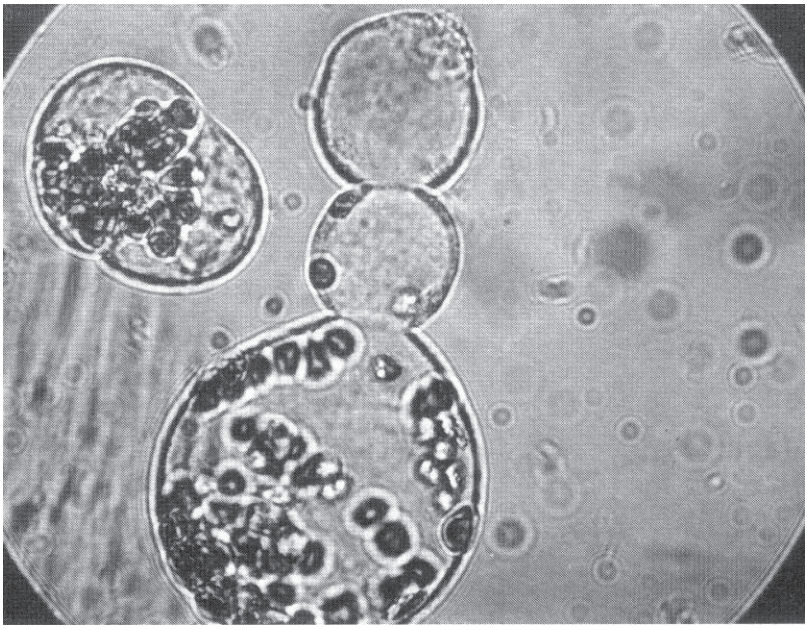

Fig. 5.6 "Budding" of a protoplast (*above*; turnip rape)

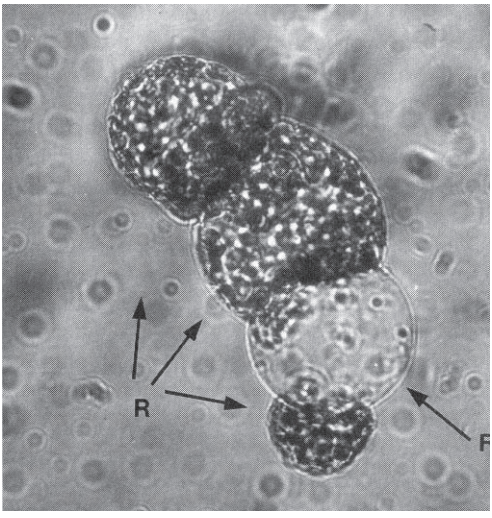

Fig. 5.7 Protoplasts from a leaf (*R* turnip rape), and from roots (*F* Fodder Kale, *Brassica oleracea* var. *viridis*) are attached to each other

Other markers are anthocyanins, or other pigments of plants. The use of foreign genetic material as markers introduced into fusion partners will be described in detail in Sect. 13.2. Markers are not required if single isolated protoplasts of different origin are fused. Here, using a micromanipulator, these two protoplasts are brought into contact in a mini-jar, and the fusion is often accomplished by electroshock.

The plasmalemma, and consequently the protoplast, exhibits an excess of negative charges on its surface. This hinders a spontaneous attachment of two protoplasts. After about 40 years of research on protoplast culture, with many attempts to overcome this problem, only two methods are generally considered as really practical. In the one case, the macromolecular polyethylene glycol is applied at high concentrations (28%), and in the other electroshock is used. Both methods can be employed in various forms, associated with various costs; for example, the common laboratory will suffice for the polyethylene glycol method, but for electrofusion the original self-made equipment is today replaced by expensive, commercially made devices.

The success of fusion experiments is related to the temperature at which the original plant material grew, with higher efficiency at lower (10°C) than at higher (25°C) temperatures. Apparently, this is related to the fluidity of membranes, which depends on their composition, particularly for membrane lipids—protoplasts characterized by membranes containing more unsaturated fatty acids exhibit an increased rate of protoplast fusion.

Protoplast fusion of two plant species aims at the production of new genomes with the genetic information of both "parents". Here, one way to create a new genome is to apply X-rays to one "parent" (50 kr). This results in chromosomes being injured, and partly eliminated in the following cell divisions (see NICA plants). The still viable chromosomes form a new genome with the untreated cells of the other "parent". Often, haploid material is employed. Using gene technology methodology, it is today possible to insert defined genetic material, i.e., single genes, which will be described later (Sect. 13.2).

Beside mixing genetic information of the nuclei of protoplasts of different origin, also plastids and mitochondria can be merged. Some more recent data have become available on the fate of the chondriom. Before first division, the mitochondria apparently elongate and then divide, causing an increase in number. After this, an actin-dependent dispersion of the mitochondria results in a uniform distribution throughout the cytoplasm. This has been observed after fusion during the first 4–8 h of protoplast culture; within 24 h, a near-complete mixing of mitochondria of the fused protoplasts was achieved. The mixed mitochondria population is passed on to daughter cells (Sheahan et al. 2005).