Protoplast cultures and protoplast fusion focused on Brassicaceae – a review

B. NAVRÁTILOVÁ

Faculty of Science, Palacký University in Olomouc, Olomouc, Czech Republic

ABSTRACT: The subjects of this article are protoplast isolations and protoplast fusions, in particular their history, a review of factors influencing the protoplasts isolation and fusion, selection of hybrid plants and utilization of somatic hybrids in plant breeding. Somatic hybridization through protoplast fusion can overcome sexual incompatibility among plant species or genera; transfer genes of resistance to diseases (viral, bacterial, fungal), pests, herbicides and others stress factors; obtain cybrid plants; transfer cytoplasmic male sterility or incease content of secondary metabolites in hybrid plants. The article is focussed mainly on the family Brassicaceae because among representatives are significant crops for the human population. Various successful combination of intraspecific, interspecific and intergeneric protoplast fusion were reported between representatives of the family Brassicaceae with the genus *Brassica* which belonged to the first agricultural crops used for the isolation of protoplast.

Keywords: Brassicaceae; *Brassica*; protoplast isolation; protoplast fusion; somatic hybridization; resistance to diseases and abiotic factors

Isolated protoplasts are a unique system for studying the structure and function of cell organelles, cytoplasmic membrane transport in plants and cell wall formation. Another possibility of their use is protoplast fusions, genetic manipulations or experimental mutagenesis.

Somatic hybridization through plant protoplast fusion enables not only to combine parent genes in higher plants but also to overcome barriers existing between plant species or genera, it is possible to obtain asymmetric hybrids and plants that are heterozygous in extranuclear genes.

Protoplast fusion enables to transfer desirable qualities, e.g. resistance to pathogens or stress factors, even between the genotypes that cannot be hybridized in a traditional way.

HISTORY

Klerceker (BAUER 1990) carried out the first mechanical isolation of protoplasts in 1892 from the tissue of *Stratiotes aloides* L. Because of the very low density of isolated protoplasts, the isolation of protoplasts was considered as an unsuitable method until the time of chemical digestion of the cell wall by the enzymes pectinase, hemicellulase and cellulase isolated from fungi (*Trichoderma viride, Rhizopus* sp.).

In 1960, Cocking obtained for the first time a high number of protoplasts at the root tips of tomato *Lycopersicon esculentum* Mill. by applying enzymes with cellulase and pectinase activity. The first plants regenerated from protoplasts were grown by NAGATA and TAKEBE (1971) in tobacco (*Nicotiana tabacum* L.). An interspecific hybrid obtained by the protoplast fusion of *Nicotiana glauca* + *Nicotiana langsdorffii* appeared in 1972 (CARLSON et al. 1972), but this hybrid could also be produced by sexual crossing. The finding was that the hybrids were characterized by spontaneous emergence of tumours without any need to add growth regulators into the culture medium.

The real breakthrough came in 1978 when, through the protoplast fusion of *Solanum tuberosum* L. and *Solanum lycopersicum* L. from the Solanaceae family, hybrid cells were formed and plants were regenerated from them which could not be produced by sexual hybridization (MELCHERS et al. 1978). Two types of hybrids were produced, one type with the chloroplasts of potato (*Solanum tuberosum* L.) and the other type with the chloroplasts of tomato (*Solanum lycopersicum* L.), they made shoots similar to potatoes but they did not flower.

ISOLATION OF PROTOPLASTS

Enzymatic isolation of protoplasts is limited to parenchymal cells with unlignified cell walls, as cells with lignified cell walls are resistant to enzymes. By applying enzymatic preparations with cellulase and pectinase activity to tissues and cells it is possible to remove the cellulose cell wall of the plant cells and to obtain spherical protoplasts.

There exist standard and general methods of isolation and cultivation of protoplasts, but the details must be modified for specific species or even variety. In a majority of cases the optimum conditions and enzymatic

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processing for the combination of genotype/explant must be defined empirically (BLACKHALL et al. 1994a).

Plant protoplasts may be isolated mechanically or enzymatically. Mechanical isolation, cutting parts of the plant and releasing protoplasts from the cut surface, is a historically important technique, but it is used scarcely as the number of isolated protoplasts is insufficient. However, its advantage is the elimination of the unknown influence of enzymes on protoplasts (AHUIA 1982; BAUER 1990).

Enzymatic isolation is advantageous because protoplasts are gained at a high quantity, cells are not damaged and the osmotic conditions may be influenced. Enzymatic isolation may be carried out in two different procedures: two-step or one-step procedure.

In the first step of the two-step procedure, individual cells are released with the help of commercial enzymatic preparations (e.g. macerozyme, macerase). The cells are released by degradation of the middle lamellas and the decay of tissue to individual cells. The free cells are then processed in the second step with the help of cellulases (cellulase Onozuka R-10, cellulysin) to protoplasts by dissolving the cell wall. The cells are exposed to the enzymes for a shorter time than at one-step isolation.

One-step isolation is used more often, during which the mechanically loosened tissue (e.g. by cutting strips) is put into a mixture of enzymes (pectinase and cellulase, commercial preparations). For each plant object the optimal composition of enzymatic mixture is necessary (POWER, CHAPMAN 1985; ONDŘEJ 1985) as well as the optimal pressure of extraction media.

FACTORS INFLUENCING THE ISOLATION OF PROTOPLASTS

A successful isolation of protoplasts depends on many factors such as the source of tissue (leaves, cell suspension), plant species and cultivar, physiological condition of the donor plant, the composition of the enzymatic mixture and the period of enzymatic action, the osmotic characteristics of the extraction mixture and the individual steps during the isolation of protoplasts.

Plant material

The protoplasts may be isolated from different tissues and organs including leaves, shoot apices, roots, coleoptiles, hypocotyls, petioles, embryos, pollen grains, calli, cell suspensions. A reliable source of protoplasts, e.g. in the genus *Brassica*, is the cells of leaf mesophyll. The leaves are a good source of protoplasts enabling the isolation of a high number of relatively uniform cells.

Material may be taken from field plants, plants grown in greenhouse or *in vitro*. The physiological condition of the plant influences the success of the isolation of protoplasts, therefore the plants are grown under controlled conditions (light, temperature) (BHOJWANI, RAZDAN 1983).

For plants grown *in vitro*, the seeds are surface sterilized, germinated on agar medium and the shoots are transferred to the culture medium, the plants are subcultured regularly and uniform clones can be obtained by their micropropagation.

Frequent objects of protoplast cultures in dicotyledonous plants are *Nicotiana tabacum, Lycopersicum esculentum, Datura stramonium,* the representatives of the genus *Brassica*.

Enzymes

Density and viability of isolated protoplasts depend on the concentration of used enzymes, the period of enzymatic action, pH of the enzymatic solution, temperature, the ratio of the enzymatic solution to the volume of the plant tissue.

Enzymes may be divided into two categories:
- pectinase dissolving the middle lamella and separating individual cells,
- cellulase and hemicellulase decomposing the cell wall and releasing the protoplast.

Enzymes are then purified and filtrated. To increase the stability of protoplasts, inorganic salts (Ca^{2+}) and organic buffer (e.g. morpholinoethane sulphonic acid) are added that minimize the changes of pH during incubation. Osmotic values of the environment into which the protoplasts are released are critically important. Seawater is used as an osmoticum or the tissues are slightly plasmolyzed prior to the isolation, e.g. in sorbitol solution. Movement and slight shaking of the mixture during enzymatic action increases the number of isolated protoplasts (UCHYMIYA, MURASHIGE 1974; DĚDIČOVÁ 1995).

Pre-action (pre-enzymatic action) prior to enzymatic action may lead to increased metabolic activity and stimulation of division after the isolation of protoplasts from the tissue. The period of action of the enzymes or enzymatic mixture is varying, short-term action (2 to 6 hours) or slower long-term action (16–24 hours) in the dark at room temperature.

The purification of protoplasts and perfect removal of the residues of cell walls, damaged protoplasts and isolation enzymes are the condition of further cultivation of protoplasts and are done by repeated centrifugation (LANDGREN 1978).

Osmotic conditions

In isolated protoplasts, the pressure of the missing cell wall on the protoplast is replaced by suitable osmotic values of used solutions and media. The osmotic potential is adjusted by adding mannitol, sorbitol, glucose or sucrose into the enzymatic mixture, washing solution and culture medium. The stability, viability and further growth of protoplasts are connected with the appropriate osmotic conditions of isolation and subsequent cultivation.

Optimal osmotic potential is between 470 and 700 mOsm. From the quantitative aspect, protoplasts are more stable in a slightly hypotonic environment rather than in

an isotonic one. A higher value of the osmotic potential prevents the bursting of protoplasts, but it can lead to the inhibition of their division (BHOJWANI, RAZDAN 1983).

Purification of protoplasts

A condition of successful cultivation of protoplasts is to remove the isolation enzymes, undigested fragments of tissues and damaged protoplasts perfectly.

Protoplasts are usually purified by a combination of filtration, centrifugation and washing. The enzymatic solution containing protoplasts is filtered through a metal or nylon sieve (50–100 μm) to remove larger parts of undigested tissue and cell clusters. Further removal of damaged cells and isolation enzymes is done by repeated centrifugation (3–10 min, 75–100 × g) and resuspension in washing medium (ONDŘEJ 1985). In the case of protoplast suspensions containing a lot of residues, the flotation of protoplasts in a gradient can be used. In such a case the protoplasts are mixed with 20% sucrose and covered with washing medium. After centrifugation, the floating protoplasts are gathered from the ring on the upper layer of sucrose, while organelles and cell residues are in the pellet at the bottom of the centrifugation test tube. The washing is repeated 2–3 times. Ca^{2+} ions in the washing medium stabilize the protoplast membrane (BHOJWANI, RAZDAN 1983). In other modifications, the gradient of mannitol and the commercial preparation of Percoll are used (SUNBERG, GLIMELIUS 1991).

The gradient separates protoplasts obtained from different tissues and leaves of the same age and enables to obtain homogeneous material.

Viability and density of protoplasts

For successful cultivation of protoplasts, their high viability and sufficient density are important. To establish the viability of plant protoplasts, several procedures are used:

a) Fluorescein diacetate (FDA), dyes the vital protoplasts and fluoresces under fluorescent microscope
b) Evans blue, living protoplasts do not let the pigment through the membranes
c) Neutral red pigment which is concentrated only in metabolically active cells
d) Observing the cytoplasmic flow as the indication of active metabolism
e) Calcofluor MR2 or calcofluor white, the renewal of the cell wall is detected with the help of fluorescent microscope.

The optimal density of protoplasts influences the division of cells and the formation of microcalli. There are usually between 1.10^4 and 1.10^6 protoplasts in 1 ml of medium (BARSBY et al. 1986), if the density is too high, uniting and interconnecting of the cell colonies may occur. The density of protoplast suspension is measured with a haemocytometer.

Methods of successful cultivation of single protoplasts or cultivation of protoplasts of minimum density have been described (SPANGENBERG et al. 1986; KELLER et al. 1997).

CULTIVATION OF PROTOPLASTS

A decisive factor of cultivation is the composition of the culture medium, especially the content of sugars, the temperature and intensity of light (DĚDIČOVÁ 1995).

Liquid medium is usually preferred as the division in solid medium is more difficult and the osmoticum in the medium is more easily regulated, e.g. after regeneration of the cell wall and during the first divisions the value of the osmotic potential must be lowered so that the cells will not stop division (KAO, MICHAYLUK 1980). In a liquid medium, the density of cells may be regulated better and changing the culture medium or isolating cells which are needed during the cultivation process is easier. However, even under suitable cultivation conditions, during 24 hours of cultivation, a part of the protoplasts may burst.

Culture media

Liquid, semi-liquid or solid media are used for cultivation of protoplasts. In liquid medium the osmotic values may be easily gradually changed, which enables quick cell regeneration.

Cultivation of protoplasts in liquid medium:

– drop cultures, cultivation in small drops 40–100 μl placed on the inside of the lid of Petri dish,
– microchamber cultures, similar to drop cultures, drops of 30 μl containing 1 to several protoplasts,
– microdroplet cultures, the drops are minimized and each can contain only one protoplast,
– protoplast suspension is in a thin layer at the bottom of Petri dish.

To prepare solid media, agar (0.8%) or agarose are most often used, they do not react with the other ingredients of the medium and become solid at low temperatures.

A combination of liquid and solid medium may be used, when the protoplast suspension is closed in agarose or agar and then cut into blocks and the cultivation of such blocks is performed in a liquid medium (ERIKSON 1986).

Requirements for nutrition

Protoplasts of different kinds and even from different sources of the same species (leaf, callus) react in different ways and may have different requirements for nutrition.

External conditions of cultivation of protoplast cultures

High intensity of light at the beginning of cultivation inhibits the growth of protoplasts, the beginning of cultivation is often done in the dark or semi-dark for only

a few days (2–10 days) before the cell wall is formed. Later it is transferred to the light, the intensity of light is between 2,000 and 5,000 lux (CHATTERJEE et al. 1985; BARSBY et al. 1986). Sensitivity to light can be genetic and some species may be sensitive to light while others are tolerant to light (e.g. legumes).

The cultivation of protoplasts takes place at temperatures between 22 and 30°C depending on the genotype (BARSBY et al. 1986; MAHESHWARI et al. 1986).

REGENERATION FROM PROTOPLASTS

Regeneration means not only the synthesis of a new cell wall but also the regeneration of the whole plant. The first visible signal of the protoplast growth includes the ordering of a majority of organelles around the nucleus and the formation of a new cell wall. The formation of the cell wall begins after the isolation of protoplasts (WILLISON, COCKING 1975).

During 1 or 2 days of cultivation, the protoplasts lose their spherical form (in protoplasts the cell cycle usually stops), which indicates that the cell wall has been renewed. Protoplasts that cannot regenerate their own cell wall are not capable of normal mitosis. The ability of protoplasts to divide may range between 0% and 80% (BHOJWANI, RAZDAN 1983), e.g. it was found in hypocotyl protoplasts of *Brassica napus* that after 6 days of cultivation only 20% of cells were divided (CHUONG et al. 1985). The cells that continue to divide form visible multi-cell colonies after 2–3 weeks and after more weeks they create calli.

The process of regeneration of plants from protoplasts was divided by NAGATA and TAKEBE (1971) into three successive stages that are defined by the composition of the culture media with different contents of plant growth regulators and osmotica:

1st stage – initial stage, the culture medium suitable for forming new cell walls and initiating the first cell division, the formation of visible colonies and microcalli; the medium contains osmoticum, growth regulators, sugars, salts and vitamins,

2nd stage – differentiation stage, the medium induces the forming of shoots on the calli, it has lower content of auxins and higher content of cytokinins,

3rd stage – rooting stage, the medium is usually devoid of growth regulators, it induces the formation of roots on the shoots of the separate calli.

To obtain the majority of diploid plants, it is necessary to begin with somatic tissues as leaf mesophyll, hypocotyl, and the callus stage must be limited to the shortest possible period. With the number of cell divisions, the chromosomal variability in undifferentiated state of calli is increased.

Successful regeneration of plants from protoplasts of different species seems to be genetically determined (ROEST, GILLISEN 1989). There are plant species and types of tissues from which whole plants were obtained and species from which only several divisions were achieved after the regeneration of the cell wall.

PROTOPLAST FUSION

Protoplast fusion leads to the formation of mixtures of genetic information – transfer of nuclear and cytoplasmic genetic information between plant species, genera, which could not be achieved during sexual crosses. It offers overcoming sexual barriers e.g. in breeding of agricultural plants. The aim of protoplast fusion is the transfer of genes controlling certain features e.g. from a wild growing plant into important agricultural crops. The characteristic aim of experiments with protoplast fusion has been to transfer:

- genes of resistance to different virus and fungal diseases from wildly growing species,
- cytoplasmic male sterility (CMS),
- resistance to stress including tolerance to salinity, cold, drought,
- resistance to insect parasites (synthesis of phytoalexins),
- genes for synthesis of reserve proteins, vitamins, secondary metabolites of pharmaceutical importance.

Using somatic hybrids created by protoplast fusion in plant breeding depends on the possibilities of selection and evidence of hybrids: morphological studies, cytological studies based on the number of chromosomes, using probes with DNA sequence when it is possible to find the size of the parent genome in hybrids, RFLP analysis and DNA-DNA hybridization, isoenzymes, chloroplast or mitochondrial DNA.

The protoplast fusion must be induced immediately after the isolation of protoplasts prior to synthesis of a new cell wall. Undamaged protoplasts, practically immediately after washing out the used enzymes from the protoplast sample, regenerate a new cell wall and within a few days the cell division is renewed. The result of the fusion is a mixture of heterokaryon, homokaryon and unfused parent protoplasts.

Somatic hybridization of plants includes four separate stages:

- isolation of protoplasts (explant, enzymes, the period of enzymatic action, isolation method),
- protoplast fusion (fusogen, viability and density of protoplasts),
- selection and regeneration of plants,
- analysis of regenerated plants.

The physiological and genetic differences of partner cells determine the ability of the hybrid cells to survive. The elimination of a part of the genome of one or both partners during the first mitotic divisions is the way in which the hybrid cell prevents the negative effects of unsuitable genetic combination. Certain chromosomes, plastids or mitochondria may be eliminated.

The method of somatic hybridization was used for the creation of genotypes of intergeneric character of "pomato" that do not exist in nature (MELCHERS et al. 1978), *Arabidobrassica* (GLEBA, HOFFMANN 1980). During the protoplast fusion of distant taxa, non-viable products of fusion often appear or products which are not able to regenerate whole plants. Protoplasts isolated

from juvenile embryos, young leaves and quickly growing calli fuse better (BHOJWANI, RAZDAN 1983).

Spontaneous protoplast fusion

Cell fusion is a process that is a regular part of the ontogenesis of plants. In plants, the fertilization of the egg, differentiation of the veins and articulated laticifers are best known. The activity of cellulytic enzymes during the formation of the veins was found. The occurrence of multinuclear cells was observed together with the enzymatic isolation of protoplasts in tissues of the same species when, after removing the cell wall, the protoplasts remained interconnected through plasmodesmas (BHOJWANI, RAZDAN 1983). Spontaneous fusion is an uncontrolled fusion of two or more protoplasts. It may be induced purposefully e.g. by centrifugation of the protoplast suspension.

Induced protoplast fusion

Fusion may be induced chemically or by an electric field (electrofusion). In both cases, the cytoplasmic membrane is destabilized temporarily during the formation of pores and cytoplasmic connections among neighbouring protoplasts.

In the case of chemical fusion, a relatively high concentration of fusogen ($NaNO_3$, $CaNO_3$, polyvinyl alcohol, polyethylene glycol) is used, combined with high pH (9.0–10.5) and Ca^{2+} ions. These factors disrupt the integrity of the cytoplasmic membrane, e.g. they change its surface charge (BAUER 1990).

Physiological and genetic differences of the fusing cells determine the ability of the hybrid cells to survive. The elimination of one genome or its part leads to the emergence of asymmetric hybrids. It is possible to achieve the purposeful creation of asymmetric hybrids if the nucleus of one genotype is inactivated e.g. by X-irradiation or γ-irradiation (BLACKHALL et al. 1994b).

Chemical fusion with polyethylene glycol (PEG)

PEG is a fusogen introduced in 1974 by KAO and MICHAYLUK to increase the frequency of the fused protoplasts of lucerne (*Medicago sativa*).

Isolated protoplasts of two donors are mixed and treated with PEG of different molecular mass (1,500–6,000) at concentrations of 15–45% for 15–30 minutes. PEG increases the frequency of forming heterokaryons (over 10% of affected protoplasts) and makes the heterokaryons viable (BHOJWANI, RAZDAN 1983). BAUER (1990) stated the fusion ratio up to 30% of affected protoplasts.

Another advantage of PEG is forming a higher ratio of binuclear heterokaryons (KAO 1977). With the help of PEG, the membranes of both donors adjoin to each other, the protoplasts make clusters, the adhesion of protoplasts is disturbed.

PEG is more suitable for mesophyll protoplasts which are not damaged so much as during electrofusion (they can burst). Ca^{2+} ions in PEG solution induce fusion.

Electrofusion

The necessary equipment for electrofusion is a pulse generator, a source of short direct current, a switching unit for the application of alternating or direct pulses and a closet equipped with electrodes for the fusion. For electrofusion, the chains of protoplasts between the electrodes are characteristic. The advantage of electrofusion compared to chemical fusion is its speed, simplicity, synchrony, ease of control and exclusion of chemical fusogens (BLACKHALL et al. 1994b). By setting the electrodes efficiently, even great numbers of protoplasts may be exposed to the pulse field at the same time.

Each method also requires the isolation of protoplasts of good quality, i.e. suspension of viable protoplasts without cell residues. Protoplasts can be fused if they are in contact and exposed to a suitable electric pulse field that induces the development of temporary holes in the plasmic membrane. The membrane functions as an insulator and has a high electric resistance. If the difference of the potential is increased through the membrane, the voltage of the membrane collapses and a hole (pore) is formed.

The critical value of voltage is between 0.5–1.5 V, depending on the composition of the cytoplasmic membrane or the type of cell. The pores are formed opposite the anode, and the formation of pores may be induced before the cells are opposite each other and can fuse. The intensity of the pulse needed for the fusion depends on the size of the protoplast. The length of the pulse is also important. Viability and efficiency are increased if shorter pulses of higher voltage are used rather than longer pulses of lower voltage (JONES 1991).

Mechanism of fusion

Protoplasts have a negative charge on the surface that helps to repulse surrounding protoplasts. They need to be in close contact prior to fusion, which can be achieved physically (by mechanical pressing with micropipette, by centrifugation) or chemically.

Protoplast fusion consists of 3 steps: agglutination, the fusion of membranes in small localized places and forming of bridges among protoplasts, rounding of fused protoplasts.

Protoplast fusion requires approaching, adhesion and joining of two different types of protoplasts. Approaching of the protoplasts is determined by many electrostatic forces arising from the potential on the cell surface. The result of the interaction is either fusion or complete failure (BHOJWANI, RAZDAN 1983; BLACKHALL et al. 1994b).

Products of fusion

The products of fusion behave in a suitable culture medium in the same way as unfused protoplasts. After 2–3 days they change their shape and form a cell wall. Experiments with the fusion of protoplasts of two differ-

ent species led to different products of fusion, including interspecies and intraspecies combinations, but also unfused products. The ratio of interspecies fusants reaches normally 0.5–10% (BHOJWANI, RAZDAN 1983), but even a ratio of 50% was reported (KAO, MICHAYLUK 1974; BAUER 1990).

By induced fusion from different sources a new category of cells is created in which there are protoplasm, organelles and genetic material from both protoplast donors. This early phase is called heterokaryon or heterokaryocyte. This phase is characterized by a mixture of cytoplasmic components, but the nuclei of both parents have not fused yet. Only by nuclear division the real hybrid cell emerges with one hybrid nucleus. The nuclear fusion depends on many factors. The frequency of intergeneric fusions is lower than that of interspecies fusions (HARMS 1986).

The products of fusion usually do not last long. Therefore there is an effort to separate the fused protoplasts from the unfused ones quickly. Elimination of cytoplasmic organelles, plastids and mitochondria or nuclear genomes may occur.

Segregation of plastids or mitochondria of one parent gives rise to homoplastic hybrid cells or cells combining chloroplasts from one parent with mitochondria of the other parent (cytoplasmic recombinant type).

Segregation of nuclear genomes may be an effect of subsequent loss of chromosomes of one parent or the result of the first cell division of heterokaryocyte if there was no nuclear fusion or one of the parent nuclei degenerated. Such early segregation, followed by proliferation of both types of daughter cells, gives rise to chimerical tissue composed of the mixture of genetically different cells. The degeneration of one parent nucleus creates a cell containing both parent cytoplasms, but only one parent nucleus. Such cells are cytoplasmic hybrids (cybrids, heteroplasmons). Further segregation of both cytoplasms may lead to alloplasmic cells containing one parent nuclear genome combined with the other parent cytoplasmic components (HARMS 1986).

Plants regenerated by somatic hybridization may differ in the number of chromosomes. There are several reasons for that:

- asymmetric hybrids may be the result of fusions of protoplasts isolated from actively dividing tissues or one genotype and from quiescent tissue of another genotype taking part in the fusion,
- more protoplasts fuse (using PEG or electrofusion),
- variability arising from *in vitro* cultivation, or somaclonal variation.

Selection of somatic hybrids

Selection of heterokaryons, heterokaryon cells or tissues or hybrid plants is important for somatic hybridization (BLACKHALL et al. 1994b). Protoplasts submitted to fusion are cultivated and checked for their hybrid or cybrid character usually as regenerated plants, which requires a greenhouse space for cultivation. Heterokaryon cells may show quick growth and visible, quickly growing colonies may be isolated mechanically and transferred to a regeneration medium.

a) Manual selection

A very precise but time-consuming method of selection of protoplast fusion products is visual identification and mechanical isolation of fusants with the help of micromanipulator ("fishing"). If the fused protoplasts are morphologically different or stained fluorescent, the products of fusion may be identified under the microscope. The products of fusion may be easily identified by fusion of green mesophyll protoplasts containing chloroplasts with colourless protoplasts of cell cultures containing vacuoles or starch grains, or with protoplasts from etiolated tissues. After such fusion, the chloroplasts are visible in one part of the cell and the vacuoles or starch grains in the other part. Additional fusion of chloroplasts in the whole cell appears shortly after the fusion. During the first division the chloroplasts are clustered around the nucleus in many hybrids. After 7–10 days of cultivation in the dark, the chloroplasts look the same as the colourless proplastids, therefore the hybrid cells may be determined only shortly after the fusion.

An advantageous modification is the use of double fluorescent staining for heterokaryons, e.g. protoplasts with chloroplasts, which are marked with green stain, are fused after staining with fluorescein diacetate with protoplasts of red fluorescence caused either by chlorophyll autofluorescence or by rhodamine isothiocyanate applied exogenously.

Although the micromanipulation with individual heterokaryons is a time-consuming method, it is direct and reliable for the production of somatic hybrids (BLACKHALL et al. 1994b).

The methods may be combined with inhibitory growth of homokaryons and, in ideal case, one or both parent protoplasts. Mutations bringing in hormonal autotrophy, thermal sensitivity, resistance against antibiotics or fungal toxins, herbicides or inactivation of one of the fusion partners by X- or γ-irradiation may be used.

Albino mutants may be used successfully when the hybrid tissues may be identified by renewed production of chlorophyll after exposing the cultures to light.

b) Flow cytometry

Flow cytometry may be used for automatic isolation of heterokaryons during which double fluorescent staining is used and selecting protoplasts during the flow through the capillary of the flow cytometer. Protoplasts flow fluently between the light source and fluorescence detectors, the flow is dispersed into droplets and the computer deflects electrostatically the droplets containing heterokaryons into different test tubes. The method is fully automatic and quick, about 10% of heterokaryons are gathered (BLACKHALL et al. 1994b). A high number of fused protoplasts may be separated from unfused protoplasts by this method quickly and effectively.

Identification of somatic hybrids

Hybrid plants may be identified in a morphological, cytological and biochemical way.

In a majority of cases, the somatic hybrid is morphologically similar to both parents, such features are included in vegetative or floral morphology. In many cases, the somatic hybrid with features of both parents may be obtained by sexual crossing. E.g. in interspecies hybrids of tobacco *Nicotiana tabacum* + *N. glauca* (EVANS 1983) morphological features of somatic hybrid and parents were compared; the shape of leaves, the size of petiole, the density of trichomes, the shape of flower, the size and colour of flower. All features were found between the parents, they were hybrid.

Morphological differences need not be observed in all hybrids. Some features present only in one parent are present in all hybrids and behave as dominant (EVANS 1983).

One of the features that need not be transitional between the parents is the viability of pollen and the number of chromosomes. The viability of pollen usually depends on the relationship of the parent species used in somatic hybridization, the somatic hybrids between more distant species have lower viability of pollen than the parent species. The number of chromosomes may be equal to the sum of chromosomes of both parents, but it can also be completely different (asymmetric hybridization, complete chromosome set, individual chromosomes, fragmentation, polyploidy).

Biochemical identification includes analyses of isoenzymes, partial proteins, secondary metabolites, resistance of plants against viral infection and antibiotics or sensitivity to herbicides and fungal toxins.

Genetic analysis can be undertaken only if the hybrid plants are fertile. Many hybrid plants of distant related species are sterile. Modern molecular technologies of RFLP (restriction fragment length polymorphism) and RAPD (random amplified polymorphic DNA) can be used for the comparison of genotypes, while flow cytometry supplies a quick analysis of nuclear DNA to establish ploidy (FAHLESON, GLIMELIUS 1999).

Variability among somatic hybrids

Intraspecific hybrids created by sexual crossing are usually uniform in the generation F1. After protoplast fusion between the hybrids, the variability is higher than in a comparable population of plants after sexual crossing (EVANS 1983). There can be variations in phenotypic features as the height of the plant, the shape and size of leaves, the length of petiole, the length and colour of flower, the viability of pollen and isoenzymes.

According to EVANS (1983), there are four potential sources of variations that can be identified in somatic hybrids: nuclear incompatibility, mitotic recombination, somaclonal variation, segregation of organelles.

Nuclear genetic instability of fusants in the combination of different species may be a result of the elimination of chromosomes which have been documented in intergeneric hybrids. Such instability can lead to the regeneration of aneuploid plants similar to that of the parent types.

Utilization of somatic hybrids

The application of the somatic fusion requires not only the regeneration of plants from protoplasts but also a successful integration into breeding programme. Somatic hybrids must be capable of sexual reproduction and must contain a mixture of genes from both parent donors while being capable of retrospective crossing into cultivated crops for the development of a new variety.

The transfer of resistance to diseases has been confirmed in somatic hybrids, e.g. the resistance to TMV in *Nicotiana tabacum* + *Nicotiana nesophyla* (EVANS et al. 1982) or the resistance to potato virus *X* in hybrids *Solanum tuberosum* + *Solanum chacoense* (BUTENKO et al. 1982).

Cybridization or cytoplasmic transfer of organelles by protoplast fusion is a possible method of gene transfer among species. Using X-radiation, the nucleus of one species is inactivated before the fusion and some economically important features controlled by cytoplasm can be transferred to the hybrid plant, e.g. male sterility, some types of resistance to herbicides and resistance to diseases, the creation of nectar and resistance to fungal toxins (YARROW 1999). In many cases, cytoplasmic markers are localized in chloroplast or mitochondrial DNA, so useful cytoplasmic markers are connected with the specific restriction enzyme model (pattern) of organelle DNA.

Cybridization has been used in successful transfer of male sterility and in creation of somatic hybrids with resistance to the herbicide atrazine controlled by cytoplasm (CHRISTEY et al. 1991).

ECONOMIC IMPORTANCE OF THE FAMILY BRASSICACEAE

The family comprises about 380 genera, 3,200 annual, two-year and perennial species, plant types of different appearance prevail. The flowers are regular, bisexual, according to the number – four, the fruits are siliquas or silicles without endosperm. Its representatives are found in temperate climate all over the world, predominantly on the northern hemisphere. Among its representatives, there are crops important for the human population. Root and stalk vegetables and greens, aromatic and medicinal species, fodder crops, oil plants and decorative plants, green fertilizers belong to this family (MAREČEK 1994).

Brassica oleracea L.

B. oleracea L. (wild cabbage, kale) includes several hundred varieties of cultivated plants nowadays, includ-

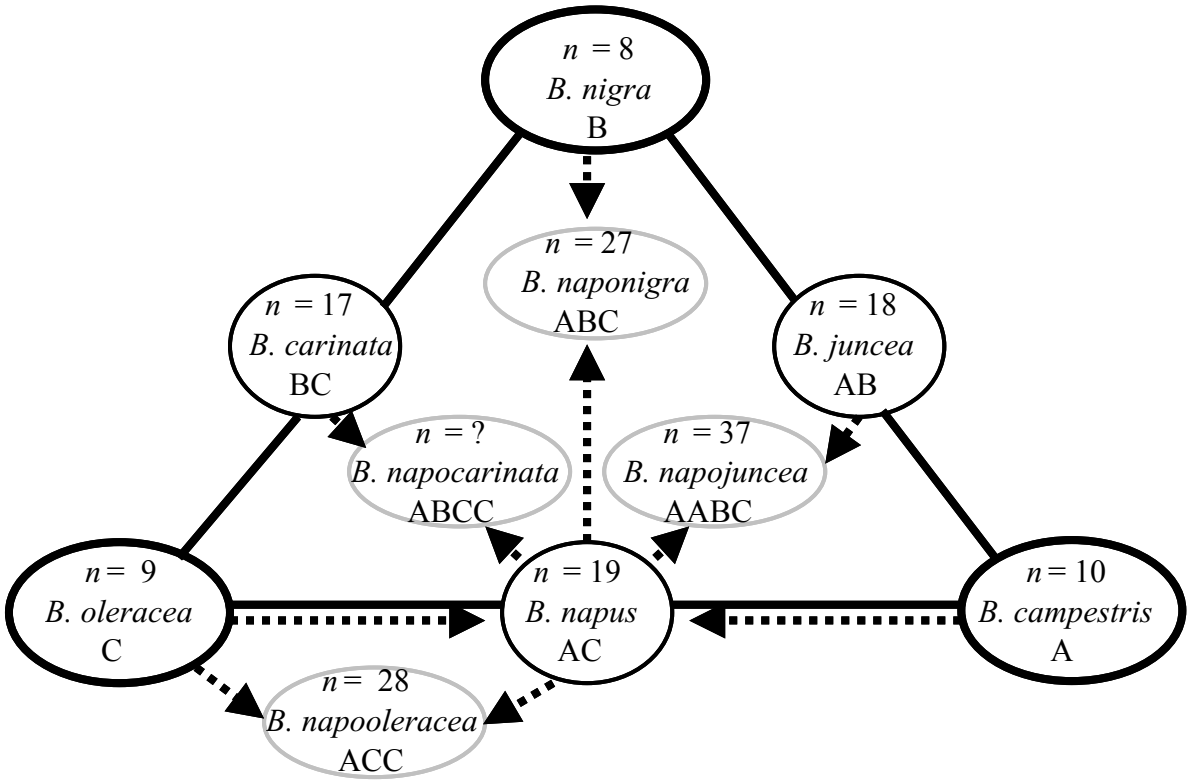

Fig. 1. Hybrid combinations between unrelated species of the genus *Brassica*. The continuous lines represent amphidiploid products of sexual recombination of diploid progenitors *B. oleracea*, *B. campestris* and *B. nigra*. Dashed arrows represent the successful combinations of somatic hybrids leading to the formation of composite genomes (adapted from U 1935; GLIMELIUS et al. 1991)

ing some important vegetables. The original gene centre is the Mediterranean coast and its stony, rocky soil. It was grown in many forms in ancient times.

It is a two-year plant with unthickened root, up to 2 m tall stalk, leaves are petiolated at the stalk base, lyrate pinnatipartite basal and median leaves or entire, crenated. The flower is sulphur yellow, rarely white, in clusters, the fruit is siliqua with a short beak. Out of the many cultivated taxa that originated also from several closely related coastal species, cultivars of 8 cultivated convarieties are grown in this country: *capitata* L. (cabbage), *sabauda* L. (Savoy cabbage), *acephala* DC. = *viridis* (kales), *gemmifera* DC. (Brussels sprouts), *botrytis* L. (cauliflower), *italica* (broccoli), *gongyloides* L. (kohlrabi), *ramosa* DC. (thousand head kale).

The six main species of the genus *Brassica* are naturally related (U 1935; GLIMELIUS et al. 1991, Fig. 1).

The exchange of useful genetic information between *Brassica oleracea* var. *capitata* (cabbage) and *Raphanus sativus* (radish) was demonstrated in 1924 by KARPEČENKO, who created by sexual crossing a synthetic genus *Raphanobrassica*. As in many distant crossings, neither the features of radish nor those of cabbage were achieved. The derived plants were strong, vital, they did not have the features of cabbage (head) or radish (root, hypocotyl), but they were suitable as fodder and green fertilizer (WILLIAMS, HILL 1986).

The relatively easy manner in which diploid and tetraploid plants may be crossed, enabled a repeated synthesis of amphidiploid species, e.g. the emergence of a new artificial synthetic form of *Brassica napus* (AACC) known as "hakuran" which was derived by sexual crossing of cabbage *B. oleracea* (CC) and china leaf *B. campestris* (AA) and is used as vegetable and fodder in Japan. The transfer of resistance to *Plasmodiophora brassicae* to sensitive species was also first achieved by interspecies crossing (WILLIAMS, HILL 1986).

PROTOPLAST CULTURES IN THE GENUS *BRASSICA*

Many successful interspecific and intergeneric fusions were carried out at the beginning of the 80's between representatives of the family Brassicaceae thanks to good regeneration of plants in this family (*Arabidopsis thaliana, B. campestris, B. napus, B. oleracea*).

The representatives of the genus *Brassica* belonged to the first agricultural crops that were used for the isolation of protoplasts (KARTHA et al. 1974; THOMAS et al. 1976) and protoplast fusion (GLEBA, HOFFMANN 1979, 1980).

The source of protoplasts for experiments with species of the genus *Brassica* were cotyledons (VATSYA, BHASKARAN 1982; KAMEYA et al. 1989; ZHAO et al. 1995a,b; SUN et al. 1998), young leaves (QUAZI 1982;

Table 1. Sources of protoplasts of the genus *Brassica*

Species	Source of protoplasts	Reference
B. alboglabra	hypocotyls, cotyledons, leaves	PUA (1987)
B. campestris, syn. *rapa*	leaves	YAMAGISHI et al. (1988)
	cotyledons	LU et al. (1982); ZHAO et al. (1995a,b); GLIMELIUS (1984)
	hypocotyls	OLIN-FATIH (1996)
	roots	XU et al. (1982)
B. chinensis	leaves	GUO, SCHIEDER (1983)
B. juncea	hypocotyls	GLIMELIUS (1984); KIRTI et al. (1991)
	leaves	CHATERJEE et al. (1985)
B. oleracea var. *acephala*	hypocotyls	LILLO, OLSEN (1989)
B. oleracea var. *botrytis*	cotyledons	VATSYA, BHASKARAN (1982)
	hypocotyls	GLIMELIUS (1984)
	hypocotyls, leaves	NAVRÁTILOVÁ et al. (1997a)
B. oleacea var. *capitata*	cotyledons	LU et al. (1982); VATSYA, BHASKARAN (1981)
	leaves	QUAZI (1982)
	hypocotyls	LILLO, SHAHIN (1986); LILLO, OLSEN (1989)
	roots	XU et al. (1982)
	hypocotyls, leaves	NAVRÁTILOVÁ et al. (1997a)
B. oleracea var. *gongyloides*	hypocotyls, leaves	NAVRÁTILOVÁ et al. (1997a)
B. oleracea var. *italica*	hypocotyls, leaves	ROBERTSON, EARLE (1986)
	leaves, cotyledons	ROBERTSON et al. (1988)
	eaves	HUAI-MING, SCHÄFER-MENUHR (1990)
B. napus	leaves	KARTHA et al. (1974); QUAZI (1982) KOHLENBACH et al. (1982); NEWELL et al. (1984) ROUAN, GUERCHE (1991) WATANABE et al. (1998)
	cotyledons	LU et al. (1982); ZHAO et al. (1995a,b) SUN et al. (1998)
	hypocotyls	SPANGENBERG et al. (1986) ORCZYK, NADOLSKA-ORCZYK (1994) PARIHAR et al. (1995)
	stems	KLIMASZEVSKA, KELLER (1987)
	cell suspensions	WEBER et al. (1983); SIMMONDS et al. (1991)
	microspores	SUN et al. (1999)
B. nigra	hypocotyls	GLIMELIUS (1984)
	cell suspensions	KLIMASZEWSKA, KELLER (1986)
B. spinensis	leaves	KIRTI et al. (1991)
B. tournefortii	leaves	LIU et al. (1995); CLARKE et al. (1999)

GUO, SCHIEDER 1983; NEWELL et al. 1984; HUAI-MING, SCHAFER-MENUHR 1990; KARESCH et al. 1991; SIGAREVA, EARLE 1997a,b,c, 1999; HANSEN 1998; WATANABE et al. 1998), hypocotyls (GLIMELIUS 1984; PAULS, CHUONG 1987; SPANGENBERG et al. 1986; KAMEYA et al. 1989; LILLO, OLSEN 1989; WALTERS et al. 1992; ORCZYK, NADOLSKA-ORCZYK 1994; YAN et al. 1999), microspores (SUN et al. 1999) and cell suspensions (SIMMONDS et al. 1991). A partial survey of the published experiments with the isolation of protoplasts from different explants in some representatives of the genus *Brassica* is given in Table 1.

Protoplasts can be a suitable material for plant transformations if their regeneration from genetically modified cells is possible (SUN et al. 1998). They are also suitable material for the acceptance of DNA through electric impulse, chemical agents or microinjection of DNA into protoplasts. Their high ability to regenerate and genetic transformation by a direct acceptance of plasmid vectors into hypocotyl protoplasts of *B. olera-*

cea var. *botrytis* were described by MUKHOPADHYAY et al. (1991).

Transformed RC *Brassica oleracea* var. *capitata* (cabbage) with the help of strains of *Agrobacterium rhizogenes* was obtained by BERTHOMIEU and JOUANIN (1992). An evidence of transformation was obtained by synthesis of opins and PCR (polymerase chain reaction). Most cabbage plants did not show *hairy-root* phenotype, which is normally expected as a criterion of transformation in roots affected by *A. rhizogenes*.

The transformed plants after using *Agrobacterium tumefaciens* were obtained e.g. in *Brassica juncea* by BARFIELD and PUA (1991) and MATHEWS et al. (1990).

SIGAREVA and EARLE (1997a,b,c, 1999) used "rapid cycling" of the plant *B. oleracea* (RC) for protoplast cultures and fusions of protoplasts. They were developed by Prof. Williams at Wisconsin University as a collection of "Rapid cycling brassicas" (RC), later known as "Wisconsin Fast Plants" (WILLIAMS, HILL 1986). RC plants were created by repeated selection of plants from different populations of the species of the genus *Brassica* (*B. oleracea, B. rapa, B. juncea, B. carinata*) with the aim of obtaining plants that show fast development in experimental conditions and flower and produce ripe seeds within 45–60 days under constant fluorescent lighting. RC plants regenerate from protoplasts well and are suitable for experiments as a fusing partner (EARLE et al. 1999).

SOMATIC HYBRIDIZATION IN THE FAMILY BRASSICACEAE

Somatic hybridization in the family Brassicaceae can overcome barriers between the representatives that cannot be crossed, thanks to the fact that nuclear, mitochondria and chloroplast genomes from different, sexually incompatible species may be combined in a single genotype. However, the restoration of fertility remains a problem that must be dealt with for a successful combination.

Introduction of genes for resistance against diseases is one of a few important goals in breeding of economic plants. In the genus *Brassica*, an improvement in resistance of the breeding material against infections by mycopathogens such as *Alternaria, Phoma, Plasmodiophora* is a problem because the genes of resistance are often accessible only in species distantly related to agricultural plants, which is a disadvantage of classic crossing, but this limitation can be avoided by protoplast fusion to a certain extent.

In protoplast fusions, mesophyll and hypocotyl protoplasts are used most often (CHRISTEY et al. 1991; NAVRÁTILOVÁ et al. 1997b; YAN et al. 1999) or mesophyll and callus protoplasts (HOFFMANN, ADACHI 1981; KAMEYA et al. 1989). The reason is a visual control of the forming fusants according to the contents of chloroplasts and vacuoles.

Intraspecific and interspecific hybridization of the genus *Brassica*

SUNBERG and GLIMELIUS (1986) resynthetized the genome of *Brassica napus* through somatic hybridization as a product of interspecies hybridization of the parent species *B. oleracea* and *B. campestris*. PEG was used for the protoplast fusion. Heterokaryons were selected manually 24 hours after the fusion with the help of micromanipulator. Their hybrid character was confirmed also by isoenzyme analysis.

Similarly, REN et al. (1999) resynthetized the genome of *B. napus* by protoplast fusion of *B. rapa* (syn. *B. campestris*) and resistant *B. oleracea* var. *acephala*, in an effort to increase their resistance to *Erwinia carotovora*. Most somatic hybrids obtained in the experiments showed resistance against *Erwinia* in tests. The results suggest that the resynthesis of *B. napus* through protoplast fusion followed by back-crossing may lead to the transfer of genes of resistance from *B. oleracea* into *B. campestris* syn. *rapa* (EARLE et al. 1999). Further possibilities of interspecies hybridization are given in Fig. 1 and the survey of interspecies and intraspecies hybridization is given in Table 2.

With the help of protoplast fusion, the combination of two cytoplasmic features was studied as well as atrazine resistance (ATR) and cytoplasmic male sterility (CMS). The resistance to the herbicide atrazine enables a selection of fusants on the medium containing atrazine. Leaf protoplasts *Brassica oleracea* var. *italica* with a petaloid type of *B. nigra* male cytoplasmic sterility were fused with the help of polyethylene glycol (PEG) with hypocotyl protoplasts of atrazine-resistant type of *Brassica campestris* var. *oleifera*. All regenerated plants were of broccoli type from the point of view of phenotype and without trichomes. Four plants, coming originally from one callus, were atrazine-resistant and grew on atrazine-containing medium. Molecular analyses proved a content of chloroplasts from atrazine-resistant *B. campestris* and the presence of mitochondria from the other parent with the petaloid *B. nigra* type of CMS at the same time (CHRISTEY et al. 1991).

SIGAREVA and EARLE (1997a) created cabbage (*B. oleracea* var. *capitata*) tolerant to cold with cytoplasmic male sterility (CMS) by the fusion of mesophyll protoplasts of cabbage *B. oleracea* var. *capitata* which is cold-tolerant with Ogura CMS line of broccoli *B. oleracea* var. *italica* (OGURA 1968), where the nuclei of CMS donor were eliminated by γ-irradiation.

Intergeneric hybridization with the genus *Brassica*

Intergeneric somatic hybridization among useful crops of the genus *Brassica* with different wild genera: *Arabidopsis, Barbarea, Camelina, Capsella, Diplotaxis, Eruca, Moricandia, Raphanus, Sinapsis, Thlaspi* etc. are listed in Table 3. The purpose was to find new sources of resistance to diseases or abiotic stressful conditions.

Table 2. Intraspecific and interspecific somatic hybridization of the genus *Brassica*

Combinations	Reference
B. campestris (syn. *rapa*) + *B. oleracea*	SCHENK, RÖBBELEN (1982) YAMAGISHI et al. (1988) LANDGREN, GLIMELIUS (1990) CHRISTEY et al. (1991) HEATH, EARLE (1996) OLIN-FATIH et al. (1996) CARDI, EARLE (1997) REN et al. (1999, 2000)
B. juncea + *B. spinescens*	KIRTI et al. (1991)
B. napus + *B. nigra*	SJÖDIN, GLIMELIUS (1989) GERDEMANNKNORCK et al. (1994, 1995)
B. napus + *B. tournefortii*	LIU et al. (1995) CLARKE et al. (1999)
B. napus + RC *B. oleracea*	HANSEN, EARLE (1995)
B. oleracea var. *capitata* + *B. oleracea* var. *italica*	SIGAREVA, EARLE (1997a)

The effort to transfer specific information led to further fusions and combinations.

The first experiments with protoplast fusion in the genus *Brassica* were purposefully aimed not only at higher yields of crops but also new intergeneric combinations on the level of new species were created, such as *Arabidobrassica* (GLEBA, HOFFMANN 1979, 1980; HOFFMANN, ADACHI 1981) from *Arabidopsis thaliana* (wall cress, 2*n* = 20). In symmetric hybrids there was no elimination of chromosomes. The created hybrids showed different morphological and cytological abnormalities. One of the problems in using *A. thaliana* in protoplast fusion lies probably in the regeneration of plants from calli (KARESCH et al. 1991). For the purpose of obtaining fertile and viable hybrids, asymmetric hybridization was tried between *Arabidopsis* and *Brassica* using UV- and iodoacetamide-treatment (FORSBERG et al. 1998a,b; YAMAGISHI et al. 2002).

Mesophyll protoplasts were used in protoplast fusion of plants *Arabidopsis thaliana* and *Brassica napus* (FORSBERG et al. 1998a), where UV- and X-radiation were used for the production of asymmetric somatic hybrids. Comparing asymmetric hybrids to symmetric hybrids, the asymmetric hybrids were a little taller and had larger leaves.

In their further work, FORSBERG et al. (1998b) applied UV-radiation as an alternative method for inactivation of nuclear components to create asymmetric hybrids. Protoplasts of *A. thaliana* irradiated with doses of UV were fused with protoplasts of *B. napus*. Out of 312 regenerated plants, in 52 the presence of DNA from *A. thaliana* was proved. With higher doses of UV-radiation the frequency of asymmetric hybrids increased. Using RFLP, they found that UV-radiation resulted rather in losing chromosome fragments than in losing whole chromosomes of *A. thaliana*.

Low regeneration ability and low fertility may be explained by great phylogenetic differences between the fusing partners. Thanks to the small size of the genome of *A. thaliana*, the somatic hybridization between *Brassica* spp. and *Arabidopsis thaliana* is successful (HANSEN 1998).

Mesophyll protoplasts of *Armoracia rusticana* were fused with hypocotyl protoplasts of *Brassica oleracea* (NAVRÁTILOVÁ et al. 1997a). The prevalent genome of asymmetric hybrids was the genome of *Brassica* containing only little fragments of the *Armoracia* genome. This work was motivated by the possibility of using the hybrids as genetic sources for breeding brassica vegetables for resistance to club root (*Plasmodiophora brassicae*).

Radish (*Raphanus sativus*, 2*n* = 18) was one of the first protoplast donors in experiments for increasing variability and transferring desirable genes of resistance into important *Brassica* crops through protoplast fusion. Hybrid plants between red cabbage (*B. oleracea*) and radish (*Raphanus sativus*) were obtained by KAMEYA et al. (1989). The selection of hybrids was achieved by using iodoacetamide induction which inhibited cell division of cabbage protoplasts and the inability of radish to grow on culture medium. Out of ten plants, only two plants flowered, they were similar to cabbage (*Brassica*), the petioles and midribs were lighter than in cabbage, the flowers were smaller, yellow as in cabbage, with degenerated anthers without ripe pollen grains. After pollination with the radish pollen, seeds did not develop, but after pollination with the cabbage pollen, seeds developed. According to morphology, number of chromosomes, isoenzyme patterns and frag-

Table 3. Intergeneric hybridization in the family Brassicaceae

Combinations	Reference
Arabidobrassica *Arabidopsis thaliana* + *B. campestris*	GLEBA, HOFFMANN (1980); HOFFMANN, ADACHI (1981)
Arabidopsis thaliana + *B. nigra*	SIEMENS, SACRISTÁN (1994, 1995)
Arabidopsis thaliana + *B. napus*	FORSBERG et al. (1998a,b); YAMAGISHI et al. (2002)
Armobrassica *Armoracia rusticana* + *B. oleracea* var. *botrytis*	NAVRÁTILOVÁ (1997); NAVRÁTILOVÁ et al. (1997a)
Barbareobrassica *Barbarea vulgaris* + *B. napus*	FAHLESON et al. (1994a)
Barbarea vulgaris + *B oleracea* var. *capitata*	RYSCHKA et al. (1999)
Camelina sativa + RC *B. oleracea*	SIGAREVA, EARLE (1997b); HANSEN (1997, 1998)
Capsella bursa pastoris + RC *B. oleracea*	SIGAREVA, EARLE (1997c, 1999)
Diplotaxis catholica + *B. juncea*	KIRTI et al. (1995)
Diplotaxis muralis + *B. juncea*	CHATTERJEE et al. (1988)
Diplotaxis harra + *B. juncea*	BEGUM et al. (1995)
Erussica *Eruca sativa* + *B. juncea*	SIKDAR et al. (1990)
Eruca sativa + *B. napus*	FAHLESON et al. (1988); LANDGREN, GLIMELIUS (1990)
Lesquerella fendleri + *B. napus*	SKARZINSKAYA et al. (1998)
Matthiola incana + *B. oleracea* var. *capitata*	RYSCHKA et al. (1999)
Brassicomoricandia *Moricandia arvensis* + *B. oleracea* var. *botrytis, capitata*	TORIYAMA et al. (1987)
Moricandia nitens + *B. oleracea* var. *italica, gongyloides, capitata*	YAN et al. (1999)
Raphanobrassica *Raphanus sativus* + *B. oleracea* var. *capitata*	KAMEYA et al. (1989)
Raphanus sativus + *B. napus*	PELETIER et al. (1983); LELIVET, KRENS (1992); SAKAI et al. (1994)
Sinapis alba + *B. napus*	LELIVET et al. (1993)
Sinapis alba + *B. oleracea* var. *botrytis*	RYSCHKA et al. (1994); NOTHNAGEL et al. (1997)
Sinapis alba + RC *B. oleracea*	HANSEN, EARLE (1994, 2002)
Sinapis arvensis + *B. napus*	HU et al. (2002)
Thlaspobrassica *Thlaspi perfoliatum* + *B. napus*	FAHLESON et al. (1994b)
Thlaspi caerulescens + *B. napus*	BREWER et al. (1999)

ments of chloroplast DNA, the plants were intergeneric hybrids containing the nucleus of cabbage and the chloroplasts of radish. Their findings suggest the possibility of inducing male cytoplasmic sterility between distant related species.

Shepherd's purse (*Capsella bursa-pastoris*, 2*n* = 32) is a small, widely spread weed, tolerant to cold, with a short life cycle, it is also highly resistant to *Alternaria brassicae* and *A. brassicola*. SIGAREVA and EARLE (1997c, 1999) fused mesophyll protoplasts of *Capsella bursa-pastoris* and mesophyll protoplasts of RC plants *Brassica oleracea* treated with iodoacetate to prevent division of unfused cells. Only 1.8% of calli regenerated plants similar to shepherd's purse. In several plants, the content of DNA was the total of the content of the parent doses of DNA. The plants were confirmed to be somatic hybrids by RAPD and isoenzymatic analysis, but they were sterile. One of the two tested hybrids was really resistant to *Alternaria brassicola* in the same way as the genus *Capsella*, which confirmed the possibility of transferring the resistance to *Alternaria* by somatic fusion.

Gold of pleasure (*Camelina sativa* L., 2*n* = 40) was also included in intergeneric protoplast fusion. It is an annual plant which draws attention as an alternative oil plant. It is highly resistant to the pathogens mentioned above, *Alternaria brassicae* and *Alternaria brassicola*. Its leaves produce glucosinolates which are not found in the other representatives from the family Brassicaceae. *Camelina* was used by HANSEN (1997, 1998) and SIGAREVA and EARLE (1997b) for protoplast fusion with RC plants *Brassica oleracea* to overcome the barriers of sexual hybridization and to transfer the resistance to *Alternaria* spp.

HANSEN (1997, 1998) proved in her studies that it is possible to obtain intergeneric hybrids. The nuclei of the fusing partner *B. oleracea* were inactivated in advance by iodoacetate in order to prevent the division of unfused protoplasts. The fusion was achieved with the help of PEG and the protoplasts were cultivated on the medium according to PELETIER et al. (1983). The regeneration of plants from calli reached only 0.5% and the plants in *in vitro* conditions were strongly vitrified, rich flowered, but their roots were necrotized. The hybrid character of the plants was proved morphologically (the stem and leaves had the same trichomes as *Camelina*, the edges of the leaves were similar to *Brassica*, the width of leaves was somewhere between the widths of the leaves of the parent components) as well as by RAPD analysis and flow cytometry.

SIGAREVA and EARLE (1997b) described the creation of intergeneric hybrid between *C. sativa* and RC plants *B. oleracea.* The formation of hybrid plants from calli was proved morphologically, by the DNA content and by analysis of isocitrate dehydrogenase and aminotransferase.

The genus *Moricandia* (2*n* = 28) from the family Brassicaceae is unique because its photosynthesis is of transitional type between C_3 and C_4 plants, it has a different expression of the enzyme glycindecarboxylase in leaf cells and a combination of the leaf anatomy with different classification of organelles (mitochondria, chloroplasts) in the cells of vascular bundle. This results in more effective inhibition of CO_2 photorespiration and lower compensatory point of CO_2 intake. The transfer of the mechanism of more effective CO_2 use by the plant into agricultural crops can improve their water economy compared to C_3 plants of the same crop, especially in conditions of water stress (O'NEIL et al. 1996).

YAN et al. (1999) carried out a protoplast fusion between *Brassica oleracea* (broccoli, kohlrabi, cabbage) and *Moricandia nitens* (wild species with C_3–C_4 photosynthesis type). 425 plants obtained were checked and 90% of them were hybrid according to morphological observance, the number of chromosomes and RAPD analysis. Morphologically, the hybrid plants were between both fusing partners (the shape of leaves, the colour or petals). In most plants, the anthers were stunted with a few fertile pollen grains. Cytologically, the hybrid plants had 46 chromosomes (18 + 28), 74, 92 chromosomes. According to measuring the compensatory CO_2 point, in several hybrid plants the character of gas exchange was expressed as transition between *M. nitens* (C_4–C_4) and *B. oleracea* (C_3) parents. That means that the genes determining the C_3–C_4 character in *M. nitens* were not completely suppressed by *B. oleracea* genes (C_3) in the somatic hybrids.

White mustard (*Sinapis alba* L., 2*n* = 24) is an important source of resistance to diseases (*Alternaria* spp., *Phoma lingam*) and abiotic stress conditions. In the wild, no natural hybrid between *Sinapis alba* and *Brassica* spp. is known (NOTHNAGEL et al. 1997). HANSEN and EARLE (1994) used protoplast fusion to overcome intergeneric barriers and transfer the resistance to *Alternaria brassicae* from *Sinapis alba* into RC plants *Brassica oleracea.* Morphologically, the somatic hybrids were between both of the parent species, with a very low production of pollen. The contents of their nuclear DNA was the total of DNA values of both parents and according to the reaction to inoculation with *Alternaria brassicae,* they showed high resistance from *Sinapis alba*.

Somatic hybrids between rape (*Brassica napus*) and field mustard (*Sinapis arvensis* L., 2*n* = 18) were also created by mesophyll protoplast fusion. 54 plants altogether were identified as symmetric hybrids and 4 plants as asymmetric hybrids. Morphologically, all 58 plants were similar to both donor genotypes. This plant material became a potential not only as a bridge for the introduction of new features from mustard into cabbage, but also for the transfer of resistance to *Phoma lingam* (HU et al. 2002).

Perfoliate pennycress (*Thlaspi perfoliatum*, 2*n* = 42) is an annual plant the seeds of which contain nervonic acid (19–20%). Nervonic acid is valued for technical purposes, but in *Brassica napus* it is present in only small volumes, therefore there is an interest in transferring the genes regulating the creation of this fatty acid

into *B. napus* (rape). FAHLESON et al. (1994b) carried out protoplast fusions between hypocotyl protoplasts of *B. napus* and mesophyll protoplasts of *Thlaspi perfoliatum*. Isoenzymes were used as markers for the testing of hybridity. The morphology of regenerated plants was between both parents. The anthers were smaller than in *B. napus*. These results show that it is possible to combine two genera into fully functional intergeneric hybrids bearing the features of both parents, including the presence of nervonic acid in some hybrids.

Pennycress (*Thlaspi caerulescens,* $2n = 14$) is a plant that is able to accumulate zinc. BREWER et al. (1999) used it as a protoplast donor for protoplast electrofusions with *Brassica napus*. The hybrids were selected on a medium with a high content of zinc that is phytotoxic to *B. napus*. Their results confirm that the feature of hyperaccumulation of metal in a plant may be transferred by somatic hybridization because some hybrid plants grew on media with high zinc contents. Such crops could be used for biological removal of metal contamination from soil.

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Protoplastové kultury a fúze protoplastů se zaměřením na Brassicaceae – přehled

ABSTRAKT: Obsahem článku jsou protoplastové kultury a fúze protoplastů, zejména jejich historie, přehled faktorů ovlivňujících izolaci a fúzi protoplastů, selekce hybridních rostlin a využití somatických hybridů ve šlechtění rostlin. Somatická hybridizace pomocí fúze protoplastů může překonat sexuální inkompatibilitu mezi rostlinnými druhy nebo rody; přenést geny rezistence vůči chorobám (virovým, bakteriálním, houbovým), pesticidům, herbicidům a dalším stresovým faktorům; získat cybridní rostliny; přenést cytoplazmickou samčí sterilitu nebo zvýšit obsah sekundárních metabolitů v hybridních rostlinách. Článek je zaměřen hlavně na čeleď Brassicaceae, protože mezi zástupci jsou plodiny významné pro lidskou populaci. Mnoho úspěšných kombinací vnitrodruhových, mezidruhových a mezirodových fúzí bylo provedeno mezi zástupci čeledi Brassicaceae s rodem *Brassica*, který patřil k prvním zemědělským plodinám použitým pro izolace protoplastů.

Klíčová slova: Brassicaceae; *Brassica*; izolace protoplastů; fúze protoplastů; somatická hybridizace; rezistence k chorobám a abiotickým faktorům

Corresponding author:

RNDr. BOŽENA NAVRÁTILOVÁ, Ph.D., Univerzita Palackého v Olomouci, Přírodovědecká fakulta, katedra botaniky, Šlechtitelů 11, 783 71 Olomouc, Česká republika
tel.: + 420 585 634 811, fax: + 420 585 634 824, e-mail: navratilova@prfholnt.upol.cz