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Investigations on the Transfer of Isolated Nuclei into Plant Protoplasts

H. Lörz and I. Potrykus
Max-Planck-Institut für Pflanzengenetik, Ladenburg/Rosenhof (Federal Republic of Germany)

Summary. Nuclei were isolated from various types of donor protoplasts and were transferred into receptor protoplasts in numerous combinations. Five percent uptake was achieved under conditions which did not interfere with viability and subsequent culture of receptor protoplasts. Methodological investigations on nuclei uptake were carried out with cereal and tobacco protoplasts. To look for biological proof of integration and replication of transferred nuclear genes, two complementing, chlorophyll-deficient, light-sensitive mutants of tobacco were used as sources of nuclei and receptor protoplasts. Ca. 5.5 $\times 10^7$ receptor protoplasts were cultured following transplantation experiments involving these complementing mutants and about 1.8×10^7 of the resulting calli were subjected to selective conditions which discriminate against the parental types. No nuclear hybrids were detected, although in control experiments somatic hybrids were obtained by protoplast fusion. Some explanations for failure of nuclear hybrid formation are discussed together with other possible approaches for selective somatic combination of plant cell genophores.

Key words: Protoplasts – Organelle transfer – Isolated nuclei – Cereals – Tobacco

Introduction

Isolated plant protoplasts are currently considered to be very suitable for genetic modification experiments and for bypassing natural barriers of incompatibility (Cocking 1977; Melchers 1977). Fusion of protoplats allows the somatic combination of the total genetic information of two cells. Transferring plant cell genophores (i.e. isolated nuclei, plastids and mitochondria) separately allows a more specific combination of parts of the plant cell genome. The latter procedure may more easily bypass cellular and molecular incompatibility barriers between distant species and favour integration of specific genetic information. Success in genetic modification of plant cells via nuclei transfer into protoplasts requires

a) the isolation of viable protoplasts (acceptors),
b) the isolation of intact nuclei,
c) the uptake of nuclei at reasonable frequencies
d) the integration of the nucleus and the formation of a nuclear hybrid,
e) the expression of the 'foreign genes'.

Protoplast isolation and culture have been developed into routine procedures for a number of plant species, predominantly *Solanaceae* (Gamborg 1977). The biochemical activity (Blaschek et al. 1974; Ohyama et al. 1977) and structural integrity (Hughes et al. 1977) of nuclei isolated from protoplasts has also been demonstrated. Lysis of protoplasts by triton was found to be a gentle and effective technique to prepare isolated nuclei. The method results in good yield and quality of isolated organelles (Nishimura et al. 1976; Rathnam and Edwards 1976). Various methods have been reported for transplantation of isolated chloroplasts into protoplasts (Carlson 1973; Potrykus 1973; Bonnett and Eriksson 1974; Davey et al. 1976; Giles 1976) together with one report on nuclei transplantation using alternating layer centrifugation (Potrykus and Hoffmann 1973). While frequencies of chloroplast uptake in the range of 20% or more were described, nuclei transfer was achieved in 0.5% only. Looking for positive results in genetic modification via organelle transfer there are two reports on uptake and integration of wild type chloroplasts into albino protoplasts of tobacco (Carlson 1973; Kung et al. 1975). These experiments, however, have been described only in a bare outline, and critically reviewed (Kleinhofs and Behki 1977, Cocking 1977).

In this communication we report some improvements of nuclei transfer conditions and the results of experiments which looked for a biological proof of genetic modification after a nucleus uptake event.

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Materials and Methods

Protoplast Isolation

Young expanded leaves from 4-6 weeks old greenhouse grown plants of *Secale cereale* (cv. 'Tero'), *Avena sativa* (cv. 'Tiger') and *Hordeum vulgare* (cv. 'Dura') were washed, surface sterilized (5 sec in 70% ethanol, 5 min in 0.5% calcium hypochlorite + 0.01% Tween 80) and then rewashed in sterile water for 30 min. The leaves were cut into diagonal strips 0.5 mm wide and preincubated for 30 min in osmoticum (0.3 M mannitol + 0.3 M sorbitol + 5 mM $CaCl_2$, pH 5.8). The protoplasts were isolated in a one-step overnight incubation at 17° C in a mixture of 0.2% Macerozyme R10 and 0.2% Cellulase R10 in osmoticum. The resulting protoplast suspensions were poured through a 100 µm stainless steel sieve, centrifuged 5 min at 35 × g, resuspended in osmoticum, washed twice by centrifugation and then resuspended again. Parenchyma protoplasts of *Zea mays* (cv. 'Prior') were isolated as described by Potrykus et al. (1977) and *Nicotiana tabacum* protoplasts (var. 'Samsun', mutants 'virescent' and 'sublethal') according to Harms and Potrykus (1978a).

Protoplast Culture

To test survival of protoplasts after transfer experiments cereal protoplasts were suspended and cultured in a cereal protoplast culture medium (Potrykus et al. 1976). Tobacco protoplasts were cultured in medium NT (Nagata and Takebe 1971) supplemented with 4 mg/l CPA (p-chlorophenoxy acetic acid) and 0.5 mg/l Kinetin (6-furfurylaminopurine) for first day in the dark at 12° C and for 3 days at 24° C (300 lux) and were finally transferred to 3000 lux. After 2-3 weeks the small calli were transferred to soft agar NT medium with reduced organic components (1/10) and BAP (0.5 mg/l; 6-benzylaminopurine) and mannitol (0.2 M). Selection of putative hybrid calli after transfer experiments using the two light sensitive mutants was carried out at 10 000 lux for at least two passages. Calli derived from the parental mutants appeared white or yellow under these conditions and stopped growing. Calli derived from the sexual and the somatic hybrid appeared green and continued to grow (Melchers and Labib 1974). Shoot regeneration was induced on MS medium (Murashige and Skoog 1962) supplemented with IAA (4 mg/l, indole acetic acid) and kinetin (2.5 mg/l) and root regeneration in half concentrated Knop solution + Thiamin HCl (1 mg/l). Plantlets with well developed roots were transferred to soil and cultured under greenhouse conditions to seed-set.

Isolation of Nuclei

All isolation procedures were carried out on a cooling plate at 5° C and cooled glassware and solutions were also used. Equal volumes of protoplast suspension and nuclei isolation medium (NIM) with 8% Triton × 100 were mixed and shaken gently (Hoffmann 1973; Blaschek et al. 1974). After the triton mediated lysis of the protoplasts (Figs. 1a-c), the solution was poured through a 25 µm sieve and the nuclei collected and washed by sedimentating three times for 10 min at 300 × g in NIM. Resulting nuclei were resuspended and used in concentrations of about 5-10 × 10^6 nuclei/ml NIM (Lörz and Potrykus 1976).

Labeling of Nuclei

Uptake of nuclei into protoplasts was examined by incubating the nuclei suspension prior to transfer experiments with 0.01% acridine orange in NIM or 0.01% ethidium bromide in NIM for 10 min. Stained nuclei were then washed twice in NIM and examined using a Leitz fluorescent microscope with filter combination BG12 and K530 (Fig. 1).

RNA Synthesis of Isolated Nuclei

RNA synthesis was measured by incubating nuclei in standard reaction mixture with 3H-UTP according to the method described by Blaschek et al. (1974).

Nuclei Transfer

Four methods were examined in more detail and in all cases a ratio of nuclei/acceptor protoplasts of at least 50/1, or higher, was used.

The method of alternating-layer-centrifugation (Potrykus and Hoffmann 1973) was carried out by centrifugation (5 min, 80-120 × g) of protoplasts and isolated nuclei to alternating layers in hypotonic mannitol solution (440 mOs/kg H_2O) + 0.03% lysozyme.

Solution of Ca $(NO_3)_2$, used for moss protoplast fusion (Schieder 1974), was used here to stimulate nuclei uptake. Protoplasts and nuclei were co-incubated for 5-20 min at pH 9 in 0.2 M $Ca(NO_3)_2$. The protoplast suspension was then diluted with mannitol (0.6 M) of higher osmolality and washed free of the remaining nuclei by centrifugation in mannitol solution.

Also the protoplast fusion technique with $CaCl_2$ and high pH (Keller and Melchers 1973) was adapted to nuclei transfer. Isolated nuclei were incubated with protoplasts in 0.4 M mannitol + 0.05 M $CaCl_2$ at various pH values (pH 7-12, 0.05 M glycin/NaOH buffer) for 5-60 min. Dilution and washing were as described above.

The method most commonly used in our experiments was a modified fusion technique with PEG (polyethylene glycol) as the uptake-inducing agent. This method and the method with $CaCl_2$ were chosen for the experiments with the two tobacco mutants. Nuclei and protoplasts were mixed on a cover slide and PEG added as previously described in detail by Kao (1975). Alternatively, 0.2 ml nuclei suspension were mixed with 1 ml protoplasts suspension in a test tube, an equal volume of PEG was added, and after 5-30 min incubation the PEG was diluted out slowly with $CaCl_2$ solution at pH 10.5. The protoplasts were washed once with the $CaCl_2$ solution at pH 7 and further washed with medium.

Uptake of nuclei was examined by scanning receptor protoplasts for fluorescence caused by the foreign nuclei and was expressed as % protoplasts with one or more foreign nuclei. By gentle rolling of the coverslip the actual position of the transferred nucleus – whether inside the protoplast or only attached – could be determined.

Results

Improvement of Uptake Procedures

Gentle isolation of nuclei from protoplasts instead of from homogenized plant tissue resulted in relatively pure

nuclear fractions (Figs. 1a-c). About 30×10^6 protoplasts were isolated from the enzymatic digestion of 10 g leaf material (average for mesophyll tissue of cereals and tobacco). From these protoplasts $5\text{-}6 \times 10^6$ nuclei could be routinely isolated. The use of Triton X 100 for lysis of the protoplasts, which could be stopped accurately at different phases, provided nuclei preparations of controlled purity (Fig. 1c). In our experiments it was not necessary to use highly pure nuclei preparations as the nuclei were labeled before use with a DNA-specific fluorescent dye (methodological investigations) or use was made of a defined nuclear gene marker in the case of the tobacco mutants (experiments for biological proof). The most suitable conditions for isolation of nuclei was 4-5 min Triton (4%) at 5° C. Using this condition there was absolutely no 'contamination' of intact protoplasts in the resulting nuclei suspension.

The method of nuclei transfer by alternating-layer-centrifugation was examined using the nuclei of barley and wheat, and predominantly corn parenchyma protoplasts as receptors. Using this material, uptake frequencies of 0.1% to 1% were achieved dependent on the condition of the receptor protoplasts which was affected by the centrifugation speed. To pellet corn parenchyma protoplasts requires ca. 600 rpm but nuclei require up to 1500-2000 rpm which damages corn protoplasts. A compromise was found in which protoplasts and nuclei were centrifuged alternatively for 5 min at 800 and 1200 rpm.

Nuclei transfer using $Ca(NO_3)_2$ as an uptake inducing agent also required a compromise of 'high physical uptake frequency' and 'physiological disposition'. Prolonged incubation in $Ca(NO_3)_2$ resulted in higher uptake frequencies – 5 min: 1.1%, 10 min: 2.3%, 20 min: 2.8% – but viability of the protoplasts was drastically reduced after long incubation. Incubation for 5 min gave 80% protoplast survival, but after 10 min only 50% and after 20 min less than 20% receptor protoplasts survived.

Results of nuclei transfer using $CaCl_2$ as uptake inducing agent at different pH values and incubation times are summarized in the table (Fig. 2). Here again prolonged incubation and higher pH values resulted in greater uptake but increased protoplast damage, that means breaking of protoplasts when the suspension was repeatedly centrifuged for washing or after transfer to culture medium

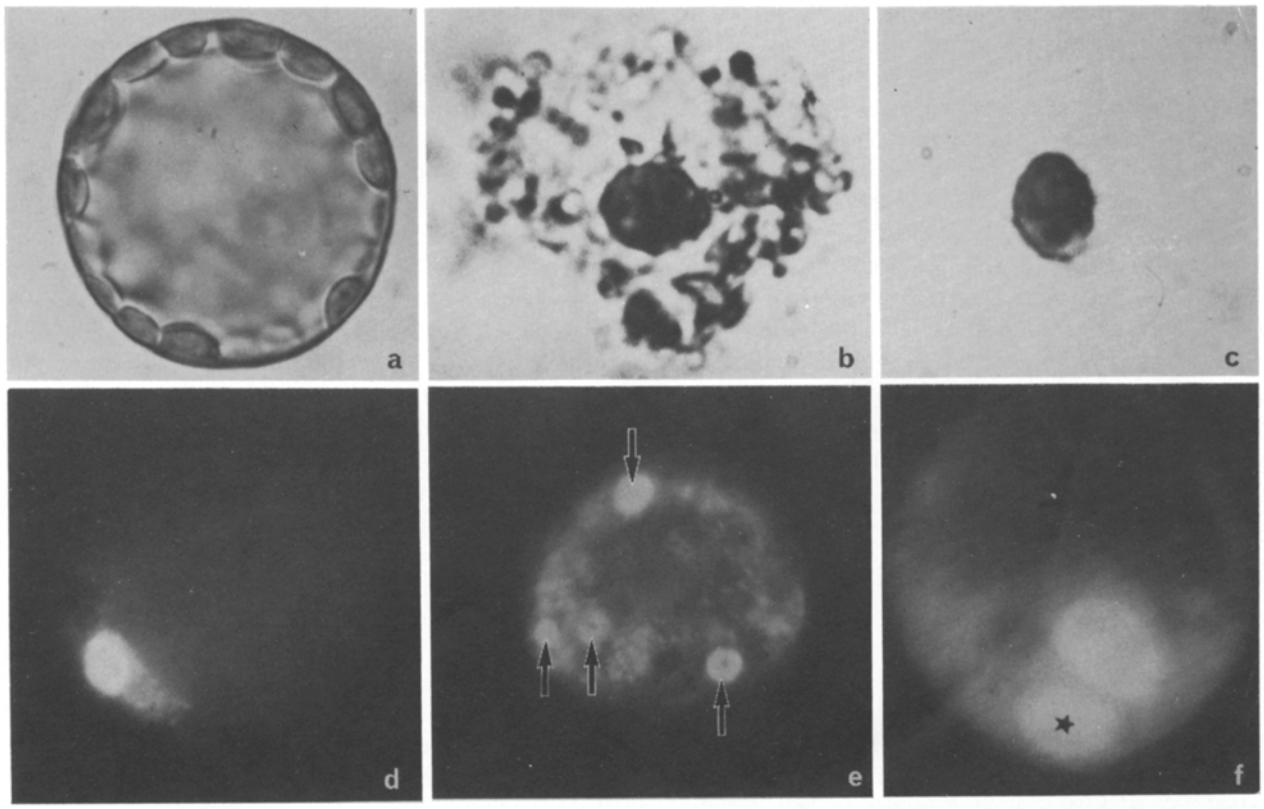

Fig. 1a–f. Isolation of nuclei (a-c) and uptake into protoplasts (d-f). **a** Isolated mesophyll protoplasts from *Hordeum vulgare*, **b** Triton-mediated lysis of the protoplast, **c** Isolated nucleus, **d** *Zea mays* protoplast with fluorescent labeled nucleus of *Secale cereale*, **e** Uptake of four nuclei (arrows) of *Hordeum vulgare* into corn protoplast, **f** *Zea mays* protoplast with one nucleus of *Triticum aestivum*. About 30-60 min after uptake of fluorescent stained nuclei the nucleus of the receptor protoplast (*) also shows some fluorescence. Magnification a, d, e X 1100; b, c, f X 2000

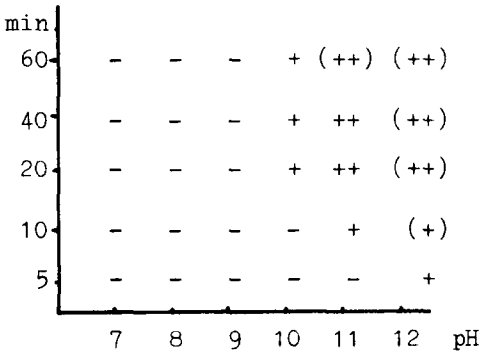

Fig. 2. Incubation of *Zea mays* protoplasts with *Triticum aestivum* nuclei at 25° C in 0.4 M mannitol + 0.05 M $CaCl_2$ + 0,05 M glycine/NaOH buffer
– no nuclei uptake
+ uptake frequency less than 0.5%
++ uptake frequency higher than 0.5%
( ) detrimental to protoplast viability

within the first hours. Routine experiments were carried out at pH 10.5 with 20-30 min incubation, resulting in 0.9 to 1.2% protoplasts with foreign nuclei and 70-80% protoplast viability.

The PEG method was the most effective of all methods examined in our studies. Both variations of the procedure described above led to the uptake of foreign nuclei into 3-5% of the protoplasts (Figs. 1d-f). By co-incubating protoplasts and nuclei on cover slides, less destruction of the receptor protoplasts occurred than by their incubation in test tubes. Isolated chloroplasts and blue green algae (*Anabaena azollae*) were used in transplantation experiments in parallel to nuclei (Lörz 1977).

To test the stress of the transfer conditions on isolated nuclei, RNA synthesis was measured. Treatment of nuclei with PEG according to transfer conditions drastically reduced the incorporation of 3H-UTP into RNA. Five min incubation resulted in 72% incorporation, 10 min: 23% and 20 min: less than 5%, compared to the 100% level determined in various control measurements without PEG treatment.

Nuclei Transfer Experiments with Complementing Tobacco Mutants

To study the fate of nuclei after uptake and to look for biological proof of integration and expression of donor nuclei, a series of experiments was performed with light-sensitive tobacco mutants. The loci of the virescent and sublethal mutations are located in the nucleus and the system has previously been used for somatic hybridisation by protoplast fusion (Melchers and Labib 1974). In accordance with the procedure for the protoplast fusion work, amphihaploid protoplasts and nuclei from microspore derived plants were used in the present experiments. However, an increased protoplast plating efficiency was achieved when NT medium was supplemented with CPA (4 mg/l) and kinetin (0.5 mg/l). In a series of 15 separate experiments ca. 2.3×10^7 virescent protoplasts were incubated with sublethal nuclei and 3.7×10^7 sublethal protoplasts were incubated with virescent nuclei. A total of 1.8×10^7 calli were recovered and placed under selective conditions in high light intensity. In an experiment using PEG, a combination of sublethal protoplasts with virescent nuclei produced more than forty green calli during a first screening under high light conditions. However, all plants derived from these calli were shown to be sublethal plants on the basis of colour and morphology under greenhouse conditions. In control experiments, sublethal or virescent protoplasts alone (ca. 2×10^7) or protoplasts and nuclei of the same type (ca. 1.7×10^7), no green calli were detected. Only protoplast fusion experiments using PEG yielded green calli and eleven fusion hybrids were isolated out of 3.1×10^6 cultured protoplasts.

Discussion

In general, methods used for protoplast fusion are also suitable for organelle uptake. The results presented above demonstrate that transfer of nuclei can be achieved with frequencies of up to 5% but that the same incubation conditions which promote uptake reduce the viability of the acceptor protoplasts and the metabolic activity of the nuclei. As shown for the use of Ca $(NO_3)_2$, $CaCl_2$ and PEG, a compromise must be found between high uptake and reduced viability of the acceptor protoplasts. When sensitive physiological selection systems are available, low frequencies of uptake may be acceptable. Using dividing protoplast systems, protoplast damage can be systematically examined. However, assessment of nuclei damage requires ultimately the expression of nuclear genetic information. Integration of nuclei and formation of nuclear hybrids are still open problems. The demonstration of adjacent interphase nuclei by differential staining does not necessarily demonstrate that the acceptor and donor nuclei will fuse and mix. But using different *Solanaceae* species in which sustained divisions are inducible and in which nuclei and chromosomes can be visually distinguished may help in overcoming this problem.

To attack the most important problem of nucleus integrity and gene expression after transfer experiments, two tobacco mutants were used. However, with this material we could not demonstrate microscopically any integration of the donor nucleus in the first divisions. Homologous fusion products of sublethal or virescent protoplasts cannot be distinguished from nuclear hybrids. Proof of nuclear hybrid formation in the form of light-resistant

calli and plants was also not obtained. The only green calli obtained in a combination of sublethal protoplasts with virescent nuclei were shown to be sublethal type when regenerated to plants. Some slight variation of the callus culture conditions under strong light, such as the amount of overlayered agar medium (light intensity, O_2-supply) could have caused this illusion. Other selection systems (e.g. auxotrophic mutants) which could be used earlier in protoplast culture than the complementation of two light-sensitive mutants may be advantageous.

As mentioned above, PEG incubation reduced nuclear RNA synthesis drastically. To overcome the problems one has to reduce the stress on the isolated nuclei, e.g. by looking for less detrimental substances or by shortening the incubation time of isolated nuclei. Immediate transfer of nuclei in a gradient system seems to be one possibility. Investigations are being carried out using a complex step density gradient system including Triton treatment where the isolated nuclei are simultaneously used as donor organelles and transferred into receptor protoplasts in a lower phase of the gradient. New step density gradient systems established in our laboratory for protoplast fractionation and enrichment of heterokaryocytes (Harms and Potrykus 1978a, b) open this approach. Another approach is the preparation of subprotoplasts (Binding and Kollmann 1976; Wallin et al. 1978). The separated organelles remain permanently in a 'natural environment' and organelle transfer is then a fusion event. Investigations on the large scale preparation of cell fractions – karyoplasts and cytoplasts – from various protoplast types are in progress.

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Literature

Binding, H.; Kollmann, R.: The use of subprotoplasts for organelle transplantation. In: Cell Genetics in Higher Plants (eds. Dudits, D.; Farkas, G.L.; Maliga, P.) pp. 191-206. Budapest: Akadémiai Kiadó 1976

Blaschek, W.; Hess, D.; Hoffmann, F.: Transcription in nuclei prepared from isolated protoplasts of *Nicotiana* and *Petunia*. Z. Pflanzenphysiol. **72**, 262-271 (1974)

Bonnett, H.T.; Eriksson, T.: Transfer of algal chloroplasts into protoplasts of higher plants. Planta **120**, 71-79 (1974)

Carlson, P.S.: The use of protoplasts for genetic research. Proc. Natl. Acad. Sci. (USA) **70**, 598-602 (1973)

Cocking, E.C.: Uptake of foreign genetic material by plant protoplasts. Int. Rev. Cytol. **48**, 323-343 (1977)

Davey, M.R.; Frearson, E.M.; Power, J.B.: Polyethylene glycol induced transplantation of chloroplasts into protoplasts: an ultrastructural assessment. Plant Sci. Lett. **7**, 7-16 (1976)

Gamborg, O.L.: Somatic cell hybridization by protoplast fusion and morphogenesis. In: Plant Tissue Culture and Its Bio-technological Application (eds. Barz, W.; Reinhard, E.; Zenk, M.H.) pp. 287-301. Berlin: Springer 1977

Giles, K.L.: Chloroplast uptake and genetic complementation. In: Plant Cell, Tissue and Organ Culture (eds. Reinert, J.; Bajaj, Y.P.S.) pp. 536-550. Berlin: Springer 1976

Harms, C.T.; Potrykus, I.: Fractionation of plant protoplast types by iso-osmotic density gradient centrifugation. Theor. Appl. Genet. **53**, 57-63 (1978 a)

Harms, C.T.; Potrykus, I.: Enrichment for heterokaryocytes by the use of iso-osmotic density gradients after plant protoplast fusion. Theor. Appl. Genet. **53**, 49-55 (1978 b)

Hoffmann, F.: Die Aufnahme doppelt-markierter DNS in isolierte Protoplasten von *Petunia hybrida*. Z. Pflanzenphysiol. **69**, 249-261 (1973)

Hughes, B.G.; Hess, W.M.; Smith, M.A.: Ultrastructure of nuclei isolated from plant protoplasts. Protoplasma **93**, 267-274 (1977)

Kao, K.N.: A method for fusion of plant protoplasts with polyethylene glycol. In: Plant Tissue Culture Methods (eds. Gamborg, O.L.; Wetter, R.L.) pp. 22-25. Saskatoon: Nat. Res. Council Canada 1975

Keller, W.A.; Melchers, G.: The effect of high pH and calcium on tobacco leaf protoplast fusion. Z. Naturforsch. C **28**, 737-741 (1973)

Kleinhofs, A.; Behki, R.: Prospects for plant genome modification by nonconventional methods. Ann. Rev. Genet. **11**, 79-101 (1977)

Kung, S.D.; Gray, J.C.; Wildman, S.G.; Carlson, P.S.: Polypeptide composition of fraction 1 protein from parasexual hybrid plants in the genus *Nicotiana*. Science **187**, 353-355 (1975)

Lörz, H.; Potrykus, I.: Uptake of nuclei into higher plant protoplasts. In: Cell Genetics in Higher Plants (eds. Dudits, D.; Farkas, G.L.; Maliga, P.) pp. 239-244. Budapest: Akadémiai Kiadó 1976

Lörz, H.: Protoplasten höherer Pflanzen als Objekte der genetischen Manipulation. Thesis, Universität Heidelberg 1977

Melchers, G.; Labib, G.: Somatic hybridisation of plants by fusion of protoplasts. I. Selection of light-resistant hybrids of 'haploid' light-sensitive varieties of tobacco. Molec. Gen. Genet. **135**, 277-294 (1974)

Melchers, G.: Kombination somatischer und konventioneller Genetik für die Pflanzenzüchtung. Naturwissenschaften **64**, 184-194 (1977)

Murashige, T.; Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**, 473-497 (1962)

Nagata, T.; Takebe, I.: Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta **99**, 12-20 (1971)

Nishimura, M.; Graham, D.; Akazawa, T.: Isolation of intact chloroplasts and other cell organelles from spinach leaf protoplasts. Plant Physiol. **58**, 309-314 (1976)

Ohyama, K.; Pelcher, L.E.; Horn, D.: A rapid, simple method for nuclei isolation from protoplasts. Plant Physiol. **60**, 179-181 (1977)

Potrykus, I.: Transplantation of chloroplasts into protoplasts of *Petunia*. Z. Pflanzenphysiol. **70**, 364-366 (1973)

Potrykus, I.; Hoffmann, F.: Transplantation of nuclei into protoplasts of higher plants. Z. Pflanzenphysiol. **69**, 287-289 (1973)

Potrykus, I.; Harms, C.T.; Lörz, H.: Problems in culturing cereal protoplasts. In: Cell Genetics in Higher Plants (eds. Dudits, D.; Farkas, G.L.; Maliga, P.) pp. 129-140. Budapest: Akadémiai Kiadó 1976

Potrykus, I.; Harms, C.T.; Lörz, H.; Thomas, E.: Callus formation from stem protoplasts of corn (*Zea mays L.*). Molec. Gen. Genet. **156**, 347-350 (1977)

Rathnam, C.K.M.; Edwards, G.E.: Protoplasts as a tool for isolating functional chloroplasts from leaves. Plant Cell Physiol. **17**, 177-186 (1976)

Schieder, O.: Selektion einer somatischen Hybride nach Fusion von Protoplasten auxotropher Mutanten von *Sphaerocarpes donnellii* Aust.. Z. Pflanzenphysiol. **74**, 357-365 (1974)

Wallin, A.; Glimelius, K.; Eriksson, T.: Enucleation of plant protoplasts by cytochalasin B. Z. Pflanzenphysiol. **87**, 333-340 (1978)

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Present address:
Dr. H. Lörz
Friedrich Miescher-Institut
P.O. Box 273
CH-4002 Basel (Switzerland)