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Full Name of Author — Nom complet de l'auteur

William Stephen SHURBEN

Date of Birth — Date de naissance

April 17, 1956

Country of Birth — Lieu de naissance

Canada

Permanent Address — Résidence fixe

8 ~~Ravaleigh~~ Avenue
Weston, Ontario
M9P 2J5

2106 STONEHENGE CRES.
GLOUCESTER, ONTARIO
K1B 4N8

Title of Thesis — Titre de la thèse

Density-Gradient Fractionation of Plant Protoplasts as a Method for Selection of Somatic Hybrids

University — Université

Carleton

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

M.Sc.

Year this degree conferred — Année d'obtention de ce grade

1984

Name of Supervisor — Nom du directeur de thèse

Dr. G. Setterfield

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DENSITY-GRADIENT FRACTIONATION OF PLANT PROTOPLASTS AS A
METHOD FOR SELECTION OF SOMATIC HYBRIDS

by

© WILLIAM STEPHEN SHURBEN, B.Sc

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfillment
of the requirements for the degree of
Master of Science

Department of Biology
Carleton University
Ottawa, Ontario

1984

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ABSTRACT

Protoplasts from Nicotiana rustica, N. tabacum and Datura innoxia cell-suspension cultures were fractionated in a six-step Percoll/mannitol discontinuous density gradient. All protoplasts had similar sedimentation distributions and mean buoyant densities despite considerable differences in size between species. Separation of protoplasts was on the basis of density although protoplast size decreased with increasing buoyant density. Morphometric analysis of electron micrographs indicated that the major factor contributing to decreased size was a decrease in vacuolar volume. Neither ploidy level, age of cell culture nor experimentally modified intracellular starch content significantly altered buoyant density. Discrete fractions from chlorophyll-deficient N. rustica and N. tabacum were fused using polyethylene glycol and recentrifuged on an identical gradient. Interspecific hybrids selected by albino complementation were enriched in an intermediate density fraction although movement of unfused protoplasts to the same fraction prevented high-level purification of heterokaryocytes. Overall frequency of hybrids recovered was improved by fractionation.

ACKNOWLEDGEMENT

Thanks go to Marcia Falconer for moral support and assistance in the lab. Thanks also to Dr. George Setterfield for his support of this research and for editing this thesis. Finally, a word of appreciation to my parents for all their support. Thankyou.

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LIST OF ABBREVIATIONS

A2C	azetidine-2-carboxylate
AEC	S-(2-amino-ethyl)-L-cysteine
BA	benzyladenine
DAPI	4', 6-Diamidino-2-phenylindole
2,4-D	2,4-dichlorophenoxyacetic acid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate
FACS	fluorescence-activated cell sorter
FITC	fluorescein isothiocyanate
KMC	$K^+ + Mg^{++} + Ca^{++}$ salt solution
KMC/S	$K^+ + Mg^{++} + Ca^{++}$ salt solution in sucrose
mOs/kg H ₂ O	milliosmol per kilogram water
MS	Murashige and Skoog
5 MT	DL-5 methyltryptophan
NAA	naphthaleneacetic acid
NR ⁻	nitrate-reductase deficient
NT	Nagata and Takebe
PEG	polyethylene glycol
PVP	polyvinylpyrrolidone
RITC	rhodamine isothiocyanate

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INTRODUCTION

Traditionally, crop improvement has relied upon sexual breeding and selection to combine desirable phenotypic characteristics (von Wettstein 1983). In general, because of sterility barriers the more distantly related plants are, the more difficult it is to produce viable embryos in sexual crosses (Sprague 1982). Such barriers to sexual hybridization may potentially be overcome through use of somatic-cell fusion techniques which include enzymatic isolation of protoplasts, fusion of protoplasts from different parents, selection of heterokaryocytes and subsequent regeneration of hybrid plants (Vasil and Vasil 1980; Zimmerman and Scheurich 1981).

Successful somatic hybridization depends on a number of factors (Keller et al. 1982): e.g., (1) production of viable protoplasts (2) induction of protoplast fusion (3) survival and subsequent division of the fusion products resulting in cultures of heterokaryocytes (cells containing non-identical nuclei) (4) selective recovery of the hybrid cells, and (5) induction of morphogenesis to give hybrid plants. Step (4), i.e. the selective recovery of true heterokaryocytes in a mixed population of regenerating protoplasts is often an important limiting factor in application of somatic hybridization. Interest has focused on selection procedures which facilitate

isolation of somatic hybrids as soon as possible after the fusion event so as to allow screening of large numbers of cultures and/or plants for desirable hybrid characteristics.

Most methods for selection have depended upon specific morphological, physiological and/or genetic factors thereby limiting the plant strains and species which may be crossed. There is a need for a more general method of hybrid selection which may be applied to all plant types. One potential solution to this problem is to use a physical property of protoplasts, such as buoyant density, as a selectable marker. The aim of the present study was to re-examine gradient fractionation of plant protoplasts as a possible selection system in somatic hybridization. The eventual objective of the work was to fuse protoplast populations and isolate hybrid protoplasts with distinct buoyant densities.

Specifically, the study attempted to evaluate salt/sucrose mixtures and Percoll as gradient materials for fractionation of protoplasts and enrichment of heterokaryocytes in mixed populations of protoplasts. The morphology and DNA content of protoplasts separating at different densities were examined in an attempt to determine cellular factors important in separation. Somatic hybridization between Nicotiana rustica var chlorotica and albino N. tabacum, developed by Douglas et al. (1981b), provided a model system to test for heterokaryocyte enrichment by density gradient centrifugation. These chlorophyll deficient parental lines form hybrid colonies which can be identified as green calli interspersed among white albino calli derived from unfused or homo-fused parent protoplasts.

Literature Review

Methods of plant heterokaryocyte selection following fusion.

Present methods for fusing mixed populations of protoplasts result in a mixture of homokaryocytes, heterokaryocytes and unfused protoplasts. Homokaryocytes arise from fusion of protoplasts with genetically identical nuclei while heterokaryocytes arise from fusion of protoplasts with genetically different nuclei. Various selection procedures have been used to differentiate hybrid protoplasts and cells from homofused or unfused protoplasts and cells. The major selection methods are addressed in the first section of this review while the second section deals specifically with gradient fractionation of protoplasts.

A. General methods for detection and selection of heterokaryocytes.

Numerous investigators have relied upon visual identification of hybrid cells either on the basis of different morphological characteristics of the parental protoplasts (Kao and Michayluk 1974; Binding and Nehls 1978; Gleba and Hoffman 1978; Gosch and Reinert 1978; Menczel et al. 1978; Hein et al. 1983) or by staining parental protoplasts differentially (Galbraith 1979, 1980; Patnaik 1982).

Once identified, heterokaryocytes are isolated mechanically by micromanipulation and cultured individually in microdroplets (Kao 1977; Gleba and Hoffmann 1978) or in "nurse" cultures (Menczel 1978; Hein 1983).

Kao (1977) was the first to introduce a successful method of mechanical selection for somatic hybrids. He fused colorless Glycine max protoplasts derived from cell suspension with green Nicotiana glauca protoplasts from leaf mesophyll. The fusion mixture was diluted and cultured in microdrops. Fusion products were distinguished microscopically from the parental protoplasts since they were of intermediate morphology. A disadvantage of this method is that dilution procedures involved require a high percentage of heterokaryocyte formation. In addition, individual cultivation of heterokaryocytes requires use of complex regeneration media which must be developed for different species (Koop et al. 1983).

As an alternative to cultivation of individual protoplasts, heterokaryocytes have been regenerated in a "nurse" culture of phenotypically different cells. Menczel et al. (1978) fused protoplasts of a chlorophyll-deficient, kanamycin-resistant cell line of Nicotiana sylvestris with green wild type protoplasts of N. knightiana. Interspecific fusion products were identified visually and transferred mechanically to protoplast cultures of the chlorophyll-deficient cell line of N. sylvestris. Somatic hybrids were identified as green, shoot-producing calli. Such cultivation of heterokaryocytes in a nurse culture of albino

protoplasts is possible only if the somatic hybrids produce chlorophyll. As an alternative, Hein et al. (1983) used protoplasts from a nitrate-reductase deficient (NR^-) cell line of N. tabacum as the nurse culture. Somatic hybrids produced by fusion of colorless NR^- N. tabacum protoplasts with green N. paniculata or N. sylvestris mesophyll protoplasts were mechanically transferred to the protoplast "nurse" culture. The culture medium was initially supplemented with nitrate and the amino acids arginine, aspartic acid and glutamine which supported growth of the auxotrophic nurse cells. After three weeks of culture the level of amino acids was reduced causing reduced growth or death of the nurse cells while somatic hybrids continued growth by metabolizing inorganic nitrogen.

The existence of only a few good cytological markers such as vacuole pigments, green vs. white chloroplasts, degree of vacuolation, and cytoplasmic strands has limited the use of microscopic identification of heterokaryocytes as a selective approach. By using fluorescent stains the range of useful combinations for hybrid production may be extended. Galbraith and Galbraith (1979) identified fused soybean protoplasts after labelling the fusion partners separately with fluorescein isothiocyanate (FITC) and rhodamine B. Similarly, Galbraith and Mauch (1980) labelled mesophyll protoplasts of Nicotiana species separately with FITC and rhodamine isothiocyanate (RITC) and used fluoromicrospectrophotometry to identify fusion products which contained both dyes. However, none of these fusion products were isolated and cultured.

Patnaik (1982) fused FITC labelled cell suspension protoplasts with mesophyll protoplasts. He located presumptive hybrids on the basis of green chloroplasts from the mesophyll protoplasts and dense cytoplasmic strands from the suspension protoplasts, and cultured them in Cuprak dishes or in suspensions of an albino Petunia hybrida nurse culture. Heterokaryocytes showed red fluorescence of chloroplasts and green cytoplasmic fluorescence from FITC, confirming their hybridity.

Despite these various approaches, visual selection is still labor intensive and limited by the number of fused protoplasts one can identify and sort per man hour. Use of fluorescence-activated cell sorting (FACS) may greatly improve this approach (Galbraith 1980).

Redenbaugh et al. (1982) have used flow cytometry and cell sorting for characterization and separation of plant protoplasts from five species: Euphorbia lathyris, Nicotiana glauca, N. langsdorfii, Petunia parodii and P. inflata (albino). Characterization was on the basis of laser-light scatter, chlorophyll fluorescence and/or fluorescence of exogenously added FITC and RITC. Light scattering properties are related to protoplast size. Heterokaryocytes were identified, characterized and distinguished from homokaryocytes and unfused protoplasts using nontoxic levels of the fluorescent dyes. Bergounioux et al. (1983) were able to apply flow cytometry in conjunction with a selective medium to isolate intergeneric heterokaryocytes of P. hybrida and Lycopersicon esculentum. Since this method of

protoplast selection is not species, genotype or explant specific it may provide a general method for identifying and selecting hybrid protoplasts. However, the FACS is expensive and not yet routinely available.

To date, most somatic hybrids have been isolated by complementation selection procedures using auxotrophic, inhibitor-resistant or chlorophyll-deficient mutants or non-morphogenic wild type lines (Schieder 1982). Several intraspecific and interspecific somatic hybrids have been reported for the genera Nicotiana, Petunia and Datura using nonallelic albino mutants (Melchers and Labib 1974; Melchers 1977; Schieder 1977; Cocking 1978; Evans et al. 1980; Douglas et al. 1981b). Albino complementation selection depends upon the availability of albino mutant genotypes (cell lines or plants). Albinos may be relatively common in some species but are extremely rare or absent in others, particularly polyploids. High frequency induction of albino mutation by nitrosomethylurea (NMU) may be exploited for the production of albino mutants for use in protoplast culture (Hanson 1983). Treatment of tomato seeds with NMU produced variegated chimeric plants from which albino progeny could be obtained by selfing or from reciprocal crosses with variegated plants. Such albino progeny could provide a source of genetically albino cells for use in protoplast culture and fusion. However, in many cases the use of albino mutants may be of limited value since hybrid selection is based on chlorophyll synthesis in vitro, a process which is often difficult to induce even with normal genotypes (Keller et al. 1982).

The use of auxotrophic mutants for selection of somatic hybrids should, in theory, be highly efficient because only hybrid lines survive. However, auxotrophic mutants in higher plants are rare, difficult to obtain and limited to only a few plant species (Maliga et al. 1982). Amongst these, only the tobacco and barley NR^- mutants have been well characterized genetically and biochemically (Muller and Grafe 1978; Kleinhofs et al. 1980; Buchanan and Wray 1982; Evola 1983). Recently, there has been rapid progress in selection of auxotrophic cell lines using cultures of haploid cells (Savage et al. 1979; King and Khanna 1980; King et al. 1980; Strauss et al. 1981; Gebhardt et al. 1981; Sidorov et al. 1981a; Negrutiu 1981; Vunsh 1982; Maliga et al. 1982; Marton et al. 1982). However, many cultures which were initially haploid have become polyploid during extended cultivation (Muller and Grafe 1978; Buchanan and Wray 1982; Evola 1983), limiting their use for hybridization.

Numerous somatic hybrids have been selected using auxotrophic mutants, some of which are finding application in genetic analysis (Glimelius et al. 1978; Sidorov and Maliga 1982; Marton et al. 1982b; Gupta et al. 1982; Lazar et al. 1983; Evola 1983b). Grafe and Muller (1983) for example, used somatic hybridization to analyze the genetics of NR^- N. tabacum auxotrophs which failed to give fertile plants. NR^- cells are unable to divide on minimal medium containing nitrate as sole nitrogen source but grew well on amino acids (Muller and Grafe 1978). Protoplast fusion between complementing NR^- cells resulted in the formation of

hybrid colonies capable of growing in nitrate only. Fusions between NR⁻ cells which belong to the same complementation group did not result in colonies which survive on nitrate alone.

Mutants resistant to chemicals such as antibiotics or amino-acid analogues are more common than auxotrophic mutants. Fusion of two different resistant mutants results in formation of a dual resistance in the somatic hybrid enabling it to grow in the presence of both inhibitors. Successful somatic hybridization has been reported in N. sylvestris (White and Vasil 1979) after fusing lines resistant to DL-5 methyltryptophan (5 MT) and S-(2-amino-ethyl)-L-cysteine (AEC) and in Daucus carota (Harms et al. 1981) using a cell line doubly resistant to 5 MT and azetidine-2-carboxylate (A2C) fused with an AEC resistant carrot cell line. Carrot+tobacco hybrids were produced after fusion of an AEC resistant carrot cell line with a 5 MT resistant tobacco cell line (Hauptmann et al. 1983). This technique is limited since naturally occurring resistance traits must be identified or a mutagenesis program must be undertaken to induce such traits.

Different growth patterns of parental cells and their hybrids, on specific media, have also been used as a method of selection (Carlson et al. 1972; Smith et al. 1976; Chupeau et al. 1978; Power et al. 1977; Butenko and Kuchko. 1980; Xianghui et al. 1982). Carlson successfully used this method to obtain the first reported somatic hybrid plants by fusing mesophyll protoplasts of wild-type N. glauca and N. langsdorfii. Selection of somatic hybrids was based on the ability of the hybrid colonies to develop on hormone-free media while parental colonies required

hormones for growth. Similar selection procedures have been used for interspecific hybridization between P. hybrida and P. parodii (Power et al. 1977) and Solanum tuberosum and S. chacoense (Butenko and Kuchko 1980). Generally, this technique can only be applied in cases where sexual hybrids are available since prior knowledge of the growth pattern of the hybrid cells is necessary in order to develop selective conditions. However, Xianghui et al. (1982) were able to obtain somatic hybrids from intergeneric fusion of tobacco tumor cells, characterized by phytohormone independence and incapacity for morphogenic regeneration, with P. hybrida cells possessing complementary characteristics. Hybrids isolated on hormone free media had the morphology and regeneration capacity of Petunia and hormone independence of the tobacco tumor cell line. Regenerated hybrid plants were verified by the acquisition of lysopine dehydrogenase activity from the tumor cells. Xianghui et al. (1983) have also used this technique to obtain interspecific hybrids with N. tabacum.

Somatic hybrids have also been selected using differential growth responses in conjunction with albino complementation and drug resistance (Cocking et al. 1977; Maliga et al. 1978; Power et al. 1976, 1979, 1980; Schieder et al. 1978, 1980; Dudits et al. 1979, 1980; Gleddie et al. 1983; Evola 1983b).

One does not have to rely upon intrinsic growth responses of fusion partners if differential growth may be artificially induced using biochemical inhibitors (Wright 1978, 1981; Nehls 1978). Parental cells exposed to either diethylpyrocarbonate or iodoacetate are unable to survive, as are homokaryocytes. However, by

using different inhibitors for each parental type heterokaryocytes may survive, since each fusion partner replaces inactive molecules from the other partner. This method has had limited success for selection of heterokaryocytes presumably because of the extreme toxicity of the inhibitors. In our laboratory the method proved to be capricious and unreliable (Wasteneys, personal communication). Gleddie (personal communication) found that iodoacetate as one inhibitor was of value when coupled with aza-uracil resistance in fusions of Solanum melongena with wild Solanum species. Irreversible inhibitors may be of greater value in promoting direct transfer of cytoplasmic organelles by lowering the competitive ability of treated organelles (Sidorov et al. 1981b). The use of antibiotics such as chloramphenicol, kanamycin, trimethoprim and gentamycin which strongly inhibit growth of cultured plant cells may have a potential use as irreversible inhibitors (Lurquin et al. 1982; Ursic et al. 1981).

Other systems for selection of heterokaryocytes are less efficient since they attempt to identify hybrids at a later stage of development. These systems depend upon recognition of morphological markers such as leaf and floral morphology (Belliard et al. 1978; Zelcer et al. 1978; Izhar and Power 1979), abnormal morphology (Melchers et al. 1978) or intermediate phenotype (Binding and Nehls 1978; Power et al. 1975; Gosch and Reinert 1978). A vigorous growth pattern of heterokaryocyte callus was successfully used for selection of a hybrid between Datura innoxia and D. quercifolia (Schieder 1978, 1980). Among about 6,000 developed calli, five demonstrated a larger size and were selected

and regenerated to shoots. One of these proved to be a true somatic hybrid (Schieder 1982). Selecting at the stage of the regenerated plant is less useful since selection occurs long after the fusion event, the number of individuals which can be handled is restricted and very few of these individuals will be hybrids.

B. Density gradient separation of protoplasts

Although the various complementation-selection systems described above allow early unambiguous identification of hybrids many are not universally applicable since they require use of protoplasts with differing genotypes, phenotypes or regenerative capacities. Preselection for any of these traits could decrease the amount of genetic variability for subsequent plant breeding and/or may include undesirable gene combinations. Furthermore appropriate genetic variants are often not available in agriculturally desirable breeding stock. Hence, there is a need for a more general method for selection which can screen a large number of regenerating protoplasts for potential hybridity, independent of genetic traits.

A well established method for separation and purification of cells, viruses and subcellular particles is centrifugal sedimentation (Anon 1980). Separation is achieved either on the basis of differences in size or differences in buoyant density between suspended particles. Velocity sedimentation has been used to separate larger animal heterokaryocytes from smaller nonfused

parental cells (Hohman and Shows 1979; Eisenberg and Migeon 1979). However, this method does not distinguish homokaryocytes from heterokaryocytes. Discontinuous density gradient centrifugation, which utilizes layers of medium differing in density, has been used for the purification of plant protoplasts from various hydrolytic enzymes and from damaged protoplasts, condensed protoplasts, organelles and cellular debris (Gatenby and Cocking 1977; Vasil and Vasil 1980b; Shahin and Shepard 1980; Hughes et al. 1978; Piwowarczyk 1979; Lin 1980; Scowcroft and Larkin 1980). More recently this method has been used for fractionation of protoplasts on the basis of differing buoyant densities (Harms and Potrykus 1978b; Hughes et al. 1978; Lorz and Potrykus 1981; Archer et al. 1982; Magnien et al. 1982; Alexander and Dubert 1983; Lesney et al. 1983; Fitzsimons and Weyers 1983).

Any gradient system designed for protoplast separation must have a suitable density range, corresponding to that of the protoplasts and should maintain iso-osmolality throughout the entire density range. In addition, it should not alter viability, or capacity for division or regeneration of plants.

Osmotically active sugars and sugar alcohols have been favoured gradient materials. Harms and Potrykus (1978b) used sucrose in combination with an iso-osmotic potassium-magnesium-calcium (KMC) solution in various ratios, to generate an iso-osmotic gradient with phases differing in density. They used this system to enrich for heterokaryocytes after fusion of protoplasts with considerably different average buoyant densities, e.g., protoplasts of maize stem internode with those of wheat

mesophyll; tobacco epidermis and stem parenchyma protoplasts (1.030 g/cm^3) with mesophyll protoplasts (1.052 g/cm^3). These protoplast types were of sufficiently different buoyant density that whole populations could be fused without prior fractionation. Fusion products were recovered in enriched fractions of intermediate density. Compared with fusion mixtures not subjected to gradient fractionation, the content of maize+wheat heterokaryocytes in the enriched fraction was increased by 2-7x. Seventy to eighty-five percent of the heterokaryocytes recovered from the intermediate interface were binucleate rather than multinucleate. Similar results were obtained for tobacco heterokaryocytes which were identified by their resistance to high-light intensities. Cell colonies originating from unfused or homofused parental protoplasts remained whitish and stopped growing while those from heterokaryocytes survived (Melchers and Labib 1974). There was no loss of viability or morphogenic capacity.

Harms (1978a) investigated other gradient materials and found that polymers including various types of Dextran, as well as silica sols such as Ficoll, Ficoll-Paque and Ludox were unsatisfactory. Most of these substances form solutions of undesirably high osmolality or viscosity. Unmodified colloidal silica sols are unstable in salt solutions at physiological pH and are toxic to cells (Wolff 1975; Pertoft and Laurent 1977), which could explain the unsatisfactory results obtained by Harms. Large excesses of polymers such as dextran, polyethylene glycol or polyvinylpyrrolidone (PVP) were added to the colloid solutions to increase stability and decrease toxicity. However, this caused

undesirable increases in osmolality and viscosity and the gradient material was difficult to remove from the biological material.

The development of Percoll overcame many of these problems (Pertoft et al. 1977, 1978). In particular, it enabled alteration of density without affecting the solute potential of the medium (Scowcroft and Larkin 1980). Percoll consists of colloidal silica particles, 15-30 nm diameter, coated with PVP which stabilizes the colloid and decreases its toxic effects. Percoll can form gradients in the range 1.0-1.3 g/ml, and is claimed to be completely non-toxic to cells (Pertoft and Laurent 1977; Kurnick et al. 1979). It has a very low osmotic pressure (20 mOs/kg H₂O) and can give density gradients which are virtually iso-osmotic. Its low viscosity (10±5 cp) facilitates rapid isopycnic banding and the gradient capacity appears quite large. Gutierrez et al. (1979) obtained successful separations of mononuclear cells with sample loading up to 80×10^6 cells/ml with 12 ml of gradient material. Finally, Percoll is readily removed from biological materials, typically using 5 volumes of wash solution to 1 volume of cell suspension (Anon 1980).

Percoll has been extensively used to separate and purify eukaryote cells, bacteria, viruses and subcellular particles. It has had limited application in isolation of plant nuclei, mitochondria and peroxisomes (Nagao et al. 1981; Willmitzer and Wagner 1981; Talbot and Russel 1982; Jackson et al. 1979; Bergman et al. 1980; Neuburger et al. 1982; Moreau and Romani 1982; Mettler and Beevers 1980; Liang et al. 1982). It has also been used successfully for selective enrichment of embryogenic

microspore populations (Wernicke 1978) and more recently for the isolation and characterization of cytoplasts and miniprotoplasts derived from protoplasts of cultured cells (Lorz et al. 1981) as well as enrichment of cytoplasts from mesophyll tissues (Archer 1982).

Lorz et al. (1981) used a Percoll/mannitol discontinuous density gradient spun at high speed to fragment protoplasts and fractionate the resultant cytoplasts and miniprotoplasts in a single step. Miniprotoplasts are small nucleated protoplasts which may be difficult to distinguish from normal protoplasts unless their cytoplasmic component is quite small. Cytoplasts are plasmalemma-limited cytoplasmic derivatives of protoplasts which lack nuclei but containing plastids and mitochondria. The upper fraction consisted of cytoplasts with only 1-7% nucleated protoplasts while non-fragmented, nucleated protoplasts and a few cytoplasts were found in the middle of the gradient. There was no detrimental effect on viability of protoplasts isolated from this layer. Miniprotoplasts were found in the lower fraction with highest specific density. The miniprotoplasts had only about 15% of the original protoplast volume. Cytoplasts and miniprotoplasts are of potential use for cybrid plant formation. Such plants display the nuclear characteristics of one parent, but the organellar traits of the other, or of both parents (Belliard and Pelletier 1978; Aviv et al. 1980).

In contrast to the findings of Lorz et al. (1981) Archer et al. (1982) found that cytoplasts formed during plasmolysis of Nicotiana mesophyll tissue were smaller and layered at a denser

step of a discontinuous gradient than nucleated protoplasts. Mesophyll protoplasts centrifuged on a two-step Percoll discontinuous density gradient separated into three distinct layers. The top layer, at the interface between the protoplast medium and Percoll, consisted predominantly of healthy nucleated protoplasts. The next layer floated at the 28%/50% Percoll interface and contained the majority of the small cytoplasts along with debris and damaged protoplasts. The last layer was a pellet of cellular debris. Protoplasts centrifuged on a continuous Percoll gradient did not separate into layers, but spread over a range of densities. Again, Percoll did not appear to be toxic since mitotic activity was observed in mesophyll protoplasts recovered from the highest Percoll concentration used in the two-step gradient.

Scowcroft and Larkin (1980) used a five-step discontinuous Percoll/ CaCl_2 gradient to obtain Nicotiana debneyi mesophyll protoplasts free of collapsed protoplasts and other debris. The division rates of these protoplasts were comparable to unfractionated protoplasts. An osmotic pressure of 600 mOs/kg H_2O was maintained throughout the gradient. The fraction at the 1.029/1.048 density interface contained fractured protoplasts, cell debris and intact protoplasts while the fraction at the 1.048/1.066 interface was essentially protoplasts free of debris. These were the only fractions obtained.

Alexander and Dubert (1983) used a gradient of Percoll in 9% mannitol and salts of Murashige and Skoog (1962) medium, consisting of 5-40% Percoll in 5% steps, to successfully separate

protoplasts of cotyledons of B. napus from harmful debris. This separation appeared to be superior to other systems such as floatation on concentrated sucrose or other density gradients (Harms and Potrykus 1978b; Scowcroft and Larkin 1980). Fitzsimmons and Weyers (1983) successfully used a Percoll/mannitol density gradient to separate protoplasts of guard and subsidiary cells from Commelina communis leaf epidermis.

MATERIALS AND METHODS

1. Plant material

Seeds of chlorophyll-deficient mutant Nicotiana rustica cv. chlorotica ($2n=48$) were obtained from Dr. R. Pandeya, Agriculture Canada, Delhi, Ontario. This mutant phenotype was the result of a recessive nuclear mutation (Douglas et al. 1981a). Seeds from Nicotiana tabacum L. ($2n=48$) were obtained from Dr. D.U. Gerstel, North Carolina State University, Raleigh, NC. These seeds were derived from plants heterozygous for white seedling (WS), a recessive nuclear mutation for lethal albinism.

Plants of N. rustica var chlorotica were grown in a greenhouse with a 16 hour light and 8 hour dark cycle at an average temperature of 15°C. The plants were watered daily and fed 2 g/l 20:20:20 (N:P:K:) nutrient solution once a week. Callus cultures were initiated from the first and second leaves of plants 6 to 8 weeks old. Explants were surface sterilized in 7% (w/v) filtered calcium hypochlorite for 20 minutes followed by four rinses of sterile distilled water. Cultures of albino N. tabacum were initiated from seedlings. Sterilized seeds from the WS heterozygote were germinated on agar-solidified hormone free B₅ medium (Gamborg et al. 1968; Appendix, Table 1) at 25°C. The first true leaves of albino seedlings were excised and used for callus initiation.

2. Initiation of cultures

To initiate callus cultures, explants were placed on solid (0.8% Difco agar) medium of Murashige and Skoog (1962) with 2% sucrose modified by the addition of 40 mg/L Sequestrene Fe 330 (Geigy) and the vitamins of B₅ medium (above), hereafter referred to as MS medium (Appendix, Table 2). The medium was supplemented with 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). The final medium was adjusted to pH 5.8 (0.1 M KOH) and autoclaved (20 min at 20 atm).

Cell suspension cultures were obtained from primary callus cultures by transferring approximately 4 gm of callus into 250 ml Erlenmeyer flasks containing 50 ml of liquid MS medium supplemented with 0.5 mg/L 2,4-D. These cultures were maintained on a gyrorotary shaker (160 rpm) at 25°C and cells were subcultured every 5-7 days initially. As vigor increased this interval was reduced to 60-84 hr. For subculture, 5-10 ml of the cell suspension was transferred to 50 ml of fresh medium.

A predominantly haploid culture of Datura innoxia (Mill.) was obtained from the Prairie Regional Laboratory, NRC, in Saskatoon. The culture (designated DI96) was maintained in B₅ medium modified by adding 0.5 g/l NH₄NO₃ and 1 mg/l 2,4-D. A polyploid culture of a pantothenate⁻ mutant of D. innoxia, (designated DIPn-4729) was maintained in modified B₅ medium supplemented with 2 mg/l Ca-D-pantothenate. This culture was obtained from Dr. J. King, Dept. of Biology, University of Saskatchewan.

3. Karyotyping of D. innoxia cultures

Approximately 1g (fr. wt.) of cells was filtered from cell suspension and transferred to 15 ml of growth medium supplemented with 0.03% colchicine. The suspension was incubated at 25°C for 4 hr to accumulate metaphases and then fixed for 10 min in glacial acetic acid:95% ethanol (1:3). The cells were washed with 5 ml 0.1M Na-acetate buffer, pH 4.5, and resuspended in 15 ml 1N HCl and incubated overnight. After incubation, cells were resuspended in 45% acetic acid. A few drops of this cell suspension was placed on a slide, 3-4 drops of modified carbol-fuchsin stain (Kao 1982) was added and cells were stained for 15-30 minutes. A coverglass was added and cells were squashed. The numbers of haploids ($n=12$) and diploid cells were scored. Any counts over the diploid number were assumed polyploid and were scored as such. One hundred cells were scored for each culture.

4. Isolation of protoplasts

For isolation of protoplasts cells were filtered from suspension using 125 μ M nylon mesh. Approximately 2g (fr. wt.) of cells were placed in 10 ml of enzyme solution in 100 x 15 mm Falcon petri plates, sealed with parafilm and incubated for 8-12 h on a reciprocating shaker (26 strokes/min) at 25°C. The enzymes used were Driselase (Kyowa Hakko Kogyo Co. Ltd., Tokyo), Rhozyme HP 150 (Rohm and Haas, Philadelphia, PA 19105) and Onozuka R-10

(Kinki Yakhu Mfg. Co., Ltd., Nishinomiya, Japan). A solution containing 1% (w/v) of each of the three enzymes plus 0.1% gelatin in 0.35 M mannitol was centrifuged for 12 min at 1500xg to remove inert material. The pH was adjusted to 5.8 with 0.1 M KOH and the solution filter sterilized (0.22 μ M Millipore filter). Following incubation in this solution protoplasts were separated from undigested tissues by successive passage through 125 μ M and 44 μ M nylon mesh then washed with the appropriate osmoticum. A salt solution consisting of equal volumes of 0.202M KCl, 0.139M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.143M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (KMC salt) adjusted to pH 5.8 was used in experiments with salt/sucrose gradients; 0.44M mannitol, 4.5mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.73mM KH_2PO_4 adjusted to pH 5.8 was used with Percoll gradients.

5. Culture of unfused N. rustica protoplasts

Washed protoplasts were sedimented by centrifugation at 100 x g for 3 minutes and resuspended at a concentration of $2-8 \times 10^4/\text{ml}$ in the medium of Nagata and Takebe (1971) with 0.35 M glucose added (NT medium) (Douglas et al. 1981a; Appendix, Table 3). The protoplasts were plated in 100-200 μ l drops in 15 x 60 mm Falcon petri dishes, 7 drops/plate, sealed with parafilm and incubated at 25°C in low light intensity. Protoplasts were observed daily using an inverted microscope. Plating efficiency was taken as the percentage of protoplasts which formed cell walls and underwent cell division. The frequency of cell colonies was scored 10 days following plating. Two random samples of one hundred individual

cells and colonies were scored in each of three drops (minimum).

To determine viability of N. rustica protoplasts they were incubated for 10 minutes in a solution of 0.1% trypan blue in NT medium. Over 1500 protoplasts per fraction were examined for uptake of dye, indicating a loss of viability.

6. Fusion of protoplasts

Samples of protoplasts to be fused were diluted to 8 ml with NT medium and centrifuged at 100 x g for 3 min to pellet the protoplasts. Supernatant was decanted and protoplasts were resuspended in 10 ml NT medium. Protoplast numbers were determined using a hemacytometer and equal numbers of protoplasts ($0.5-1.0 \times 10^6$) were mixed. The mixed protoplasts were sedimented at 100 xg for 3 min, most of the supernatant was removed, the protoplasts were gently resuspended in 0.5 ml of NT medium and 0.5 ml of polyethylene glycol (PEG) solution was added along the wall of the tube. The protoplasts were gently dispersed by slowly rotating the tube between the palms of the hands. The PEG solution was that of Kao and Michayluk (1974) using 50% (w/v) PEG 1540 (Baker Chemicals) (Appendix, Table 7). Following 20 min incubation the PEG solution was diluted successively by gentle addition of 0.5, 1.0, 2.0 and 5.0 ml aliquots of NT medium at 5 min intervals (Power et al. 1976). Following the last addition protoplasts were sedimented by centrifugation, the supernatant was removed and replaced by 3-4 ml of fresh NT medium and the suspension was incubated 45-60 min at room temperature.

7. Selection of Hybrid Colonies

For post-fusion culture, protoplasts were suspended in 1.5-4.0 ml NT medium at $2-8 \times 10^4$ /ml and plated in 50-100 μ l drops in 15 x 60 mm Falcon petri dishes, maintained under low light intensity, 50 lux at 25°C. To promote chlorophyll synthesis in potential hybrid colonies the cultures were transferred to higher light intensity, 500 lux after 10-15 days. After three weeks individual culture drops were supplemented with 50 μ l of NT medium with glucose concentration reduced to 0.175 M. After a further 1-2 weeks calli were transferred to solid shoot-induction medium without osmotic stabilizer, i.e. NT medium supplemented with 2 mg/L benzyladenine (BA) and 0.05 mg/L naphthaleneacetic acid (NAA). The number of green colonies in all drops was scored after about a week on solid medium (Douglas et al. 1981b).

8. Preparation and use of density gradients

a) K^+ + Mg^{++} + Ca^{++} /sucrose (KMC/S) gradients

Iso-osmotic concentrations of the solutions used to prepare these gradients were calculated from data given by Weast (1978). Following the procedure of Harms and Potrykus (1978b), a KMC salt solution containing 67.3 mM KCl, 46.3 mM $MgCl_2 \cdot 6H_2O$ and 47.7 mM $CaCl_2 \cdot 2H_2O$, pH 5.8, was mixed in various ratios with 0.317M sucrose to produce iso-osmotic gradient medium with graded densities (Table 1). Specific densities were calculated from the concentrations of gradient components. The osmotic pressure of all

solutions as determined in an Osmette osmometer (Precision System Inc., Sudbury, Mass.), was 370 ± 20 mOs/kg H_2O , corresponding to 0.35 M mannitol.

b) Percoll/mannitol gradients

Iso-osmotic Percoll/mannitol solutions at 470 ± 20 mOs/kg H_2O of different densities, were prepared using various concentrations of mannitol and Percoll (Pharmacia Fine Chemicals, Upsala, Sweden) as given in Table 2. Densities were calculated from data of Anon (1980) and Weast (1978). The pH of all solutions was adjusted to 5.8 using 0.1 M KOH.

c) Formation of gradients and protoplast fractionation

All manipulations using density gradients were performed in a cold room at $5^\circ C$ under aseptic conditions. Gradients were formed using a Pasteur pipette to add 1.5 ml aliquots of medium of successively greater density at the bottom of 15 ml glass centrifuge tubes (16 x 115 mm i.d., Corning No. 8442).

Protoplast samples (1-1.5 ml), containing $2-8 \times 10^5$ protoplasts/ml were layered on the top of the gradient. Gradients were centrifuged for 5 minutes at $100 \times g$ in a Danon/IEC clinical centrifuge equipped with a swinging bucket-head ($R=12$ cm). After centrifugation protoplasts were removed from each gradient interface, beginning at the top, using Pasteur pipettes. The volume of suspension removed was measured and the concentration of protoplasts determined in a Fuchs-Rosenthal haemocytometer. The density-dependent distribution of protoplasts was expressed as the

Table 1 Composition of the standard iso-osmotic KMC/S density gradients
(370 ± 20 mOS/kg H_2O) at $20^\circ C$

Number of Gradient Interface	Specific Density (g/cm^3) ⁺	Ratio KMC*/Sucrose v/v	Final concentration of Components (mM)			
			KCl	MgCl ₂ .6H ₂ O	CaCl ₂ .2H ₂ O	Sucrose
1	1.001	7:0	67.3	46.3	47.7	0
2	1.007	6:1	57.7	39.7	40.9	45.3
3	1.013	5:2	48.1	33.1	34.1	90.6
4	1.019	4:3	38.5	26.5	27.3	135.9
5	1.024	3:4	28.8	19.8	20.4	181.2
6	1.030	2:5	19.2	13.2	13.6	226.5
7	1.036	1:6	9.6	6.6	6.8	271.8
	1.042	0:7	0	0	0	317.1

* KMC = 67.3 mM KCl + 46.3 mM MgCl₂.6H₂O + 47.7 mM CaCl₂.2H₂O, pH 5.8

+ Calculated from data given by Weast (1978)

Table 2 Composition of iso-osmotic Percoll/mannitol discontinuous
gradient (470 ± 15 mOs/kg H_2O) at $20^\circ C$

Number of Gradient Interface	Specific Density (gm/cm ³)*	Percent Percoll in Mannitol (v/v)	Mannitol Concentration (M)
1	1.026	0	0.441
2	1.030	3.0	0.455
3	1.035	6.9	0.473
4	1.040	10.7	0.493
5	1.045	14.5	0.515
	1.050	18.3	0.540

* Calculated from figures given by Anon (1980) and Weast (1978).

number of protoplasts isolated from each interface as a percentage of the total protoplasts recovered from the gradient. The figures are expressed as mean $\pm$ standard deviation from at least three separate gradients. The total number of protoplasts recovered from the gradient was expressed as a percentage of the protoplasts layered onto the gradient to give percent recovery.

Quantitative data was supplemented with photographs of the gradients. Following fractionation the centrifuge tubes were clamped in an upright position and a light beam was directed vertically down on the gradient to reveal the protoplast bands by scattered light. Photographs were taken horizontally from a position 90° from the direction of the light beam.

9. Analysis of protoplasts

a) Determination of volume

After gradient fractionation protoplasts isolated from each interface were washed once with NT medium, resuspended in the same medium and plated in 100-200 μ l drops on plastic petri dishes. After a 10 min settling the protoplasts were photographed at a magnification of 40 x in an inverted microscope. Protoplast diameters were measured by projecting the photographic negatives onto plain white paper. A photograph of a stage micrometer was used for calibration. This method minimized the time protoplasts were in NT medium before measurement. Volume of each protoplast was calculated using the formula for a sphere (volume = $\frac{4}{3} \pi \times \text{diameter}^3$). The variance ratio, F was determined from a 1-way

analysis of variance to test for significance ($p < 0.01$) (Snedecor and Cochran 1980). Over 1000 protoplast diameters were determined for each gradient fraction.

b) Electron microscopy and morphometry

N. rustica protoplasts were fractionated using four replicate Percoll/mannitol density gradients. Individual interface fractions from each gradient were combined and washed (centrifugation for 5 min at 100 x g) with NT medium. Subsequent changes of solution used the same centrifugation. Protoplasts were sedimented, resuspended in NT medium and 3% glutaraldehyde in NT medium (pH 5.8) was added with gentle mixing to give a final concentration of 1% glutaraldehyde. After 1 h at room temperature the fixative was replaced with 3% glutaraldehyde in NT medium for 1 additional h. The protoplasts were then washed with three changes of NT medium, 20 min each, post-fixed in 1% osmium tetroxide in NT medium (pH 5.8) for one hour, and washed once in NT medium and twice in distilled water, 15 minutes per wash.

For all further changes of solution protoplasts were allowed to sediment by gravity. Protoplasts were dehydrated in dimethylaminoethanol and ethanol and embedded in Spurr's resin (Spurr 1968, 1969). Thin sections were cut using a diamond knife on a Reichert OM-U2 microtome and stained with 3% methanolic uranyl acetate (w/v) for 1 hour followed by lead citrate (Reynold 1963) for 7 minutes.

Volumes of nuclei, vacuoles and remaining cytoplasm (including organelles) were assessed by morphometric analysis of electron micrographs. A regular lattice of points 5 μ m apart was superimposed on random electron micrographs (mag. 3500 - 5000 x) for each gradient sample. The number of micrographs examined was 41, 22, 21 and 38 from interfaces 2, 3, 4 and 5 respectively. The number of points lying over the three regions of a protoplast profile were counted and expressed as an area fraction of the total points over the entire protoplast. Mean volumes of individual regions were obtained by multiplying mean area fractions by the mean protoplast volume as determined by light microscopy (above) (Weibel 1963; Dardick and Setterfield 1976).

c) Determination of DNA content

Protoplasts from individual interfaces in Percoll density gradients were collected, washed and counted with a haemocytometer. Identical fractions from four gradients were combined to obtain approximately 5×10^5 protoplasts. Replicate samples from three separate experiments were used for each mean DNA content determined.

Protoplasts were pelleted by centrifugation at 60 g for 10 min. The supernatant was removed and the pellet stored at -15°C . Prior to assay the pellet was thawed on ice, suspended in 0.3 ml of cold 2 TNE buffer (2.0 M NaCl, 10 mM EDTA, 10 mM Tris, pH 7.0), and sonicated for 15 s. Chloroform (1.5 volumes) was added to the homogenate and mixed with a Vortex mixer. The phases were separated by centrifugation at 1,000 x g for 10 min and the aqueous supernatant was removed, stored on ice and used for fluorimetric measurements.

Fluorescence was measured according to Brunk et al. (1979) modified by Baer et al. (1982) for plant extracts using a Varian SF-330 spectrofluorometer with excitation and emission wavelengths of 350 nm and 470 nm, respectively. A stock solution of 4', 6-Diamidino-2- phenylindole (DAPI) at 1 mg/100 ml water was prepared and stored at 4°C. DAPI was used at a final concentration of 50 ng/ml in 0.1 TNE buffer (0.1 M NaCl, 10 mM EDTA, 10 mM Tris, pH 7.0). Calf thymus DNA was used as an internal standard at a concentration of 20-25 µg/ml 0.1 TNE buffer. Methods for preparation of buffers and solutions are given in the Appendix, Table 5.

To 3 ml of final DAPI solution three 20 µl aliquots of homogenate were added in succession followed by three 10 µl aliquots of the DNA standard solution. Fluorescence was measured at the beginning of the assay and after addition of each aliquot. Increase in fluorescence was recorded in mm from the baseline to the emission level using a chart recorder. Two straight lines were obtained when fluorescence was plotted against the cumulative volume of aliquots added to the DAPI solution (Fig. 1). One line corresponds to the increase in fluorescence resulting from addition of homogenate and the other from addition of the DNA standard. Slopes of these lines were determined and the concentration of DNA in the homogenate was calculated by multiplying the concentration of DNA in the standard solution by the ratio of the slope for the homogenate to that for the standard DNA solution. Taking the curves of Figure 1 as an example, the ratio of the slope of fluorescence enhancement for the extract to that for

standard DNA (in the presence of extract) is 0.567. This value times the DNA standard concentration (26.8 $\mu\text{g/ml}$) yields 15.2 $\mu\text{g/ml}$, the DNA concentration of the extract. Comparing the slope for the DNA standard in the presence of extract with the slope for the DNA standard in the absence of extract, the ratio is 0.797. This ratio represents the quenching of fluorescence of the DAPI-DNA complex by the extract.

Measurement of the slope of fluorescence enhancement for several DNA standards gave a linear relationship over a DNA concentration from 5-80 $\mu\text{g/ml}$ (Fig. 2). The DNA concentration of the extracts were all within this range. Kapuscinski and Skoczylas (1977) demonstrated the assay to be linear over DNA concentrations ranging from 5-1000 ng/ml .

FIGURE 1 Quantitation of DNA in protoplast extracts. Addition of three 20 μ l aliquots of protoplast extract (●) followed by three 10 μ l aliquots of standard DNA solution (○). (■) Standard DNA added in the absence of extract. (S) Slope of the curve (mm/ μ l). Fluorescence observed without added extract or standard DNA (blank) was due to fluorescence of DAPI.

FIGURE 2 Quantitative relationship between the slope of fluorescence enhancement of DAPI by DNA and the DNA concentration. The slope of the fluorescence enhancement was determined for four DNA standards as described in Materials and Methods.

SLOPE OF
FLUORESCENCE
ENHANCEMENT
mm/μl

RESULTS

1. Fractionation of protoplasts in KMC/S density gradients

Initially, N. rustica and N. tabacum protoplasts were fractionated using the iso-osmotic KMC/S density gradient system described by Harms and Potrykus (1978b). These workers found that for cereal and Nicotiana protoplasts isolated from primary cultures a specific density increase of 8 mg/cm^3 from one layer to the next was optimal for resolution. In the present studies good resolution of interfaces between density layers was achieved with a specific density increase of 8 mg/cm^3 but the distribution indicated that more than 60% of the protoplasts were in a single interface (Table 3). Numerous other ratios of salts and sucrose were tried and a specific density increase of 5.7 mg/cm^3 from one phase to the next was selected as optimum (Table 1). This gradient separated both N. rustica and N. tabacum protoplasts into four density interfaces with fairly even yields of protoplasts in each (Table 4). Gradients with specific density increases less than 5.7 mg/cm^3 per layer were too difficult to make while those with density differences greater than this gave less suitable fractionation.

The distribution and mean buoyant densities in Table 4 indicate only minor variations between protoplasts isolated from N. rustica and N. tabacum. Visually, the gradients after centrifugation were identical. Gradients formed at 20°C were difficult

Table 3 Distribution of N. rustica protoplasts in iso-osmotic KMC/S density gradients with a density increase of 8 mg/cm^3 between successive layers.

Specific Density (g/cm^3)	Mixing Ratio KMC/Sucrose	Number of Gradient Interface	% Total Protoplasts
1.001	15:0	1	-
1.009	12:3	2	7 ± 10
1.018	9:6	3	66 ± 5
1.026	6:9	4	24 ± 11
1.034	3:12	5	3 ± 4
1.042	0:15		

The figures represent the means $\pm$ S.D. of three separate determinations.

Table 4 Distribution and mean buoyant densities of protoplasts from N. rustica and N. tabacum cell cultures fractionated in iso-osmotic KMC/S density gradients under standardized conditions (370 ± 20 mOsmol/kg H₂O, 5°C, centrifugation for 5 min. at 100 x g, protoplast load $2-8 \times 10^5$ protoplasts per gradient, specific density increase 5.7 mg/cm^3 between successive layers).

% Total Protoplasts			
Species		<u>N. rustica</u>	<u>N. tabacum</u>
	1	-	-
	2	-	-
Number of	3	16 ± 10	34 ± 4
Gradient	4	36 ± 3	41 ± 9
Interface	5	34 ± 11	19 ± 6
	6	14 ± 4	7 ± 10
	7	-	-
Mean			
Buoyant		1.024	1.021
Density			
(g/cm ³)			
Percent			
Recovery		44 ± 9	48 ± 4

to make and unstable; after centrifugation interfaces were indistinct and diffusion of fractionated protoplasts was rapid. All subsequent gradient fractionations were performed at 5°C.

Douglas et al. (1981a) noted that protoplasts derived from cells grown with 2,4-D had fewer starch grains than those derived from cells grown with NAA. This offered a potential method for modifying the buoyant density of protoplasts which might result in altered distributions on gradients. There was an increase in the frequency of protoplasts in the interfaces at lower densities for both species when grown with NAA (Table 5). NAA-derived protoplasts obtained from interface 3 had a number of dense bodies, presumably starch grains which were not present in 2,4-D derived protoplasts. In addition, NAA-derived protoplasts were larger than 2,4-D derived protoplasts from this same density interface. Both NAA and 2,4-D protoplasts in the interfaces of greater densities had similar appearance and size (Fig. 3). No attempt was made to repeat this experiment since NAA did not produce a marked change in the sedimentation distribution of protoplasts and cells grown with 2,4-D have superior morphogenetic potential (Douglas et al. 1981a). Cells grown with 2,4-D were used for subsequent fusion experiments.

Generally, protoplasts in the upper layers of the KMC/S gradients were in excellent morphological condition with well developed cytoplasmic networks and active streaming of the cytoplasm. More debris was observed in denser interfaces and there appeared to be a trend towards a decrease in protoplast diameter with increasing buoyant density (Fig. 4), in agreement with the results of Haras and Potrykus (1978b).

Table 5 Distribution of protoplasts derived from cell cultures grown on either 0.5 mg/l 2,4-D or 1 mg/l NAA, for two successive subcultures, in iso-osmotic KMC/S density gradients under standardized conditions (as in Table 4).

% Total Protoplasts

Species		<u>N. rustica</u>		<u>N. tabacum</u>	
		NAA	2,4-D	NAA	2,4-D
	1	-	-	-	-
	2	-	-	-	-
Number of	3	42	28	60	38
Gradient	4	49	34	19	34
Interface	5	8	19	13	15
	6	2	18	8	14
	7	-	-	-	-
Percent					
Recovery		60	65	40	45

FIGURE 3 Phase contrast microscopy of N. rustica protoplasts isolated from discontinuous KMC/S gradients as in Table 5. Bars = 100 μ m

3a Protoplasts from cell culture supplemented with 2,4-D; density interface 3.

3b As in 3a; density interface 6.

3c Protoplasts from cell culture supplemented with NAA; density interface 3.

3d As in 3c; density interface 6.

The KMC/S gradients presented several difficulties. Clumping of protoplasts was a frequent problem as seen in Figure 5. The gradients were difficult to form without mixing the phases and only one or two gradients could be formed at any one time. Diffusion of the fractionated protoplasts occurred rapidly even at 5°C, making it difficult to remove protoplasts from one interface without cross contamination. These factors made this gradient system unattractive as a routine method and further use was discontinued.

2. Fractionation of protoplasts in Percoll/mannitol gradients

After considerable preliminary experimentation, a six step discontinuous gradient of Percoll in mannitol, with a density range from 1.026 g/cm³ to 1.050 g/cm³ and osmotic pressure of 470±20 mOs/kg H₂O was found most effective in fractionating protoplasts of both Nicotiana species (Table 2). Gradients were formed at 5°C and 1.5 ml samples containing 5-8 x 10⁵ protoplasts were fractionated by centrifuging at 100 x g for 5 minutes. With samples containing more than 10⁶ protoplasts clumping and poor fractionation resulted.

Percoll gradients were easier to form and much more stable than the KMC/S gradients. Diffusion between density layers occurred very slowly and interfaces were not easily perturbed. Protoplasts separated into well defined bands at the interfaces (Fig. 6), and diffused only slowly, enabling removal without cross-contamination.

FIGURE 4a-f N. rustica protoplasts isolated from consecutive interfaces, 1-6, respectively from a KMC/S density gradient. Bars = 100 μ m

FIGURE 5a KMC/S gradient after fractionation of N. rustica protoplasts.

FIGURE 5b KMC/S gradient after fractionation of N. tabacum protoplasts. Note the clumping and diffuse banding of protoplasts.

Visually, the distribution of protoplasts in the gradient interfaces appeared similar for both species, and this was confirmed by counts (Table 6). The fractionation for both species was improved over KMC/S gradients. Protoplasts were spread into five interfaces with relatively equal frequency in the three central interfaces. As with the KMC/S density gradients, the calculated distributions of both Nicotiana species were similar with little difference in mean buoyant densities. Mean buoyant density of all species did not vary with different treatments.

Two Datura innoxia cell cultures were also fractionated on Percoll/mannitol gradients (Table 6). Despite marked differences in the nuclear condition of these cultures (DI96; 93% haploid; DIPn-4729; 94% polyploid) they both gave protoplasts with density profiles similar to the Nicotiana cultures.

Recovery from these gradients was $73 \pm 7\%$ of the protoplasts initially layered onto the gradients. Up to 85% of the protoplasts layered on the gradients could be re-isolated from the gradient interfaces without mixing of the individual bands. This was a significant improvement over the recoveries in the 40% range obtained with KMC/S density gradients.

Some of the loss of protoplasts during fractionation can be attributed to mechanical damage. Damaged and collapsed protoplasts as well as other debris sedimented to the bottom of the centrifuge tubes. Fractionation of preparations initially containing substantial debris resulted in enrichment of debris in the higher density fractions.

FIGURE 6 Percoll/mannitol discontinuous density gradients
(conditions as in Table 6) after fractionation of
protoplasts. a) N. rustica; b) N. tabacum

Table 6 Distribution and mean buoyant densities of protoplasts fractionated in Percoll/mannitol discontinuous density gradients under standardized conditions: (470 ± 20 mOsmol/Kg H_2O , $5^\circ C$, centrifugation for 5 minutes at $100 \times g$, protoplast load $2-8 \times 10^5$ protoplasts per gradient, specific density increase of 5 mg/cm^3 between successive phases).

% Total Protoplasts				
Species			Haploid	Polyploid
	<u>N. rustica</u>	<u>N. tabacum</u>	<u>D. innoxia</u>	<u>D. innoxia</u>
			DI96	DIPnI-4729
	1	6 ± 2	1 ± 1	2 ± 3
Number of	2	32 ± 4	20 ± 1	18 ± 9
Gradient	3	36 ± 3	41 ± 4	33 ± 4
Interface	4	21 ± 7	29 ± 5	28 ± 8
	5	6 ± 2	9 ± 1	18 ± 8
Mean				
Buoyant	1.037	1.039	1.039	1.040
Density				
(g/cm^3)				

These figures are means $\pm$ S.D. of at least three gradients run separately; four samples of protoplasts from each interface were counted.

3. Viability of protoplasts following fractionation in Percoll/mannitol gradients

Protoplasts isolated from the gradients remained spherical and most showed active streaming of the cytoplasm. Over 60% of the N. rustica protoplasts from each fraction lost their spherical shape and divided within 3-4 days after being plated. No difference was found in the division capacity of protoplasts isolated from different interfaces of the gradients. All drops of plated protoplasts produced white callus, hence, viability of these protoplasts did not seem to be adversely affected by fractionation in Percoll. In addition, Table 7 indicates that approximately 80% of protoplasts removed from each interface excluded trypan blue, a value comparable to the unfractionated control. Only 50% of the protoplasts taken from the pellet at the bottom of the gradient, i.e., interface 6, excluded the dye.

4. Factors affecting the buoyant density of protoplasts

Examination of the protoplasts isolated from consecutive Percoll/mannitol gradient interfaces revealed a trend, as with KMC/S gradients, towards decreasing diameter with increasing buoyant density (Fig. 7). To examine this situation more closely, protoplasts from each species were fractionated, protoplast diameters were determined for each fraction and volumes were calculated. The results (Table 8) show that mean protoplast volume decreased as buoyant density increases. This was true for all species which were fractionated in this gradient system. The

Table 7 Viability, as determined by exclusion of 0.4% trypan blue, of N. rustica protoplasts isolated from Percoll/mannitol density gradients.

Number of Gradient Interface	% protoplasts unstained
1	-
2	89±2
3	80±3
4	82±5
5	80±4
6	51±15
Unfractionated Protoplasts	82±4

Values represent percent of protoplasts which showed no uptake of dye. Over 500 protoplasts were counted per interface for each of three replicate gradients.

trend was statistically significant as determined from the variance ratio, F. Interestingly, protoplasts of the two Nicotiana species isolated from the same density interfaces had significantly different sizes. The same was true for the two Datura cultures.

Electron micrographs (Fig. 8 and 9) showed that isolated N. rustica protoplasts were approximately spherical and well fixed, as indicated by the presence of thin intact trans-vacuolar cytoplasmic strands. Typically, protoplasts from all fractions had from 1-5 large primary vacuoles and numerous smaller secondary vacuoles. This pattern was also observed in the non-fractionated protoplasts (Fig. 8a and 8b). The degree of secondary vacuolation of protoplasts varied within a fraction. Note the large number of secondary vacuoles in Figs. 9b and 9d in contrast to Figs. 9a and 9c. Secondary vacuolation may have resulted from vesiculation of the canalicular system during fixation. Mersey and McCully (1978) found that all fixatives in common use convert the canalicular system of tomato petiolar hair cells to vesicles. A densely staining material was observed within many of the larger primary vacuoles of both fractionated and non-fractionated protoplasts. Most secondary vacuoles appeared to be free of this material. Mitochondria dispersed throughout the cytoplasm were numerous in protoplasts from all fractions. Plastids were not uniformly dispersed but were closely associated with the nucleus, usually fitting its contours. A few of these plastids contained starch deposits (Fig. 9a). The nuclei were close to spherical and there was no apparent difference in the state of condensation of

FIGURE 7a N. rustica protoplasts before fractionation. Note the variation in protoplast size. Bars = 100 μ m

FIGURE 7b-7f N. rustica protoplasts isolated from interfaces 1 to 5, respectively, of a Percoll/mannitol discontinuous density gradient. Bars = 100 μ m.

Table 8 Mean volume ($\pm$ S.D.) of protoplasts isolated from Percoll discontinuous density gradients.

Number of Gradient Interface			Polyploid	Haploid
	<u>N. rustica</u>	<u>N. tabacum</u>	<u>D. innoxia</u>	<u>D. innoxia</u>
1	11.0 $\pm$ 8.1	3.8 $\pm$ 2.4	12.6 $\pm$ 5.7	6.5 $\pm$ 5.4
2	8.1 $\pm$ 4.7	3.4 $\pm$ 1.9	9.1 $\pm$ 5.0	5.3 $\pm$ 4.4
3	6.2 $\pm$ 3.2	2.2 $\pm$ 1.2	5.6 $\pm$ 3.1	3.9 $\pm$ 3.1
4	4.7 $\pm$ 2.5	1.7 $\pm$ 0.9	4.4 $\pm$ 2.3	3.1 $\pm$ 2.4
5	4.2 $\pm$ 2.2	1.3 $\pm$ 0.7	3.5 $\pm$ 1.7	2.4 $\pm$ 2.0

Volumes ($10^4 \mu\text{m}^3$) were calculated from light microscope measurements of protoplast diameter. Diameters were determined for over 700 protoplasts isolated from each interface.

FIGURE 8a, 8b Electron micrograph of non-fractionated N. rustica protoplasts. Bars = 10 μ m.

FIGURE 8c-e Electron micrographs of N. rustica protoplasts fractionated and isolated from interface 2 of a Percoll/mannitol discontinuous density gradient. Bars = 10 μ m.

FIGURE 9a-d Electron micrographs of N. rustica protoplasts fractionated and isolated from interface 4 of a Percoll/mannitol discontinuous density gradient. Bars = 10 μ m.

9a

9b

9d

the chromatin of fractionated and non-fractionated protoplasts. Protoplasts from different fractions differed morphologically. Interface 2 protoplasts (Figs. 8c-e) have very thin peripheral cytoplasm whereas interface 4 protoplasts (Fig. 9) have quite thick peripheral cytoplasm. Note also the different degrees of vacuolation as born out by Table 9.

Morphometric analysis of N. rustica protoplasts isolated from different density interfaces revealed a marked decrease in vacuolar volume with increasing density (Table 9). Overall, the decrease between interfaces 2 and 5 was approximately 65% with the sharpest decline of 35% between interfaces 2 and 3. Cytoplasmic volume decreased only 20% between interfaces 2 and 5; little change occurred between interfaces 2 and 3 or 4 and 5. Nuclear volume remained constant.

The DNA content of N. rustica and N. tabacum protoplasts isolated from each interface was examined by fluorimetric assay (Table 10). There was no difference in the average DNA per protoplast for consecutive interfaces of the gradients for either species. Hence, the protoplasts were not fractionated into populations differing in ploidy. This result agreed with that obtained for gradient fractionation of the two D. innoxia protoplasts (Table 6) where despite major differences in ploidy levels similar gradient profiles were obtained.

To study possible effects of age of cell culture on buoyant density, N. rustica protoplasts prepared from cells 3 and 5 days after subculture were fractionated. The density-dependent distribution (Experiment 1, Table 11) indicated that culture age had no

Table 9 Mean volume ($10^4 \mu\text{m}^3$) and % of total volume of the vacuole, cytoplasm and nucleus of N. rustica protoplasts isolated from Percoll discontinuous density gradients.

Number of Gradient Interface	Vacuole		Cytoplasm		Nucleus	
	Volume	% Total	Volume	% Total	Volume	% Total
2	5.1	63	2.6	32	0.4	5
3	3.2	51	2.7	43	0.4	6
4	2.2	46	2.2	46	0.4	8
5	1.8	41	2.1	49	0.4	10

The cytoplasmic volume includes true cytoplasm, organelles and small secondary vacuoles.

Table 10 Deoxyribonucleic acid content (picograms/protoplast) determined by fluorimetry: Protoplasts were fractionated in Percoll discontinuous density gradients under standardized conditions (Table 6). Fractions from four gradients were collected and combined for each assay. Assays were performed in triplicate.

Number of Gradient Interface	DNA/Protoplast (pg)	
	<u>N. rustica</u>	<u>N. tabacum</u>
2	12.7 $\pm$ 2.5	11.3 $\pm$ 3.1
3	13.0 $\pm$ 1.9	16.6 $\pm$ 2.4
4	16.3 $\pm$ 2.1	17.1 $\pm$ 1.8
5	12.9 $\pm$ 0.8	16.3 $\pm$ 2.3

major effect on the buoyant density of protoplasts. A slight difference in the distribution of protoplasts fractionated after the next subculture, (i.e., experiment 2, Table 11), was evident. Auxin was not added to this culture and growth slowed considerably. These cells were probably entering stationary growth phase.

Gamanetz and Gamburg (1981) reported that stationary-phase tobacco cells cultured for 3 days in fresh medium supplemented with 0.1 mM adenine increased their starch content several times as compared to cells grown without adenine (from 1.9 gm/ g fr. wt. to 11.6 mg/g fr. wt). This effect was accompanied by severe inhibition of growth. In an attempt to alter buoyant density of protoplasts experimentally, N. rustica cells were grown under the conditions described by Gamanetz and Gamburg (1981). A stationary phase N. rustica cell suspension (7th day of subculture) was diluted 1:100 with fresh MS medium. Ten-ml aliquots of diluted cell suspension were transferred to large petri plates with or without 0.1 mM adenine, and incubated in the dark at 60 rpm for 72 hours. The distribution of protoplasts derived from these suspensions was determined. Although, an accumulation of dark granules was observed in the adenine treated cells no effect on distribution of protoplasts was observed (Table 12). In addition, the yield of protoplasts decreased from $2.9 \pm 1.4 \times 10^5/\text{ml}$ for non-treated controls to $7.5 \pm 1.3 \times 10^4/\text{ml}$ for cell suspensions supplemented with 0.1 mM adenine. Presumably this was due to reduced growth in adenine.

Table 11 The effect of age of culture on distribution (%) of N. rustica protoplasts in Percoll density gradients. Protoplasts were isolated 3 and 5 days after subculture (Experiment 1). Experiment 2 used the next subculture into medium lacking auxin.

		% Total Protoplasts			
		Experiment 1		Experiment 2	
Days after					
Subculture		3	5	3	5
Number of Gradient Interface	1	4±1	6±1	16±2	15±3
	2	27±3	32±3	34±2	30±2
	3	33±2	34±4	30±5	39±3
	4	29±6	21±5	17±1	15±3
	5	7±1	6±2	4±1	1±1

Table 12 Effect of adenine upon the distribution (%) of N. rustica protoplasts fractionated in Percoll/mannitol discontinuous density gradients.

Number of Gradient Interface	% Total Protoplasts	
	Control	0.1 mM Adenine
1	7±2	4±3
2	34±2	29±4
3	39±2	40±6
4	16±4	19±4
5	4±1	8±2

These figures represent the means ± S.D. of four separate determinations.

5. Reproducibility of gradient separations

To assess the reproducibility of the gradient fractionation, N. rustica protoplasts isolated from interface 3 were washed, counted and recentrifuged in an identical Percoll gradient. After refractionation, protoplasts were removed from each interface, washed and counted. Mean results from three separate determinations were expressed as a density-dependent distribution (Table 13). Photographs of typical gradients are also provided (Fig. 10). Upon recentrifugation 80% of protoplasts in the isolated fraction (interface 3) rebanded in the same position. The remaining 20% of the protoplasts redistributed in the other interfaces. Increasing the time of centrifugation to 10 minutes did not alter the banding pattern of these protoplasts in the gradient.

The same procedure was repeated using N. rustica protoplasts isolated from interfaces 2 and 4 which were mixed prior to recentrifugation on a new Percoll gradient (Table 15). Note that Table 13 gives the distribution (%) of all protoplasts on the initial gradient before recentrifugation of all interface 3 protoplasts while Table 15 gives the % of interface 2 and 4 protoplasts which were mixed prior to recentrifugation. The initial overall distribution of protoplasts was similar to that in Table 13. Again, some loss of protoplasts to adjacent interfaces occurred. Loss seemed greater from interface 2.

Recovery of interface 3 protoplasts from the second gradient in Table 13 was 40-60% (mean $48 \pm 9\%$) of the total number of

Table 13 Reproducibility of density-dependent distribution of N. rustica protoplasts in Percoll/mannitol density gradients.

Total Protoplasts		
Gradient	Before isolation	After recentrifugation
Interface	of interface 3	of interface 3
	protoplasts	protoplasts
1	7±3	-
2	19±3	10±4
3	29±5	81±3
4	29±5	8±5
5	16±4	1±1

Protoplasts were fractionated in a Percoll gradient, interface 3 was removed, washed, counted and recentrifuged on an identical gradient.

FIGURE 10a, 10c Percoll/mannitol gradient after primary fractionation of N. rustica protoplasts.

FIGURE 10b Percoll/mannitol gradient after recentrifugation of interface 3 protoplasts isolated from gradient in Figure 10a. .

FIGURE 10d Percoll/mannitol gradient after recentrifugation of interfaces 2 and 4 protoplasts isolated from gradient in Figure 10c.

interface 3 protoplasts isolated from the first gradient. Thus, there was a 60-40% loss of the original interface 3 protoplasts both to other interfaces in the second gradient and through manipulations following isolation from the first gradient. In one experiment, protoplasts isolated from interface 3 of the second gradient were recentrifuged on a third gradient in an attempt to improve purification. Eighty-five percent of the protoplasts recovered from the third gradient were in interface 3. However, 64% of the interface 3 protoplasts isolated from the second gradient were lost through manipulations. This represented an overall loss of 81% of the original interface 3 protoplasts. Therefore, a third centrifugation step was judged not practical since the slight enrichment in interface 3 did not compensate for additional loss of protoplasts.

Since fusion of protoplasts isolated from gradients would require about 2h the effect of incubation for this time on the buoyant density of protoplast fractions was determined. Similar protoplast fractions isolated from selected gradient interfaces were either recentrifuged as soon as possible or after incubation for 2 hours at room temperature. Protoplasts recentrifuged after 2 hours incubation had distributions similar to fractions recentrifuged without incubation (Table 14 and 15) although there was a tendency for more loss from the original interface with greater incubation time. Interface 3 protoplasts re-isolated from interfaces 2 and 3 of the second gradient, with or without incubation, were in excellent morphological condition. However, there was an increased number of misshapen and collapsed protoplasts in interfaces 4 and 5 when compared to the 0 hour control (Fig. 11).

Table 14 Effect of incubation time on distribution (%) of N. rustica protoplasts in Percoll gradients. Interface 3 protoplasts were isolated after fractionation in a Percoll gradient and were recentrifuged after incubation at 20°C in NT medium.

Incubation(h)	% Total Protoplasts	
	0	2
Gradient		
Interface		
1	-	-
2	7±1	11±1
3	75±15	68±13
4	18±14	18±9
5	-	3±3

Table 15 Effect of incubation time on the distribution (%) of N. rustica protoplasts in Percoll density gradients. Interfaces 2 and 4 were isolated, counted, mixed, incubated in NT medium and recentrifuged in identical gradients.

% Total Protoplasts				
Incubation(h)	0		2	
	% Mixed	After recentri- fugation	% Mixed	After recentri- fugation
Gradient				
Interface				
1	-	-	-	1±2
2	41±5	29±18	42±3	16±14
3	-	15±6	-	27±15
4	59±5	49±17	58±3	37±19
5	-	7±1	-	20±11

FIGURE 11a Percoll/mannitol density gradient of N. rustica interface 3 protoplasts recentrifuged without prior incubation.

FIGURE 11b-d N. rustica protoplasts re-isolated from interfaces 2-4, respectively of the gradient in Figure 11a.

FIGURE 11e Percoll/mannitol density gradient of N. rustica interface 3 protoplasts recentrifuged after 2 hours incubation in NT medium.

FIGURE 11f-i N. rustica protoplasts re-isolated from interfaces 2-5, respectively of the gradient in Figure 11e.

To determine the effect of protoplast fusion procedures on gradient reproducibility, protoplasts were isolated from interface 3, treated with PEG as used for fusion (Douglas et al. 1981b) and recentrifuged on an identical gradient. Both PEG treated and non-treated control protoplasts had visually identical distributions as seen in the photograph in Figure 12. Microscopic examination revealed a greater number of misshapen and collapsed protoplasts in interfaces 4 and 5 of the PEG treated sample than in the control.

Similar distributions resulted when protoplasts of interfaces 2 and 4 isolated from a primary gradient were incubated with or without PEG and recentrifuged (Table 16). Again in these experiments substantial numbers of protoplasts reappeared in interfaces other than 2 and 4, particularly in interface 3, even without PEG treatment.

6. Density gradient analysis of protoplast fusions

A direct measure of successful heterofusion between N. rustica var chlorotica and albino N. tabacum is the number of green colonies produced through genetic complementation (Douglas et al. 1981). This system was used to test the effectiveness of gradient fractionation in enriching for heterokaryocytes.

Many factors influence the rate and frequency of protoplast fusion. Protoplast concentration in the fusion mixture is one such factor (Kao 1978). Approximately $0.5-1.5 \times 10^5$ viable

FIGURE 12a, 12c Percoll/mannitol discontinuous density gradient
after fractionation of N. rustica protoplasts.

FIGURE 12b Percoll/mannitol discontinuous density gradient
after recentrifugation of interface 3 protoplasts
isolated from gradient in Figure 12a.

FIGURE 12d Percoll/mannitol discontinuous density gradient
after homologous fusion and re-centrifugation of
interface 3 protoplasts isolated from gradient in
Figure 12c.

Table 16 Effect of fusion procedure on distribution (%) of N. rustica protoplasts. Protoplasts were isolated from interfaces 2 and 4, counted, mixed with or without PEG then recentrifuged in identical Percoll/mannitol discontinuous density gradients.

% Total Protoplasts				
-PEG			+PEG	
Gradient	%	After	%	After
Interface	Mixed	recentri- fugation	Mixed	recentri- fugation
1	-	1±1	-	1±1
2	65±7	34±8	66±6	33±1
3	-	26±16	-	35±6
4	35±7	36±26	34±6	22±2
5	-	5±4	-	10±3

protoplasts having a distinct buoyant density could be isolated from a single gradient interface. This number is determined by the capacity of the gradient, distribution of protoplasts in the gradient, recovery from the gradient and viability of the recovered protoplasts. Keeping these limitations in mind, control fusions without prior gradient fractionation were performed (Douglas et al. 1981) using 5×10^4 protoplasts from each species. The results of four fusion experiments gave 2, 8, 12 and 32 green calli per 10^5 total protoplasts in the fusion mixture. In the experiment which gave 32 green foci, the hybrids were quickly overgrown by the parentals giving the callus a pale green tinge. To increase the number of heterokaryocytes protoplasts from several identical gradient interfaces were combined for subsequent fusions using fractionated protoplasts.

A series of control fusion experiments were run. Approximately 5×10^5 protoplasts from each parental species were fused and cultured without gradient fractionation. Similar numbers of protoplasts from both parental species were mixed and co-cultured without PEG fusion and protoplasts from each parental species were cultured after homologous fusion. As expected, green colonies appeared only when the parental species were fused with each other using PEG. Only white or light brown calli formed in unfused co-cultures. Homofused N. rustica protoplasts survived PEG treatment and gave rise to white or cream-coloured colonies. Most N. tabacum protoplasts treated alone with PEG for homofusion died. Those that survived produced only white cell colonies which eventually became light brown in colour.

22
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Enrichment of heterokaryocytes by centrifugation was attempted using N. rustica protoplasts isolated from interface 2 and N. tabacum protoplasts from interface 4. For each species four aliquots containing $5-8 \times 10^5$ protoplasts per aliquot were fractionated in separate Percoll density gradients (four gradients per species) and a single interface was isolated from each gradient. N. rustica protoplasts from interface 2 and N. tabacum protoplasts from interface 4 were collected mixed, fused with PEG and re-fractionated.

Protoplasts isolated from the initial gradients (Fig. 13) and those re-isolated from gradient interfaces 2, 3, 4 and 5 after fusion treatment (Fig. 14) appeared well hydrated although many of the latter remained in clumps. On protoplast culture medium cell colonies became visible macroscopically 17-30 days postfusion. The majority of these colonies were white. In virtually all fusion experiments, protoplasts isolated from gradient interface 5 and the pellet did not form callus when cultured. Visually these interfaces appeared to contain condensed and misshapen protoplasts with considerable debris.

The distribution of heterofused protoplasts on gradients after recentrifugation (Table 17) was similar to that found with unfused and homofused protoplasts (Table 16). The distribution gave no indication of a large increase in heterokaryocytes with density intermediate between interfaces 2 and 4.

Heterofusions were identified as bright green colonies interspersed among the albino colonies. The number of such

FIGURE 13a N. rustica var chlorotica protoplasts isolated from interface 2 after fractionation in Percoll/mannitol discontinuous density gradients prior to heterologous fusion.

FIGURE 13b Albino N. tabacum protoplasts isolated from interface 4 after fractionation in Percoll/mannitol discontinuous density gradients, prior to heterologous fusion. Bars = 50 μ m.

FIGURE 14a-d Protoplasts isolated from Percoll/mannitol gradient interfaces 2-5 respectively, after heterologous fusion and recentrifugation of N. rustica interface 2 protoplasts and N. tabacum interface 4 protoplasts (see Figures 13a and 13b).

Bars = 50 μ m.

Table 17 Distribution (%) protoplasts after fusion of N. rustica protoplasts from interface 2 with N. tabacum protoplasts from interface 4 on Percoll discontinuous density gradients.

Gradient Interface	% Mixed	After fusion and recentrifugation
1	-	-
2	44±14	13±10
3	-	42±18
4	56±14	34±11
5	-	11±10

colonies was scored 30 days post-fusion (Table 18). Scattered green colonies without particular enrichment in any density interface was observed in experiments 1-6. In these experiments protoplasts isolated from the post-fusion gradient were diluted with 3 volumes of NT medium and centrifuged to remove Percoll. Prolonged centrifugation failed to sediment many of the protoplasts in this suspension, particularly those that remained clumped. In one experiment the supernatant was removed, diluted with one volume only of NT medium and plated as a monolayer. Thirty-two green colonies subsequently appeared suggesting that most heterokaryocytes had been lost in previous experiments where only sedimented protoplasts were used for plating.

In subsequent experiments, 7-10, (Table 18) the re-isolated protoplasts were diluted with one volume of NT medium, giving good sedimentation. In these experiments distinct enrichment of hybrid protoplasts in the intermediate density interface 3 was obtained. In the best experiment, i.e., no. 10, 19 hybrids were obtained from 7.5×10^4 protoplasts in interface 3 out of 22 hybrids from the total 9.6×10^5 protoplasts on the entire gradient.

In all of these experiments the fusion mixture was divided into equal aliquots and one aliquot was re-fractionated while the other aliquot was plated directly as a control. Surprisingly, in experiments 7-10 the non-refractionated aliquots gave substantially fewer total hybrid calli than did the re-fractionated aliquots (eg. 6 as against 22 in Experiment 10).

In general the fusion frequency was low compared to previous fusion frequencies ($150/10^6$) obtained by Douglas et al. (1981b). Use of the high calcium-high pH buffer of Keller and Melchers

Table 18 Number of green *N. rustica* + *N. tabacum* hybrid cell colonies scored 30 days post-fusion in fractions isolated from Percoll discontinuous density gradient. Equal numbers of protoplasts ($0.5-1.0 \times 10^6$) from fractions 2 and 4 of the Percoll gradient were mixed, fused using PEG and divided into two equal aliquots. One aliquot was re-fractionated then plated and the other aliquot was simply plated (non-refractionated aliquot).

Treatment	Experiment Number									
	Gradient									
	Interface	1	2	3	4	5	6	7	8	9 10
Re-fractionated Aliquot	1.	0	0	1	3	0	0	0	0	0
	2	0	3	3	2	2	0	1	1	5 1
	3	1	1	1	2	1	1	4	5	13 19
	4	0	0	0	1	0	0	0	0	2 2
	5	0	0	0	2	0	0	0	0	0 0
Non-refractionated Aliquot	-	-	-	15	3	3	0	2	2	4 6

(1973) as eluant to increase fusion frequency resulted in excessive clumping of the protoplasts and sedimentation to the pellet on Percoll gradients. Thus, NT medium was used as eluant in all fusions.

In the time available four of the hybrid calli produced shoots when placed on shoot induction medium (Figs. 15c and 15d). These calli were from both fractionated and non-refractionated aliquots indicating that morphogenic potential of protoplasts is retained after exposure to percoll.

FIGURE 15 Albino callus derived from homofused protoplasts of N. rustica (15a); Albino calli derived from a mixture of N. rustica and N. tabacum protoplasts (15b); Green callus derived from N. rustica and N. tabacum heterokaryocyte demonstrating shoot differentiation (15c, 15d). All colonies on glucose-free NT medium with 1.0 mg/L BA and 1% sucrose.

3-----
COLOURED PICTURES
Images en couleur

DISCUSSION

Density gradients composed of K^+ + Mg^{++} + Ca^{++} in sucrose (KMC/S) were found to be unsuitable for fractionating protoplasts derived from Nicotiana cell cultures. Fractionation was not reproducible at 20°C and was also difficult to reproduce at 5°C.

Harms and Potrykus (1978b) were able to achieve reproducible fractionation of protoplasts isolated from various tissues of tobacco, barley, wheat, rye, oat and maize using KMC/S. They fractionated protoplasts in an eight step discontinuous gradient of constant osmolality (600 ± 20 mOs/kg H_2O) with a specific density increase of 8 mg/cm^3 between successive phases. The KMC salt and sucrose concentrations used here were adjusted for a lower osmotic pressure, 370 ± 20 mOs/kg H_2O , compatible with our cells. This gave an 8 step discontinuous gradient with a specific density increase of 5.7 mg/cm^3 between successive phases. Density was lowered to an extent where easy handling (especially the layering of the gradient phases) was difficult. Attempts at changing the mixing ratio to compensate for this (eg. having 5 instead of 8 phases) gave a distribution in which over 60% of the protoplasts were in a single interface (Table 3).

The need to use low density increments to adequately fractionate the protoplast population contributed to gradient instability seriously decreasing value of the system.

Clumping of the protoplasts was also a serious problem with KMC/S medium. This probably resulted from high salt

concentrations, in particular, calcium chloride . Although Harms and Potrykus reported no deleterious affects of these high salt concentrations on protoplast viability there is evidence to suggest that prolonged culture in high salt concentrations causes abnormalities in cytokinesis of regenerating cells (Meyer 1974; Meyer and Abel 1975).

Fractionation of protoplasts in Percoll/mannitol gradients was superior to fractionation in KMC/S gradients. The distributions were highly reproducible as judged from both the standard deviations in Tables 6 and 9 and from the rebanding experiments in Table 10. Gradient phases were easily layered and mixing of the layers occurred only slowly.

Protoplasts from four distinct cultures, i.e., N. rustica, N. tabacum, haploid D. innoxia and polyploid D. innoxia had similar distributions and identical mean buoyant densities when fractionated in Percoll gradients (Table 6). Several factors which might influence protoplast behaviour on gradients were investigated.

A determination of mean volume of protoplasts in each gradient fraction showed that protoplast size decreased with increasing buoyant density (Table 11). This was true for protoplasts from each culture. However, with different species protoplasts from the same density interface may differ considerably in size (compare N. rustica with N. tabacum volumes). Thus fractionation must be based upon differences in density although size does have some role. Morphometric analysis indicated that the decrease in size with increased buoyant density could be attributed mainly to a decrease in vacuolar volume, although a decrease

in cytoplasmic volume may also have contributed (Table 9). There was no apparent change in nuclear volume through the gradients.

Increased nuclear ploidy levels are frequently correlated with changes in several cellular features, for example cell volume and the amount of DNA per nucleus have been shown to increase with an increase in genome size, although not in exact proportion (Dean and Leech 1982; Molin et al. 1982). Magnien et al. (1982) demonstrated that N. plumbaginifolia mesophyll protoplasts could be fractionated on a sucrose gradient into two populations differing in DNA content. G2-phase protoplasts (4C DNA content) had a higher average cell size and accumulated in the low-density fraction of the gradient while G1-phase protoplasts (2C DNA content) accumulated in a denser fraction. S-phase protoplasts were distributed in both fractions. This suggests that fractionation is affected by ploidy level although this could be an indirect effect due, for example, to changes in vacuole size through the cell cycle.

Results of the current study gave no evidence that DNA content per se influenced sedimentation of protoplasts. Fluorimetric assay of N. rustica and N. tabacum protoplasts did not detect any differences in the DNA content/cell between successive fractions for either species (Table 10). D. innoxia cultures with significantly different ploidy levels gave similar sedimentation distributions (Table 6). Fractionation of these protoplasts was not determined by ploidy level, which agrees with results obtained by Harms and Potrykus (1978a). It is interesting to note that the protoplasts with 4C DNA content observed by Magnien et al. (1982)

could only be obtained from plantlets cultured in vitro on synthetic substrate; plantlets cultured in vitro but in sterilized turf did not show a class of protoplasts with doubled DNA content.

Several factors were varied in an attempt to modify protoplast buoyant density. Napthaleneacetic acid and adenine, both reported to increase starch content (Douglas et al. 1981a; Gamanetz and Gamborg 1981) did not alter mean buoyant density or significantly change sedimentation distribution of protoplasts over 2,4-D controls (Tables 5 and 10). Buoyant density was unaffected by culture age since protoplasts in the exponential phase of growth had the same distributions regardless of time after subculture (Table 9). It is interesting to note that protoplast mean buoyant density is lower in KMC/S than in Percoll/mannitol gradients. Protoplasts in KMC/S are subjected to lower osmotic pressure and are probably more swollen than in Percoll/mannitol. Thus density in KMC/S is reduced. It is possible that manipulation of osmotic concentration of gradients could be used to vary sedimentation patterns. Certainly, the morphometric results indicate that any treatment which alters relative vacuolation of protoplasts will probably change their density and sedimentation behaviour.

Harms and Potrykus (1978b) found that mesophyll protoplasts isolated from etiolated leaves had distributions and mean buoyant densities in KMC/S intermediate between those of mesophyll and epidermal protoplasts from illuminated leaves. Mesophyll cells have fully differentiated chloroplasts, epidermal cells lack

chloroplasts while etiolated mesophyll cells have partially differentiated etioplasts. From this they suggest that the number of differentiated plastids affects protoplast buoyant density. However, they found a negative correlation between age, represented by relative leaf position, and the mean buoyant density of mesophyll protoplasts, which indicated that differentiation of the tissues affected protoplast buoyant density. This suggests that factors other than number of differentiated chloroplasts are important in determining protoplast buoyant density. These protoplasts are derived from tissues which have undergone different degrees and patterns of differentiation and may differ with respect to relative volumes of the cytoplasm, and vacuoles in addition to chloroplast numbers.

In Percoll the distributions of N. rustica and N. tabacum are congruent, hence an initial fractionation of each population was necessary to isolate protoplasts with distinct differences in buoyant density. Most isolated protoplasts rebanded in the same position in identical gradients (Table 13 and 15), however, loss of protoplasts to adjacent interfaces and poor overall recovery associated with fractionation and refractionation indicate problems for use of gradient fractionation in selecting heterokaryocytes following protoplast fusion. For example, in Table 16 where interface 3 might be expected to contain mainly fusion products following PEG treatment the unfused sample also shows substantial numbers of protoplasts in interface 3. Therefore, clean segregation of heterokaryocytes from parental protoplasts is not possible but the sedimentation technique may be useful for enrichment of heterokaryocytes.

When N. rustica var chlorotica and albino N. tabacum protoplasts from different density fractions were fused and then refractionated in identical Percoll gradients a partial separation of the mixed population was achieved resulting in enrichment of hybrids in a fraction of intermediate density (Table 16). Development of hybrids in the non-refractionated fusion mixture was hindered when compared to the development of hybrids in the refractionated fusion mixture. Perhaps application of this technique favours development of heterologous fusion products by removal of undesired parental protoplasts and debris. Wright (1981) found that survival of heterokaryocytes increased if viable cells were separated from non-viable cells by centrifugation onto a cushion of Ficoll-sodium diatrizoate. Similarly, Wernicke (1978) noted that embryogenic microspores enriched by discontinuous density gradient centrifugation in Percoll undergo embryogenesis more rapidly than unfractionated populations.

Generally, hybrid recovery after fusion of fractionated protoplasts was lower than expected. Control fusions with non-fractionated protoplasts gave approximately a 24 fold greater hybrid recovery (mean of 13.5 per 10^5 protoplasts fused) than that obtained with fractionated protoplasts (mean from experiments 7-10, Table 18 of 5.7 per 10^6 protoplasts fused). This could be the result of either reduced fusion frequencies using fractionated protoplasts or reduced recovery of colonies from heterokaryocytes, or both.

Fractionation of the protoplasts prior to fusion increases the time interval between removal of the cell wall degrading

enzymes and induction of fusion. Increasing this preincubation prior to fusion has been shown to reduce fusion frequencies (Kao and Michayluk 1974; Weber et al. 1976). The yield of heterokaryocytes decreased from 9% with 5 minutes preincubation to 1% after 2 hours. The most rapid decrease occurring during the first 15 minutes (Weber et al. 1976). Klein et al. (1981) detected cellulose synthesis within minutes after protoplasts were separated from cell-wall degrading enzymes hence decrease in fusion frequency may be due to rapid cell-wall regeneration during preincubation. Prior fractionation increases preincubation before fusion and increases the possibility of regeneration of cell-wall.

Three more general factors influence the frequency of fusion of isolated protoplasts (Power et al. 1970). Briefly, protoplasts undergoing fusion must touch and remain touching at the same point of surface contact. This point of contact must have sufficient 'depth' of cytoplasm before fusion can occur and the presence of plastids or any organelle which excludes cytoplasm from a point of contact inhibits fusion. Hence, the nature and distribution of cytoplasm in the protoplast as well as protoplast size and the extent of vacuolation will influence the efficiency of fusion.

Analysis of protoplast volumes demonstrated a trend towards decreasing protoplast size with increasing buoyant density (Table 11). Attempted fusion of protoplasts from different fractions of the gradient may exaggerate differences in protoplast size between parental species. This in turn could reduce the area of surface contact between the two protoplast species, thus reducing fusion frequencies.

Morphometric analysis of protoplast fractions indicated that the vacuolar volume relative to the cytoplasmic volume decreased from a value of 2 for interface 2 protoplasts to a value of 1 for interface 4 protoplasts (Table 12). Thus, highly vacuolated protoplasts from interface 2 might not have a sufficient depth of cytoplasm for efficient fusion with the more moderately vacuolated protoplasts from interface 4. This could also account for reduced fusion frequencies with fractionated protoplasts.

The effects of Percoll upon membrane function are unknown, but it appears that Percoll changes the capacity of the cell surfaces for adherence. Dettman and Wilbur (1979) demonstrated a low adherence (1-6%) of mouse spleen cells to the walls of centrifuge tubes when centrifuged in Silica-PVP density gradients compared to (52- 94%) adherence in Ficoll or bovine serum albumin gradient material. Wakefield et al. (1982) reported that a 30 min exposure to 45% (v/v) Percoll decreased the percentage of macrophages adhering to plastic by about 30% when compared to non-exposed controls. They suggested this may result from a loose association between Percoll particles and the cell surface. It is likely that other surface related phenomena are also affected by exposure of cells to Percoll and this could also affect fusion frequencies.

Low recovery of colonies from heterokaryocytes may be due to (a) reduced viability of heterokaryocytes as a result of excessive handling, (b) lack of nuclear fusion and complementation, (c) nuclear instability in hybrid cells.

The mechanics of protoplast fractionation prior to induction of fusion made this procedure time consuming, tedious and difficult to manage for a single individual. In addition to increasing the possibility of contamination it may also reduce fusion frequencies. It seems therefore that gradient fractionation is neither a practical nor efficient means for selection of heterokaryocytes produced by fusion of protoplasts. However, it might be more useful for enrichment of heterokaryocytes in cases where initial fractionation of the protoplast populations was not necessary, i.e., using protoplasts with intrinsically different mean buoyant densities. For example, use of hypocotyl and mesophyll tissue may provide protoplasts with different buoyant densities which would give significant enrichment of heterokaryocytes in an intermediate fraction of a single gradient separation.

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APPENDIX

TABLE 1. Nutrient composition of modified B₅ medium

Component		Concentration
Vitamins	Nicotinic acid	1.0 mg/L
	Thiamine . HCl	10.0 mg/L
	Pyridoxine . HCl	100.0 mg/L
	Myoinositol	10.0 mg/L
Micronutrients	MnSO ₄ .H ₂ O	10.0 mg/L
	H ₃ BO ₃	3.0 mg/L
	ZnSO ₄ .7H ₂ O	2.0 mg/L
	Na ₂ MoO ₄ .2H ₂ O	0.25 mg/L
	CuSO ₄ .5H ₂ O	0.025 mg/L
	CoCl ₂ .6H ₂ O	0.025 mg/L
	KI	0.83 mg/L
Macronutrients	NaH ₂ PO ₄ .H ₂ O	150 mg/L
	KNO ₃	2500 mg/L
	(NH ₄) ₂ SO ₄	134 mg/L
	MgSO ₄ .7H ₂ O	250 mg/L
	CaCl ₂ .2H ₂ O	750 mg/L
	Iron (Sequestrene-Fe330 -Geigy)	40 mg/L
Sucrose		20000 mg/L

TABLE 2. Nutrient composition of modified MS medium

Component		Concentration
Vitamins		As in Table 1
Micronutrients	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	22.3 mg/L
	H_3BO_3	6.2 mg/L
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6 mg/L
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25 mg/L
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025 mg/L
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025 mg/L
	KI	0.83 mg/L
Macronutrients	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	1650.0 mg/L
	KNO_3	1900.0 mg/L
	$(\text{NH}_4)_2\text{SO}_4$	440.0 mg/L
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370.0 mg/L
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	170.0 mg/L
	Iron (Sequestrene-Fe330 -Geigy)	40.0 mg/L
Sucrose		20000.0 mg/L

TABLE 3. Nutrient composition of NT medium used for culturing protoplasts

Component		Concentration
Vitamins	Meso-inositol	100.0 mg/L
	Thiamine - HCl	1.0 mg/L
Micronutrients		As in Table 2
Macronutrients	NH_4NO_3	825.0 mg/L
	KNO_3	950.0 mg/L
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	220.0 mg/L
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1233.0 mg/L
	KH_2PO_4	680.0 mg/L
Sucrose		10000.0 mg/L
Hormones	1-Naphthalene acetic acid	3.0 mg/L
	6-Benzylaminopurine	1.0 mg/L
Osmoticum	Glucose	0.35 M

TABLE 4. Composition of medium used for fusion of protoplasts

	<u>Amount/100 ml</u>
Glucose	1.98 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.15 g
$\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	0.009 g
PEG 1540	50.0 g

pH adjusted to 5.5 prior to autoclaving

TABLE 5. Preparation of buffers and solutions for
fluorimetric DNA assay

Stock solutions

1 M Tris stock (pH 7.0)

Dissolve 121.1 gm Tris base in 800 ml of H₂O. Adjust to pH 7.0 with concentrated HCl. Allow solution to cool to room temperature before making final adjustments of pH. Make up the volume to 1 L. Dispense into aliquots and sterilize by autoclaving.

0.5 M EDTA stock (pH 7.0)

Add 190.1 gm of EDTA tetrasodium salt to 800 ml H₂O. Stir vigorously on a magnetic stirrer. Adjust the pH to 7.0. Dispense into aliquots and sterilize by autoclaving.

5 N NaCl stock

Dissolve 292.2 gm of NaCl in 800 ml of H₂O. Adjust volume to 1 L. Dispense into aliquots and sterilize by autoclaving.

Buffers

0.1 TNE

To 1 ml of 1 M TRIS stock (10 mM) add 2 ml of 0.5 M EDTA stock (10 mM) and 2.0 ml of 5 M NaCl stock (0.1 M). Make the volume up to 100 ml with double distilled water and filter sterilize with a 0.22 μ m Millipore filter.

2TNE

To 1 ml of 1 M Tris stock (10 mM) add 2 ml of 0.5 M EDTA stock (10 mM) and 40 ml of 5 M NaCl stock (2.0 M). Make the volume up to 100 ml with double distilled water and filter sterilize with a 0.22 μ m Millipore filter.

DAPI solution, 50 ng/ml

Take 500 μ l of 1 mg/100 ml DAPI stock and dilute to 100 ml with 0.1 TNE buffer.

DNA standard

Weigh 2.00-2.50 mg of calf thymus DNA and dissolve in 10 ml of 0.1 TNE buffer. Take 1,000 μ l of this solution and dilute to 10 ml with 0.1 TNE buffer. Maintain on ice.

END

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