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The Isolation, Fractionation and Uptake of
Plant Chromosomes

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Robert James Griesbach

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THE ISOLATION, FRACTIONATION AND UPTAKE OF PLANT CHROMOSOMES

By

Robert James Griesbach

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ABSTRACT

THE ISOLATION, FRACTIONATION AND UPTAKE OF PLANT CHROMOSOMES

By

Robert James Griesbach

Plant chromosomes can be efficiently isolated from several different tissues--root tips, microspores and suspension cultures. All these tissues have two characteristics in common. They have a high mitotic index and are easily converted into protoplasts. The procedure for isolating chromosomes involves exposing protoplasts to a buffer which ruptures the cell membrane and inhibits the activity of nucleases and proteases. The procedure is efficient with yields of over 50 percent of the available chromosomes. Sucrose gradients then allow the chromosomes to partially fractionated into several different size classes. Chromosome uptake can be measured by fluorescence microscopy. Isolated chromosomes which are stained with 4'6-diamidino-2-phenylindole fluoresce green. These chromosomes are easily seen when taken up by mesophyll protoplasts which fluoresce red due to their chlorophyll. About 1 percent uptake is obtained when a chromosome/protoplast suspension is incubated for 20 minutes in 35 percent polyethylene glycol.

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LIST OF ABBREVIATIONS

TRIS	=	TRIS-(Hydroxymethyl)Aminomethane
CAPES	=	Cyclohexylaminopropanesulfonic Acid
MES	=	2(N-Morholino)ethanesulfonic Acid
HEPES	=	N-2-Hydroxyethyl piperazine-N-2 ethanesulfonic Acid
EDTA	=	Ethylenediaminetetraacetic Acid
EGTA	=	Ethyleneglycol-Bis-(B Aminoethyl Ether) N_1N^1-Tetraacetic Acid
IAA	=	Indoleacetic Acid
2,4-D	=	2,4-Dichlorophenoxyacetic Acid
DTT	=	Dithiothreitol
SDS	=	Sodium Dodecyl Sulfate
BIS	=	N_1N^1-Methylene-Bis-Acrylamide
TEMED	=	$N_1N_1N_1^1N^1$-Tetramethylethylenediamine
2XSSC	=	3.0M NaCl + 0.3M Sodium Citrate
DAPI	=	4'6 Diamidino-2-Phenylindole
PEG	=	Polyethylene Glycol

INTRODUCTION

The improvement of horticultural and agronomic plants depends upon selecting better producing varieties and also on increasing the amount of genetic variability that is available, so that there is a broad base from which to choose new desired types. Plant breeders have for a long time been improving most of the economic plant species. They have already efficiently utilized a large quantity of the available genetic variability, thus increasing the difficulty in gaining major, genetic improvements in many traits. This situation has caused many breeders to turn to various non-conventional methods of breeding, such as somatic hybridization and _in vitro_ mutagenesis, in order to obtain new sources of desirable genetic variability.

During the last 25 years, the techniques for the genetic manipulation of plant somatic cells have been developing. Methods are now available for the production of haploids (Kasha, 1979) and the regeneration of whole plants from a wide variety of tissue cultured cells (Pierik, 1979). Additionally, procedures for the isolation, fusion and regeneration of whole plants from protoplasts are available for a more limited range of species (Butenko, 1979). The ability to regenerate plants from tissue cultured cells or protoplasts is paramount in the application of the newer techniques of genetic engineering to plant breeding, for the breeder generally requires more than one sexual cycle beyond the initial engineering for further selection. During the mid-1970's it was often considered that somatic cell genetics would solve many of the breeder's problems for it could theoretically allow an unlimited gene pool (Heyn, *et al*., 1974). The use of *in vitro* cell selection and somatic hybridization would allow the geneticist an opportunity to create or introduce new sources of variability. This variability could then be funnelled into a classical, practical, plant breeding program.

Some of the problems in applying somatic cell genetics to plant breeding are now being recognized (Carlson, 1980). One of the major problems stems from a lack of well characterized genetic systems in which mutants can be rapidly selected. Perhaps the most severe limitation is an insufficient amount of biochemical and genetic characterization of many horticultural and agronomic traits. For example, many economic traits like heterosis and yield are now only abstractly and statistically defined. These traits need to be broken down into their individual biochemical components and genetically analyzed. Without a more precise characterization these traits cannot be analyzed *in vitro*. Another problem which is also very serious concerns the tissue specificity of many of the horticultural and agronomic traits. Many of these traits like leaf angle are exclusively expressed at the whole plant level; thus it is impossible to do *in vitro* selection.

Ever since the original report of fusing plant protoplasts to create a somatic cell hybrid (Carlson, *et al.*, 1972), there has been a great deal of interest in using this method to increase genetic variability and to create new genetic combinations. It is now theoretically possible to fuse any two somatic cells; they need not belong to the same species, family or kingdom (Butenko, 1979). There are, however, several problems in applying somatic hybridization to plant breeding. The most severe difficulty lies in selecting and regenerating the hybrid cells from the parental cells. Even if one is able to select and regenerate the hybrids, there are several reasons why such plants may not be of much use to the breeder. First, most of the hybrids which have been regenerated from cell fusions can also be produced via sexual means (Vasil, *et al.*, 1979). Second, if the parents are only distantly related, then the hybrid progeny will be developmentally unstable. The resulting somatic hybrids will have many morphological abnormalities which will lead to low fertility and lack of vigor (Melchers, *et al.*, 1978; Gleba and Hoffman, 1980). Third, whole genomes, instead of individual desirable genes, are transferred. This means that several subsequent, sexual generations are required before the undesirable genes are eliminated from the hybrid; however, in many somatic hybrids advanced sexual generations are impossible. Finally, in many of the wide hybrids the chromosomes of one parental type can be preferentially eliminated (Kao, 1977).

The problems inherent in somatic hybridization make DNA-mediated transformation an attractive alternative; however, there has never been reported an example of a stable, long-term, DNA-mediated transformation in higher plants. All the reported data on successful transformation in higher plants are very weak and in some cases the appropriate controls are even absent (Lurquin, 1977; Kleinshoff and Behki, 1977). It is fairly well established that foreign DNA can be taken up by plant cells and even expressed for a very brief period (Lurquin, 1977; Kleinshoff and Behki, 1977). Almost all the DNA, however, is eventually degrated (Slavik and Widholm, 1978). Another problem in DNA-mediated transformation stems from the lack of specific genetic markers and selection systems. This makes it almost impossible to confirm transformation, for there are vitually no markers to transform.

A new approach, chromosome-mediated transformation, might be able to overcome many of the difficulties associated with applying somatic cell genetics to plant breeding. It offers the geneticist a unique means for introducing into a genome small amounts of foreign information without potentially affecting developmental processes, relative vigor, fertility or gene balance. The first, physiologically active chromosomes were isolated from cultured mammalian cells (Chorazy, et al., 1963; Summers, et al., 1963). Since then considerable progress has been made in isolating mammalian chromosomes.

Figure 1 outlines the procedure generally used. Cultured cells are first exposed to an agent which holds the cells in mitotic metaphase. Colchicine and its derivatives are the most widely used agents. Once sufficient quantities of mitotic cells are obtained, the cells are placed in a hypotonic solution and swollen. This process helps disperse the metaphase chromosomes. The cells are then lysed by adding a suitable buffer and the cellular debris removed via differential centrifugation. Finally, the chromosomes are centrifuged down.

The most critical step in the isolation procedure is the composition of the lysis buffer (Hanson, 1973). Table 1 lists the chemicals found in several of the more successful buffers used in isolating mammalian chromosomes. The selection of a specific buffer depends upon how the chromosomes will be subsequently used after isolation. Certain properties are required of the chromosomes if they are to be used in transformation studies. One, the chromosomes must contain high molecular weight DNA which is unnicked or modified. Two, the acidic and basic proteins must be unmodified and correctly associated with the DNA. Finally, the number of contaminating interphase nuclei and chromatin should be kept to a minimum.

The method of cell lysis affects chromosome morphology. For example, prolonged incubation in hypotonic solutions prior to lysis or lysis in hypotonic solutions can lead to irreversible chromosome expansion or disintegration

Figure 1. Procedures for Isolating Chromosomes

A. <u>Mammalian Cells (Hanson, 1973)</u>

1. expose tissue-cultured cells to colcemid
2. hypotonically swell the cells
3. add lysis buffer
4. pass cells through a hypodermic needle
5. spin down debris
6. spin down chromosomes

B. <u>Plant Cells (Malmberg & Greisbach, 1980)</u>

1. expose tissue to colchicine and cell wall, digestive enzymes
2. collect and wash protplasts
3. add lysis buffer
4. pass through a hypodermic needle
5. spin down debris
6. spin down chromosomes

Table 1. Several of the More Common, Mammalian Chromosome Isolation Buffers.

Sommers, *et al.*, 1963	Maio & Schildkraut, 1967
0.5mM $MgCl_2$	1mM $CaCl_2$
0.5mM $CaCl_2$	1mM $MgCl_2$
0.5M sucrose	1mM $ZnCl_2$
unbuffered	0.02M Tris (pH 7.0)
	1% Triton X-100

Mendelsohn, *et al.*, 1968	Stubblefield, *et al.*, 1978
1mM $MgCl_2$	1mM $CaCl_2$
1mM $CaCl_2$	1M hexylene glycol
0.1M sucrose	1mM CAPS or HEPES
0.1M sodium acetate	pH 10.5 or 6.5
pH 3.0	

Blumenthal, *et al.*, 1979	
15mM Tris (pH 7.2)	0.5M hexylene glycol
0.2mM spermine	0.34M sucrose
0.5mM spermidine	0.1% digitonin
2mM EDTA	80mM KCl
0.5mM EGTA	20mM NaCl
14mM mercaptoethanol	

(Mendelsohn, *et al.*, 1968; Bak and Zeuthen, 1977). The use of non-ionic detergents should also be avoided; because they can cause nuclei to rupture, as well as, increase the chromosomes' sensitivity to shearing forces (Blumenthal, *et al.*, 1979). In lysing the cells, the use of mechanical means, such as passage through a hypodermic needle, prove to be the least disruptive of chromosome morphology (Hanson, 1973).

Chromosomes with a high molecular weight DNA component (6.5×10^7 daltons) were isolated by Wray (Wray, 1973). He found that a basic lysis buffer (pH around 10) could reduce most of the nuclease activity without some of the harmful side-effects found when using low pH or metal chelators to reduce the enzyme activity. Low pH can reduce nuclease degradation; however, it can also lead to depurination of the DNA, as well as, remove histones and other basic, chromosomal proteins (Lewin, 1980). A high pH may prevent DNA degradation; however, it does not preserve the protein integrity of the chromosome. Up to 25 percent of the acidic chromosomal proteins can be removed by high pH (Hearst and Botchan, 1970). Thus, basic and acidic pHs can preserve the integrity of the DNA; but they destroy the chromosomal protein composition. As a consequence, chromosomes must be isolated at near physiological pH if one wishes to preserve the protein composition. It is possible at neutral pH to reduce the degradative effects nucleases have on DNA. For example, DNA which is bound to protein is protected from nuclease attack. The greater the quantity of protein bound, the less likely nucleases can bind and digest the DNA (Lewin, 1980). By adding protein stabilizers, such as DTT and hexylene glycol, it is possible to help preserve the protein composition of the chromosomes (Wray, 1973). Metal chelators like EDTA can also restrict nuclease activity by binding Mg^{++} and Mn^{++} cations. These cations are the cofactors which are needed for nuclease activity (Lewin, 1980). Agents like colchicine, cold temperature, high ionic strength and polyamines can also reduce nuclease binding by helping to condense the chromosomes (Lewin, 1980). Chromosomes with highest molecular weight DNA (2×10^8 daltons) were isolated from neutral buffers containing polyamines, chelators and protein stabilizers (Blumenthal, et al., 1979).

Isolated, mammalian chromosomes have been fractionated based upon their mass (Huberman and Attardi, 1967), size (Hanson, 1973), density (Stubblefield and Wray, 1973) and electrical charge (Landel, et al., 1972). In order to separate or fractionate chromosomes based upon their charge or density, the protein/DNA composition of the chromosomes must be selectively modified prior to fractionation. For example, unmodified HeLa cell chromosomes all band at the same density of 1.31 g/ml (Huberman and Attardi, 1967) and all have the same pK of about 4.0 (Landel, et al., 1972). If, however, the chromosomes are treated with trypsin before fractionation, it is possible to selectively change the density and the electrical charge of the individual chromosomes (Stubblefield and Wray, 1973). One very serious problem with these methods of fractionation is that they permenantly alter the composition of the chromosomes. Although separation based upon size via selective filtration does not damage the chromosomes' composition, it does have severe limitations. The major problem lies in the limited availability of filters which do not absorb chromosomes and which have a sufficient range of uniform pore sizes. As a result, selective filtration is only of use in very crude separations or in selecting chromosomes of unusually large or small size. The most widely used technique for fractionating unmodified chromosomes is based upon their density or differential sedimentation through sucrose gradients. In this method, an unfractionated chromosome mixture is layered onto a preformed sucrose gradient and centrifuged for a brief period of time. The exact centrifugal force and sucrose concentrations depend upon the species (Table 2).

It has only been very recent that studies in isolating higher plant chromosomes have begun. Plant chromosomes have now been isolated from a mixture of tissues from various species (Malmberg and Griesbach, 1980). Figure 1 shows the procedure used to isolate plant chromosomes. From this preliminary study several things were noted. First, the use of techniques developed to separate mammalian chromosomes will not work with plant tissues since plant chromosomes respond differently to these extraction procedures. Second, in order to isolate chromosomes from a given tissue, protoplasts with a fairly high mitotic index ($<$ 15 percent) must be easily obtained.

Table 2. Several Methods of Fractionating Isolated, Mammalian Chromosomes via Differential Sedimentation through Sucrose Gradients.

Huberman & Attardi, 1967

1. HeLa cell chromosomes
2. 0-30% sucrose (w/w)
3. 30-0% glycerol (w/w)
4. 450 x g, 40 minutes
5. 4 fractions collected
6. able to locate rRNA genes to the smaller chromosomes

Stubblefield, *et al.*, 1978

1. chicken chromosomes
2. 20-40% sucrose
3. 1500 x g, 50 minutes
4. 26 fractions collected
5. complete visual fractionation

Mendelsohn, *et al.*, 1968

1. Chinese hamster chromosomes
2. 10-40% sucrose
3. 50 x g, 40 minutes
4. 3 fractions initially collected which were subsequently resedimented
5. almost complete visual fractionation

The following study is an attempt at improving the chromosome isolation procedure in higher plants. The first attempt (Malmberg and Griesbach, 1980) was only partially successful, for condensed chromosomes were only isolated from a limited number of species and then only from suspension cultured cells. This report describes a method which makes it possible to isolate condensed higher plant chromosomes from a wide range of species and tissues. Studies were then undertaken to develop procedures to separate the isolated chromosomes into several size classes and to find the best method of incorporating the chromosomes into foreign cells.

MATERIALS

Cell Cultures

Cells from *Nicotiana tabacum* cv. Wisconsin 38 were maintained in liquid suspension culture on Murashige-Skoog medium (Murashige and Skoog, 1962) with 3 mg/L IAA and 0.3 mg/L kinetin. There are approximately 92 chromosomes per cell in this line. Cells from *Lycopersicon esculentum* cv. Cherry were maintained in liquid suspension culture on Murashige-Skoog medium with 5 mg/L IAA, 0.3 mg/L kinetin and 0.5 mg/L 2,4-D. There are approximately 72 chromosomes per cell in this line.

Root Tips

Root tips were obtained from various *Lilium regale* hybrids, *Lilium* x Black Beauty, various *Lilium* x Mid Century hybrids, *Vicia faba*, *Zea maize*, *Pisum sativum*, *Lycopersicon esculentum*, various *Hemerocallis* hybrids and *Allium cepa*.

Microspores

Microspores were obtained from various *Lilum regale* hybrids, *Lilium henryii*, *Lilium* x Black Beauty and various *Hemerocallis* hybrids by cutting lengthwise an anther in the desired meiotic stage. The microspores were subsequently released into the medium by applying gentle pressure on the cut surface.

METHODS

Chromosome Isolation

Figure 1B diagrams the chromosome isolation procedure used on either root tips or microspores. The tissue was first exposed at room temperature to a solution of 0.05 percent colchicine, 2 percent cellulysin (Calbiochem), 1 percent macerase (Calbiochem), 0.25 percent hemicellulase (Sigma) and 10 percent mannitol at pH 5.7. When using non-sterile tissue, 50 mg of Benlate, 50 mg of gentamycin-s, 25 mg of nystatin and 100 mg of penicillin-G were added to each milliliter of enzyme solution (Thurston, et al., 1979). After a 45 minute incubation for the meiotic cells and an 18 hour incubation for the mitotic cells, the tissue was teased apart, passed gently through a pasteur pipette and incubated at room temperature for an additional hour. Large debris was then removed by filtering through two layers of cheese cloth and the protoplasts or cells with weakened cell walls were collected via centrifugation at 200 x g for 15 minutes. The cells were then washed twice with 20 volumes each time of 5mM MES (pH 6.5) and 10 percent mannitol. The washed protoplasts or cells were then resuspended in lysis buffer (Table 3) and passed gently through a 27 gauge hypodermic needle. Cellular debris was removed by centrifugation at

Table 3. List of the Major Isolation Buffers Tried.

1. 5mM MES (pH 5.7)
   1mM $CaCl_2$
   1mM DTT

2. 5mM MES (pH 5.7)
   1mM $CaCl_2$
   1mM DTT
   0.01% SDS

3. 5mM MES (pH 5.7)
   1mM $CaCl_2$
   1mM DTT
   0.1% Triton X-100

4. 5mM MES (pH 6.5)
   1mM $CaCl_2$
   1mM DTT
   0.1% Triton X-100

5. 5mM (pH 6.5)
   5mM $CaCl_2$
   5mM DTT
   0.1% Triton X-100
   10% mannitol

6. 15mM Tris (pH 7.2)
   5mM $CaCl_2$
   5mM DTT
   0.1% Triton X-100
   10% mannitol

7. 15mM Tris (pH 7.2)
   5mM $CaCl_2$
   15mM DTT
   0.1% Triton X-100
   10% mannitol

8. 15mM Tris (pH 7.2)
   1mM EDTA
   15mM DTT
   0.1% Triton X-100
   10% mannitol

9. 15mM Tris (pH 7.2)
   1mM EDTA
   5mM Mg^{++} acetate
   15mM DTT
   0.1% Triton X-100
   300mM sucrose

10. 15mM Tris (pH 7.2)
    1mM EDTA
    15mM DTT
    0.1% Triton X-100
    1mM spermidine
    300mM sucrose

Table 3. (Continued)

11. 15mM Tris (pH 7.2)
    1mM EDTA
    15mM DTT
    0.1% Triton X-100
    1mM spermidine
    0.25mg/ml bovine serum albumin
    300mM sucrose

12. 15mM Tris (pH 7.2)
    1mM EDTA
    15mM DTT
    0.5M hexylene glycol
    1mM spermidine
    300mM sucrose

13. 15mM Tris (pH 7.2)
    1mM EDTA
    15mM DTT
    0.5mM spermidine
    0.5mM spermine
    80mM KCl
    20mM NaCl
    300mM sucrose
    0.5M hexylene glycol

200 x g for 15 minutes and the chromosomes were finally collected after 10 minutes at 2500 x g.

The procedure for isolating chromosomes from cells in suspension culture was as follows. Actively growing cells were exposed for between 12 and 24 hours to 2μg/ml fluorodeoxyuridine and 1μg/ml uridine. These chemicals inhibit DNA replication without affecting RNS transcription. The inhibition was then relieved by washing the cells in tissue culture medium supplemented with 2μg/ml thymidine. After a few hours, the cells were exposed to colchicine and cell wall digestive enzymes. The procedure described above was then followed.

Chromatin Isolation

The procedure for isolating chromatin was modified from Hamilton, *et al*. (1972) and Towill and Nooden (1973). Five hundred grams of leaves were ground in a blender in 2 liters of 10mM Tris (pH 7.6), 1.14M sucrose, 5mM DTT, and 5mM $MgCl_2$. The suspension was filtered through two layers of cheese cloth and centrifuged at 4^oC for 5 minutes at 750 x g. The pellet was washed twice with a total of one liter of TRIS/DTT/$MgCl_2$/sucrose buffer (see above). After washing, the pellet was resuspended in 100ml of isolation buffer plus 0.1 percent Triton X-100 and washed three times with a 100ml each time of this buffer. The nuclei were then resuspended in 100ml of 10mM Tris (pH 8.0), 3mM EDTA, and 50mM $NaHSO_3$. After incubating for 30 minutes at 4^oC, the suspension was centrifuged for 10 minutes at 4^oC at 1000 x g. The chromatin was finally precipitated by making the supernatant 10mM $CaCl_2$ and collected by centrifugation at 4^oC at 1000 x g for 10 minutes.

Histone Isolation

Histones were isolated using the procedure of Towill and Nooden (1973). A chromosome preparation or interphase chromatin was adjusted to 0.2 H_2SO_4 and homogenized (at low voltage in a dounce homogenizer) for 20 minutes at 4^oC. The suspension was centrifuged for 10 minutes at 10,000 x g. Five volumes of cold, absolute ethanol was then added to the supernatant. After 2 days at -20^oC, the protein was collected via centrifugation at 1000 x g for 5 minutes.

Histone Electrophoresis

Separation of histone proteins was accomplished following the procedure of Hardison and Chalkey (1978). The histones were resuspended in 2ml of glycerol and 15.5ml of water containing 50mg of DTT, o.5gm SDS and 0.9gm of Tris (pH 8.8). The histones were then disassociated from one another by incubating the mixture at 100^oC for 5 minutes. The mixture was loaded on a 15 percent polyacrylamide gel containing 0.35 percent BIS, 0.1 percent SDS, 15.5 percent Tris (pH 8.8), 0.05 percent ammonium persulfate and 0.08 percent TEMED. The electrophoresis buffer was 0.05 Tris (pH 8.3), 0.38M glycine and 0.1 percent SDS. The gels were run at a constant voltage (50 volts), stained overnight in 0.1 percent Commassie blue R in 5 percent acetic acid and 20 percent methanol and destained in 5 percent acetic acid and 20 percent methanol.

Chromosome Fractionation

Extracts of isolated chromosomes (0.5 ml) were sedimented at 2000 x g through a 5ml 5-40 percent sucrose gradient. The time of centrifugation (between 30 and 45 minutes) depended upon chromosome size. Ten, 0.5 ml, fractions were collected via tube puncture.

rRNA Isolation

Ribosomal RNA was extracted from 200ml of chopped roots which were added to 500ml of 0.25M sucrose, 200mM Tris (pH 8.5), 500mM KCl and 15mM $MgCl_2$, homogenized and filtered through two layers of cheese cloth. The suspension was adjusted to 2 percent Triton X-100 and centrifuged twice at 10,000 x g for 10 minutes. Ribosomes were then pelleted after 2 hours at 70,000 x g. The ribosomes were resuspended in 10mM Tris and an equal volume of phenol-saturated Tris was added. The aqueous phase was re-extracted until clear of protein. The rRNA was then precipitated by adding two volumes of cold, absolute ethanol.

RNA Iodination

The isolated rRNA was kindly radioiodinated to 100,000 dpm/mg by Dr. Asher at Michigan State University.

DNA Isolation

The DNA isolation procedure was modified from Blumenthal, et al. (1979) and Lambert and Daneholt (1975). An equal volume of 0.5N NaOH, 0.02 EDTA and 0.1 percent triton x- 100 was added to a chromosome suspension. After one hour at room temperature, the solution was neutralized with 0.5N HCl and made 2XSSC. The DNA was then filtered through $1.0mm^2$ strips of cellulose nitrate at a concentration of 0.1 μg of DNA per strip. Each strip was subsequently washed with 2XSSC. After drying overnight at room temperature, the strips were incubated at 80^oC for 2 hours and stored in a dessicator until used.

DNA-RNA Hybridization

Four-tenths of a microliter of 2XSSC was added to each $1.0mm^2$ strip of DNA-bound nitrocellulose. After the excess buffer was blotted off, 5ug of rRNA at 2000dpm/ug was added. The mixture was then incubated at 63^oC for 12 hours. After the incubation, the rRNA was blotted off and the strips submerged in 100μg/ml RNAase (DNAase free) for 1 hour at 37^oC. The strips were thenwashed in 2XSSC and counted. Bacterial DNA served as the control.

Chromosome Uptake

Isolated lily chromosomes were stained in 0.1 percent DAPI in the dark for 1 hour. They were then washed free of the stain via centrifugation and resuspended in a chromosome isolation buffer (Table 3).

Tobacco, mesophyll protoplasts were obtained by incubating leaf tissue overnight in 2 percent cellulysin (Calbiochem), 1 percent macerase (Calbiochem) and 10 percent mannitol at pH 5.7. Protoplasts were washed free of enzymes using 1mM $CaCl_2$, 5mM MES (pH 6.5) and 10 percent mannitol at 200 x g for 15 minutes.

Protoplasts at 10^6/ml were incubated for various periods of time with isolated, stained chromosomes at several PEG 4000 concentrations in 4 percent mannitol and 12 percent $CaCl_2$ at pH 6.0. The reaction was stopped by adding 4 volumes of 50mM $CaCl_2$ and 10 percent mannitol at ph 8.5. The protoplasts were then pelleted at 200 x g for 15 minutes and washed in 5mM MES (pH 6.5), 1mM $CaCl_2$ and 10 percent mannitol.

RESULTS AND DISCUSSION

Chromosome Isolation

Higher plant chromosomes were isolated from a large number of species (lily, onion, pea, tomato, corn, daylily, tobacco and broad bean) and from a wide range of tissues (root tips, cells in suspension culture and microspores). Several criteria were used to confirm that the structures isolated were indeed chromosomes. First, the isolated structures morphologically resembled chromosomes with primary and secondary constrictions (Figures 2, 3, 5, and 6). Second, they contain DNA, for they stain with the DNA-specific dyes DAPI and Schiff's reagent (Brunk and James, 1978) (Figure 10B). Third, the only basic proteins they contain are histones (Figure 4). Finally, they have the correct 260/280 ratio of about 1.1 (Table 5). A crude approximation of the relative protein and nucleic acid composition can be obtained by taking the optical densities at 260nm and 280nm. For isolated, mammalian chromosomes a ratio of about 1.2 or about 4 times more protein than nucleic acid is obtained (Hanson, 1973).

The recovery of chromosomes in terms of the percent of the total chromosomal material available varies depending upon the species (Table 4). The highest yields were obtained from those species having the highest chromosome numbers and

Figure 2. Isolated Mitotic Chromosomes Containing Multiple Constrictions:

A. onion, 15um long

B. daylily, 10um long

C. pea, 5um long

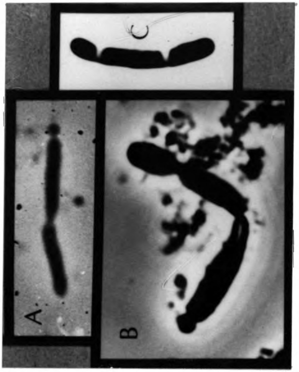

C
A
B

Figure 3. Collection of Isolated Meiotic Chromosomes:

A. daylily, 10um long
B. lily, 35um long
C. lily, 8um long
D. lily, 10um long
E. daylily, 5um long
F. lily, 10um long
G. lily, 8um long
H. daylily, 8um long
I. lily, 10um long

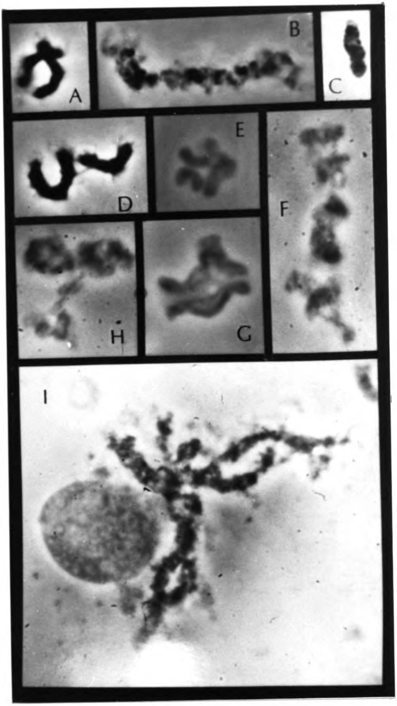

A
B
C
D
E
F
H
G
I

Figure 4. Histone Gels (Extracts Were Incubated at 100^{o} C for Two Minutes Before Loading onto the Gels:

A. meiotic complex from isolated, daylily chromosomes

B. histones from isolated, mitotic, daylily chromosomes

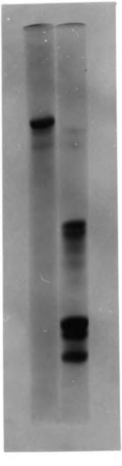


Table 4. Chromosome Yields for Various Species

SPECIES	YIELD
daylily	42 percent of the available chromosomes 1000 chromosomes/root tip
pea	24 percent of the available chromosomes 100 chromosomes/root tip
broad bean	71 percent of the available chromosomes 1000 chromosomes/root tip
lily	25 percent of the available chromosomes 50,000 chromosomes/anther

Table 5. Ratio of the Optical Densities at 260 nm and 280 nm for Isolated Chromosomes.

	RATIO OF $\frac{\text{O.D. } 260}{\text{O.D. } 280}$
DNA	2.0
protein	0.55
daylily	
root tip chromosomes	1.11
microspore chromosomes	1.11
leaf chromatin	1.07
onion	
root tip chromosomes	1.01
pea	
root tip chromosomes	1.16

the largest chromosomes. Because of these two factors, there is a higher yield of onion, lily and broad bean chromosomes as compared with the yield for tobacco, pea and tomato. Another factor involves protoplast formation, for the highest yields were obtained from those species with the most efficient yield of protoplasts. This factor allows one to obtain higher yields of onion chromosomes than with lily chromosomes. The yield not only depends upon the species but also upon the condition of the tissue prior to isolation. For example, under some conditions there can be a 50 percent conversion of onion root tip cells into protoplasts; while under different conditions the conversion may not even reach 1 percent. In these cases, there can be a 100 fold difference in chromosome yields. The only problem is that the best conditions cannot be routinely defined. The chromosome yield is also indirectly affected by the tissue. Two variables are involved. First, the mitotic index of some tissues is higher. For example, the mitotic index of colchicine-treated root tips is about 15 percent; while the mitotic index of untreated microspores is 100 percent. The higher the mitotic index, the higher the yield. Second, the efficiency of protoplast conversion varies between tissues. Thus, cells from suspension cultures generally have a higher chromosome yield than cells from root tips.

The most difficult aspect of isolating chromosomes is in keeping them condensed. The mammalian buffers are not at all effective in initially constricting the chromosomes;

however, they are partially effective in keeping the chromosomes compacted after they are initially condensed. The most important factor then is in initial condensation of the chromosomes prior to isolation. An 18 hour incubation in 0.05 percent colchicine is the most effective at initially constricting the chromosomes. Without this pretreatment it was impossible to obtain significant yields of condensed chromosomes. Once the chromosomes are initially compacted, it is important that the isolation buffer preserve this condensation. The difference in isolation buffers lies not in their short term ability to keep chromosomes constricted but in their long term ability to maintain them in a condensed state. The most efficient buffer is #13 found in Table 3. This buffer can keep the chromosomes in very good morphology for one month at $4^{o}C$; while the other buffers cannot keep the chromosomes condensed for more than a few hours (Figure 5). It is extremely interesting to note that buffer #13 is almost identical with that of Blumenthal's (Table 1). We both independently observed the effects of polyamines, hexylene glycol, EDTA, sucrose, Tris and DTT. These simultaneous observations, however, are not too surprising since both buffers are partially composites of several other mammalian workers' buffers. In buffer #13, the EDTA is present to remove Mn^{++} and Mg^{++}, nuclease cofactors. The EDTA, while reducing nuclease activity, also prevents normal, chromosome condensation. This is why the polyamines spermidine and spermine were added. They tend to reduce the charge on the DNA, thereby allowing

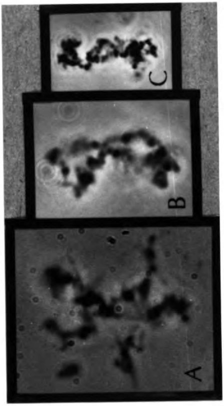
Figure 5. Mitotic Tobacco Chromosomes after Exposure to Various Buffers:

A. buffer #1 from Table 3

B. buffer #5 from Table 3

C. buffer #10 from Table 3

Note: The magnification for all three chromosomes equals 15,000 x.


C
B
A

it to supercoil. Likewise, the KCl and NaCl are needed to obtain the correct ionic strength which maintains chromosome condensation. DTT is added to preserve protein structure, as well as, help reduce nuclease and protease activity. Hexylene glycol, besides rupturing membranes and disaggregating chromosomes, also helps maintain protein structure. The sucrose is added to keep the interphase nuclei intact. Tris is needed to maintain the pH at near physiological conditions.

One unique aspect of isolating mitotic chromosomes is seen in Figure 6. It appears that isolated chromosomes can become quite sticky. In this case, the chromosomes have become associated at both the primary and secondary constrictions. This secondary association is quite strong, for the physical strength needed to break the association shears the chromosomes at their centromere or primary constriction. In some preparations up to 10 percent of the chromosomes can be linked in such a way.

Unlike mammalian systems, meiotic chromosomes can be isolated from higher plants (Figure 3). It is even possible to isolate quadrivalents in which the nucleolus is still attached to the nucleolar organizer (Figure 3 I). Once again, the yield depends upon several factors. The most important factor is the stage of meiosis. From prophase I to anaphase II, microspores were easily converted into protoplasts. After anaphase II, it was not possible to convert the tetrads into protoplasts. This was probably due to the build up of callose and the beginning of the exine formation. These compounds have β 1-3 linkages instead of the β 1-4 linkages that the standard cell wall digestive enzymes degrade. Eventhough protoplasts can be isolated from most stages of meiosis, some stages produce more fragile protoplasts. For example, prophase I and metaphase I protoplasts are extremely weak and can easily be ruptured in the centrifugation process. Extreme care must be taken when handling these cells. The other stages produce protoplasts which are less fragile but not as resilient as somatically derived protoplasts. Meiotic chromosomes are also much more fragile than their mitotic counterparts. A quick passage through a hypodermic needle can completely destroy all but the most highly condensed, metaphase I univalents.

In terms of the general biochemical and structural analysis of chromosomes, plant systems potentially offer more than mammalian systems, for meiotic chromosomes have so far only been isolated from plants. Much information can be gained from a comparison of meiotic and mitotic chromosomes. For example, the way in which histones bind to meiotic chromosomes seems to be different than the way they bind to mitotic chromosomes. In chromatin, all 4 histones are non-covalently linked together into 2 tetramere- $(H2a\text{-}H2b)_2$ and $(H3\text{-}H4)_2$ (Lewin, 1980). These tetrameres can be disassociated by heating at $100^{o}C$ in the presence of SDS. A two minute incubation is sufficient to break the mitotic, histone complex (Figure 4); while a longer exposure is required to disassociate the meiotic complex. Another minute of heating is needed before the meiotic histone complex separates. This suggests that in meiotic chromosomes the histones are somehow more tightly attached to each other. There are many other structural and biochemical differences between meiotic and mitotic chromosomes which can also be studied at the isolated chromosome level. For example, one very important question concerns the difference between meiotic and mitotic chromosome condensation. Meiotic metaphase I chromosomes are generally about ten times more compacted than mitotic metaphase chromosomes. Additionally, meiotic metaphase I chromosomes have a banded appearance and lack primary and secondary constrictions. Besides comparing the two types of chromosomes, meiotic chromosomes in themself have a great potential for research. Using isolated chromosomes, it will be much easier to study the underlying biochemistry of meiosis. It will also be simplier to look at synapsis formation and crossing over at a *in vitro* level, rather than at the typical *in vivo* level.

Chromosome Fractionation

The techniques for chromosome fractionation are based upon differential sedimentation through sucrose gradients. The more uniformly condensed the chromosomes, the more efficient is fractionation. One problem in isolating uniformly condensed chromosmes is in the colchicine pretreatment. Excessive colchicine is required to initially condense the chromosomes, as well as, increase the mitotic index. The colchicine also helps disrupt the spindle, thereby separating the chromosomes. The long colchicine treatment, however, has one serious drawback, differential condensation. The cells in which mitosis began at the start of the colchicine treatment will contain chromosomes which have been exposed to the chemical for 18 hours. These chromosomes will be highly condensed at the end of the incubation. Other cells which start mitosis at the end of the colchicine treatment will contain chromosomes which have only been exposed to the chemical for a short period of time and will be less condensed. An example will illustrate this phenomenon. _In vivo_, onion chromosomes are all about the same size. Figure 6 shows several isolated onion chromosomes in which there is up to a ten fold difference in chromosome condensation. This causes some very serious problems in chromosome fractionation, for a given chromosome will be present in varying degrees of condensation in an unfractionated chromosome mixture. When this mixture is sedimented through sucrose, fractionation does occur (Figure 7); however, the gradient besides separating given chromosome types also separates the different degrees of condensation within a given type.

If chromosome fractionation is to have any practical significance, the influence of differential condensation must be less than the influence due to true chromosomal size differences. An experiment can test this difference. An ideal marker is the chromosomal region called the nucleolar organizer. In daylilies it is a secondary constriction located on only one chromosome type. In this region, the genes for the

Figure 6. Differentially Condensed, Mitotic, Onion Chromosomes:

A. & B. the same chromosome at different positions

C. & D. the same chromosome at different positions

E. & F. the same chromosome at different positions

Note: The magnification for all chromosomes equals 12,500 x.

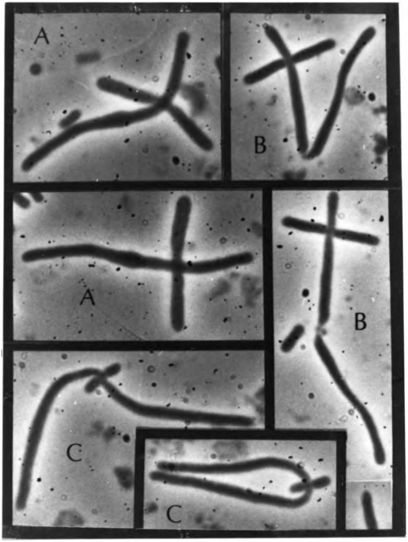
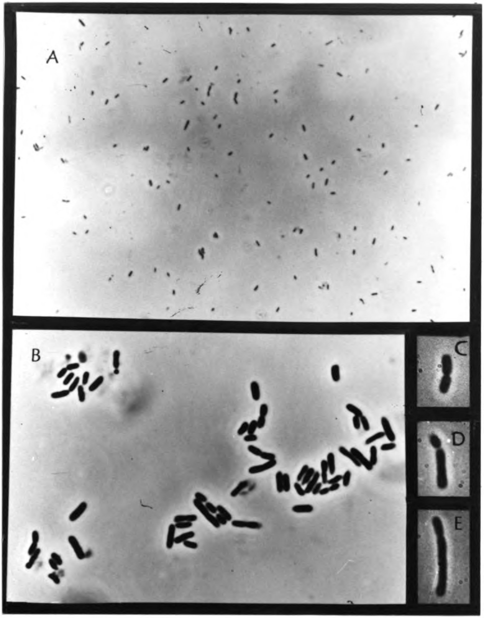

A
B
A
B
C
C

Figure 7. Daylily Chromosome Fractionation:

A. unfractionated mixture at 800 x

B. unfractionated mixture at 3200 x

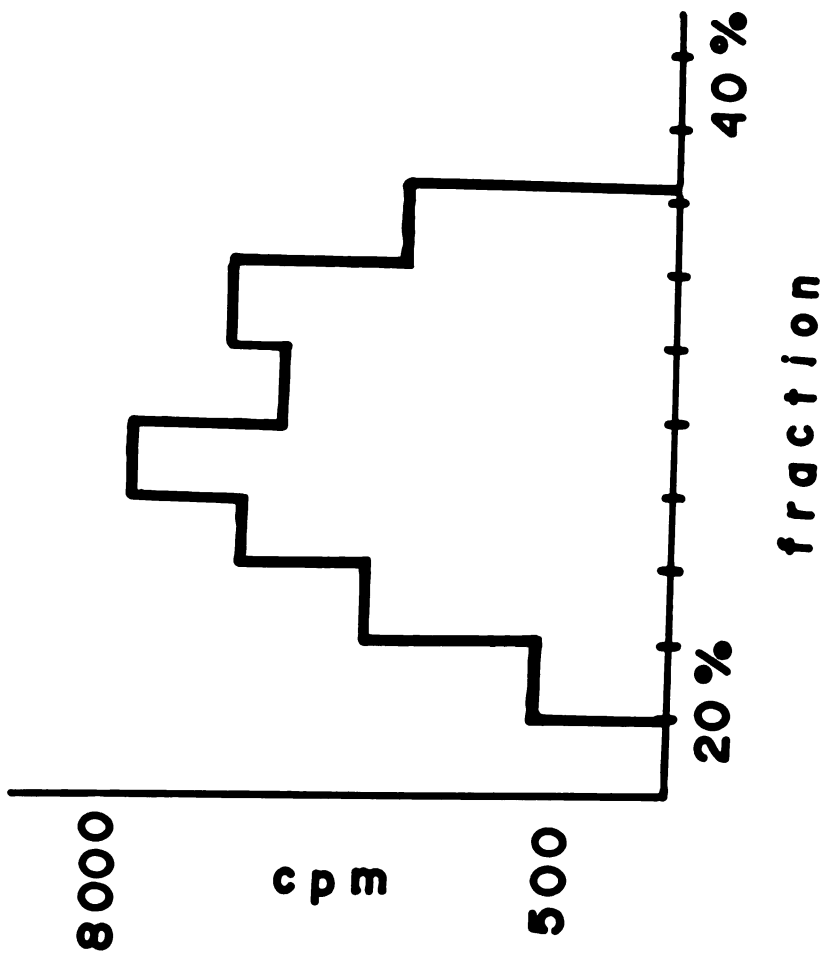
C., D. & F. the major chromosome in three fractions


A
B
C
D
E

25S, 18S and 5.8S rRNA are located. These rRNAs were extracted from ribosomes and radioiodinated to 100,000 dmp/ug. Likewise, DNA from each chromosome fraction was isolated, denatured and bound to nitrocellulose filters. The radioactive RNA was then used as a probe to determine which fraction or fractions contained the chromosomes with nucleolar organizer. The data is presented in Figure 8. Several things can be observed. First, the rRNA cistrons appeared in all but two of the seven fractions which contained chromosomes. The first and last two fractions did not contain any chromosomes. Second, the rRNA cistrons seemed to be more prevalent on the medium to large chromosomes. This is in agreement with the in vivo cytogenetic evidence. This experiment suggests that it is not possible to completely fractionate higher plant chromosomes using buffer #13 and the above chromosome isolation procedure.

Chromosomes can efficiently be fractionated in size, but the resulting in vitro size does not completely reflect the in vivo size because of a lack of uniform condensation during the isolation procedure. Some method needs to be developed which either condenses the chromosomes more uniformly or synchronizes the cells more efficiently. Attempts to modify the buffers after isolation either have no effect or lead to complete decondensation. Another method, besides a long colchicine pretreatment, is needed to increase the mitotic index and initially condense the chromosomes. A similar problem, although not quite as

Figure 8. Hybridization of DNA from Fractionated Chromosomes with ^{125}I-rRNA


8000
cpm
500
20%
40%
fraction

serious, also occurred in the mammalian (chromosome isolation) system. Their problem was solve in one of two ways. The first involves resedimenting the fractionated chromosomes (Mendelsohn, et al., 1963). This procedure initially requires a large number of chromosomes (at least 10^7). It is impractical in our system to obtain that quantity of plant chromosomes. Assuming an efficiency of obtaining 1000 chromosomes/root tip (Table 4), it would require approximately 10^4 root tips to obtain 10^7 chromosomes. This translates into about 200 man-hours of cutting root tips!! The second way of overcoming the differential condensation problem involves the use of flow microfluorometry (Stubblefield and Wray, 1978). In this system stained fractionated chromosomes are individually passed through a beam of light. As the chromosomes move through the light they fluoresce with an intensity proportional to their DNA content. The fluorescence data are then analyzed by a computer which subsequently sorts the individual chromosomes and channels them into the appropriate container. The one problem with this high technology system is its cost.

Chromosome Uptake

In order to monitor chromosome uptake into isolated protoplasts a visual system seemed appropriate. The system developed relied upon the natural fluorescence of chlorophyll and the fluorescent ability of DAPI, a DNA-specific dye. DAPI is a stain which only fluoresces when bound to

DNA. It fluoresces most intensely when bound to double-stranded, AT-rich regions (Brunk and James, 1978). Mesophyll protoplasts served as the recipient cells, because their chlorophyll fluoresces red when irradiated with ultaviolet light at 360nm (Figure 9 A). The red color was an ideal background for DAPI-stained chromosomes which fluoresce yellow-green (Figure 9 B). The selection of the appropriate plants is very important in this visual system. Two types of plants are required. One type must produce small protoplasts and the other type must have large chromosomes. Without the large chromosomes and small protoplasts, it would be extremely difficult to see a green chromosome in a red cell. Tobacco protoplasts were selected as the recipient cells due to their small size; while the foreign chromosomes were isolated from lily because of their large size. Because mesophyll protoplasts were used, the foreign chromosomes when taken up are restricted to the small peripheral area of cytoplasm surrounding the large central vacuole. This limited area of cytoplasm causes the chromosomes to appear distorted (Figure 9 C), for they are tightly pressed against the plasma membrane and take on the curvature of the cell. If one squashes these cells, the foreign chromosomes take on their normal morphology (Figure 9 D). This limited area of cytoplasm also causes some difficulties in determining if the foreign chromosomes are indeed inside, instead of on top of, the protoplasts. Figure 9 E and F show optical sections of a cell with a foreign chromosome. Another confirmation that the chromosomes are inside the host cells involves dispersal of the

Figure 9. Chromosome Uptake:

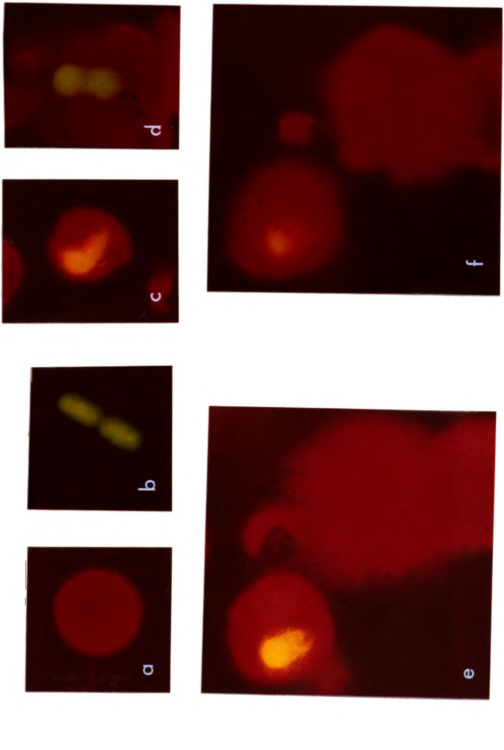
A. mesophyll, tobacco protoplast under ultraviolet light of 360nm

B. DAPI-stained, lily chromosome under ultraviolet light of 360nm

C. lily chromosome taken up by a tobacco protoplast

D. squashed protoplast containing a lily chromosome

E. & F. optical section through a protoplast containing a lily chromosome


a
b
c
d
e
f

stain. Chromosomes which have not been taken up retain after several hours their initial fluorescent intensity; while chromosomes which have been taken up gradually lose their fluorescence.

This visual system was used to monitor chromosome uptake under various conditions. In the past decade numerous procedures were developed which allowed protoplasts to take up a wide variety of objects (Vasil, et al., 1979). One technique involving PEG appeared to be the most efficient and consistent (Figure 10). This procedure involves exposing protoplasts and the object the cells are to take up to PEG at low calcium concentrations and under low osmotic strength. After the exposure, the reaction is stopped by increasing the calcium concentration, pH and osmotic strength. The PEG is then removed by washing the cells in a standard protoplast buffer. Beginning with this procedure, the optimal conditions for chromosome uptake were defined. There are three variables which can be studied--concentration of PEG, length of PEG exposure and the ratio of chromosomes to protoplasts. From previous work (Vasil, et al., 1979) the optimal protoplast concentration was determined to be 1×10^6 per milliliter. Two ratios of chromosomes to protoplasts were tested (Figure 11 A). It appears that the higher the chromosomes to protoplasts ratio, the higher the uptake. A ratio greater than 10:1 was impractical because of the difficulty in obtaining the large quantities of chromosomes needed. The next variable tested was time of exposure. In Figure 11 B it can be

Figure 10. Generalized Procedure for PEG-Mediated Uptake.

some object + 10^6 protoplasts/ml

↓

PEG + low CA^{++} + low mannitol

↓

high Ca^{++} + high pH + high mannitol

↓

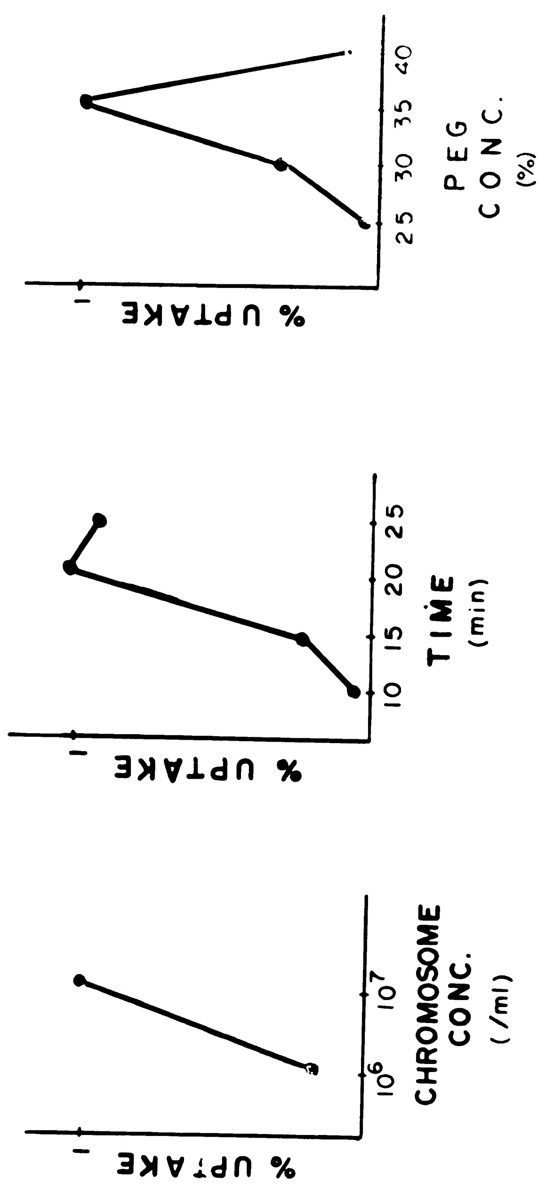
wash

Figure 11. Conditions for Optimal Uptake of Chromosomes by Protoplasts:

A. chromosome concentration vs. percent uptake

B. time of incubation in PEG vs. percent uptake

C. PEG concentration vs. percent uptake


% UPTAKE
1
25 30 35 40
PEG
CONC.
(%)
% UPTAKE
1
10 15 20 25
TIME
(min)
% UPTAKE
1
10^6 10^7
CHROMOSOME
CONC.
(/ml)

seen that the highest uptake was obtained after a 20 minute incubation in PEG. The uptake was still higher after a longer exposure; however, the morphology of the cells deteriorated considerably. The last variable was the concentration of PEG. The maximum uptake appeared after an exposure to 35 percent PEG (Figure 11 C). The optimal conditions for the uptake of lily chromosomes by tobacco mesophyll protoplasts are an incubation for 20 minutes in 35 percent PEG at a chromosome-to-protoplast ratio of 10:1.

A tobacco protoplast which has taken up a large lily chromosome has not been seen to divide; even though PEG-trated control protoplasts which have not incorporated a chromosome divide. This does not seem unreasonable, for the foreign chromosome is almost as large as the entire host nucleus. A more physiological situation would have been to incorporate smaller chromosomes into tobacco protoplasts; however with the DAPI-chlorophyll system it is below the limits of resolution to see small green chromosomes in large red protoplasts. Although the large chromosomes when incorporated represent an unphysiological situation, this system does give one a rough idea as to the optimal conditions needed for a successful transformation.

We now have a system for isolating chromosomes from almost any higher plant species, as well as, a procedure for introducing them into protoplasts. The next step will be to attempt transformation. In mammalian cell culture there are numerous examples of successful chromosome-mediated transformation (Willecke, 1978; Shows and Sakaguchi, 1980). In some instances large foreign chromosome fragments (about 1 percent of the genome) can become stablily integrated into the host genome. The frequency of such an event is about 50 percent of all the transformants or between 5×10^{-6} and 5×10^{-8}. If all the transformants, both the stable and unstable, are cultured under selective conditions for a prolonged period of time, up to 95 percent of the foreign chromosome fragments can become stablily integrated into the host genome (Klobutcher and Ruddle, 1978). Whole chromosomes, instead of fragments, can be transferred when liposomes or artificially created lipid vesicles are used (Mukherjee, _et al_., 1978). When the vesicles are made in a chromosome suspension, chromosomes are entrapped as the vesicles form. These vesicles are then fused with recipient cells in a manner similar to somatic hybridization. Besides increasing the size of the introduced genetic material, liposomes also allow a higher frequency of transformation. With cultured mammalian cells a frequency of 1×10^{-5}, as compared with 1×10^{-7} for non-liposome mediated gene transfer, can be obtained (Mujkerjee, _et al_., 1978). It is also possible to transfer unselected genes (Wigler, _et al_., 1979). If mammalian cells are exposed to a mixture of two types of DNA, about 95 percent of the transformants in which one of the DNAs was selected for will contain the other DNA. A similar system also operates in chromosome-mediated transformation (Klobutcher and Ruddle, 1978).

Although chromosome-mediated genetic transformation has not yet been attempted in plant systems, I fully expect the phenomenon to occur. One problem in looking for transformation is the lack of suitable genetic markers; however, there might be a marker. In Agrobacterium-transformed strains of tobacco, tissue-cultured cells can be produced which synthesize the unusual amino acid derivatives octopine and nopaline and which do not require an exogenous source of hormones for growth. In such transformed cells part of the bacteria's tumor inducing plasmid is integrated into the tobacco genome (Chilton, et al., 1980). This system could be used to study chromosome-mediated transformation. For example, chromosomes isolated from a Agrobacterium-transformed strain of tobacco could be introduced into non-Agrobacterium-transformed tobacco cells. Chromosome-transformed cells would be expected to synthesize nopaline or octopine and be able to grow in the absence of hormones.

Chromosome-mediated transformation should alleviate many of the problems plant breeders will face in the future, for it allows the breeder to overcome many of the difficulties of developmental incompatibility and extensive introgression faced when making either somatic or sexual wide hybridizations. Before chromosome-mediated transformation can have an impact upon plant breeding several things are needed. First, one needs to be able to regenerate whole plants from single protoplasts. It is still not possible to produce mature plants from the protoplasts of many of the most economically important horticultural and agronomic species. Second, there needs to be a more indepth, biochemical analysis of all the horticultural and agronomic traits. The molecular geneticist cannot select mutants in vitro if the desirable characteristics are only defined at a whole plant level. Finally, there needs to be a serious attempt at finding new agronomic and horticultural characteristics which can be expressed in vitro, for many of the important traits are tissue specific and are not expressed at the tissue culture level. Let us hope that chromosome-mediated genetic transformation will be of more use than the current, molecular genetic technology in future plant breeding.

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