MOLECULAR GENETIC MODIFICATION OF EUCARYOTES

Edited by

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Preface

Significant advances in molecular biology have been made since Watson and Crick described DNA and suggested its mode of replication. Certainly, molecular genetics has been one of the most exciting areas of science of this century. The worldwide food situation and the demands being placed on agriculture for increased production of high quality food have led to an intense interest in applying the scientific advances in molecular genetics, developed mostly with microbial systems, to plant and animal improvement for the betterment of mankind.

A transition appears to be occurring in the biological and agricultural sciences. In the biological sciences there is a shift from the investigation of model systems, principally procaryotic, to the study of applied systems such as economic crops. The intent is to apply the basic information generated over the past few decades to these higher plants. In the agricultural sciences, methods are being explored to reduce the complex economic crop plant to the cellular level. The intent is to manipulate and select for favorable phenotypes at the cellular level and then to regenerate complete plants that could be used in plant breeding programs. These transitions appear to be effecting a bridge that could result in a better utilization of the world's genetic resources. Researchers in the animal sciences might want to view these new approaches with plants as pilot systems that may provide future applications for the genetic engineering of animals. Indeed, the flow of information between plant and animal research is and will continue to enhance knowledge in the field.

With the foregoing ideas in mind, an interdisciplinary group of University of Minnesota faculty and graduate students commenced regular meet-

ings in 1973. Members from eight departments in three colleges (Agriculture, Biological Sciences, and Health Sciences) participated in discussions on the molecular genetic modification of eucaryotes. Annual workshops on the topic have ensued, and this book represents the updated proceedings of the first two held in 1974 and 1975. The basic goals of the workshops were to review the current state of knowledge and techniques potentially useful for the molecular genetic modification of eucaryotes and to consider such questions as the following: What existing molecular biological techniques might be useful in carrying out genetic modification of eucaryotes? What techniques need to be developed? In what ways might molecular biological techniques be applied to plant improvement? Can techniques developed for plant improvement be applied to animal breeding and the treatment of human disease? What fundamental questions of cell biology and genetics need to be answered to facilitate the application of these techniques? How can these techniques be used to answer fundamental questions of cell biology and genetics? What are the main obstacles in the culture of plant cells and tissues to the successful application of molecular biological techniques? What are the advantages and disadvantages of the various plant materials presently available?

The editors hope that this book will serve to collect in one volume some independent judgments on the potential of molecular genetic modification, assess some of the tools at hand for such research, expose the more obviously deficient areas of knowledge, and present additional data. The common thread of the workshops, and of this book, was the interfacing of molecular genetics, plant cell and tissue culture, and plant improvement. The workshops were not widely publicized beyond the University of Minnesota and were attended by approximately 150 persons each year. It is our hope that this book will make available to the larger scientific community the philosophy and information presented at the workshops. We anticipate that the readers of this volume may have as diverse interests as those faculty members involved in the University of Minnesota "Molecular Genetic Modification Discussion Group" who organized the workshops. Thus the book will be of interest to many geneticists, cell biologists, plant breeders, plant physiologists, plant pathologists, and biochemists. Throughout the book the usefulness of molecular genetic techniques is obvious in three aspects: as an alternative approach to currently available genetic methods, as a new approach to currently unfeasible ideas, and as an approach to the study of the basic biology of the eucaryotic genetic system.

Papers presented at the workshops are integrated into two sections reflecting aspects of cell biology and genetics. Specific topics in the cellular aspects section include cell and tissue culture, protoplasts, somatic cell fusion, cellular mutagenesis, regeneration of new plant types, and the ap-

plicability of these techniques to plant improvements. Specific topics in the genetic aspects section include viruses and viral integration; integration and expression of foreign genetic material in human cells, *Drosophila*, plant cells, and legumes; biophysical studies of DNA uptake by plants; and genetic engineering for plant protection against diseases. Four presentations are not included in this volume due to the prior commitment of the authors to publish their papers, or similar papers having been published elsewhere; these include "Differentiation and morphogenesis of cell cultures into plants" by T. Murashige, "Techniques for the insertion of eucaryotic DNA into bacterial and viral DNA's—biochemical tools for genetic modification" by R. W. Davis, "Isolation of ovalbumin synthesizing polysomes—methods for the isolation of a gene" by R. T. Schimke, and "Molecular biology of nitrogen fixation" by R. C. Valentine.

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Section II. GENETIC ASPECTS

Protoplasts and Somatic Cell Hybridization in Plants

O.L. Gamborg, F. Constabel, K.N. Kao, L.C. Fowke, K. Ohyama,
L. Pelcher and K.K. Kartha

Somatic cell hybridization in plants by fusion has been the object of intense investigation because of the extraordinary potential of such a process. The interest has been motivated by the need to expand genetic accessability of desirable characteristics of plants and thereby accelerate progress in crop improvement (Witwer, 1974; Gamborg *et al*, 1974).

Somatic cell genetics and hybridization in seed plants have developed relatively slowly. A serious obstacle to rapid advances has been the lack of materials and a system that would be amenable to cellular genetic analyses and rapid cloning of desirable phenotypes. The use of whole plants requiring months to complete a life cycle makes genetic analysis a slow and tedious process. Somatic cells cultured *in vitro* may alleviate this problem.

In the last few years, significant advances have been made in the technology of plant cell culture (Street, 1973). Large populations of cells can be cultured under controlled environment in simple nutrient media on agar or in liquid suspension. These cells can be subjected to mutagenic treatments and plated under conditions for selection and cloning of desired genotypes.

Somatic plant cells possess the capacity for totipotency. Regeneration of complete plants from somatic cells has been reported for many species (Murashige, 1974). Recently, this technology has been extended to include plant protoplasts which are formed by means of enzymatic removal of the cell walls. The availability of protoplasts and a variety of tissue culture procedures make it possible to investigate somatic hybridization and other forms of genetic manipulation in higher plant cells (Gamborg and Wetter, 1975).

PROTOPLAST ISOLATION AND CULTURE

Procedures for the isolation and growth of protoplasts from cultured cells, leaves, and other organs are developing rapidly (Table 1). Protoplasts have been isolated from plant tissues by enzymatic removal of the cell walls (Fig. 1). Tissues or cells have been incubated in a solution containing osmotic stablizers, calcium salts, phosphate and a mixture of commercial enzyme preparations

TABLE 1
Examples of protoplasts in which cell regeneration and division has been observed.

Systematic	*Common Name*	*Cell Origin*
Ammi visnaga		culture
Bromus inermis	Brome grass	culture
Cicer arietinum	Chick pea	leaf
Brassica napus	Rapeseed	culture, leaf
Daucus carota	Carrot	culture, leaf
Glycine max	Soybean	culture
Linum usitatissimum	Flax	leaf, hypocotyl
Medicago sativa	Alfalfa	leaf, culture
Melilotus alba	Sweet clover	leaf
Phaseolus vulgaris	Bean	leaf, culture
Pisum sativum	Pea	leaf
Pisum sativum	Pea	culture, shoot tip
Vicia hajastana	–	culture
Vigna sinensis	Cow pea	leaf

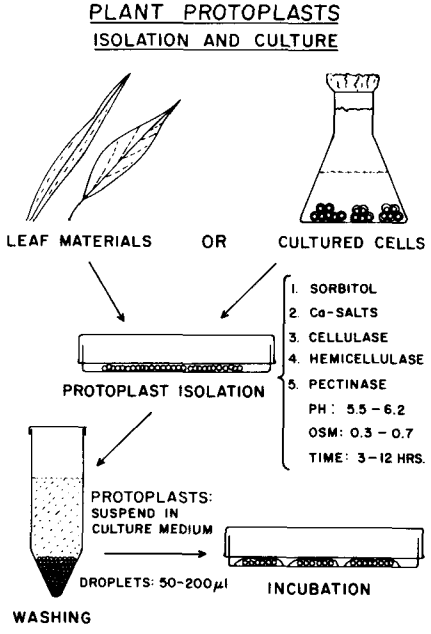

Fig. 1. Protoplast isolation and culture.

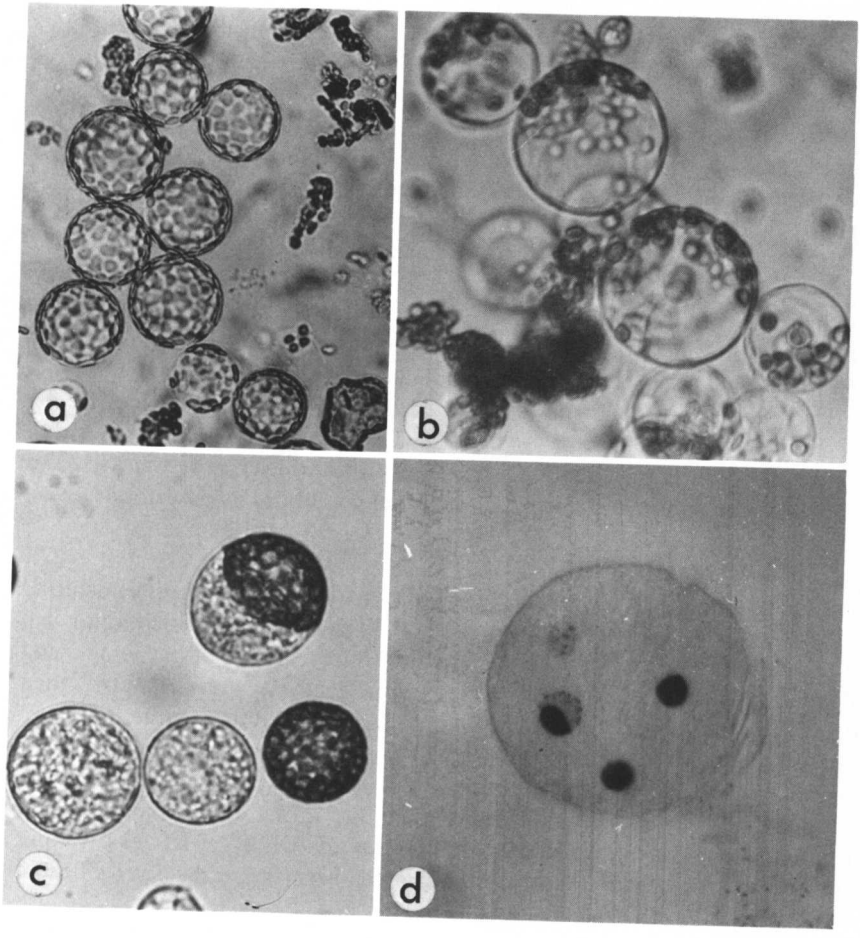

Fig. 2. a) Protoplasts from leaf tissue of rapeseed (Brassica napus). b) Protoplasts from corn leaf tissue. c) Protoplasts and a fusion product of carrot (light) and barley (dark) protoplasts. d) Heterokaryon showing nuclei of carrot (light) and barley (dark) and a nuclear fusion product (synkaryon).

which are predominantly cellulases, hemicellulases and pectinases (Constabel, 1975). After incubation for 3-12 hours, the debris was removed by filtration and the protoplasts were washed by centrifugation (Fig. 2, a, b).

Isolated protoplasts have been suspended in a nutrient medium and cultured as droplets in petri dishes (Kao *et al.*, 1971). The nutrient medium was similar to that used for plant tissue culture, but was modified to contain in addition sorbitol or similar osmotic stablizers. A suitable medium may consist of mineral salts, vitamins, glucose, ribose, casein hydrolyzate, 2, 4-dichlorophenoxyacetic acid, a cytokinin, and the osmotic stablizers. Other components, such as additional calcium salts, naphthaleneacetic acid and glutamine, may improve the survival rate and the possibility of cell wall regeneration and division.

A suitable pH may range from 5.5 to 6.2, and a suitable osmolality may vary from 0.3 to 0.7 depending upon the source of the protoplasts in culture. The isolated protoplasts were cultured as droplets with 10^4 -10^5 protoplasts per ml in petri dishes and incubated in humidified chambers at 25-28°C.

The cultured protoplasts have reformed the cell wall immediately after the enzymes were removed (Fowke *et al.*, 1974). The first division may occur within 1-3 days. After further incubation, cell clusters form which can be transferred to agar plates with similar nutritional and cultural conditions as used for droplets.

Uses of Plant Protoplasts

Plant protoplasts can be obtained in large quantities. They provide materials for new approaches to a variety of structural, physiological, biochemical and genetical problems. For example, the structure and biogenesis of membranes and cell walls might be studied more precisely and effectively with protoplasts than with other plant tissues. Cell wall removal has also permitted studies on membrane surface properties (Hartman *et al.*, 1973). The action of some plant pathogenic toxins has been elucidated through the use of protoplasts (Strobel, 1975). Protoplasts also have been employed successfully in the development of plant cell mutants and for uptake studies with viruses and DNA. Of all the attributes of protoplasts, perhaps the most remarkable has been their capacity for fusion.

Protoplast fusion

Spontaneous fusion between isolated protoplasts rarely occurs. Agents have been discovered, however, which promote fusion of protoplasts, apparently by increasing the area of membrane contact. The most successful techniques have employed alkaline-high calcium conditions (Keller and Melchers, 1973) or polyethylene glycol (PEG) (Kao *et al.*, 1974; Wallins *et al.*, 1974). The alkaline-high calcium method has been used in the fusion of tobacco protoplasts (Melchers and Labib, 1974), while PEG has proven effective in fusing protoplasts from a large number of plant taxa (Kao *et al.*, 1974).

Fusion has been initiated by adding a concentrated solution of PEG (M.W. 1540-6000) to protoplasts contained in a droplet placed in a petri dish. After 15-30 minutes, the PEG was gradually diluted out and replaced by culture medium. Fusion products were seen (Fig. 2, c). Unfused protoplasts and fusion products adhere to the surface of the petri dish. In most experiments, one of the protoplast species was obtained from leaf tissue and the other from cultured cells (Fig. 2, c).

Since the survival of unfused leaf protoplasts are low during culture, the heterokaryon fusion products could be recognized by the presence of green chloroplasts (Fig. 3, a). Up to 30% of the surviving protoplasts may be heterokaryons. The heterokaryons contained different proportions of nuclei from the two protoplast species (Constable *et al.*, 1975a). Large heterokaryons arising by multiple fusions and containing several nuclei often failed to survive. A proportion of the heterokaryons regenerate a cell wall and divide (Kao *et al.*, 1974; Constable *et al.*, 1975b; Kartha *et al.*, 1974b). The fusion of nuclei from the two parental species may occur during mitosis in the heterokaryon (Kao *et al.*, 1974). Recent results support this, indicating that interphase nuclei of two different species within the heterokaryon can fuse (Constable *et al.*, 1975a; Dudits *et al.*, 1976) (Fig. 2, d). The process may be initiated by the formation of nuclear membrane bridges (Fowke *et al.*, 1975). It has not been possible to establish that such hybrid nuclei enter mitosis.

Division has been observed in heterokaryons encompassing a wide range of plant genera and families (See Table 2) and there has been no indication of cellular incompatibility (Fig. 3, b). Fusion combinations such as pea x soybean and corn x soybean have yielded fusion products where both sets of parental chromosomes have been identified in metaphase plates (Kao *et al.*, 1974; Constable *et al.*, 1975a). In the systems where heterokaryons divided and formed cell clusters, the hybrids could be recognized visually. Cells with appropriate phenotypic markers for selection were required to isolate hybrid clones (Constable *et al.*, 1975b).

Uptake studies

Various reports describe the uptake by protoplasts of nuclei, chloroplasts, and DNA. Bonnet and Eriksson (1974) have investigated the uptake of algal chloroplasts by protoplasts from carrot cell cultures. They employed PEG to facilitate the process. Uptake consistantly occurred in the presence of 28% PEG (Modepeg, M.W. 1500). Up to 16% of the viable protoplasts contained one or more chloroplasts which entered the cytoplasm. The PEG activated the plasma membrane but did not appear to affect the chloroplast membranes (Bonnet and Eriksson, 1974). Although carrot protoplasts tolerated the PEG treatment, the fate of the algal chloroplasts was not ascertained.

TABLE 2.
*Examples of plant genera in which protoplast fusion and heterokaryocyte division has been observed.**

SOURCE OF PROTOPLASTS		
Leaf mesophyll		*Cell Culture*
Barley *(Hordeum vulgare)*	x	soybean *(Glycine max)*
Pea *(Pisum sativum)*	x	*Vicia hajastana*
Corn *(Zea mays)*	x	soybean *(Glycine max)*
Pea *(Pisum sativum)*	x	soybean *(Glycine max)*
Rapeseed *(Brassica napus)*	x	soybean *(Glycine max)*
Alfalfa *(Medicago sativa)*	x	soybean *(Glycine max)*
Sweet clover *(Melilotus alba)*	x	soybean *(Glycine max)*
Chick pea *(Cicer arietinum)*	x	soybean *(Glycine max)*
Angelica archangelica	x	carrot *(Daucus carota)*

*References
(Kao and Michayluk, 1974; Kao *et al.*, 1974; Kartha *et al.*, 1974b; Fowke *et al.*, 1975; Dudits *et al.*, 1976; Constable *et al.*, 1975a, 1975b).

Uptake of bacterial DNA has been reported in protoplasts of several plant species (Ohyama, *et al.*, 1972). This DNA appeared to be taken up in the double-stranded form. Uptake was enhanced by polycations. This bacterial DNA in protoplasts was subject to degradation, the extent of which varied between plant species. Using homologous DNA, Hoffman and Hess (1973) observed that DNA taken up by petunia protoplasts became associated with the nucleus.

The success of transformation in bacterial cells has been related to the absence of particular nucleases (Heyn *et al.*, 1974). Conceivably, plants may vary with respect to enzymes which recognize and degrade non-homologous DNA. The information on plant DNase is scant. A comprehensive study of these enzymes, and of DNA uptake and incorporation mechanisms would be extremely valuable (Holl, 1973).

The uptake of DNA and genetic transformation also has been investigated with seeds and other plant organs (Holl *et al.*, 1974). These systems have the advantage that complete plant formation, after DNA uptake, may be achieved.

The uptake of nuclei has been observed in petunia protoplasts (Potrykus and Hoffman, 1973). In the future organelle uptake may be extended to metaphase chromosomes, a method which has been employed successfully in mammalian systems (Willecke and Ruddle, 1975).

The choice of plant systems for genetic transformation studies by DNA or organelle uptake has been restricted. Phenotypic markers detectable at the cellular level which would permit effective selection and cloning are not yet available.

Selection systems

Somatic hybridization in mammalian cells has been greatly aided by drug-resistant mutants (Littlefield and Goldstein, 1970; Davidson, 1974). One cell line is resistant to 5-bromodeoxyuridine (BUdR) and its cells lack thymidine kinase (TK). Another mutant cell line is resistant to 8-azaguanine and it lacks hypoxanthine-guanine phosphoribosyl transferase. When cells of these two lines were fused, the hybrids were isolated in a selective medium containing hypoxanthine, aminopterin and thymidine. Neither of the parental lines survived since aminopterin blocks *de novo* synthesis of thymidine monophosphate and the exogenous compounds could not be utilized. Hybrids contained both enzymes and grew in the presence of aminopterin. Mutants of this type would be particularly suitable for plant protoplast fusion studies because a positive selection system for the hybrids could then be employed.

BUdR-resistant plant cell mutants have been reported (Chaleff and Carlson, 1974). A similar resistant line from soybean suspension cultures also has been isolated and studied (Ohyama, 1974). Cells of a more recent BUdR-resistant clone grow at a very high rate in the presence of 1 mg/ml BUdR. In contrast to the BUdR-resistant mammalian cells, the soybean mutant contained active thymidine kinase and incorporated the BUdR into DNA. The cells retained resistance during culture in the absence of BUdR. The metabolic basis for the resistance has not been entirely clarified.

A number of plant tissue cultures with resistance to natural amino acids and analogs of amino acids have been reported (Chaleff and Carlson, 1974; Green and Phillips, 1974; Widholm, 1974). Until the biochemical basis for the resistance has been fully established, it is not possible to predict the usefulness of these mutants in selection procedures.

Some toxins produced by plant pathogens exhibit a high degree of specificity. For example, the toxins from *Helminthosporium maydis* race T selectively destroyed protoplasts from susceptible but not resistant lines of maize (Pelcher *et al.*, 1975). In a fusion experiment, perhaps hybrid cells could be selected by using appropriate cell lines and a combination of toxins and antimetabolites. Such a system would have potential of a practical nature, assuming toxin-resistant lines could regenerate plants which retained resistance to the pathogen. Albino and light-sensitive mutants of species where plant regeneration can occur from protoplasts provide an effective hybrid selection system (Melchers and Labib, 1974).

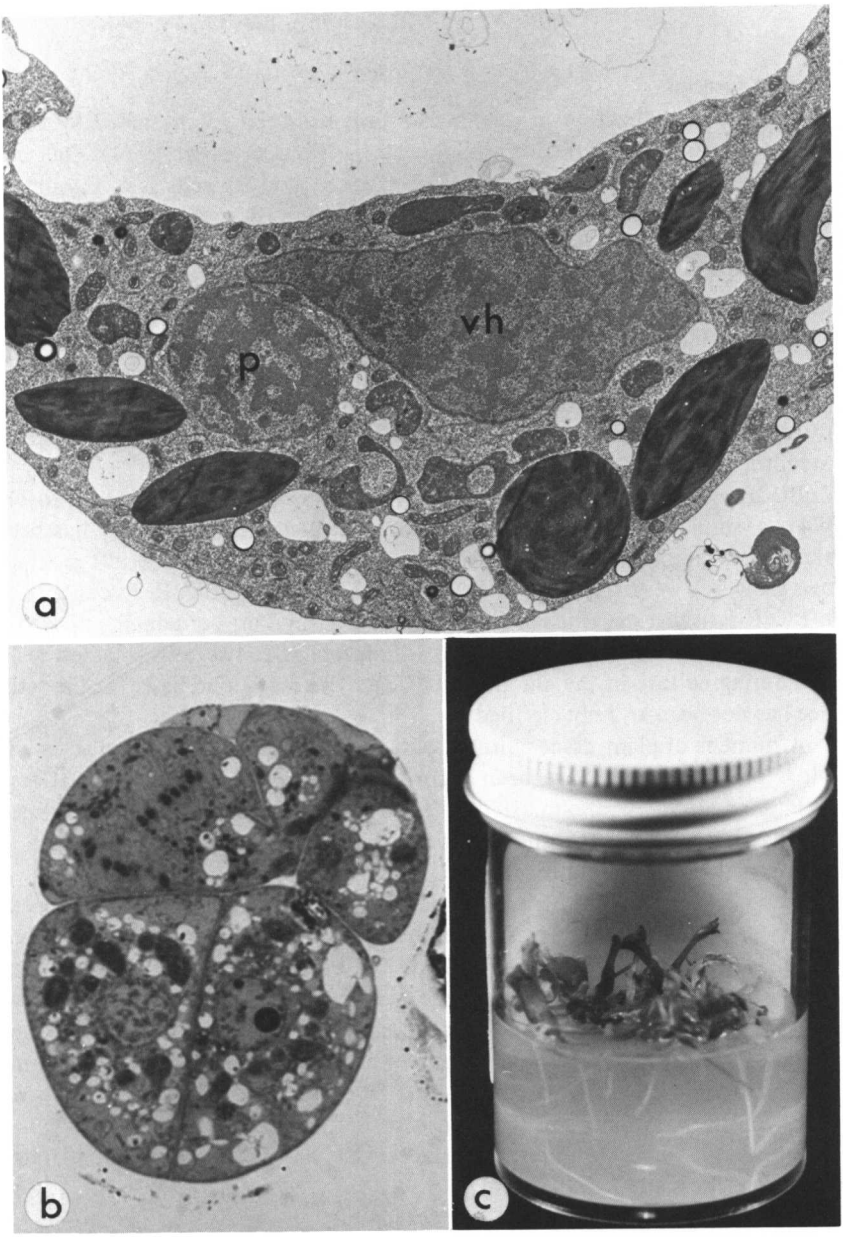

Fig. 3. a) Section of fusion product showing nucleus and chloroplasts from pea leaf (p) and nucleus from Vicia hajastana (vh). b) Cell hybrids of soybean and sweet clover. c) Plantlets obtained from carrot protoplasts by embryogenesis.