Monographs on
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Protoplast Fusion

Genetic Engineering in Higher Plants

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Edited by R. Shoeman

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Preface

Although only about 3 years have passed since the preparation of the original manuscript of this book for the Russian edition, the number of successful experiments on somatic hybridization of higher plants has doubled. Although the main inferences of the first edition still remain in force, most of them have received conclusive experimental support and, moreover, some new conclusions have been drawn. It can be expected that these inferences and conclusions will constitute a more or less durable foundation for somatic cell genetics of higher plants. We thus hope this book will also remain useful over the next years, in spite of the rapid progress of experiments and the increase in the number of scientific reports in this field.

Though it might appear strange to an uninvolved observer, the principal progress in hybridization of somatic cells of higher plants has been due to plant physiologists (who entered the field by elaborating methods and techniques for plant cell and, later, for isolated protoplast culture) rather than plant geneticists. However, further qualitative improvement in this field is inconceivable without the instillation of genetic ideology and the strict logic of genetic experiments. The main purpose of this book is the attempt to organize the available experimental data in terms and categories of genetic analysis. For this reason, this book lays no claim on being a comprehensive treatise on somatic hybridization. The consideration of the principally genetic aspects of somatic hybridization seemed indispensible to us, thus, numerous important questions of physiology of protoplasts and hybrid cells are not discussed. Although we tried to include all of the significant studies in this field, not all of the available literature has been cited. Unfortunately, the unnecessary publishing of the same results several times (in a scientific journal, then in proceedings of congresses or symposia, and then in combination with other data, etc.) is very widespread. Therefore, we have attempted to cite the same data only once.

We are aware that this book is subjective to a certain extent and we apologize beforehand to our colleagues whose works are cited perhaps less frequently than they merit. In describing the experimental data, we have given preference to the works of those colleagues whose investigations were known to us not only from

published reports. Similarly, our own data have been reported in more detail. It is easier to discuss and critically analyze data which are better known. As was mentioned earlier, this book represents a geneticist's point of view and the conclusions herein may therefore appear to be at odds with the view of those dealing with somatic cell hybridization in higher plants (mostly physiologists or sometimes "culturists").

Certainly, we bear complete responsibility for the possible successes and, first and foremost, for all failures of this book. However, it should be acknowledged that the appearance of this book is the result of the work of many people. This book is rather profusely illustrated and contains the illustrations from the works of numerous investigators. We wish to thank Prof. G. Melchers, Dr. K. N. Kao, Dr. K. Glimelius, Dr. L. R. Wetter, Dr. L. C. Fowke, Dr. F. Constabel, Dr. O. Schieder, Dr.L. Menczel, Dr. V. A. Sidorov, and Dr. F. Nagy for their kind permission to incorporate their illustrations into this book and for providing the originals of these illustrations.

Dr. V. A. Sidorov kindly read the manuscript and we are thankful to him for a number of valuable remarks.

This book would not have appeared without the work of the editor of Springer-Verlag, Dr. R. L. Shoeman, who revised and completely rewrote the English text.

We would also like to express our gratitude to our colleagues and collaborators for their tremendous help in the preparations of the manuscript and especially to M. N. Moskalenko, I. F. Kanevsky, and V. P. Momot.

Y. Y. Gleba
K. M. Sytnik

Still no forbidden fruits on the tree of knowledge: one of the pioneers in somatic hybridization research,

Professor GEORG MELCHERS

with his creation – potato (x) tomato somatic hybrid plant.

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Contents

1 Introduction

The research described in this book elaborates the production of higher plant hybrids by using the experimental technique of parasexual hybridization by means of protoplast fusion. Additionally, investigations into the genetic novelty of the resulting hybrid plants will be described.

The main distinction owned by this new hybridization method is that somatic plant cells, rather than sexual ones (gametes), are used as the parental cells. Treatment of these cells with specific enzymes results in the removal of their rigid polysaccharide cell walls: thus, "naked" plant cells (i.e., isolated protoplasts) are obtained. Certain, specific experimental treatment of these protoplasts results in cell fusion. Hybrid cells produced in this way may, in many cases, be subsequently regenerated into entire hybrid plants. This parasexual hybridization via protoplast fusion has been made possible by the elaboration of two experimental approaches in plant physiology: (1) methods of plant cell and tissue culture and (2) techniques of production and manipulation of isolated protoplasts. The avenues opened by the first of these methods may be summarized as follows. Special nutrient media have been developed and conditions have been described in which individual cells or isolated groups of cell dedifferentiate (i.e., they lose the characters of the initial tissue) and begin dividing as independent unicelluar organisms. This sort of unorganized growth and cell reproduction can be practically maintained in vitro for tens of years. The organized growth of the cells can be restored and, as a result, the entire plant can be produced de novo by altering the culture conditions, in particular by modifying the hormonal content of the media. As matter of fact, these techniques enable one to step from the organism level down to the cellular level, making feasible experiments on millions of cells in a test tube that would otherwise be prohibitive or impossible if attempted on a similar number of plants. At the same time, these techniques permit the regeneration of the entire plant at the end of an experiment on isolated cells, thus allowing the visualization of the manipulation at the organism level as well.

The techniques of production and manipulation of isolated protoplasts that have been described in the last 10 years depend upon the enzymatic hydrolysis of the cell walls, making possible not only the fusion of the resulting naked cells (protoplasts), but also enabling the cells to take up macromolecules (proteins, nucleic acids, etc.) and particles (isolated cell organelles, microorganisms, etc.) from the surrounding solution. Cultivation of protoplasts under defined conditions results in synthesis of a new cell wall. The resulting cells behave like "normal" cells cultured in vitro and, in particular, are capable of dividing and forming cell colonies and/or even entire plants. Thus, to reiterate a crucial point, these two methods permit the experimenter to easily change from the organism level to the

cellular level and back again, with the added bonus that the contents of the plant cell may be manipulated at the isolated protoplast stage. The combination of these techniques clears the way for hybridization of higher plants without sexual crossing, i.e., parasexual hybridization. Substantial progress has been made up to now in only one of the above discussed realms of parasexual hybridization, namely, in hybrid production by fusion of isolated protoplasts. The full realization of the other aspects, such as the transplantation of organelles or the introduction of foreign DNA into protoplasts, depends largely on the refinement and application of existing methods.

The subject of this book, the hybridization of plant somatic cells by fusion of protoplasts, is a method similar to the previously elaborated method of hybridization of animal somatic cells. There is, however, one fundamental distinction between these two methods–hybridization of animal somatic cells enables the production of *hybrid cells*, whereas the fusion of plant somatic cell protoplasts results, in many cases, in the production of *hybrid plants*. Thus, parasexual hybridization is not only a novel pathway for genetic analysis, but may also develop into a powerful tool for industrial plant breeding in the future. Up to now, both the geneticist, investigating the mechanics of plant cell inheritance, and the breeder, attempting to feed mankind by creating more productive plant varieties, possessed only sexual crossing as the sole tool for the engineering of novel plants. Our purpose is to equip them with a new method to achieve their diverse goals.

The necessity of elaboration of a new method of hybridization is also dictated by the limitations of the old method: sexual crossing is a strictly determined system where only a defined group of organisms in restricted combinations may be used as parental forms. The resulting progeny from such a sexual cross possess defined and limited genotypes. A short review of the general characteristics of sexual crossing will illustrate the restrictions imposed on genotype engineering by conventional methods of hybridization and also serve to point out the potential advantages offered by parasexual hybridization.

Highly specialized plant cells, gametes, are involved in sexual crossing. Gameteogenesis involves meiotic reduction and segregation of chromosomal material. Only plants which are capable of normal gametogenesis can be sexually crossed. The zygote is a product of the fusion of two gametes. This process results in nuclear fusion and in the restoration of the sporophytic chromosome number. Nuclear genetic determinants are inherited biparentally. Sexual crossing is symmetrical in the sense that both the male and female gametes contribute equally and supply a gametophytic set of nuclear genetic material to the zygote. The segregation of nuclear characters is in accordance with the Mendelian laws of inheritance. Extranuclear genetic determinants are inherited strictly uniparentally and maternally in most higher plants. Sexual crosses are limited to only a few genotype combinations out of many theoretically possible combinations, because of inherent systems of incompatibility. Sexual crosses are limited to phylogenetically closely related species.

Plant hybridization by protoplast fusion may therefore be of interest if it can be used:

1. to perform hybridization between species which are too distantly related to be crossed sexually;
2. to obtain different assymetric hybrids, in which the progeny would have all the chromosomal material from one of the parents, but only a few chromosomes, a few genes, or only the cytoplasm and organelles from the other parent;
3. to devise hybridization systems involving simultaneous fusion of more than two parental cells;
4. to obtain hybrids genetically representing a sum of parental idiotypes;
5. to obtain plants heterozygous for extranuclear genes;
6. to bypass restrictions on hybridization imposed by inherent systems of incompability;
7. to produce hybrids between plants that are not sexually crossable because of abnormalities in the morphogenesis (gametogenesis) of the parents;
8. to obtain hybrids between cells containing different epigenetic programs.

The most important distinction of parasexual hybridization as compared to the usual sexual crossing is the completely artificial nature of this new method. Theoretically, it may be assumed that many restrictions inherent in sexual crossing (for example, the restrictions due to abnormal gametogenesis in the parent or due to reactions of inherent incompatibility) are not a problem in parasexual hybridization, since gametes and their exchange are not involved in this new method. Moreover, since parasexual hybridization is a complex process consisting of a number of events (cell fusion, fusion/nonfusion of cell nuclei, fusion/nonfusion of organelles, etc.), each of which may lead to genetic alterations and since the genotype of the progeny is a function of all these events, it is quite evident that by defining and standardizing some parameters of the artificial process of parasexual hybridization, one can predetermine, to a certain extent, the genetic results. The great importance of this presumable control of the parasexual hybridization process is stressed by the fact that we deliberately chose to include the term "genetic engineering of plants" rather than "plant hybridization" in the title of this book. We believe the term genetic engineering emphasizes a creative human presence and accentuates the synthetic rather than the analytic nature of the scientific methodology utilized in these investigations of plant inheritance. Thus, parasexual hybridization is not only a way of producing novel plant forms, but is also a new synthetic method for investigations of biological systems.

Not all methodological problems of plant cell genetic analysis can be solved by using the techniques of parasexual hybridization. We would like to point out two of the major limitations to the use of parasexual hybridization. First, the technique of parasexual hybridization, although dealing with isolated plant cells rather than entire plants, is nonetheless relatively time-consuming since plant cells divide more slowly than animal cells and microorganisms (the duration of the cell cycle in plant cells in vitro is typically 25–40 h); moreover, the regeneration of entire plants takes several months in most cases. For this reason, one experiment for production of *Nicotiana* hybrids by protoplast fusion and subsequent analysis of their progeny takes 1–1½ years and no foreseeable improvements in ex-

perimental technique are likely to reduce the time necessary for this process. Second, the plant cell is very labile and responds readily to external signals, producing the undesirable consequence of a great genetic heterogeneity of the plant cells via external induction. This heterogeneity complicates the proper analysis of genetic events caused by hybridization, since the spectrum of induced genetic alterations are superimposed upon those resulting from parasexual hybridization. This heterogeneity may also become a grave obstacle to practical work using protoplast fusion to achieve the goal of production of material for plant breeding.

2 Techniques of Parasexual Hybridization

2.1 Culture Techniques

2.1.1 Culture of Isolated Plant Protoplasts

Methods of hybridization based on the phenomenon of induced fusion of somatic cells have been used successfully in animal cell genetics for over 20 years (see reviews of Ephrussy 1972; Harris 1974; Ringertz and Savage 1976). Until recently, the principal obstacle to the introduction of analogous methods in plant cell genetics was the cellulose-pectin cell wall which completely excluded the possibility of plant cell fusion. Although naked plant cells (isolated protoplasts) were mechanically obtained as early as 1892 (Klercker 1892), the improvement of the method of producing isolated protoplasts became possible only after the elaboration of the techniques of enzymatic isolation of a large quantity of naked protoplasts (Cocking 1960). Over 2000 papers concerning isolated protoplasts have been published on a world scale. The present state of the art and the potentials of this method have been reported in detail in the proceedings of several symposia and congresses. The latest of these have been: The Fourth Cell and Tissue Culture International Congress, August 20–25, 1978, Calgary, Canada (Frontiers of Plant Tissue Culture 1978, 1978); Fifth International Protoplast Symposium, July 9–14, 1979, Szeged, Hungary (Abstracts of the Fifth International Protoplast Symposium, 1979); Second International Congress on Cell Biology, August 31-September 5, 1980, Berlin (West); and the Fifth International Congress of Plant Tissue and Cell Culture, July 11–16, 1982, Tokyo, Japan. The more or less modern data are reported in the following books (full bibliographic data are contained in the references): *Cell genetics in higher plants,* 1976; *Microbial* and *plant protoplasts,* 1976; *Plant cell and tissue culture*, 1979; *Plant cell, tissue and organ culture*, 1977; *Advances in Protoplast Research*, 1980; and *International Cell Biology* 1980–1981.

This chapter contains a brief enumeration of the techniques, methods, and conditions which determine the modern trends in the development of genetic engineering of higher plants.

A list of the plant species for which the techniques of protoplast culturing and cell colony or entire plant regeneration have already been described is given in Table 1. In most experiments, leaf mesophyll or callus protoplasts were used as the initial material. The regeneration of tobacco mesophyll protoplasts and *Arabidopsis* callus protoplasts are illustrated in Fig. 1–4. As can be seem from the data contained in Table 1, techniques of protoplast culture and plant regeneration are available for a great number of species.