PLANT GENETIC ENGINEERING
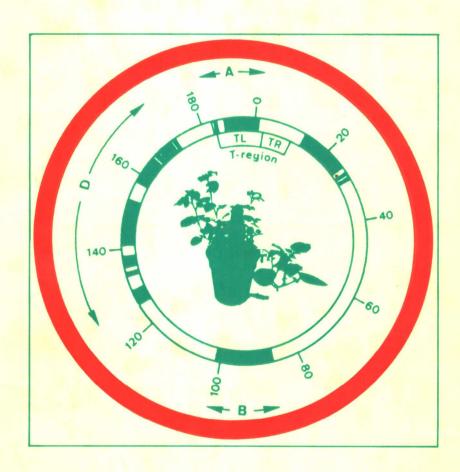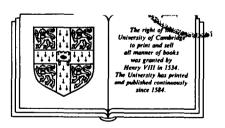

EDITED BY
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Plant genetic engineering

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1   Introduction

J. H. DODDS

For centuries humans have been trying to improve the quality and yield of their cultivated plants by conventional breeding and selection programs. The value of those efforts should not be underestimated, for they pointed the way toward what has come to be known as the "green revolution."

Although the history of plant tissue culture goes back to the turn of this century (Haberlandt 1902), it is only since the 1950s that practical applications have been considered. The field of plant tissue culture uses many different techniques, some of which have long been functionally applied in agricultural and horticultural science. A good example is the eradication of virus infections by meristem tip culture and subsequent micropropagation of the material. Micropropagation of potatoes and fruit trees, for example, is now a routine procedure. The meristems or shoot tips are incubated on a suitable medium to induce the outgrowth of axillary meristems, which can then be excised and subcultured again. The rates of propagation in these systems are so rapid that it is easily possible to produce a million plants from a single source in one year.

There are other areas of plant tissue culture that are still very much in experimental stages. The process of isolation, culture, and fusion of protoplasts falls into this category. It is possible to regenerate whole plants from isolated protoplasts in a number of genera; however, the rate of success is still low, and the results in cereals, for example, have been disappointing.

Nevertheless, it is these experimental areas that seem to hold enormous promise for improvements in crop plants. Over the last decade we have seen tissue culture methods used in conjunction with the molecular sciences to investigate the fundamental structures and actions of plant genomes and to analyze the possibilities of modifying plant genomes.

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The new possibilities in this area of plant molecular biology have proven interesting not only to scientists in universities and government research centers but also to a wide range of industrial and agrochemical companies. It is obvious that any new development, any "novel" plant or transformed plant carrying a new characteristic, such as resistance to a herbicide or higher photosynthetic efficiency, could have significant economic value. Such new plants would be patentable, with royalty payments to the company producing them.

An important unknown factor in the area of plant genetic manipulation and engineering is the time scale of the operation. It may be only a few years before commercial companies achieve a breakthrough that will lead to large-scale production of these novel plants; on the other hand, such developments could take decades.

Recent publications have indicated that in model systems such as the tobacco plant, it is now possible to insert and translate new genetic information (Chilton et al. 1978, 1980; Herrera-Estrella et al. 1983*a*, 1983*b*, in press). We now face two immediate problems. First, the technology used in these model systems must now be transferred to economically important crop plants. This may prove to be relatively straightforward for the potato, a solanaceous plant that exhibits a high degree of plasticity in culture. However, for other plants, such as cereal grains, the situation is more difficult, and much more basic research will be required. Second, a consensus must be reached among agriculturalists, physiologists, and molecular technologists to determine what genes to include in order to produce improved plants. A number of ideas are currently being discussed. For obvious economic reasons, the agrochemical companies are greatly interested in the possibility of including genes conferring resistance to specific herbicides, in order to produce crop lines with specific resistance factors.

There is also the possibility of improving the nutritional value of a crop, either by improving the photosynthetic efficiency of the plant (see Chapter 7) or by modifying the quality of the food component, that is, their storage proteins (see Chapter 8). However, the complexities of plant improvement still pose difficult questions, and if we consider the feasibility of improving a plant simply by addition or modification of a few genes, the number of possibilities would appear to be few.

In this volume we try to put into perspective the two major aspects of these technologies. The first part deals with tissue culture

or, to be more specific, protoplast culture work. It describes the problems associated with this work and presents ideas regarding the potential for these techniques in the future. The second part deals with the molecular biology involved and concentrates on two model systems: one looking at overall photosynthetic efficiency and a second looking at specific modification of an important storage protein. Chapter 9 gives an overview of the possible implications of genetic engineering for agricultural science.

It is hoped that this volume will stimulate further interest in the area of plant genetic engineering. The problems we face are considerable, but if the expected breakthroughs are achieved, their applications will be of great importance. We hope that this volume will encourage more people to join in these studies.

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2 Isolation and culture of plant protoplasts

J. H. DODDS

An isolated protoplast is a plant cell in which the outer wall has been mechanically or enzymatically removed. The result of this wall removal is that the plasma membrane is the only barrier between the cell cytoplasm and the external environment. Isolation of plant protoplasts is not a new technique. Mechanical isolation of protoplasts was carried out as early as 1893 (Rechinger 1893). At that time, isolation and observation of protoplasts was primarily of academic interest, and the number of protoplasts that could be liberated was extremely small, on the order of a few hundred per hour. In the early 1960s, many workers became interested the synthesis and structure of the plant cell; as a means of studying its composition, work was carried out on the activities of fungal enzymes known to cause digestion of the cell wall. The purification of these enzymes opened up the possibility of isolating plant protoplasts by an enzymatic method. By appropriate selection of the enzyme mixture it is possible to liberate billions of protoplasts in a few hours (Cocking 1960, 1972, 1973), as will be described later.

In this chapter we shall look at the culture conditions and techniques required for isolation and culture of protoplasts and eventual regeneration of a whole intact plant. The demonstrated ability of a single isolated protoplast to regenerate a whole plant (Raveh and Galun 1975; Takebe et al. 1971) is a perfect demonstration of the hypothesis of totipotency of plant cells (Haberlandt 1902). In this chapter, each stage of the culture process will be analyzed, and indications will be given regarding the problems that are normally encountered. One of the fundamental problems with this aspect of plant tissue culture, a problem that also arises in other areas, is that what will work for one plant genus often will not work for another. In general, the Solanacea family provides an excellent model system for protoplast work; this family of important crop plants includes

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both potatoes and tomatoes (Melchers 1978, 1982; Melchers et al. 1978; Shepard 1982; Thomas 1981). Unfortunately, success with cereal protoplasts has thus far been relatively limited (Vasil 1982).

Physiological state of the plant

The physiological state of the starting plant material for protoplast isolation apparently has a significant effect on the yield and survival of the resulting protoplasts. In general, it is advisable to grow the material in a room in which the environmental conditions can be closely controlled, and the use of systemic herbicides or pesticides should be avoided (D. Roscoe, personal communication). If possible, it is better to start with material in the form of axenic shoot culture. Axenic shoot cultures have a very thin cuticle, thus permitting easy access of the enzyme mixture, and their inherent sterility removes the need to subject the material to the possibly damaging effects of surface sterilization.

There is some evidence that plant material that is slightly drought-stressed to cause leaf wilting a couple of hours before isolation helps improve the isolation procedure. An alternative to this is to plasmo-lyze the detached leaves in a 13% (w/v) mannitol solution for a few minutes prior to the enzyme treatment.

Enzymology and osmoticum

Once the protoplast has been isolated, either by mechanical methods or by enzymic methods, the plasma membrane is the only boundary between the cell contents and the external bathing environment. Thus, the osmotic protection of the cell wall has been removed. To compensate for this, an osmotic agent must be included in the surrounding medium to keep the cells in an isotonic state. Various osmotic agents are available. Mannitol, an osmotic sugar alcohol, is the most common and is usually employed at a concentration of approximately 12–14% (w/v). Sorbitol can be used as an alternative osmotic sugar alcohol. It is possible to use sucrose as an osmotic agent, but the problem with using a conventional sugar is that it is metabolized by the cytoplasm of the cells, thus constantly dropping in concentration and osmotic value.

In the last few years a number of enzymes have become available

Table 2.1. *Types of enzymes available for isolation of single cells and protoplasts*

Enzyme	Source
Cellulase R-10	Kinki Yakult Biochemical, Nishinomiya, Japan
Cellulase (Cellulysin)	Calbiochemicals, San Diego, California
Cellulase (Drieselase)	Kyowa Hakko Co., Tokyo, Japan
Pectinase macerozyme R-10	Kinki Yakult Biochemical, Nishinomiya, Japan
Pectinase	Sigma Chemical Co., St. Louis, Missouri
Hemicellulase HP-150 rhozyme	Rohm and Haas Co., Philadelphia, Pennsylvania

Note: The listing of a company does not signify any commercial preference. Other suppliers are available.

that have wide-ranging wall degradation properties and varying degrees of purity. Table 2.1 lists some of the enzymes that are available. The purity of the enzyme can be a critical factor. For example, if the enzyme preparation contains impurities of proteases or lipases, they will act on the plasma membrane of the protoplast and will result in bursting. It is important to remember that the enzyme digestion mixture should always include the osmotic agent, usually mannitol. After the enzyme is dissolved in the standard culture medium (Murashige and Skoog 1962), the normal method for sterilizing enzyme mixtures is by ultrafiltration through a 0.45-μm filter.

Method of protoplast isolation

The basic technique for isolation has been described by many workers (Cocking 1972; Dodds and Roberts 1982; Gamborg et al. 1981; Yeoman and Reinert 1983). Figure 2.1 is a diagrammatic representation of the typical procedure that is followed. After surface sterilizing (not necessary for axenic shoot material, which is highly recommended), the leaves are rinsed and plasmolyzed for 1 hr in 13% mannitol. The lower epidermis is then carefully teased away to allow penetration of the enzyme mixture. If there are technical problems with removal of the lower epidermis, penetration can be achieved simply by scratching the leaf with a needle or scalpel to break the epidermal and cuticular surfaces.

After an appropriate period of incubation, the leaf will appear to dissolve. The time period involved will vary greatly, depending on the type and physiological state of the material; it can vary from 30 min to

Sterilize in 10% chlorox
for 10 minutes

Crude protoplast
suspension

Remove lower
epidermis

Rinse 3 times with sterile
distilled water

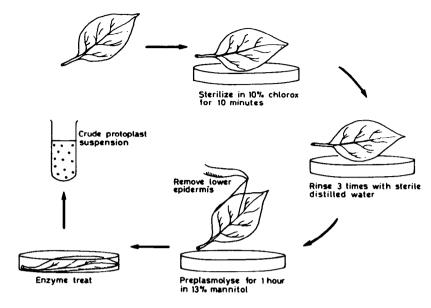Enzyme treat

Preplasmolyse for 1 hour
in 13% mannitol

Figure 2.1. Diagrammatic scheme for isolation of crude protoplast preparation.

overnight incubation. In some cases it helps to gently agitate the petri dish during this incubation period at a rate of 40 strokes/min.

After the incubation is over, the protoplasts are released, leaving a crude protoplast suspension floating in a medium containing enzymes. The next step is to remove the enzyme mixture and purify the protoplast preparation.

Purification of protoplast preparation

Several possible methods are available for purifying a crude protoplast preparation. Figure 2.2 shows a centrifuge pelleting technique (Dodds and Roberts 1982), although flotation methods are also available (Gamborg et al. 1981). The pelleting technique simply involves *gently* pelleting the protoplasts to the bottom of a conical-tipped centrifuge tube and discarding the supernatant. At this stage the supernatant will be a mixture of enzymes and cell wall debris. The protoplasts are resuspended at each stage with fresh, osmotically buffered medium without enzymes. After an appropriate num-
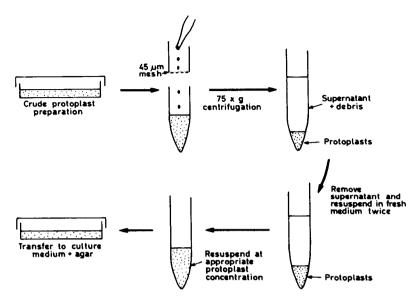
Figure 2.2. Diagrammatic scheme for purification of crude protoplast preparation.

ber of washing steps, normally two or three, purified protoplasts similar to those in Figure 2.3 are obtained.

Planting density and viability determination

The purified suspension will probably contain a mixture of viable and nonviable (damaged, but alive) protoplasts. It is important to determine what percentage of the cells are going to live in order to be able to work out how many protoplasts to inoculate in order to achieve minimum planting density (m.p.d.), as will be described later. The two methods normally used are exclusion of Evans blue stain (Dodds and Roberts 1982) and FDA (fluorescein diacetate) staining. These two staining methods will give an indication whether or not the plasma membrane is still intact (Widholm 1972).

In order to obtain successful growth of the isolated protoplasts, it is important that a sufficiently large number (concentration) of them be obtained. For tobacco, for example, the m.p.d. is $1 \times 10^4/cm^3$. If the protoplasts are cultured at a lower concentration than this, wall regeneration and cell division will not take place.
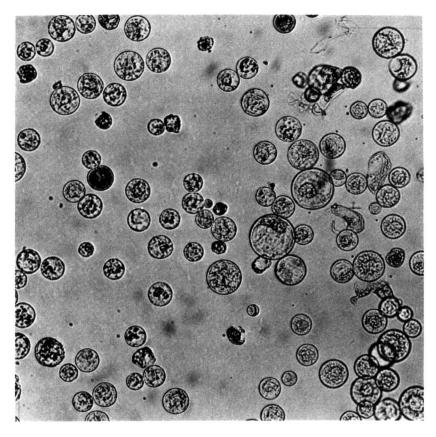
Figure 2.3. Isolated potato mesophyll protoplasts.

Wall regeneration and cell division

After a few hours in culture the isolated protoplasts will begin to develop a new cell wall (Cocking 1970; Davey et al. 1974; Pojnar et al. 1967; Willison and Cocking 1972). This wall formation can be detected by ultraviolet microscopy and the use of calcaflor stain. In a few days a perfectly normal cell wall will have formed. Given the appropriate culture conditions, the cells will then begin to divide and will eventually give rise to a small callus colony (Figure 2.4). It is from these small callus colonies that new intact plants will be regenerated.
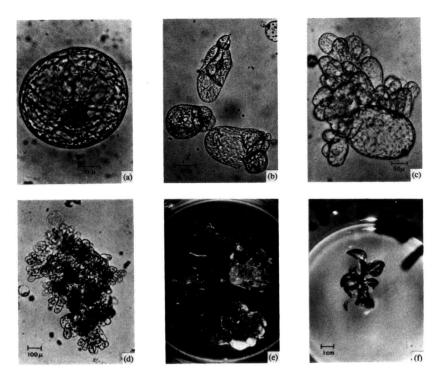
Figure 2.4. Photographic sequence of whole-plant regeneration from single isolated protoplast: (a) freshly isolated protoplast; (b) cell division; (c) small clumps of cells formed; (d) formation of small callus colonies; (e) shoot formation; (f) plantlet. (Courtesy H. Lörz.)

Organogenesis and whole-plant regeneration

Demonstration of the ability to induce organ formation on callus clumps by altering the composition of the culture medium is one of the classic studies in plant tissue culture. Figure 2.5 shows the effects of various hormone combinations on callus segments to promote the inducation of either shoots or roots (Skoog and Miller 1957). By manipulation of the culture medium it is possible to induce shoot formation on protoplast-derived callus (Gamborg et al. 1981; Raveh and Galun 1975).). The regenerated shoots can then be excised and rooted and transferred to sterile soil, thus returning to the normal whole-plant state. The stage of rooting and transfer to