John G. Torrey

The Development of Plant Biotechnology

Building on early discoveries about plant nutrition, researchers have produced an array of techniques for exploiting the genetic potential of plants

Although "biotechnology" has become a familiar term only in the last decade, plant biotechnology has a long, sustained, and exciting history with a remarkable degree of continuity. In this article I plan to follow the major lines of thought that lead up to what most people mean when they refer to plant biotechnology today—that is, the application of existing techniques of plant organ, tissue, and cell culture, plant molecular biology, and genetic engineering to the improvement of plants and of plant productivity for the benefit of man.

The origins of plant biotechnology can be traced back to the 1840s and the ideas of Justus von Liebig concerning the chemical basis of plant nutrition, especially as applied to agriculture. But perhaps a more reasonable beginning date for this history is about 1860, when the work of two German plant physiologists, von Sachs and Knop, led to important new insights. Using an experimental procedure that has come to be called water culture, they demonstrated that many kinds of plants could be grown by placing their roots not in soil but in simple solutions containing only a few salts dissolved in water. Sachs's formulation (1860) of a nutrient solution specified the following salts: calcium nitrate, or ($Ca(NO_3)_2$, magnesium sulfate ($MgSO_4$), potassium dihydrogen phosphate (KH_2PO_4), and a trace of iron sulfate ($FeSO_4$). Provided with these essential elements as salts dissolved in water and with oxygen and carbon dioxide from the air and energy from the sun, most plants can flourish, showing that they are the master biochemists among living organisms.

In the 1860s "pure" salts were not highly purified, and it was another fifty years before plant biologists could demonstrate experimentally that plants require very small traces of micronutrients in addition to the macronutrients used empirically by Sachs. The micronutrients were also shown to be necessary for completion of the life cycle in the case of plants grown from seed. Considering the fact that the earth's surface is made up of more than 100 elements, it is surprising that the list essential for plant growth is quite short, consisting of only 16 elements in the form of gases or dissolved salts: carbon, hydrogen, oxygen, phosphorus, potassium, nitrogen, sulfur, calcium, iron, magnesium, molybdenum, boron, copper, manganese, zinc, and chlorine.

This knowledge of mineral requirements formed the basis of all later plant nutritional research, including the formulation and application of chemical fertilizers for agriculture. By the middle of the twentieth century, Hoagland and Arnon (1950) could feel confident that almost all kinds of plants could be grown with "Hoagland's solution," a balanced mixture of 11 salts containing all the essential mineral elements in appropriate concentrations. With a few exceptions, we know in considerable molecular detail the role of each of these elements, whether they are used in plant structure (carbon, hydrogen, and oxygen), in the structure of proteins (sulfur and phosphorus), or in the energy metabolism of the plant (phosphorus). Calcium is a part of cell walls and enhances the action of enzymes, iron plays a role in respiratory enzymes and in the formation of pigments for photosynthesis, and nitrogen is an important component of amino acids, proteins, and many other constituents of plant cells and tissues. Trace elements serve largely to facilitate the work of enzymes, and the fact that they are essential for growth can be demonstrated only with the most meticulous techniques (see Epstein 1972).

The biotechnological spin-off of these basic scientific discoveries included techniques that were developed in time for use during World War II. "Hydroponics" became the biotechnology of the 1940s. United States military troops stationed on the soilless atolls of the South Pacific were fed green vegetables grown hydroponically with the help of Hoagland's solution and techniques developed by plant physiologists trying to understand the mineral nutrition of plants. These same techniques continue to be used today in agricultural research and production and are still being explored for use in space travel.

Since the early applications of water culture all kinds of ingenious modifications have been developed (Hewitt 1966). In our own laboratory we use such culture extensively in studies of symbiotic nitrogen fixation, omitting nitrogen from the nutrient solution and introducing pure cultures of bacteria which invade the roots and form root nodules. The bacteria then "fix" nitrogen by using N_2 gas from the atmosphere; the gas is reduced to NH_3 and incorporated into amino acids, and thence into proteins and plant structure. In

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another variation on these early techniques we have devised a procedure called "aeroponics," in which the nutrient solution is atomized into a fine mist confined in a dark box. From this mist the roots absorb the elements essential for their growth. The roots are clean and easy to observe, and the plants flourish (Zobel et al. 1976).

Hydroponics and aeroponics are, however, expensive ways to grow plants and are economically feasible only under limited circumstances. Most plant production for man's benefit continues to use the soil as the source and substrate of plant growth, supplemented by organic or chemical fertilizers.

Plant organ culture

Once plants had been freed of their dependence on soil per se for growth and development, it was natural to ask whether it was possible to grow plant parts independent of each other. To what extent do given plant organs depend on other parts of the plant for their survival and growth? Roots, for example, normally grow in the dark, and do not participate in photosynthesis. They must get sugars—the products of photosynthesis in the leaves—by transfer from the leaves via the stem to the root.

In the early 1930s, using information available from studies of mineral nutrition, plant physiologists like White, Robbins, and Bonner (see White 1943) began to investigate the growth of excised root tips in nutrient solutions. Starting with mixtures of inorganic salts that included trace elements, they added sucrose, the most common soluble sugar in plants. All kinds of microorganisms will grow well in such a solution, so they had to work under sterile conditions—that is, they sterilized both the solutions and the surface of the seeds, and germinated the seeds in sterile water. They then excised root tips and transferred them to the sterile solution.

They found, however, that the excised root tips did not grow. They added complex mixtures such as yeast extract, casein hydrolysate, or beef broth extract to stimulate growth, searching for the unknown essential factor. They eventually discovered that the excised root tips of tomato or pea would grow in nutrient solutions very well and in normal form if specific vitamins, including the B vitamins, thiamin, nicotinic acid, and pyridoxine, were added (Fig. 1). These were needed only in tiny amounts—0.1 to 0.5 parts per million—but they were essential for the elongation and development of excised roots in culture.

It was later shown that these vitamins are normally synthesized in the leaves of the intact plant and transported to the roots along with sugar via the phloem, the tissues of the plant that conduct organic solutes. Excised root tips, deprived of their normal sources of these organic compounds, would fail to grow. The sugar must be provided in substantial amounts, comprising 1 to 4% of the solution, whereas the vitamins need be present only in trace amounts.

Since those early studies, other vitamins and organic factors have been demonstrated to be required by some excised roots in culture. For example, in our own experiments with the excised roots of woody dicotyledonous plants (Goforth and Torrey 1977), we found that the sugar alcohol myoinositol markedly stimulated elongation when it was present in addition to the B vitamins. Still other factors have also been proposed as essential for the growth of excised roots. Research on root culture, reviewed in 1966 by Street, has recently been rejuvenated by molecular geneticists, who have found cultured roots useful for experiments in gene transfer (Fig. 1).

The 1930s also saw the beginning of an intensifying pursuit of previously hypothesized but not then identified organic substances believed to serve as plant hormones—that is, substances that act at very low concentrations to control cell and tissue processes leading to normal growth and development. This vast and complex area of plant research can be touched on only briefly here, but the discovery of numerous plant hormones and their isolation and chemical characterization from about 1940 onward form an integral part of the work leading to the biotechnological achievements of today.

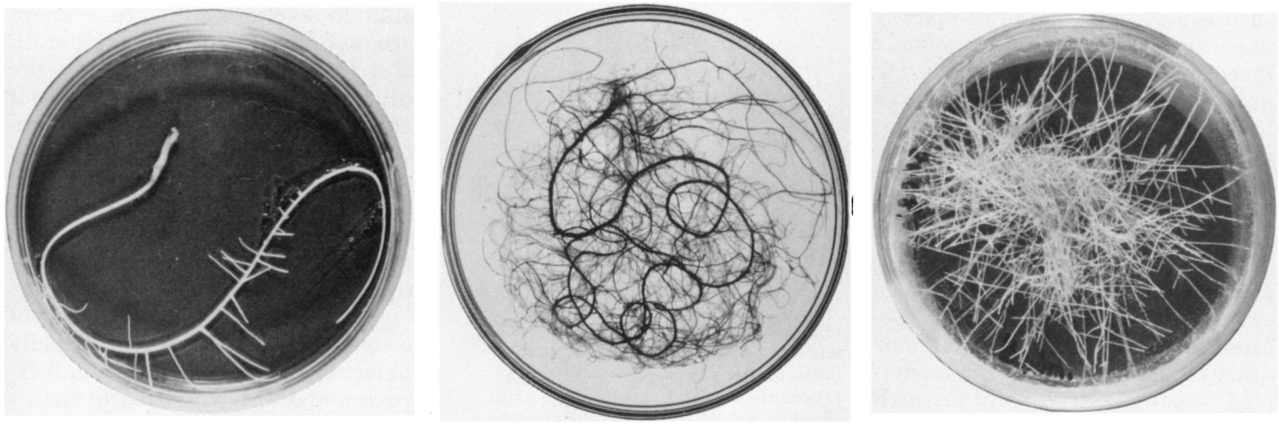


Figure 1. As early as 1860, studies of plant nutrition demonstrated that plants could be grown successfully in solutions of dissolved mineral salts, paving the way for hydroponics and for later experiments in which plant parts such as the excised root tips shown here were cultured separately. At the left, a three-week-old root tip of a garden pea (*Pisum sativum*) grown in a sterile nutrient medium displays the typical branching pattern of a seedling root. The root tip in the center, excised from a germinated seed of sheep sorrel (*Rumex acetosella*), exhibits extensive branching and thickening after several months of growth. The culture at the right represents a further step in plant biotechnology, in which foreign DNA is introduced into plant tissues. The root tip of bindweed (*Convolvulus sepium*) cultured here has been excised from tissues transformed by genetic material introduced from *Agrobacterium rhizogenes*, producing elaborate branching and accelerated growth. All cultures are shown in 10-cm petri dishes. (Photos by author unless otherwise noted; photo at right courtesy of J. Mugnier.)

A major breakthrough occurred when discoveries by Went and Thimann (1937) and other early workers resulted in the identification of indole-3-acetic acid (IAA) as one of the hormones controlling cell enlargement in the shoots of plants. Since then, a whole group of organic compounds with similar biological activity, known as auxins, has been identified. Some of these substances occur naturally, while others are only produced synthetically in the laboratory. The hormone IAA seems to occur naturally at levels ranging from 0.01 to 1.0 parts per million in most plant tissues. It moves around the plant from cell to cell and plays a regulating role in many processes, especially at the cell level—for example, influencing cytoplasmic streaming, cell enlargement, cell division, and cytodifferentiation. The herbicide 2,4-dichlorophenoxyacetic (2,4-D) falls into the class of synthetic auxins. Applied at levels somewhat above the normal physiological ones, it seriously disrupts cell and tissue metabolism, ultimately causing the death of the plant.

A second class of plant hormones called cytokinins includes derivatives of a universally occurring organic compound, the purine base adenine in DNA. Cytokinins such as isopentenyladenine (IPA) and zeatin are synthesized at low concentrations in different parts of the plant and serve as organic regulators of cell division, cell greening, and cell enlargement, among other processes, often acting with other hormones such as IAA in ways not completely understood. There is strong evidence that cytokinins are preferentially, but not exclusively, manufactured in root tips and move from there to other parts of the plant, where they regulate developmental processes.

Other classes of plant hormones discovered and studied since the 1950s include the gibberellins (GAs), abscisic acid (ABA), ethylene (a puzzling and unusual hormone because of its gaseous nature), and some other substances less well characterized. A panoramic view of research on plant hormones is provided by Thimann (1977).

By mid-century two important facts about plant hormones were clear: they are crucial to normal plant development; and since they move about the plant from one organ to another, they are involved in the behavior of isolated plant parts that researchers are interested in growing and studying in vitro. Whenever a plant part is excised so that its development can be studied in isolation, the flow of hormones, inorganic and organic nutrients, and even water from one organ, tissue, or cell to another is inevitably interrupted. To sustain the excised plant part it is necessary to provide all these essentials, including hormones.

If a tiny growing leaf of a plant such as a tobacco or a fern is excised and placed on a nutrient medium like that used for culturing root tips, the primordium, or embryonic leaf, will turn green, photosynthesize, and grow into a mature leaf, smaller than normal but typical of the species from which it comes. Sometimes a root will develop from the base of the petiole and the individual will live for years as a small leaf. Sometimes a small bud will form at the petiole base and then a root, and the leaf will grow into a whole plant. Unlike excised root tips, leaves do not require special supplements, either in the form of vitamins or hormones; they are able to synthesize all they need for near-normal development.

Figure 2. One of the most useful and widely applied techniques of plant biotechnology is meristem culture, a procedure in which the apex of a plant shoot is excised and grown in a nutrient medium to produce a whole plant. When trace amounts of plant hormones are added to the medium, not one but multiple shoots form, as in this culture of French mulberry (*Callicarpa*). Each shoot is capable of growing into a whole plant, or mericlone, making it possible to increase a single plant to a million almost identical plants in a year. (Photo courtesy of J. W. Einset.)

If the tiny apex of the shoot itself is excised and placed in a sterile mineral-sugar medium in the light, it too will develop, provided it is not too small. Shoot tips measuring about 1 to 2 mm in length and possessing 2 or 3 primordiums will elongate and develop leaves, often forming a root and going on to grow into a whole plant. This "meristem culture," a technique pioneered by Morel (1975), has been used commercially to rid plants of viral diseases. Such diseases may limit growth in the field or cause deformed leaves and flowers, resulting in severe losses in agriculture and floriculture.

Meristem culture is effective in controlling disease because viruses well established elsewhere in the infected plant do not grow into the shoot apex. If the apex is excised and grown in culture, virus-free plants can be produced and the crop saved. This method is also used to improve plants that are usually propagated by rooted stem cuttings, such as chrysanthemum and carnation, and to increase productivity in crops such as white potatoes. A great increase in yield results from using virus-free stocks of seed potatoes, normally persisting for three to four harvests, after which reinfection in the field usually requires a repetition of meristem culture to produce new virus-free stock.

It is interesting to note that in the laboratory, researchers have been able to excise and culture shoot apexes only 100 μm in length (Smith and Murashige 1970)—that is, the ultimate apical dome of the shoot apex. Such tiny apexes usually require some supplementary hormones such as gibberellic acid and perhaps enriched levels of inorganic ions such as potassium, in addition to the nutrients needed by excised root tips.

If appropriate trace amounts of an auxin and a cytokinin (usually determined empirically through experimentation) are added to such a medium for culturing shoot apexes, a surprising thing happens. Instead of a single elongated shoot, several new buds or shoot apexes often develop around the base of the initial apex. In place of one shoot, within a few weeks one may find four or five or over longer periods dozens of tiny

shoots (Fig. 2). These tiny plants can either be subdivided and transferred to a fresh batch of medium, where the process can be repeated, or transplanted to a different medium, where each bud can be made to grow into a whole plant. If this process is performed with a rare orchid, a possibility first demonstrated by Morel (1964), a single plant can potentially be increased to a million essentially identical plants, or mericlones, within a year. A summary of these uses of cultured plant parts in horticulture is presented by Hartmann and Kester (1983).

Plant tissue culture

At about the same time that experiments with excised seedling root tips were being carried out, a French scientist, Roger Gautheret, began experimenting with the culture of excised mature root and stem pieces. He excised segments of young, growing tissues from roots such as carrot, the stems of tobacco, and even the woody stems of willows. At first Gautheret used a medium containing inorganic salts, sucrose, and vitamins, but he found that one more component was necessary—a plant hormone such as an auxin. From the stem tissues there developed unorganized "callus tissue," reminiscent of the tissues formed around wounds on the stems of trees. The callus tissue grew quickly as cells divided, enlarged, and divided again. Cultured under sterile conditions on appropriate hormone mixtures, these tissues could potentially be grown indefinitely (Gautheret 1959). Gautheret's carrot callus tissues, started in the mid-1930s, were subcultured continuously for 40 years or more.

Such callus culture, studied also by White and others, is possible with tissues from almost any plant part or plant group—herbaceous or woody, monocot or dicot, gymnosperm or fern (Murashige 1974; Bhojwani and Razdan 1983). The proliferative capacity, rate of development, and cellular characteristics expressed may vary, but the general requirements for the development of plant callus in vitro are now well defined (Fig. 3). One of the most widely used mediums for the culturing of callus tissue is that devised by Murashige and Skoog (1962).

Later experiments demonstrated that tobacco stem tissue cultured in the presence of an auxin such as IAA would grow much better if provided with certain complex organic supplements such as yeast extract. Skoog and his co-workers used the growth of the callus tissue itself as an assay for the unidentified growth-stimulating substance contained in these supplements (Miller et al. 1956). They ended up purifying and chemically characterizing the first cytokinin, which they called kinetin. Thereafter, a number of naturally occurring cytokinins were identified. It became apparent that in the presence of an auxin, cytokinins stimulated mature cells to subdivide and proliferate. Skoog and Miller (1957) later found that by modifying the concentration of cytokinin in the presence of a fixed concentration of IAA, they could control the morphogenetic expression of the tobacco tissue in culture. At a given concentration of cytokinin and auxin, the proliferation of callus tissue could be perpetuated. If the cytokinin level was lowered, roots developed from the tissue; if it was raised, buds and developing shoots would form. If no cytokinin was added, no growth occurred. This remarkable discovery of the hormonal control of morphogenetic expression is the foundation of much of our present understanding of higher plant morphogenesis at the physiological level (Fig. 4).

As these discoveries were being made, another question began to preoccupy researchers: Can any single plant cell, subjected to appropriate nutrient and hormonal stimuli, develop into a whole plant? First it was necessary to show that a single mature plant cell could divide in isolation. That this was possible was demonstrated by the use of a simple but ingenious technique known as

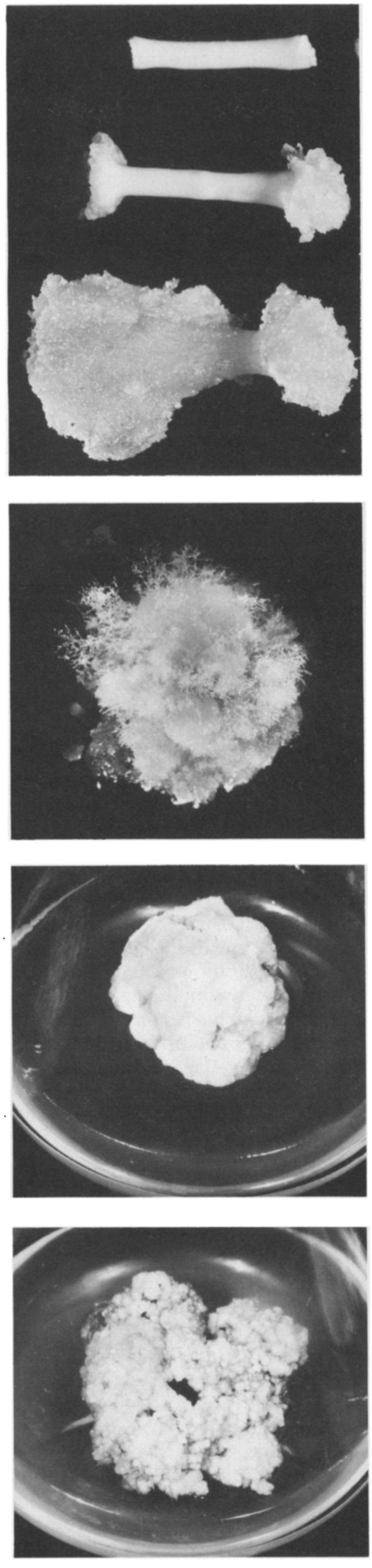


Figure 3. Experiments with excised root and stem pieces led to the discovery that tissues from almost any plant part, cultured in the proper medium, will produce unorganized "callus tissue" which provides valuable material for further manipulation. In the photograph at the top, segments of root tissue of the common bindweed (*Convolvulus arvensis*) cultured in a medium containing yeast extract and 2, 4-D show the typical progression of callus formation. Immediately below, a mutant strain of this callus tissue cultured on agar produces elaborate filamentous branching. Callus tissue may be either dense or friable in texture, as shown in the two bottom photographs of two forms of *P. sativum* callus tissue cultured on complex mediums.

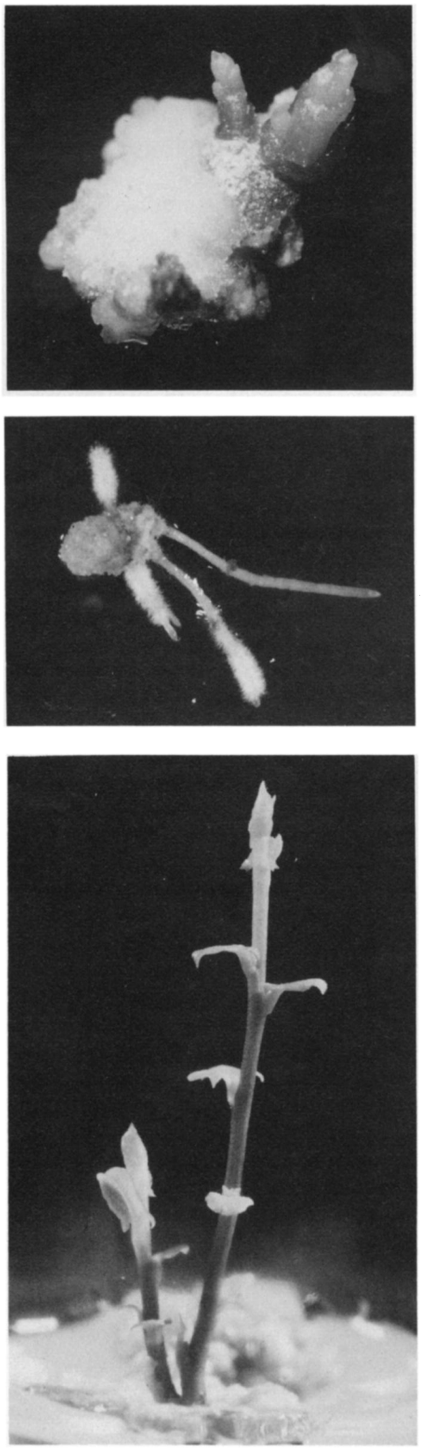


Figure 4. The hormonal control of the morphogenetic expression of tissues in culture, first demonstrated in 1957, was found to depend on the presence of a cytokinin in addition to an auxin. If the level of cytokinin is raised above a certain point, buds will form, as in this colony of root callus tissues of *C. arvensis* grown on an agar medium (*top*). A similar colony shows the growth of roots triggered when levels of cytokinin are lowered (*center*). If buds are allowed to develop in the light, elongated shoots will appear, as in the *C. arvensis* culture at the bottom.

nurse culture (Muir et al. 1958). After pieces of callus tissue were grown on an agar nutrient medium, a small raft of filter paper was placed atop the tissue, where it became saturated with nutrients both from the medium and from fluids conditioned by products of the tissue's metabolism. Single cells from the callus tissue were then isolated by micropipette and placed on the raft, separated from the callus tissue itself. There, after a period of time, the single cell divided repeatedly to form a new mass of callus tissue which could ultimately be subcultured separately from the nurse tissue.

By using this technique of culturing single cells and then inducing morphogenesis by the manipulation of hormones, it became possible to demonstrate conclusively that living plant cells of diverse types from different plant tissues have the genetic capacity to form all the parts of a whole plant through successive cell divisions and cell enlargements—a capacity that came to be known as cellular totipotency. This basic characteristic of living plant cells was elegantly demonstrated and amplified by later studies that used suspensions of carrot cells grown in complex mediums containing coconut milk (Steward et al. 1964).

Callus tissues produced on a solid medium can be transferred to a liquid medium and grown with constant stirring. Under favorable hormonal conditions the cells separate and divide repeatedly, forming a cell suspension that can then be passed through a fine nylon filter to produce a dense suspension of single living plant cells. Such cells can be plated out on a solidified agar nutrient medium, just as one plates out suspensions of bacterial cells, so that single cells can grow into colonies which can then be induced to form organs and a whole plant (Fig. 5). This method of demonstrating cellular totipotency was pioneered by a number of researchers (Bergmann 1960; Earle and Torrey 1965), who showed that single cells had nutrient requirements still more complex than tissue callus—requirements met by the addition of such organic growth factors as myoinositol, vitamins, purines, pyrimidines, or mixtures of amino acids. These nutrients act as least in part by surrounding the cells with those metabolic components which they lose by leaching and thus sustaining them until a tissue mass large enough to perpetuate itself on a less complex nutrient medium is formed.

A final technical feat, accomplished at the start of the 1960s, joined with these past achievements to set the stage for the modern era of plant biotechnology. This was the success of Cocking (1960) in obtaining isolated single cells for culture by separating cells from each other in a living, growing organ such as a root tip. He did this by using enzymes that dissolved the cell walls, so that the membrane-bound living matter within the cells was released and formed a suspension of spherical, wall-less cells, or protoplasts. Given a change in medium and some time, each of these protoplasts formed a new surrounding wall and began to divide, creating a new cell colony (Fig. 6). Protoplasts could be produced in this way from tissues of root, stem, leaf, or other plant parts, and the technique was widely used to produce cell suspensions capable of being plated out and followed to new morphogenetic forms.

One astounding observation (Takebe et al. 1971), anticipated in part by researchers experimenting with tissue culture, was that under appropriate nutrient conditions, including optimum concentrations of hormones, cells that had been regenerated from protoplasts and stimulated to divide could differentiate directly into embryo-like structures capable of developing into whole plants—by the thousands or tens of thousands! In recent years embryogenesis from protoplasts, cell suspensions, or cultured callus has been achieved (e.g., Vasil and Vasil 1982) in a wide variety of tissues from flowering plants (Fig. 7).

In 1964 Guha and Maheshwari reported that when they excised whole anthers, or stamen tips, from young developing flowers and cultured them, young plantlets grew from inside the anthers on a variety of mediums. Some of these plantlets were haploid, and it was deduced that they were derived from pollen cells formed after the meiotic, or reduction, division in the anther had reduced the chromosome number to half—typical of the haploid number of the pollen cell and the male gametes to which they give rise. Pursuing this line of inquiry, other researchers (Nitsch and Nitsch 1969) were able to

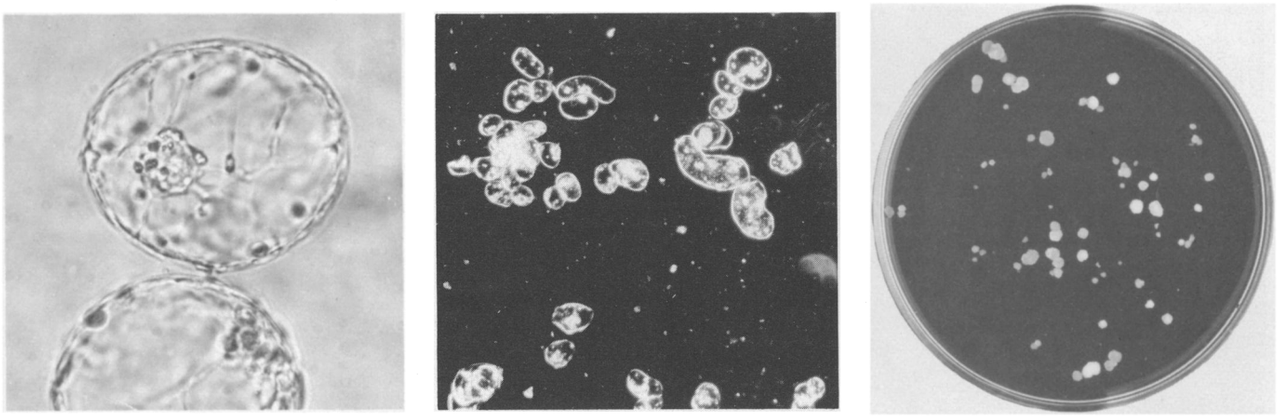


Figure 5. The ability to cause single cells to multiply through the technique of nurse culture allowed researchers to determine for the first time that living plant cells have the genetic capacity to form all parts of a whole plant. This cellular totipotency can also be demonstrated by creating a suspension of single cells in a liquid nutrient (*left*), which is then filtered to form populations of single cells (*center*) that can be plated out on an agar medium. The cells thus isolated divide repeatedly to develop into cell colonies like those shown in the photograph at the right, and these colonies in turn can be induced by hormonal treatment to form whole plants. From left to right: *P. sativum*, × 730; *Acer pseudoplatanus*, × 760; *C. arvensis* in a 10-cm petri dish. (Center photo courtesy of G. Becker.)

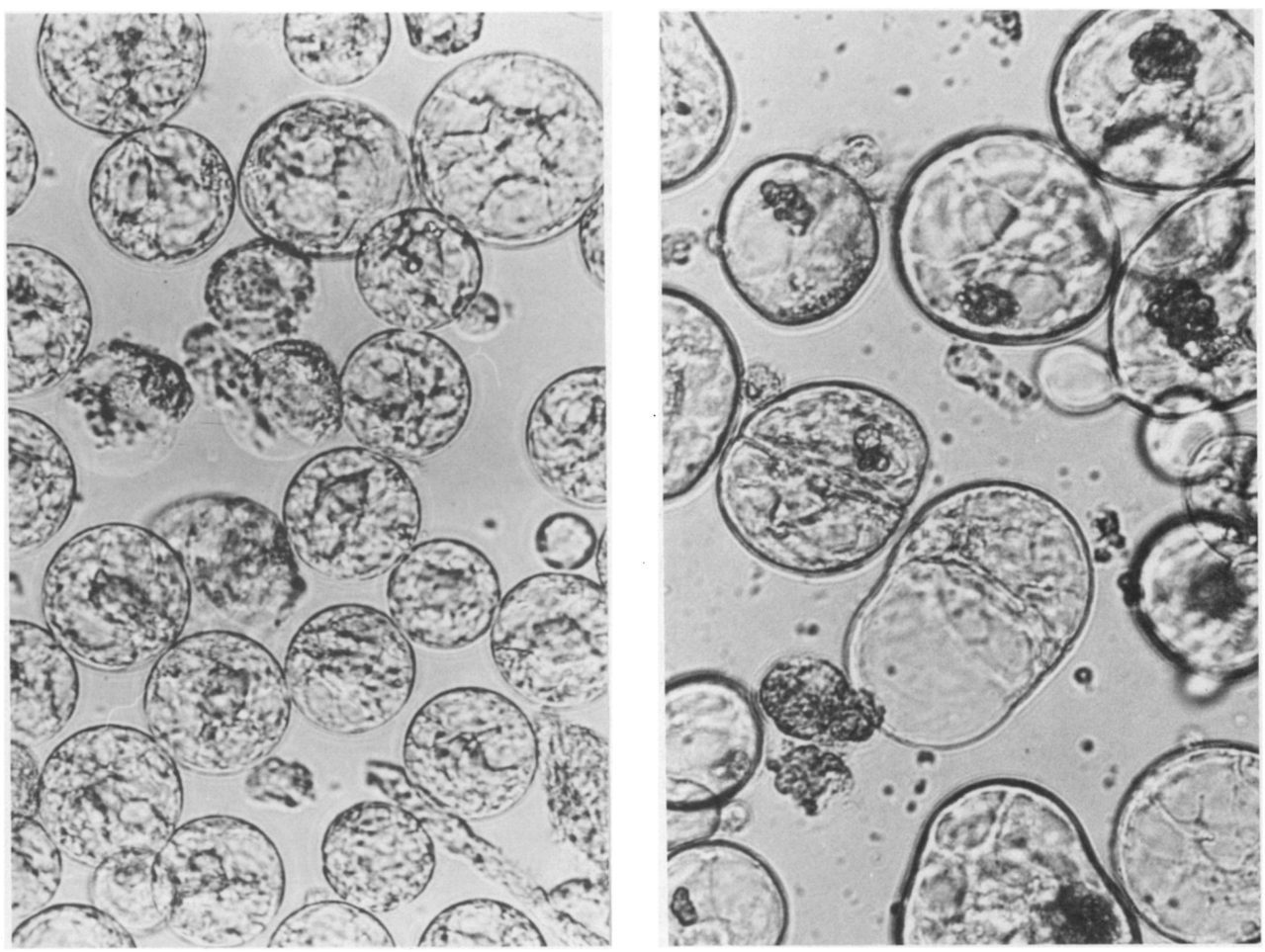
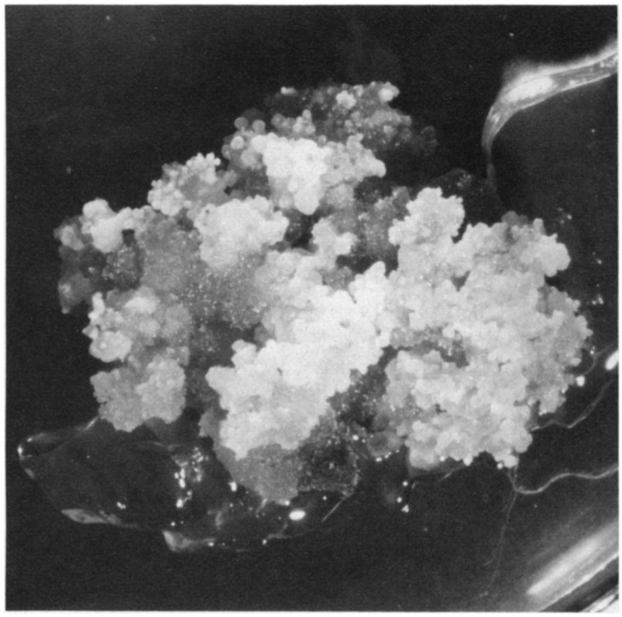
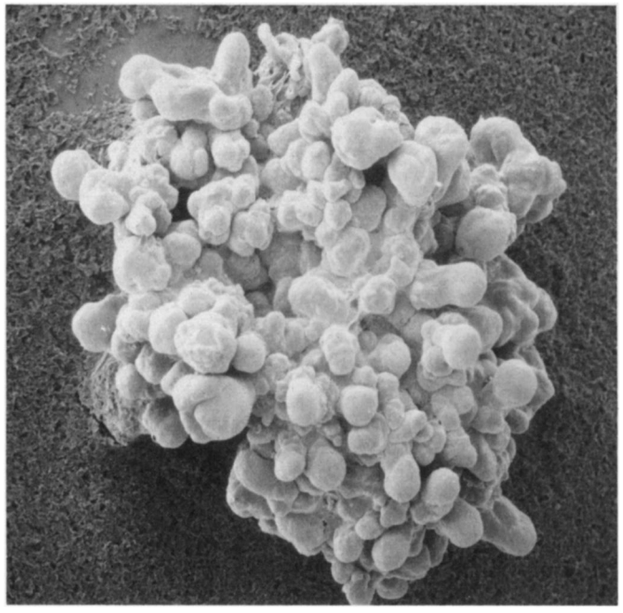


Figure 6. The discovery that hydrolytic enzymes such as cellulases and pectinases could be used to dissolve cell walls in a living plant organ prepared the way for current experiments in which the living, wall-less cells, or protoplasts, of different plants are fused to create new plant hybrids or are used for the introduction of new genetic material. In the freshly prepared culture of cortical cells of a pea root (*P. sativum*) shown at the left, the spherical protoplasts are seen suspended in a liquid nutrient medium with a raised osmotic pressure. In a similar protoplast culture 12 days after preparation (*right*), new cell walls have been formed, as is reflected in the changed cell shapes, and a number of the cells have divided, creating new colonies. Both cultures are seen at a magnification of × 900. (Photos courtesy of C. F. Landgren.)





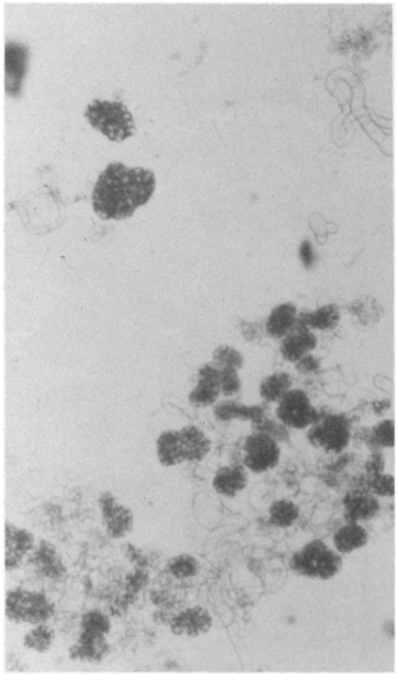


Figure 7. A particularly striking phenomenon is the ability of cells from protoplasts, callus tissue, or cell suspensions to differentiate directly into embryo-like structures in remarkable numbers under certain conditions. Here such embryogenesis is seen taking place in cultures derived from floral tissues of pearl millet (*Pennisetum americanum*). Immediately above, dense aggregates of embryonic cells develop in a liquid nutrient medium. In the photograph at top left, a vigorous growth of young embryoids is visible on the surface of callus tissues cultured in an agar medium; a scanning electron micrograph of a similar culture is seen at the top right. Magnifications are × 184, × 6, and × 25, respectively. (Photos courtesy of I. Vasil.)

prepare suspensions of single isolated haploid pollen cells from tobacco anthers that could be grown to form embryos directly in nutrient culture, producing haploid plants. The haploid material provided by this method of propagation later proved valuable in studies of protoplast fusion in which the bringing together of haploid cells resulted in "normal" diploid progeny. The haploid plants themselves are not able to reproduce sexually and must therefore be propagated vegetatively.

In the relatively short period between the time protoplasts are produced by enzyme treatment and the time they form new cell walls—a matter of hours or perhaps a day—the naked cytoplasmic membranes of protoplasts can be made to fuse, either by the addition of appropriate agents such as polyethylene glycol (Kao and Michayluk 1974; Wallin et al. 1974) or by the use of electric shock (Senda et al. 1979). Protoplast fusing brings together the entire living contents of two cells, and more if care is not taken to prevent it. With careful attention to conditions, the fused protoplasts form a new cell wall and their nuclei enter into mitosis together and divide, forming two cells, each with a nucleus possessing twice the chromosome number of the protoplasts from which they are derived.

This cell fusion is most successful when the two cells share a single parental source. The further apart the protoplasts are genetically, the greater the difficulty—not of cell fusion, which usually does occur—but of the survival of the fused product. A great deal of experimentation, continuing to the present (Potrykus et al. 1983), has been directed toward producing new plant hybrids using somatic hybridization—i.e., the fusion of two different types of protoplasts. In the course of this research we are learning much basic biology, but thus far not many startling new plants of use to man have been created.

Genetic experimentation

A logical next step was the introduction of foreign genetic material into the plant protoplast to attempt to modify its course of development or to cause the cellular derivatives to express new genetic information. Research showed that with the cell wall gone, viruses could easily pass through the protoplast membrane into the cytoplasm. Even whole bacteria such as *Rhizobium*, the symbiotic microorganism in legume root nodules, could be introduced into protoplasts before new cell walls sealed them in. Cells that were infected in this way usually did not survive very long. Other experiments in which genetic material such as purified DNA extracted from various sources was introduced directly into plant protoplasts also resulted in high mortality.

Success in introducing foreign

DNA into plant cells came from another, parallel line of research focusing on the special problem of crown gall, a tumorous disease caused by the soil bacterium *Agrobacterium tumefaciens* which affects a wide range of plants, including many agricultural crops. Using microbial techniques combined with plant tissue culture, Braun (1954, 1962) showed that although the tissues of the initial tumors produced by *A. tumefaciens* in plants such as tobacco contained the bacteria, secondary tumors elsewhere on the plants were bacteria-free. Moreover, he found (1958) that he could culture tissue from these tumors on nutrient medium alone, without adding plant hormones. That is, the tissue itself was synthesizing all the auxin and cytokinin necessary for its own growth—a situation which was perpetuated indefinitely as long as the tissue was being cultured.

In the course of a long series of experiments Braun discovered that a particulate part of the bacterium—material which was inherited and which carried the genetic instructions for the synthesis of plant hormones, among other capacities—had been transferred to the plant cells, thereby conferring the tumorous character. More recently, it has been demonstrated that Braun's tumor-inducing principle (TIP) is in fact a plasmid (Schell 1975), which is carried by the bacterium *A. tumefaciens* and transferred to the plant cell upon infection. A piece of plasmid DNA, the T-DNA, is incorporated into the DNA of the host cells and is thereafter transmitted to the progeny, carrying properties of the plasmid (Chilton et al. 1977). In a comparable way, part of the plasmid DNA in *A. rhizogenes* can be transferred to plant cells. The genetic information conveyed by the Ti-plasmid includes opine synthesis, but instead of tumor formation, lateral root formation is transferred (Fig. 1). This transformation by a bacterium is the perfect mechanism for introducing DNA into a plant cell, and serves as a model for much of the research directed toward genetic engineering of plant cells (Fig. 8).

Problems and prospects

Unresolved problems still remain in research on plant tissue culture, limiting its usefulness as a tool for agricultural improvement (Day et al. 1977). First is the general problem of getting plant tissues to respond in the way the model system behaves. Although it is easy to organize plants from single cells or callus tissues of tobacco and carrot, it is much more difficult in the case of soybean, corn, and important cereal grains, and more difficult still, if not impossible, with many other plants. We simply do not know the basic mechanisms whereby nutrients and hormones elicit these responses.

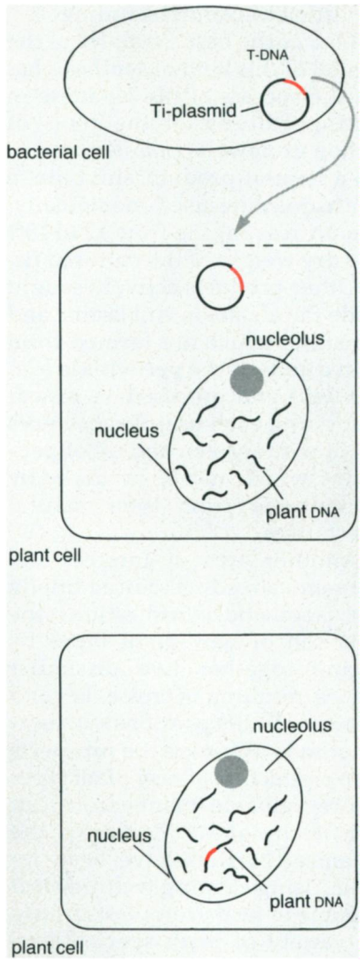


Figure 8. The process by which the soil bacterium *Agrobacterium tumefaciens* transforms plant cells, shown here diagrammatically, offers a promising mechanism for the introduction of new genetic materials. Within the bacterial cell (*top*) is the Ti-plasmid, a large, circular molecule of DNA containing a region known as the T-DNA – the portion of the DNA that normally acts as a vector for the tumor-forming traits. The bacterium infects the plant through a wound, inserting the Ti-plasmid into the plant cell cytoplasm (*center*). The T-DNA then enters the plant nucleus and becomes incorporated into the nuclear DNA of the host cell (*bottom*). When the transformed cell divides, the T-DNA is replicated along with the nuclear DNA. Tissues derived from the transformed cell may express the genetic material of the T-DNA, and thus also any foreign genes spliced into the T-DNA to produce desirable traits.

Similarly, although the use of protoplasts to regenerate whole plants by embryogenesis or organ initiation works well for tobacco, petunia, carrot, rapeseed, asparagus, and *Datura*, effective procedures have not as yet been established for most species of plants. Even more basic is the problem of the instability of the chromosomal constitution of callus tissues grown in culture. Callus tissues tend to double chromosome numbers or lose individual chromosomes, introducing deleterious genetic changes that make them less useful for plant propagation, as a source for plant cells, or as material for protoplast manipulation.

Other problems include the lack of an easy method of producing genetic markers for cell selection, the difficulty of generating and maintaining haploid tissues readily, and the uncertainty of routinely initiating embryogenesis from cells. Barriers have also been encountered in attempting to induce plant tissues in culture to express their known genetic potential, for example by forming valuable secondary metabolites.

These difficulties should be seen, however, against a background of successful or emerging applications of the techniques of biotechnology. The methods of meristem culture are already in use today by hundreds of growers for the production of large numbers of plants of relatively high commercial value which are otherwise difficult to propagate. Orchids are the example par excellence. Ornamental plants such as begonia, Boston fern, day lilies, and others lend themselves to the technique and, when grown to maturity, bring a price justifying the somewhat greater expense of using in-vitro culture. For crops such as potato, where virus-free stock makes a dramatic difference in productivity, meristem culture has proved a remarkable tool.

In another application, the methods of plant tissue culture have been adapted to make the long-term storage of plant parts economic and effective. Plant structures, usually cultured shoot apexes or embryos, are placed in cold storage at 4 to 9°C or

frozen in liquid nitrogen at −196°C after treatment with a protective substance. They are held at these temperatures for months, even years, and then thawed and returned to subculture and propagation. This storage, which replaces expensive propagation in the field, is economic with regard to the cost of space and maintenance, allows rapid recovery for further propagation, and retains the plant material in a genetically stable state free of pests, pathogens, and viruses. Such aseptic materials can be shipped around the world without problems of quarantine or the need for disinfection. Although still experimental, these methods also show great promise for the preservation of germplasm.

Cultured plant tissue itself has been useful in a number of ways. The conversion of cultured plant tissues to cell suspensions makes it possible to plate out millions of plant cells, each theoretically capable of forming a whole plant. At these high numbers it is reasonable to seek spontaneous mutations which might prove valuable, and the possibility of useful mutations is sometimes enhanced by treating the cells with mutagens such as ethylmethane sulfonate or nitrosoguanidine. By a choice of appropriate mediums, researchers can select cells tolerant of special conditions, such as the presence of high concentrations of salt or specific herbicidal substances. Most cells will die, but cells with natural resistance will grow, allowing workers to single out the exceptional cells which will develop into plants better adapted to a given field situation. Such selections have been made not only for tolerance of saline soils and herbicides but for a number of other traits—for example, resistance to drugs or the ability to grow in the absence of certain metabolites—desirable either in the laboratory or the field. Although these methods have proved to be effective in field trials, so far none of the mutations selected has led to significant economic success.

Plant tissue culture has also been used to harness the special, sometimes unique, biosynthetic capacities of selected plant cell populations. This approach involves culturing callus tissues in bulk under conditions that allow the cells to form (ideally in large amounts) a secondary product that has economic value—such compounds as drugs, oils, fragrances, pigments, and the like, which have usually been collected from plants grown in the wild, often in exotic, out-of-the-way places. Here the problem is how to make cultured tissues switch on the genes leading to the synthesis of the special product desired. In a sense this means trying to induce the specific cytodifferentiation of cell populations, a process about which we still have little basic understanding.

One of the best examples of the potential of this kind of synthesis has been the success of the Japanese in inducing cultures of the roots of common gromwell (*Lithospermum*) to form a natural product, shikonin, a naphthaquinone used medicinally, in quantities ranging from 12 to 15% of the dry weight of the cultured tissue. Other products actively sought include the alkaloids vinblastine and vincristine, which are formed from cultured cells of the periwinkle (*Catharanthus*) and are used in cancer therapy, and such useful substances as oil of wintergreen and oil of peppermint, which may be produced by cells cultured from their natural plant sources.

Another area of interest and excitement, already discussed briefly above, is somatic hybridization—the production of new plant types by bringing together two dissimilar genomes, resulting in crosses beyond the normal limits possible with sexual methods. What are the prospects for the production of new plant types or more valuable plants from cell fusion techniques? Certainly the mechanical barriers have been removed. Using existing techniques it is possible to fuse protoplasts from a wide variety of plant species. Further, there appear to be no major blocks to the fusion of protoplasts across generic or family lines. Yet in spite of the success of cell fusions across generic lines, few such crosses have gone on to form tissues capable of regenerating whole plants. Thus far interspecific somatic hybrid plants have been obtained from five genera: *Nicotiana*, *Datura*, *Solanum*, *Petunia*, and *Daucus*; some of these crosses involve species that are not sexually compatible. As Harms (1983) states in his recent review of somatic incompatibility in plants, however, on the basis of our present knowledge it seems doubtful whether remote somatic hybrids can produce regular, functional, competitive, and fertile plants.

One of the most attractive prospects for the future is the use of cultured plant tissues, cells, or protoplasts as receptors for selected cloned DNA conveying specific genetic information which can thereafter be expressed either in the cultured tissue or cells or in organized plants derived from the cultured cells. This approach combines the methods of plant tissue culture with the capabilities of genetic engineering, leading to a number of interesting possibilities (Chaleff 1981; Kahl and Schell 1982; Kosuge et al. 1983; Varner 1983). To date the best model available is that based on the mechanism used by the soil bacterium *A. tumefaciens* to induce crown gall disease, outlined above. The current strategy is to attempt to use the Ti-plasmid, depleted of its tumor-forming genes, as a vector for other, favorable genes selected and cloned from bacterial or higher plant cells or other gene sources and carried in the transformation process to the host plant cell. Thereafter the newly inserted genes will be expressed, it is hoped, in desirable and useful ways.

Rapid progress is being made in achieving these goals. Although other vectors are being studied, the greatest effort among a number of laboratories has been in developing the use of the Ti-plasmid from *A. tumefaciens*. Plants studied as possible hosts are relatively few, including tobacco, petunia, carrot, potato, and flax. Work with these model systems has now demonstrated (Zambryski et al. 1983) that it is possible to modify the Ti-plasmid by gene deletion to allow efficient T-DNA transfer without tumor formation—that is, a non-oncogenic Ti-plasmid derivative has been constructed, which acts as a general vector capable of transferring any gene of interest into cells of higher plants. Further gene constructions use the regulatory regions of the nopaline synthase, or *nos*, genes, which control and direct the expression of specific gene traits carried by the T-DNA. These genes serve as donors for transcriptional signals which allow the expression of the foreign genes integrated into the T-DNA.

Thus specially engineered foreign genes can be introduced via the non-oncogenic Ti-plasmid and transferred as T-DNA into the DNA of host plant cells, which are thereby transformed. Whole plants can be

regenerated from these selected transformed cells, using the techniques of cell and tissue culture. The regenerated plants are phenotypically normal and fertile, and the newly transferred genes are inherited as Mendelian traits. Such traits may be expressed throughout the tissues of the plant (as is the case, for example, with resistance to drugs such as kanamycin, a trait which has recently been studied in two different laboratories), in specific tissues or organs, or in response to specific environmental cues.

Caplan and his colleagues have noted (1983) that the successful use of DNA transfer vectors for plants depends on advances in several major areas of plant research, namely, the isolation of particularly interesting genes, the analysis of their control, and the improvement of the techniques of plant tissue culture to make it feasible to study more agronomically important species. Progress in these areas will make it possible to study the mechanisms of plant-specific gene regulation in both plant tissues and whole plants, and may lead to an understanding of other functions that promote resistance to pathogens or increase nutritive or medicinal value (DeBlock et al. 1984).

What does seem certain is that the ability to identify, clone, and transfer genes not only for specific biochemical steps but for whole sequences of steps and even whole morphological events will open new avenues to an understanding of normal processes of plant biochemistry, physiology, and development. From this knowledge will come further applications in agriculture of benefit to man.

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