nologies are only at the developmental stage and, unlike fusion, are not yet routine.

The manipulations described are eminently feasible and in some cases have been successfully accomplished. However, we are only at the beginning of somatic genetic manipulation of crops, primarily because many important crop plants behave poorly in culture. For example, corn protoplasts usually cannot divide; soybean callus does not regenerate to plants. Also, too little is known about the fundamental processes of plant development and gene regulation. Once a foreign gene is introduced into a plant, one faces the next stage of problems: will the gene express in the appropriate organ; will it cause side-effects to weaken the plant? These questions must be addressed and solved before somatic methods can be employed to produce better crops.

Several aspects of tissue culture are currently being applied to agriculture. For example, the orchid industry now relies almost exclusively on tissue culture to propagate orchids that are difficult to breed. Also, tissue culture multiplication can often be used to eliminate virus contamination in seed stock.

Vegetative propagation by tissue culture from a single plant might be expected to yield identical plants, because all cells would be of identical genetic constitution, barring very rare mutational events. However, quite striking variability has been found in regenerated plants. This variation may offer a new source of valuable genetic traits for plant breeding.

Another application of tissue culture is in the increasing use of haploid plants. Germ cells, usually the immature pollens either enclosed in (another culture) or isolated from the anther (pollen culture) are cultured to produce new plants. Since these germ cells are haploid, the derived callus or plants are also haploid—having only half the chromosome number of the diploid parent. Such plants can be treated with colchicine and made diploid again. During the processes, the plant becomes homozygous at all loci. Plant breeders often have to self-pollinate strains for many generations to produce "true-breeding, pure" lines. The anther or pollen culture provides a quick way to produce homozygous lines in a single step. Finally, somatic genetic systems based on haploid cultures have the advantage of allowing isolation of recessive mutants.

We believe we can look forward to tissue culture making a modest contribution to plant breeding and agriculture in the next few years. This contribution might be expected to increase radically as the capability of manipulating crops in culture improves, gene transfer and cloning technologies develop, and the knowledge of plant growth and development increases.

Protoplast regeneration

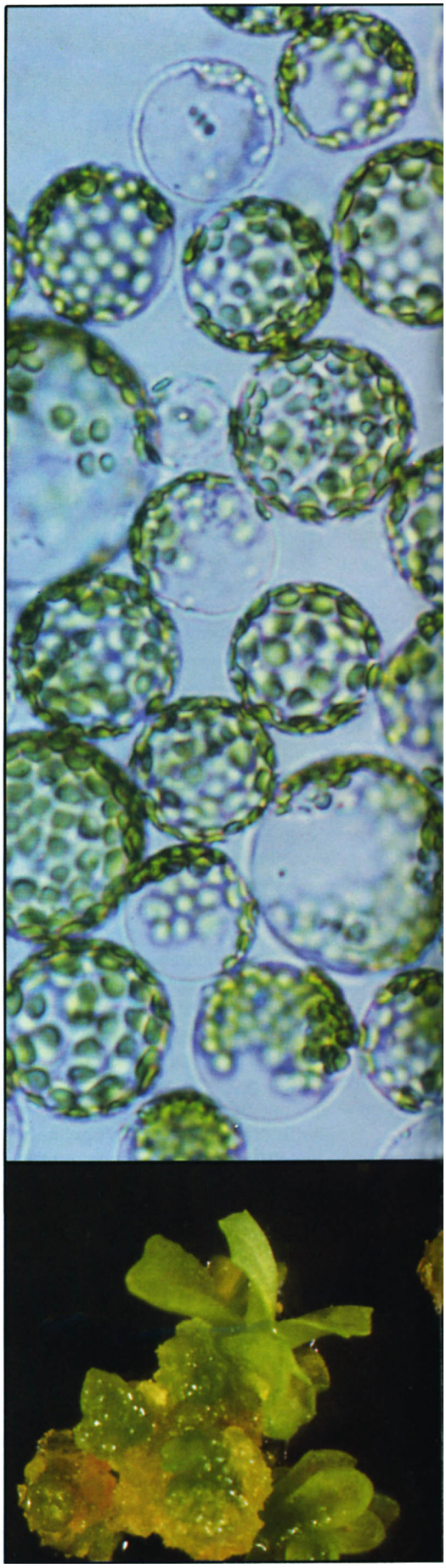
Dean E. Engler
Raymond G. Grogan

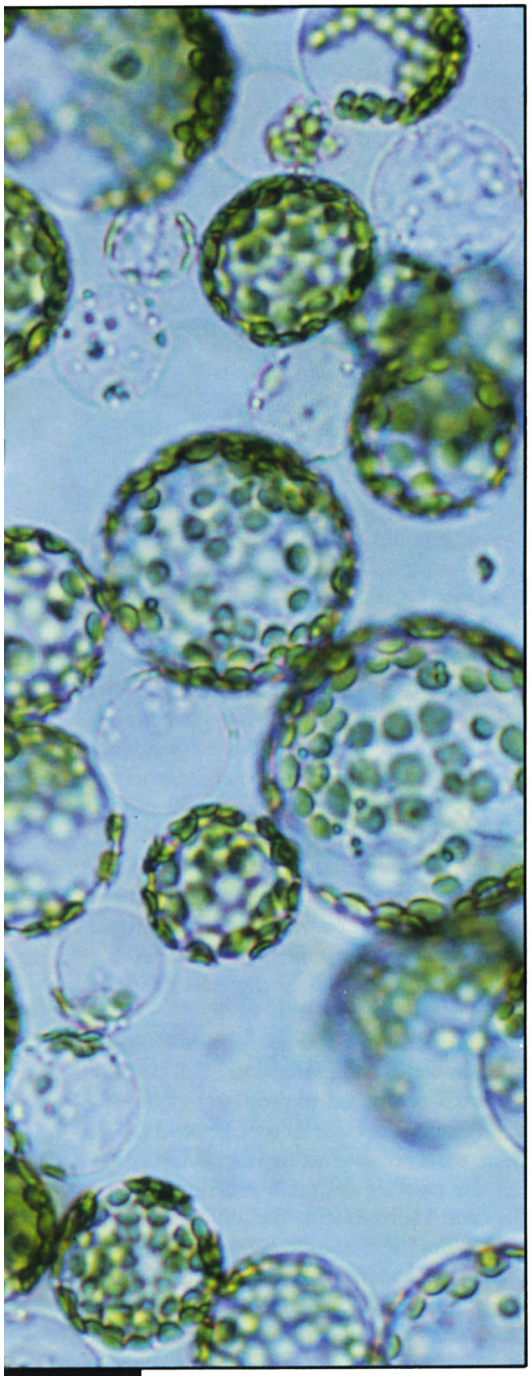
Plant cells without walls (protoplasts) can be isolated from leaves by a process of enzymatically digesting away the middle lamellae between cells and the cell walls. Tremendously large numbers of protoplasts can be isolated from a single leaf; yields are typically two to four million protoplasts per gram of leaf tissue. Development of techniques and procedures causing isolated protoplasts to reform their walls, proliferate, and regenerate into whole plants is essential for the utilization of the new genetic technology.

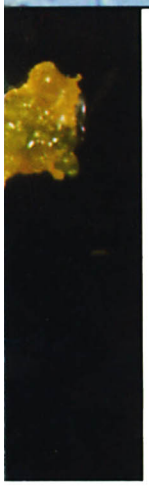
In recent months we have succeeded in developing culture media and the methodology for regeneration of lettuce protoplasts into whole plants. When isolated lettuce protoplasts are maintained in the right conditions, they can be induced to reform their walls and divide to form unorganized clumps of cells (P-calli). These P-calli are transferred to media with the proper balance and concentration of plant hormones and other ingredients to induce the formation of shoots. The shoots then are transferred to media for further growth and eventual root production. These regenerated plants then can be transplanted into a greenhouse for seed production and, finally, the progeny are evaluated and selected for desirable characteristics in the field.

One would expect all plants regenerated from a single lettuce leaf to be identical, since their production involves no sexual process. Observations of regenerates, however, reveals the astonishing result that many of them are different from the source plant and from each other. Other researchers have found a similar frequency of variation in potatoes, which has proved to be stable over many generations. Although the reasons for genetic variation among regenerates are not fully understood, it may be possible to obtain desirable improvements in horticultural characteristics, such as enhanced green color, uniform maturity, and resistance to diseases, because of this inherent variability of protoplast regenerates.

The millions of protoplasts that can be cultured in a single petri dish can be subjected to specific selection pressures that will eliminate all but the very few tolerant protoplasts. For example, many plant-disease-causing organisms produce toxins that can be incorporated







Above: Micrograph of freshly isolated lettuce leaf protoplasts containing bright green chloroplasts.

Left: Protoplast-derived calli giving rise to lettuce plants.

Above right: Lettuce plants ready for transplanting. Each plant originated from one protoplast.

into protoplast regeneration media, thus allowing the few resistant cells to grow into calli, while killing the millions of sensitive cells. In lettuce this technique has been used to select cells capable of surviving exposure to oxalic acid (the toxin produced by the lettuce drop organism) and ethylene gas (the chemical responsible for injury resulting in russet spotting). Selection pressures also can be applied to select for characteristics other than disease resistance. Cold and heat tolerance could be selected for, as well as tolerance to chemicals such as salts and herbicides.

We have used this selection pressure technique to identify the few lettuce protoclones capable of proliferating on media containing greatly reduced calcium levels. We hope that plants regenerated from these protoclones will be resistant to tipburn, a disease caused by a deficiency of calcium in the interior tissues of head lettuce.

Crops have been improved over the years by crossing plants with desirable traits, but the parent plants must be sexually compatible. The use of protoplast fusion can remove this restriction. Protoplasts from any two plants, regardless of species, can be fused. In many cases it has been possible to regenerate fused protoplasts into whole plants, resulting in "somatic hybrids" or asexual crosses. In theory, this technique opens up the potential "gene pool" of a plant to every other biological organism. In lettuce, this means that we can now tap the vast resources of wild lettuce species that contain desirable characteristics, such as genes for disease resistance, but that have previously been unavailable to breeders because of sexual incompatibility.

Crop improvement with this new methodology seems promising. Field testing and selection for desirable characteristics in head lettuce plants derived from protoplasts will be done for the first time in 1982.

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Regeneration of plants

Toshio Murashige

Reporting his pioneering experiments on plant cell culture to the German Academy of Science in 1902, G. Haberlandt predicted that someday "in this way one could successfully cultivate embryos from vegetative cells." Had IAA and kinetin been at his disposal, Haberlandt might have realized his prediction, and there is no telling how much further plant cell culture might be today.

Widespread success with plant cell cultures was made possible when the plant hormones auxin and cytokinin were discovered and when F. Skoog and C. O. Miller revealed in 1957 that regeneration of shoots and roots in cultured cells could be manipulated simply by varying the proportions of these hormones in the nutrient medium.

Genetic engineering of crop plants must usually begin with single cells or protoplasts as the objects of molecular manipulations. The effort must culminate with reconstituted plants. Regeneration of plants from isolated cells currently follows one of two pathways. In the first, plants are obtained through a sequence of shoot formation followed by rooting of the shoot. In the other, embryos—that is, structures with simultaneously differentiated shoots and roots—are initiated.

A series of nutrient formulations is usually required by either path. In the method of separate shoot- and root-forming steps, a critical cell mass, or callus, is prerequisite to any organ formation. An auxin and sometimes also a cytokinin must be provided for callus development. When transferred to a medium containing a relatively high level of cytokinin and a low level of auxin, the callus differentiates shoots. Shoots of suitable size are separated and recultured in still another medium, one lacking cytokinin but containing some auxin, to generate roots. Additional supplements, such as adenine and tyrosine, may enhance the shoot initiation step. Similarly, rooting may be improved by reducing the salts and by including cofactors, such as phloroglucinol and caffeic acid.

In the embryo method, the cells are first stimulated to divide and induced, or otherwise prepared for eventual development into embryos. The induced cells are then allowed to proceed with embryo formation. The medium for the cell division and induction phase often contains an auxin, such as 2, 4-D; a high level of nitrogen, preferably NH_4^+; and sufficient potassium. For the embryo devel-