WILLIAM BROKAW: No, but you could certainly get full year-round production by planting two cultivars and heavy two-year production with three cultivars. I would recommend planting three trees of different cultivars in one hole.

VOICE: Where does DNA recombination fit into tissue culture research in the future and in plant hybridization.

HUDSON HARTMANN: We are going to have a talk on Friday on this subject so if you can stay until then you will, perhaps, get all your questions answered. Cell and tissue culture research is a new field but DNA recombination is probably quite a ways down the road for plant propagators, although it is something coming up in the future. Genetic engineering, where you might develop your own plant, is a fascinating idea, but it is not in the immediate future for plant propagators.

VOICE: What is the strength of the Clorox solution that you use for avocado seeds; a second question is whether or not you condition the seed before dipping in Clorox.

WILLIAM BROKAW: We use 10% of the 3% household bleach. The answer to the conditioning is essentially — no. What we have is kind of a cold water dip, then we dip them into the Clorox solution. Shortly after that we give them a Captan treatment.

VOICE: At what time, if ever, do you excise the original nurse seedling in your nurse root grafts?

WILLIAM BROKAW: Never, it is automaticaly aborted. With the nurse root graft procedure we use now the root is automatically cut off and we have never found a failure.

POTENTIAL APPLICATION OF PROTOPLASTS FOR FUTURE PLANT IMPROVEMENT

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INTRODUCTION

Traditional plant breeding methods for commercial improvement of plants are restricted to hybridizing plants that are closely related. With few exceptions, hybrids combining desired qualities derived from both parents can only be made between different species of plants. These F_1 hybrids are usually self-sterile and require doubling of the chromosomes before they can be used for further breeding purposes. Intergeneric hybrids are very rare. In recent times, some notable discoveries have come out of fundamental research on plant tissue cultures.

Some of these discoveries have already given rise to practical applications, such as embryo cultures of seeds, which would not otherwise germinate, to obtain rare hybrids, and creation of virus-free stock and rapid vegetative propagation of rare plants. Furthermore, production of haploid plants by anther culture and subsequent doubling of the chromosomes have provided pure line materials. More recently, special attention is being focused on the potentialities of introducing new genetic information directly into protoplasts. Protoplasts are individual plant cells which have been treated to remove their outer cell walls. Without the barrier of the cell wall, a naked protoplast is vulnerable to virus infection, fusing with protoplasts of an entirely different species of plant, and taking up genetic materials such as DNA. In this brief paper, an assessment of recent progress will be made as well as the prospective value of this research in regard to future plant improvement.

Isolation and Culture of Protoplasts. Whereas numerous investigators throughout the world have made important contributions, the results emanating from Cocking's laboratory in England and Takebe's laboratory in Japan have been in the forefront. The Cocking group demonstrated the mass production of protoplasts after enzymatic treatment of cells of tomato seedlings (4). Takebe and collaborators were the first to show what conditions were necessary for protoplasts to regenerate new cell walls and then divide to form a callus of new cells from which entire plants could be created (15,23). Thanks to the previous pioneering efforts of Steward, Skoog, and their collaborators, it was already known what conditions were required to transform the undifferentiated cells of a callus tissue culture into entire plants indistinguishable from the plants that gave rise to the tissue culture cells in the first place (20,22).

The successful isolation of protoplasts has now been reported for more than 50 plant species (16). Of these, regeneration of the protoplasts back into plants have been reported for several cultivars of tobacco (*Nicotiana tabacum*), *Petunia* × *hybrida*, carrot (*Daucus carota*), etc. (26). In many plants, even though protoplasts can be formed, it has still not been possible to cause them to regenerate back into entire plants. Because of this difficulty, this is a very active field of investigation particularly in regard to regeneration of plants from protoplasts of crop plants such as soybean, rice, corn, wheat, etc.

Techniques Being Explored on How to Introduce New Genetic Information into Protoplasts. Three methods are currently under intensive investigation in various laboratories throughout the world: Fusion of protoplasts derived from two different species of plants; Introduction of nuclei or chloroplasts of one plant species into protoplasts of another; Introduction of par-

ticular genes in the form of DNA molecules isolated from one species of organism into the protoplasts of another species.

1. *Somatic hybridization through protoplast fusion.*

There are now two recorded instances of creating entirely new species of plants by fusion of protoplasts (2,19). In addition, three new cultivars of *N. tabacum* have been created by fusing protoplasts of two different cultivars (6,9,14). The first creation of completely self-fertile plants by fusion of protoplasts of *Nicotiana langsdorffii* with protoplasts of *N. glauca* by Carlson, Smith and Dearing (2) is recognized as an exciting achievement of modern plant genetics. This success came about because it was possible to select for fused protoplasts containing the *N. langsdorffii* + *N. glauca* genomes because they required non-hormones to grow as a callus culture rather than homologous fusions of either *N. langsdorfii* or *N. glauca* protoplasts alone. The further exploitation of this success by Smith, Kao and Combatti (21) came about by a discovery made by Gamborg and his associates in Canada that polyethylene glycol was a very effective agent in promotion fusion of protoplasts (10). Besides the new species of *Nicotiania*, fusion of *Petunia* × *hybrida* and *P. parodii* protoplasts have created a new species of *Petunia* (19). These successes have promoted a great deal of experimentation to see whether hybrids could be made between protoplasts belonging to different genera such as soybean and wheat with the hope of combining the soybean's nitrogen fixing capacity with wheat's capacity to form grain. There has been success in causing the fusions to occur but so far there are no published reports that the fused protoplasts will continue growth to produce a callus which is capable of regenerating a new intergeneric hybrid plant species.

In the case of the new species of *N. langsdorffii* and *N. glauca* plants, fusion of protoplasts resulted in combining the genes contained in the DNA of the nuclei of both of the species and subsequent fusion of the nuclei. Thereafter, the cells derived from the fused protoplasts contained a nucleus with one set of chromosomes derived from *N. glauca* and one set from *N. langsdorffii*. This permitted the new hybrid to become self-fertile because during meiosis, the *N. glauca* chromosomes could pair with each other and the *N. langsdorffii* chromosomes with themselves and both sets undergo an independent, but a normal reduction division which would result in fertile eggs and pollen. In addition to the genetic information contained in the genes of the chromosomes of nuclei, there also exists genetic information contained in the DNA found in chloroplasts and mitochondria. It is this extranuclear genetic information which is the basis of cytoplasmic inheritance. The genetic factors in cytoplasmic inheritance can have profound effects on the

expression of nuclear genes. Also the genetic factors of cytoplasm can, in most cases, only be inherited by the maternal line. They are therefore not subject to change by the conventional breeding techniques whereby genes in nuclei can be manipulated more or less at the convenience of the plant breeder.

Fusion of protoplasts would seem to offer the promise whereby plant breeders could begin to control the cytoplasmic factors. However, in the *N. langsdorffii* + *N. glauca* case, there is a difference in cytoplasmic factors between the two species of plants. but it is a disappointment so far that in all of the hybrid plants created, there has been no evident mixing of the genetic information in the cytoplasms as compared to the complete mixing of the nuclear genetic information. The new hybrids contain cytoplasms either of the *N. glauca* or the *N. langsdorffii* type, but not both (3). Consequently, more research will have to be done before the plant breeder can expect to control the genetic factors in cytoplasm.

2. *Transfer of the genetic information contained in chloroplasts and mitochondria of one species into protoplasts of another species.*

This approach is being tried as one possibility of overcoming the barrier in changing the genetic information in cytoplasms. The idea is to use the chloroplast or mitochondrion as the vehicle to place new cytoplasmic genetic information in a protoplast. Experimentation has advanced to the point where it can be demonstrated that isolated chloroplasts can be caused to be absorbed into the interior of protoplasts (1,18). While the chloroplasts appear to survive after mitosis of protoplasts resumes, there has been only one report of a plant regenerated containing chloroplasts identical to those introduced into the protoplast (11). Efforts to duplicate this finding have so far been unsuccessful (Uchimiya, H., unpublished data). There are many problems to solve such as whether the DNA of the chloroplasts survives in the protoplasts and, if so, what causes it not to function in forming new chloroplasts of its own type.

The practical importance of being able to change the genetic information in cytoplasm is illustrated by recent experience in hybrid corn production. Practically the entire hybrid corn agriculture of the U.S. was based on a high yielding corn containing a particular cytoplasm known as Texas cytoplasm. However, in recent times, this type of hybrid corn was destroyed by a fungus. The ability of the fungus to attack this corn was directly traced to the presence of the Texas cytoplasm and it is believed that the fungus secretes a toxin which interferes with activity of the mitochondria in the Texas cytoplasm (13). It is probable that nothing can be done about this problem until it

can be learned how to introduce new mitochondria resistant to the fungal toxin.

3. *Uptake of DNA as a means of introducing new genetic information into protoplasts.*

There are a few reports that a foreign DNA applied to callus, roots or seeds has produced changes in the plant that could only be attributed as having come from the genetic information contained in the foreign DNA (7,12,24). However, repetition of these various experiments has not occurred with enough frequency to encourage their use as a means for improving plants.

Another possible means of unconventional altering the genetic make-up of plants involves the uptake of foreign DNA itself by protoplasts (5,8,17). The hope would be that the foreign DNA could integrate with the host DNA and use the host's mechanism to get the new genetic information expressed and create a change in the plant phenotype. Conditions have been established whereby protoplasts have taken up as much as 10% of a foreign DNA supplied them (25). It could be shown that some of the foreign DNA survived for a prolonged period of time within the host protoplasts (25). It remains to be seen, however, whether the genetic information in a foreign DNA is ever expressed in plants regenerated from protoplasts. It is also clear that a great deal more experimentation will be required before any easily reproducible transformation of a plant's phenotype by treatment with a foreign DNA will be attained.

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