The Use of Protoplasts for Genetic Research

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Protoplasts isolated from cells of higher plants have provided a new, much heralded, experimental material for cell biologists and physiologists (1, 2). Though the potential of plant protoplasts as a system for genetic research has often been stressed, very little has been written about the kinds of unique genetic questions that can be resolved by the use of protoplasts. This paper describes the attributes of plant protoplasts relevant to their use in genetic experimentation, and reviews recent work from the author's laboratory using protoplasts in a diverse array of experimental contexts. For a discussion of the method involved in protoplast isolation, manipulation, and culture, several recent review articles may be consulted (2–4).

Protoplasts have several characteristics that make them a unique material for the study of the genetics of higher eukaryotes.

(i) It is possible to generate large, homogeneous populations of protoplasts of known genetic composition from single plants. Protoplasts of any genotype, be it haploid or diploid, mutant or aneuploid, can be easily obtained. This property is in contrast to single cells derived from in vitro cell cultures, that vary markedly in their genetic composition and physiological state.

(ii) Protoplasts are devoid of the normal plant cell wall, and can be treated experimentally much as cultured mammalian cells. The absence of a wall permits protoplasts to fuse with one another, and allows the uptake of externally supplied particles or objects.

(iii) Protoplasts can be cultured with high efficiency. It is common to have plating efficiencies of 50% or over with protoplasts, while this is a rare event with single cells derived from in vitro cell cultures.

(iv) Protoplasts of some plant species are capable of regenerating into entire organisms. This attribute makes it possible to genetically analyze the mechanisms and processes of development. Other plant cell cultures also accomplish this feat. However, the possibility of combining regeneration to whole plants with the ease of working with haploid single cells makes plant protoplasts an unequaled material for genetically dissecting and analyzing biological processes.

Perhaps these features are best understood by considering examples of the kinds of genetic experiments that have successfully used protoplasts. All of the work described below has been made possible or easier by the availability of protoplasts. Protoplasts used in the following experiments were derived from leaf mesophyll cells and were subsequently cultured according to the methods described by Nagata and Takebe (5).

Production of interspecific plant hybrids (6)

The observations that protoplasts are easily obtained and cultured from several different species and that they can be stimulated to fuse (7) suggest that it should be possible to produce a hybrid individual without having to involve a normal sexual cycle.

A hybrid between two different species of Nicotiana, N. glauca Grah. and N. langsdorffii Weim., has been produced by parasexual means. The amphiploid hybrid between these two species had been previously produced by sexual means, and the characteristics of the hybrid plant have been thoroughly studied. Known biological differences between the hybrid and its parental species have been utilized in a selective screen to preferentially recover regenerated fused hybrid protoplasts from a mixed population of protoplasts. The distinctive characteristics of the hybrid tissue were used to verify that parasexual hybridization was achieved.

Populations of protoplasts derived from N. glauca and N. langsdorffii were mixed in an approximate 1:1 ratio and were stimulated to fuse by suspension in 0.25 M NaNO3 followed by pelleting by low-speed centrifugation. This pellet was then resuspended and plated in petri dishes. After the fusion procedure the population consisted of protoplasts of both parental types and fused clumps of protoplasts involving various numbers of cells. More than 10^6 protoplasts of N. glauca and 10^6 protoplasts of N. langsdorffii were taken through the fusion procedure and plated on a regeneration medium that permits the growth only of cells containing the genetic information of both parental species (i.e., the amphiploid hybrid). 33 Regenerated calli were recovered after 6 weeks, and placed on a medium containing no added hormones. This procedure constitutes a further selective screen since tissue from neither parental species is able to grow on a medium without added hormones, while the amphiploid hybrid grows vigorously in the absence of exogenously supplied hormones. All 33 isolates grew vigorously with no exogenously supplied hormone source. This observation provided circumstantial evidence that the recovered calli had a hybrid genetic composition. Several of the recovered calli that were presumed to be parasexual hybrids were chosen for further tests to confirm their hybrid genetic composition.

Morphology of the tissue in culture

The characteristic tissue morphology and growth requirements of the somatically produced hybrid are identical to those of sexually produced hybrid. Tissues from both sources grew vigorously in culture in the absence of exogenous hormones. On an agar medium containing no hormones, both tissues form a semi-friable callus. In a liquid medium containing no hormones, both tissues will regenerate shoots and leaves. Tissue from neither parental species is capable of growth and differentiation in medium lacking added hormones.

Morphology of the Leaf and Flower. The morphology of leaves and flowers regenerated on the somatically produced
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hybrid are identical to those of the sexually produced hybrid, and distinct from either parental type (Fig. 1).

Trichome Characteristics. The leaves of *N. langsdorffii* are densely covered with trichomes, while leaves of *N. glauca* are glabrous without trichomes. On leaves of both the somatically and sexually produced hybrids, trichomes are present but in a lower density.

Tumor Formation. The somatically produced hybrid spontaneously forms tumorous outgrowths on the stem. Spontaneous tumor formation is a genetically determined trait that is characteristic of the F1 hybrid and amphiploid, but is not found in either parental species and is not transmitted across a graft union.

Chromosome Numbers. A somatic chromosome number of 42 was determined for the somatically produced hybrid. This figure is a summation of the diploid somatic numbers of the parental species (24 + 18) and is distinct from a whole ploidy change in either parental type. The sexually produced amphiploid had previously been shown to contain a chromosome number of 42. Although the somatically produced hybrids all demonstrated a chromosome number of 42, deviations from simple addition of the parental chromosome numbers might be expected to occur due to the complexity of the fusion event and postfusion divisions.

Peroxidase Isozymes. The leaf peroxidase isozymes in the somatically produced hybrid are identical to those of the amphiploid. The isozyme bands of the hybrid are a summation of those found in the parental species.

The characteristics of the somatic hybrid are not due to a chimeral association of cells. Single cells derived from calli of the somatically produced hybrid were regenerated into calli and their characteristics were analyzed. In every case the regenerated callus displayed characteristics of hybrid tissue and was distinct from either parental type. Hence, the characteristics of the somatically produced hybrid are not due to a chimeral association of cells of the parental species. All cells of the somatically produced hybrid contained only one nucleus. The possibility that the somatically produced hybrid is due to contamination by sexually produced amphiploid cells is ruled out by the experimental procedure used.

A summation of these lines of evidence leaves no doubt that the calli and plants recovered from fused cells are of a hybrid genetic constitution corresponding to the sexually produced amphiploid.

The successful recovery and analysis of a parasexually produced hybrid has depended primarily on the availability of a selective technique to permit preferential recovery of fused hybrid cells, and recognition of known distinctive characteristics of the hybrid amphiploid. Further attempts to produce a somatic interspecific hybrid and hybrids between more distantly related species have been hampered by a lack of familiarity with the kind of characteristics the tissue will display. Preliminary attempts to preferentially recover intra- and interspecific and intergeneric hybrids using the available auxotrophic mutants of *N. tabacum* have been inconclusive. Many of the auxotrophic protoplasts will grow at a reduced rate in minimal medium when mixed with other auxotrophic mutants or with wild-type protoplasts of other species. This leakiness of the selective procedure is presumably due to the effect of cross-feeding between the cell types. One further method for preferentially recovering parasexually produced hybrids is being investigated. Protoplasts containing recessive nuclear albino mutations that are potentially complementing from different genotypes or species are isolated and fused. Only those cells that have regenerated from hybrid protoplasts will appear as green colonies. Preliminary work in several different plant species suggests that this technique will provide a general selective system for the recovery of parasexually produced hybrids.

Somatically produced hybrids should be of importance to plant breeders, both as a possible source of new crop species and as a method for the interspecific transfer of important agronomic traits.

Organelle transfer

It may be possible to define a system for the characterization of nuclear-organelle interactions by stimulating the uptake of foreign chloroplasts by mutant protoplasts containing no functional chloroplasts.

Protoplasts lacking green functional chloroplasts are isolated from leaf mesophyll cells of a maternally inherited, variegating, albino mutant of *N. tabacum*. Since the albino trait is transmitted cytoplasmically, it is a mutant of the chloroplast DNA and not of a nuclear gene. The nucleus of such a cell is wild type and will support the replication and functioning of a nonmutant chloroplasts. These albino protoplasts are placed in a medium containing wild-type tobacco chloroplasts. We have observed that the nonmutant chloroplasts are taken into the cytoplasm of the albino protoplast, and that occasionally they are able to replicate and function in this new cytoplasmic environment. The externally supplied chloroplasts escape destruction in pinocytotic vacuoles and enter the cytoplasm of the cell. Whole plants have been regenerated from the albino protoplasts containing the "foreign" chloroplasts. Since it has been established that albino mu-

<table>
<thead>
<tr>
<th>Mutant</th>
<th>No growth</th>
<th>Slight growth</th>
<th>Normal growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>31</td>
<td>51</td>
<td>18</td>
</tr>
<tr>
<td>B</td>
<td>36</td>
<td>42</td>
<td>22</td>
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</table>
tant chloroplasts remain phenotypically mutant (albino) in the presence of wild-type chloroplasts in the same cell, the observation that whole green plants (containing green, functional chloroplasts) can be regenerated from these protoplasts is a positive demonstration that the incorporated chloroplasts replicate and function. Hence, the chloroplasts are able to utilize information encoded by the protoplast nucleus.

Chloroplasts from several different Nicotiana species can be utilized by the albino N. tabacum protoplasts. However, this same ability does not appear to exist when chloroplasts are used from different genera, even though they are taken into the protoplasts. The interaction between nuclei and chloroplast derived from diverse genera should provide interesting experimental material for geneticists. Perhaps chloroplast transfer will provide a method for plant breeders to incorporate highly efficient chloroplasts into important crop species.

**Mutant isolation.**

Recent advances in the somatic cell genetics of higher plants have demonstrated that it is possible to utilize selective techniques to recover mutant individuals from populations of single haploid cells cultured in vitro (8). These experiments were initiated to pose two questions: (a) Is it possible to select mutants of a higher plant that have an altered response to a pathogen by recovery of cells that are resistant to the toxin produced by that pathogen? (b) Is it possible to selectively increase the level of a nutritionally important component in a plant by selecting mutants resistant to a toxic analogue of that component? Both of these questions can be resolved by recovering and analyzing mutants of N. tabacum that are resistant to the methionine analogue, methionine sulfoximine. It has been demonstrated by Braun (9) that toxin produced by Pseudomonas tabaci, the bacterial pathogen that causes the wildfire disease of tobacco, is a structural analogue of methionine. Braun observed that the sulfoximine, though not the true bacterial toxin, would elicit an identical response from tobacco leaves, and that mutants of Chlorella vulgaris resistant to this analogue were also resistant to the toxin. Methionine is also an essential amino acid in human nutrition.

Haploid protoplasts were isolated, treated with 0.25% ethyl methanesulfonate for 1 hr, and plated in an agar regeneration medium. The cultures were permitted to grow for 2 weeks, overlaid with an equal volume of medium containing 10 mM methionine sulfoximine, and incubated for 3-4 months. Surviving calli were recovered and placed on medium containing no sulfoximine. Each callus was grown for several months, divided, and retested for resistance to 10 mM methionine sulfoximine. Those calli that retained resistance were diploidized and regenerated into whole plants for further genetic and physiological analyses.

An analysis of about 2.7 \( \times 10^3 \) viable protoplasts after mutagenesis yielded 33 presumptive analogue-resistant calli. Most of the presumed mutants were unstable in expression of this resistance. After further growth of the calli on medium lacking the analogue, 31 of the 33 presumed mutants were found to segregate tissue that was no longer resistant to methionine sulfoximine. The phenotype of the unstable tissue exhibits many characteristics common to presumed mutants recovered from animal cell cultures (10).

Diploid plants were regenerated from both mutant calli. Each plant was crossed to a wild-type plant to give F\(_1\) progeny. The F\(_1\) progeny were self-fertilized to yield an F\(_2\) generation. 100 F\(_2\) seedlings from each cross were germinated under sterile conditions and tested for resistance to the sulfoximine. The results are presented in Table 1. The patterns of transmission of the mutants appears to be best explained by a single semi-dominant locus, yielding a 1:2:1 ratio. Since the mutants were derived from mutagenic treatment and there is the possibility of extensive genetic damage, the ratios must be considered preliminary. Crosses between the mutants indicate that they are probably allelic. Since these two

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**Fig. 2.** Reaction of tobacco leaves to infection by *Pseudomonas tabaci* and to application of methionine sulfoximine. (A) Control leaf from a wild-type plant; (B) leaf from mutant A; (C) leaf from mutant B; (D) leaf from cv. Burley 21, which carries a naturally occurring resistance to *P. tabaci*. Each leaf was infected twice in the apical region with 0.1 ml of a 3- to 4-day-old culture of *P. tabaci* in nutrient broth. 0.1 ml of a 0.1 mM solution of methionine sulfoximine was applied to the left side of the basal portion of the leaf. Uninoculated nutrient broth was applied to the right basal region. Leaves were examined for their reaction after 9 days.
TABLE 2. Concentration (nmol/g of fresh weight) of certain free amino acids in tobacco leaves

<table>
<thead>
<tr>
<th></th>
<th>Methionine</th>
<th>Glycine</th>
<th>Alanine</th>
<th>Proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Havanna</td>
<td>0.4 ± 0.2</td>
<td>1.3 ± 0.3</td>
<td>1.8 ± 0.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Wisconsin</td>
<td>1.9 ± 0.5</td>
<td>1.7 ± 0.5</td>
<td>2.0 ± 0.4</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Mutant A</td>
<td>2.4 ± 0.6</td>
<td>1.2 ± 0.2</td>
<td>1.5 ± 0.3</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>

Fully expanded young leaves with veins removed were homogenized at room temperature. An equal volume of 10% trichloroacetic acid was added to the homogenate, which was then centrifuged. The acid-soluble supernatant was analyzed on an amino-acid analyzer. Each value was calculated from three replicates. Concentrations of glycine, alanine, and proline are included to demonstrate that the increases in methionine in mutants A and B are specific for that amino acid.

TABLE 3. Expression of the bacteriophage T3 genome

<table>
<thead>
<tr>
<th>Host</th>
<th>Infected with T3</th>
<th>Time of infection</th>
<th>Cleavage enzyme</th>
<th>T3 RNA polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>S-Adenosylmethionine hydrolyzed per hr per mg of protein</td>
<td>pmol Uracil polymerized per hr per mg of protein into acid-insoluble material</td>
</tr>
<tr>
<td>E. coli</td>
<td>No</td>
<td>10 min</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>E. coli</td>
<td>Yes</td>
<td>10 min</td>
<td>360,000</td>
<td>—</td>
</tr>
<tr>
<td>H. vulgare</td>
<td>No</td>
<td>24 hr</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>H. vulgare</td>
<td>Yes</td>
<td>24 hr</td>
<td>5,200</td>
<td>970</td>
</tr>
<tr>
<td>H. vulgare (osmotically ruptured cells)</td>
<td>Yes</td>
<td>24 hr</td>
<td>970</td>
<td>115</td>
</tr>
<tr>
<td>H. vulgare (+ 1 mg/ml of acid of cycloheximide)</td>
<td>Yes</td>
<td>24 hr</td>
<td>1,250</td>
<td>300</td>
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<tr>
<td>H. vulgare (+ 50 μg/ml of acid of chloramphenicol)</td>
<td>Yes</td>
<td>24 hr</td>
<td>2,800</td>
<td>62</td>
</tr>
<tr>
<td>H. vulgare (+ E. coli, 10⁸ cells per ml)</td>
<td>Yes</td>
<td>24 hr</td>
<td>6,800</td>
<td>1400</td>
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<tr>
<td>H. vulgare (gene-1 amber mutation)</td>
<td>Yes</td>
<td>24 hr</td>
<td>4,100</td>
<td>74</td>
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Fig. 3. Appearance of S-adenosylmethionine cleaving enzyme in protoplasts of Hordeum vulgare infected with T3.

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plant pathologists to be a variety of P. tabaci that does not produce toxin. Both mutant types are susceptible to infection by P. angulata, while Burley 21 is resistant. The mutants are distinctly more resistant to the effects of infection by P. tabaci than is the wild-type variety from which they were selected. The naturally occurring resistance of Burley 21 is superior to that of the mutants.

The concentration of free amino acids in fully expanded leaves of wild-type and mutant tobacco is presented in Table 2. The mutants both show specific increases in the concentration of free methionine.

The answer to both questions posed in this work is affirmative. Mutants of agricultural importance can be recovered by selection at the level of the single cell.

Uptake and expression of viruses

Protoplasts from N. tabacum have proven to be a suitable system for studying the mechanism of infection, replication, and assembly of tobacco mosaic virus (TMV), since a large fraction of the protoplasts are infected synchronously, and

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mutants were derived from protoplasts isolated in two different experimental series, they must be derived from two different mutational events.

Fig. 2 demonstrates the reaction of the leaves upon infection of P. tabaci or application of methionine sulfoximine. After infection with P. tabaci, the susceptible plant forms chlorotic halos that surround a brown necrotic spot at the site of inoculation. Methionine sulfoximine causes a similar reaction, except that the necrotic spot is not as pronounced. Infection of the homozygous mutant plants and a variety of N. tabacum carrying a naturally occurring genetic resistance (Burley 21) does not lead to production of the characteristic chlorotic halo. Leaves of the mutants show small dark areas at the site of infection with P. tabaci. Burley 21 is unaffected by infection. The small dark areas on the mutants are similar to the blackfire disease caused by P. angulata. This observation is quite striking, since P. angulata is considered by many
mature particles are released from the infected cells (11). These observations led to an attempt to demonstrate the expression of bacteriophage genes in plant protoplasts. Bacteriophage T3 was chosen because of its relatively simple composition and because it encodes for several unique enzymatic activities. After infection of Escherichia coli with phage T3, the RNA polymerase of the host cell transcribes a group of "early" phage genes. Hence, the "early" region of the T3 genome might be transcribed by a plant RNA polymerase and subsequently translated by the plant protein synthetic machinery. Two enzymatic activities that are encoded in the "early" region of the T3 genome and are normally absent from plant cells were assayed after infection of Hordeum vulgare protoplasts with a high titer (10^4 plaque forming units per protoplast) of T3. The S-adenosylmethionine cleaving enzyme and T3 RNA polymerase, both early gene functions, were produced at very low levels in the protoplasts after infection with T3. Control experiments demonstrated that this activity was not due to bacterial contamination and was not carried by the phage particles. The levels of the enzymes found in plant protoplasts are between two and three orders of magnitude less than that found in E. coli. The data in Table 3 demonstrate that intact protoplasts are necessary for the appearance of activity. Cycloheximide is more effective than chloramphenicol in inhibiting the production of activity. An amber mutation in the structural gene encoding for T3 RNA polymerase does not permit the production of an active enzyme. The deliberate contamination of the incubation mixture with E. coli does not lead to strikingly increased levels of activity. This negative finding is presumably because of the differences in the time periods required for expression of enzymatic activity (10 min versus 24 hr), and because the infected protoplasts are routinely washed before they are extracted. The rate of appearance of S-adenosylmethionine cleaving enzyme and T3 RNA polymerase are diagrammed in Figs. 3 and 4. Both activities show similar increases in the first 24 hr after infection. These observations provide strong circumstantial evidence that bacteriophage genes can be transcribed and translated in plant cells. Work with defective lambda phages that carry known genes from E. coli confirms these findings (C. Doy, personal communication). Insertion of defined genetic material into higher plant cells should play a central role in the definition of molecular mechanisms in higher organisms, and should provide a powerful tool for plant breeders in transferring defined genetic characteristics.

These four examples point out the types of genetic problems that can be approached by use of the unique properties of protoplasts. There is, however, one primary problem facing the general use of protoplasts in genetics. Current techniques allow the culture and regeneration of protoplasts derived from only a few species of higher plants. Characterization of the requirements for culturing protoplasts from many different species, especially the important crop species, must occupy a central place in future research.

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