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Opportunities from the Use of Protoplasts

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Phil. Trans. R. Soc. Lond. B 1981 **292**, 557-568
doi: 10.1098/rstb.1981.0050

Phil. Trans. R. Soc. Lond. B **292**, 557–568 (1981)
Printed in Great Britain

Opportunities from the use of protoplasts

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Plant protoplasts of several horticultural and crop species can now be readily regenerated into plants. There are now available several opportunities for their use in the manipulation of genetic systems in plant breeding. Protoplast cloning has recently been shown to produce additional genetic variation in potatoes; the possibility is examined for protoplast cloning of seed producing crop species for new genetic variation. Fusion of protoplasts of different species is now providing an additional method of hybridization; sexually incompatible species can be hybridized and horticulturally useful hybrids are now being produced. Many possibilities exist for hybridization assessments, both nuclear and cytoplasmic, between various crop species; however, the extent to which these wider hybridizations will produce useful genetic variation is not yet clear, and in many instances plant regeneration from these cultured cell hybrids is not yet possible.

Plant protoplasts are also providing an opportunity for the transfer of genes between different species. This may be by fusion with an irradiated protoplast system, or by direct transformation. Transfer of genes by using *Agrobacterium* plasmid as a vector system appears promising, and fusions with wild-type protoplasts will ensure the regeneration of non-tumorous plants.

1. Introduction

At this Discussion Meeting it will be most useful to clarify what has already been accomplished with the use of protoplasts, to indicate the main obstacles impeding further developments and to forecast the most likely future applications of this new technology to plant breeding. Comprehensive earlier assessments of the role of plant tissue culture and protoplasts have already been provided (Riley 1979; Cocking & Riley 1981), but recent advances in various aspects of protoplast manipulation are providing additional results and new vistas.

Because protoplasts are single cells they provide opportunities to clone plants at the single cell level. Also, because the cell wall is absent they can be fused with other protoplasts, thereby providing opportunities for somatic hybridization. The absence of the cell wall also facilitates the uptake of DNA enabling evaluations of transformation to be carried out at a level comparable with those of microbiological transformations. These manipulations are not restricted to the cellular level because sometimes whole plants can be regenerated by suitable tissue culture procedures, and then subjected to a conventional breeding programme.

In this survey of the opportunities in plant breeding from the use of such protoplast systems, it will be assumed that the reader is familiar with the techniques involved; these have been fully described previously (Evans & Cocking 1974), and more recently in the practical handbook from this laboratory (Power & Davey 1979).

2. Cloning of plants from protoplasts

Phenotypic diversity appears to be a ubiquitous occurrence in cell culture, which is manifested among plants regenerated from cell culture. As recently discussed by Scowcroft & Larkin (1980), there is reason to suspect a genetic, or at least an epigenetic, basis for this variation. As also emphasized by these investigators, plant tissue culture *per se* appears to be an unexpectedly rich and novel source of genetic variation, which is already being utilized in plant improvement. All of the major crop species are included among the 300 or so species from which plants have been regenerated from cell cultures, but not necessarily from protoplasts.

Table 1. Somaclonal variation in *Solanum tuberosum*
(From Scowcroft & Larkin (1980).)

source material	number of somaclones screened	characters displaying enhanced phenotypic variation	reference
Leaf mesophyll protoplasts (Russet Burbank)	> 10000	tuber shape, yield, maturity date, photoperiod requirement, plant morphology, enhanced resistance to *Alternaria solani* and *Phytophthora infestans*	Shepard *et al.* (1980)
Suspension culture protoplasts of a dihaploid line	< 211	tuber shape	Wenzel *et al.* (1979)

The ability to dissect whole plants into single cells, through the use of protoplasts, has now stimulated a considerable effort to find out whether such genetic variation can be enhanced by protoplast cloning. Procedures such as protoplast cloning, which have the objective of enhancing a popular variety rather than creating a new one, are particularly attractive to the plant breeder for obvious reasons. This approach has been pioneered by Shepard and his collaborators, particularly for potatoes and more recently for other vegetatively propagated species. Shepard *et al.* (1980) have, however, cautioned regarding the interpretation of the results relating to improvement in any one character as a result of protoplast cloning (table 1). In any such breeding programme, clones with an acknowledged improvement in one character must be exposed to adequate environmental pressures (both physical and biological) to eliminate any that are simultaneously deficient in some critical feature. Nevertheless, the results obtained so far have been sufficiently encouraging to convince plant breeders in several countries of the need to evaluate their own potato varieties in this respect. The need for evaluations to be performed on varieties suitable for particular countries highlights the need for the procedures for protoplast isolation, culture, division and regeneration into whole plants to be applicable to a very wide range of potato varieties. Plant breeders in the U.K. will rapidly lose interest if the protoplast cloning procedures are only applicable to the Russet Burbank variety. Fortunately this does not appear to be the situation. Several U.K. recommended varieties respond well under the sophisticated growth and cultural conditions developed by Shepard and his collaborators (P. Day, personal communication). It should be noted that Wenzel *et al.* (1979) obtained a rather high uniformity of plants regenerated from their potato mesophyll protoplasts. This suggests that different varieties of potato may possess markedly different levels of inherent genetic variation.

Meaningful evaluation of protoplast cloning for potato improvement depends on reproducible regeneration of plants from protoplasts and the field testing of the plants obtained. Recurrent

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selection from clonal material may further enhance selection. With potato, and with any other species subject to this new breeding technology, it is important to keep the cultural procedures as simple as possible; there is a need to compare the rather simple shoot tip culture isolation procedures with the far more sophisticated procedures employed by Shepard *et al.* (1980).

At present there is little evidence to suggest that this type of somaclonal variation could make a significant contribution to plant improvement of seed propagated species. However, there has been very little work comparable with that using potato protoplasts. Since, also, at present, the origin of somaclonal variation is not fully understood, even in potatoes, it is worth while to explore the situation in seed propagated species. Recently the ability of *Medicago sativa* (alfalfa, lucerne) leaf protoplasts to regenerate into plants at high frequency, under relatively simple growth conditions (Santos *et al.* 1980), has stimulated work towards such an evaluation of this important forage legume.

As discussed by Scowcroft & Larkin (1980), in polyploid species such as sugar cane and potatoes, aneuploidy could account for some variation, but the reasons for the variation may be more fundamental. Recent molecular studies of genome organization in eukaryotes has indicated that the somatic genome is not static but highly variable. Moreover, in vegetatively propagated species this variation might be enhanced by cryptic chromosomal changes such as small deletions, additions, transpositions or inversions, which would normally be screened out during meiosis. There is no doubt that somatic variation in plants will prove to be an exciting area of research in which the use of protoplasts will feature to a large extent. The regeneration of crop plants from protoplasts is still very much the acid test for regeneration capability (Bhojwani *et al.* 1977), and we still lack this capability for the cereals and grain legumes. There are indications, now that callusing and plant regeneration from cereal leaves have been obtained (Wernicke & Brettell 1980), that further work may resolve this present deficiency. In the meantime, quite apart from protoplast studies, detailed studies on the genetic variation ensuing in plants regenerated from *in vitro* cultures of our major crop species could be particularly rewarding to the plant breeder.

3. Somatic hybridization by protoplast fusion

In higher plants, just as in the fungi, the first essential step in any parasexual cycle will be heterokaryosis as a result of fusion of somatic protoplasts. If this is followed by the fusion of nuclei and the development of hybrid cells after mitosis, then this somatic hybridization component of the parasexual cycle will be complete. Moreover, if diploidization associated with the fusion process results in the formation of amphiploid hybrid plants, then protoplast fusions will be providing an alternative to gametic fusions for plant hybridization (Cocking 1979).

Practical details of the various procedures involved in protoplast isolation and fusion to produce heterokaryons have been fully described previously (Power & Davey 1979). Although there is still much current work aimed at increasing the percentage of heterokaryons that can be obtained when protoplasts of different species are fused, it is clear that fusion itself is not a major incompatibility consideration. Usually approximately 1% heterokaryon formation can be obtained, and provided the culture conditions are satisfactory, and provided an adequate selection pressure can be put on the system, hybrid callus (or hybrid embryoids) can be selected. Sometimes it is possible to select heterokaryons manually.

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Perhaps it should be re-emphasized that the somatic hybridization approach need not be restricted to wide crosses. For instance, in potato breeding it has been suggested by Wenzel *et al.* (1979) that an ideal procedure would be to fuse protoplasts of different dihaploid hybrids. The heterozygous dihaploids would be combined to a completely heterozygous tetraploid plant, without any meiotic segregation: propagated vegetatively it could immediately become a stable variety.

Results obtained so far on the consequences of fusion of protoplasts have provided good evidence that, in many instances, fusions between those from sexually compatible species will result in the formation of heterokaryons in which both nuclear genomes are capable of forming stable amphiploid nuclear hybrids. Organogenesis from hybrid callus will then result in the regeneration of somatic hybrid plants with both sets of parental chromosomes. In our studies within the *Petunia* genus (Cocking 1979) we have produced a large percentage of amphidiploid somatic hybrid plants by fusing wild-type leaf protoplasts of one species with albino protoplasts from cell suspension cultures of the other species. This has resulted in the production of flowering plants, with 28 chromosomes, of the somatic hybrid *Petunia parodii* ($2n = 14$) $\otimes$ *Petunia hybrida* ($2n = 14$), and of *Petunia parodii* ($2n = 14$) $\otimes$ *Petunia inflata* ($2n = 14$). While *P. parodii* and *P. hybrida* can be crossed sexually, it is known that *P. parodii* and *P. inflata* cannot be crossed sexually by standard procedures, since they possess prezygotic unidirectional sexual incompatibilities.

Recently we have investigated the consequences of fusion of *Petunia parodii* leaf protoplasts with albino protoplasts from cell suspensions of *Petunia parviflora*. These two species are sexually incompatible, probably due to both prezygotic and postzygotic isolation mechanisms, and all previous attempts at sexual hybridization have failed. Our somatic protoplast fusions have enabled us to produce somatic hybrid plants with 32 chromosomes, *P. parodii* ($2n = 14$) $\otimes$ *P. parviflora* ($2n = 18$) which are amphidiploids ($4n = 32$) and some with 31 chromosomes (Power *et al.* 1980). Differences in size and morphology of the chromosomes of these two species have proved sufficient to enable us to establish the presence of both parental sets in the somatic hybrid nuclei. Other somatic hybrids have also been produced between species that are very difficult or impossible to hybridize conventionally, e.g. *Lycopersicon esculentum* and *Solanum tuberosum* (Melchers *et al.* 1978), *Datura innoxia* and *Atropa belladona* (Krumbiegel & Schieder 1979); *Arabidopsis thaliana* and *Brassica campestris* (Gleba & Hoffmann 1979).

It would seem likely that the main opportunities for the plant breeder centre on the use of protoplast fusions for hybridizations within the same genus, or between closely related genera, particularly if fertile, seed-producing somatic hybrids are desired. An example where tetraploids from somatic fusion would be useful is the *Lolium–Festuca* hybrid. In this cross the diploid, even if obtained sexually, often lacks fertility, and chromosome doubling by colchicine treatment is frequently impossible. Thus tetraploid production, somatically, between these species might produce fertile hybrids. The present main impediment to finding out if this would be so is our present inability to regenerate plants from protoplasts in these grass species. Many intergeneric hybrids are desirable in programmes of crop improvement between sexually incompatible species. A typical example within the *Vicias* is the desirability of crossing *V. faba* with *V. narbonensis* to convey chocolate spot resistance from *V. narbonensis* into the cultivated disease-sensitive *V. faba*. Progress at the protoplast culture level has already reached the stage of callus formation from protoplasts of the parental species, but regeneration of plants still remains unresolved. Numerous examples in other genera have previously been itemized

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(Sanchez-Monge & Garcia-Olmedo (eds) 1977), and more recently in legumes (Razdan & Cocking 1981).

As has been discussed by Dewey (1977), wide hybridization can be used in two different ways by the plant breeder. One approach involves the transfer of one of several characters from one species to another (controlled introgression), and the other is the merging of two or more species into a new synthetic species. Both of these approaches are currently being explored by using sexual hybridization: noteworthy successes have resulted in the incorporation of leaf rust resistance of *Aegilops umbellulata* into wheat, *Triticum aestivum* L. (Sears 1956), and the development of the new economically important species triticale, an amphiploid hybrid between *Triticum* and *Secale*. Perhaps it is salutary to note that hybrids between wheat and rye were known as early as 1875; success has not come easily as evidenced by over 40 years' effort by breeders in several countries. What opportunities do protoplasts provide for wide hybridizations such as these? The answer is an opportunity to experiment with the fusion of protoplasts to determine the outcome. These attempts should not be restricted to crosses that are currently impossible sexually. Recent work on the sexual hybridization of rice and sorghum (Deming *et al.* 1979) has highlighted the need for parallel efforts by protoplast fusion. The main difficulty in attempting somatic hybridization between rice and sorghum is the fact that there is at present no regeneration of whole plants from protoplasts of either of these species. There is no difficulty in fusion and limited culture of heterokaryons, but until the present culture difficulties have been overcome we cannot undertake realistic attempts at somatic hybridization. Recent work on the embryo culture of sexual hybrids between maize and sorghum at Cimmyt (James 1980) has also re-emphasized the need for such somatic crosses. Yet again our work in this direction is being impeded by an inability to regenerate whole plants from maize or sorghum protoplasts. There is no difficulty in fusing these protoplasts and obtaining heterokaryons with inherent viability (Brar *et al.* 1980). However, regardless of the method of hybridization, sexual or somatic, the question still remains as to whether the top productive varieties of the main crops of today can still take advantage of the quantitative diversity present in the natural resources. Have we, as suggested by Röbbelen (1979) passed the 'point of no return' at which further addition of foreign variation does nothing but spoil the achieved high level of performance?

What is of importance for plant breeding is that the cytoplasmic mix obtained from protoplast fusions is novel. It would seem likely, as emphasized by McKenzie (1979), that while it is relatively simple to bring together two plastid genotypes in a common cytoplasm by protoplast fusion, it is difficult to keep them together. The inevitable result will be the eventual somatic segregation of the two types into two 'cytoplasmic subspecies', each with only one of the two plastid genotypes in its tissues. Belliard *et al.* (1978) observed that only one species-type of chloroplast DNA remained in the somatic hybrid between two sexually compatible tobacco species. From the studies of Kumar *et al.* (1981) on the polypeptide profiles of fraction 1 protein isolated from somatic hybrid plants, ensuing from the fusion of mesophyll derived protoplasts of wild-type *Petunia parodii* with albino protoplasts of *P. parviflora*, which contained only the chloroplast-coded large subunit polypeptides of *P. parodii*, it has been suggested that the use of albino mutants for selection may cause unidirectional segregation. Clearly an ability to manipulate cytoplasmic (chloroplast) segregation in this way would be very useful, particularly in sexually incompatible species. Opportunities for increasing cytoplasmic variability by protoplast fusions may be greater with mitochondria than with chloroplasts. Belliard *et al.* (1979)

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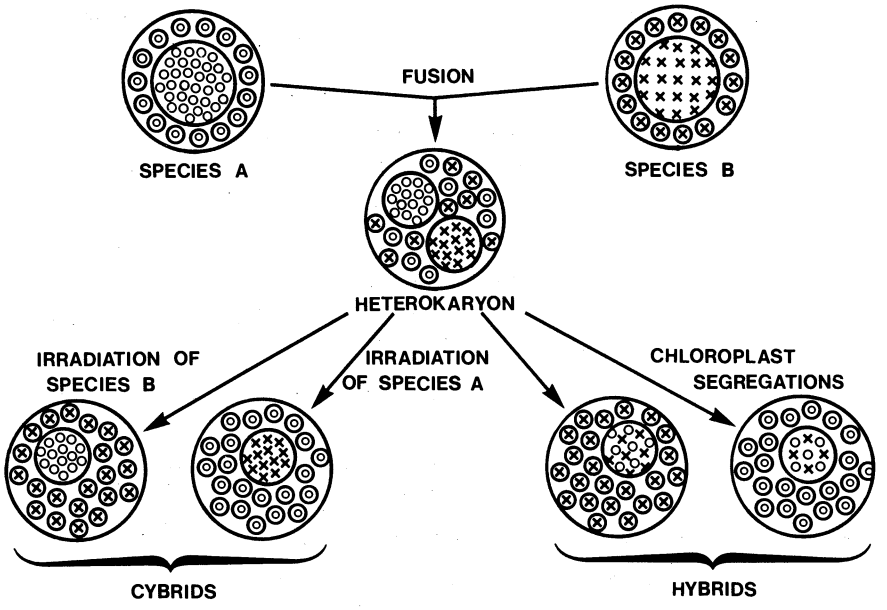

FIGURE 1. Protoplast fusion and some possible chloroplast segregations.

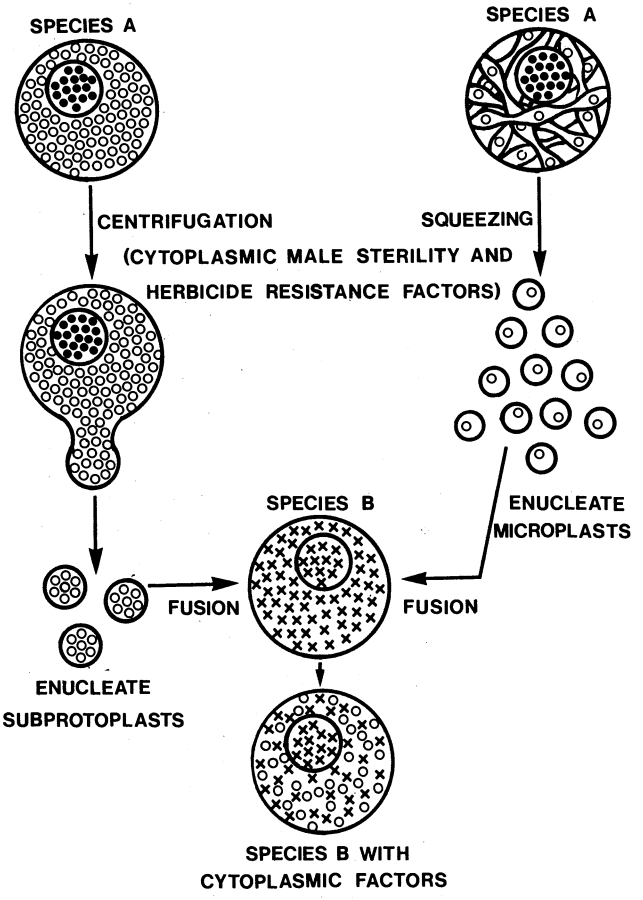

FIGURE 2. Transfer of cytoplasmic factors by fusion.

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obtained evidence for mitochondrial recombination in cytoplasmic hybrids of *N. tabacum* by protoplast fusion. The mitochondrial DNAs of cybrids were different from those of the parent and from the mixture of the two.

If this fusion of protoplasts is also coupled with procedures for the inactivation of the nuclear genome of one of the species, and suitable selection procedures, a range of novel cybrids can theoretically be obtained (figure 1). Aviv *et al.* (1980) have already taken significant steps in this direction in their progeny analysis of the interspecific hybrids *N. tabacum* (cytoplasmic male sterility) and *N. sylvestris* with respect to nuclear and chloroplast markers. Transfer of cytoplasmic male sterility in *Nicotiana* to *N. sylvestris* by protoplast fusion was reported by Zelcer *et al.* (1978), and transfer of cytoplasmic male sterility by protoplast fusion in *Petunia* by Izhar & Power (1979). Rather than irradiating protoplasts to inactivate nuclei, fractionation of protoplasts into enucleate subprotoplasts or enucleate microplasts (Bilkey & Cocking 1980) may provide suitable enucleate units for fusions to produce this novel range of cybrids, thereby avoiding any irradiation effects on the cytoplasm (figure 2). Fractionation of protoplasts may also facilitate the transfer of cytoplasmically based herbicide resistance between sexually incompatible species, as has recently been demonstrated for sexually compatible species with respect to atrazine resistance (Beversdorf *et al.* 1980). The opportunities for the plant breeder are considerable in this respect since cytoplasmic influences are generally quite important in crop yield, for instance in the triticales (Hsam & Larter 1974).

4. Gene transfer

Both sexual and somatic hybridization involve an extensive intermixing of genes, and as emphasized by Riley (1979), there are often disadvantages in attempting to combine the total genetic structures of two species and greater advantage in attempting to incorporate in the recipient crop species some limited, perhaps single, genetic attribute of a donor species. Our earlier work on the fusion of *Petunia* and *Parthenocissus* protoplasts indicated that this might be possible (Power *et al.* 1975).

Currently, advances towards this goal with the use of protoplasts are taking place from several directions. Szabados *et al.* (1980) have recently described the isolation of plant metaphase chromosomes in bulk from highly synchronized cell suspensions and have provided cytological evidence for incorporation of chromosomes into plant protoplasts. Earlier, Malmberg & Griesbach (1980) had described the isolation and purification of chromosomes from plant protoplasts. The demonstrations that, in *Nicotiana*, pollination with highly irradiated (10^5 rad†) killed compatible pollen can cause certain genes from the pollen to be transferred to the egg without proper fertilization has also greatly stimulated work in this direction. This phenomenon, which is in effect transformation of the egg, has resulted in egg transformation for the flower colour gene and the *S* gene (Pandey 1979) and other genes (Virk *et al.* 1977). A particular advantage of this form of sexual transformation is that parthenogenetic plants are produced, avoiding the whole plant regeneration problems currently inherent in the use of protoplasts for many of our crop species. Is the egg special in this respect, or could somatic protoplasts behave similarly? Currently, we are investigating, in collaboration with J. L. Jinks and his colleagues at Birmingham, whether leaf protoplasts of *N. rustica* fused with irradiated

† 1 rad = 10^{-2} Gy = 10^{-2} J kg^{-1}.

protoplasts will yield plants with similar genetic traits exhibited by those obtained as a result of sexual transformation. We are also currently investigating (in collaboration with A. Müller and his colleagues in Gatersleben, G.D.R., and E. J. Hewitt and his colleagues at Long Ashton) whether such somatic transformation could be used to transfer nitrate reductase genes between different species. We are utilizing the nitrate reductase minus mutants of tobacco isolated by the Gatersleben group and fusing with X-ray irradiated cereal and legume protoplasts, and then selecting for nitrate reductase proficiency among the tobacco cells. Such markers should enable selection of any somatic transformants; in future work, say between a nitrate reductase minus legume or cereal and a wild-type tobacco (or some other species with a highly active nitrate reductase), this selection for limited gene transfer could result in a legume or cereal with improved nitrate reductase capability, and perhaps improved yield. The requirement for auxotrophic mutants for these evaluations emphasizes the need for a greater range of auxotrophic mutants, in a wide range of crop species capable of plant regeneration.

The most direct approach to the introduction of genetic information is afforded by the model of bacterial transformation with purified DNA. This procedure for gene transfer is particularly attractive to the molecular biologist. The opinion of certain plant breeders, however, has been that the utilization of these techniques (even if successful) has definite limitations in scope. For instance, Nelson (1977) has pointed out that these approaches are unlikely to allow selection for traits that arise as a consequence of differentiation or selection in the polygenic complexes that condition yield. It is, however, also recognized that qualitative traits that are expressed in cell cultures enable considerable experimental leverage to be put at the disposal of the investigator. In 1977, summarizing the situation for the prospects for plant genome modification by non-conventional methods, Kleinhofs & Behki concluded that the present science of transformation in plants (including protoplasts) was still vague and poorly defined. They stressed that more and better-controlled experiments were needed to make the results convincing and reproducible in other laboratories, using gene markers that could be specifically identified at the protein or RNA level.

The main deficiencies in previous approaches to plant transformation with purified DNA arose largely because the transforming molecules were not of the type that could be expected to replicate in the plant cell or become integrated into the plant's DNA. Langridge (1978) suggested that a molecule with reasonable chances of acting as a vector would need to be a double-stranded DNA, circular in form, to allow for replication and integration; however, our present knowledge of these requirements is still very limited (Cocking *et al.* 1981). It is well recognized that there are several natural barriers to the introduction, maintenance and expression of 'foreign' DNA in plant cells and that the use of protoplasts greatly facilitates the introduction, but not necessarily the maintenance and expression, of 'foreign' DNA. With cultured animal cells these barriers appear to be less, since it is possible to introduce specific genes into such cultured cells by DNA-mediated gene transfer and to detect the rare transformant by biochemical selection. Such cells can also be co-transformed with two physically unlinked genes. Wigler *et al.* (1979) have shown that co-transformed cultural animal cells can be identified and isolated when one of these genes codes for a selectable marker, such as thymidine kinase; ligation to a viral vector was not required.

The singular attraction of *Agrobacterium tumefaciens* is that when interacting with plants it naturally manages to transfer, maintain and express its prokaryotic DNA in plant cells. Numerous workers are now busy trying to exploit this natural capability of the *Agrobacterium*

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bacterium to use the *Agrobacterium tumefaciens* Ti plasmid as a host vector system for introducing foreign DNA in plant cells (Drummond 1979; Hernalsteens *et al.* 1980). In many of these experiments on plant transformation it is not necessary, at least initially, to use protoplasts.

Aiding this work has been the demonstration that plasmids isolated from *Agrobacterium* can be used to transform protoplasts, transformed cells being selected by their independence and associated proliferation on hormone-free media, overgrowth formation when grafted onto host plants, octopine synthesis and lysopine dehydrogenase activity (Davey *et al.* (1980*a*), and that intact *Agrobacterium* will transform cells that have been formed by the regeneration of a wall on isolated protoplasts of wild-type *N. tabacum* (Davey *et al.* 1980*b*) (figure 3).

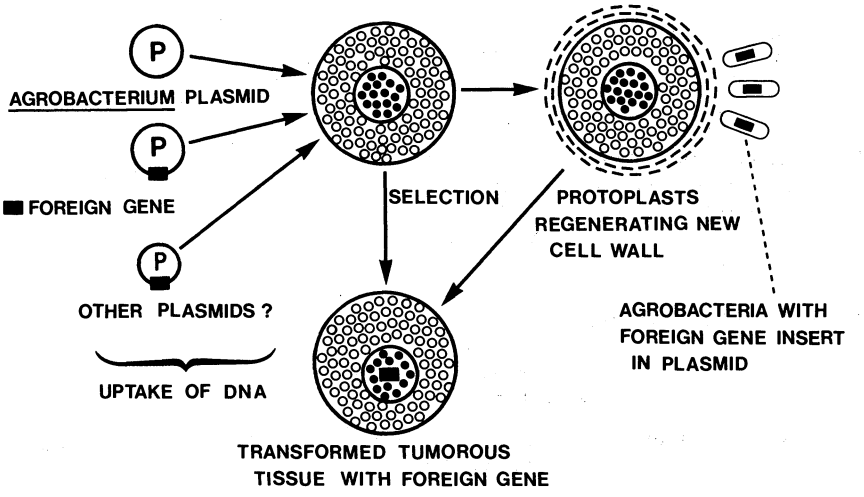

FIGURE 3. Strategy for transformation with the use of protoplasts.

Opportunities for the use of protoplasts will arise in this work when an inadequate percentage of the cells are transformed in the tumours from plant–*Agrobacterium* transformations. This will necessitate cloning procedures, either from the transformants, or by initially using protoplast-regenerant cells interacting with intact agrobacteria (Davey *et al.* 1980*b*). If, with further work, it is established that foreign gene transfer and expression into dicotyledonous plants is possible with such an *Agrobacterium* vector system, the need to extend this approach to the cereals will be paramount. It would seem probable from the work of Davey *et al.* (1980*a*, *b*) that cereal protoplasts interacted with isolated *Agrobacterium* plasmids will be capable of being transformed, since the removal of the cell wall is likely to remove the natural barrier to infection. Protoplast-regenerant cells from cereals are also likely to be transformed by intact agrobacteria since the newly synthesized cell wall formed on protoplasts is likely to facilitate bacterial interaction.

It has often been suggested that it is of little practical value to the plant breeder to engineer plant tumours, however enriched they might be with foreign genes. Regeneration of plants is an essential prerequisite for uses in plant breeding. Protoplasts will undoubtedly play a key role in resolving this type of difficulty. It has been shown in fusion experiments between protoplasts from wild type and tumour cell lines (which frequently fail to regenerate into plants) that fusion between the morphogenic and non-morphogenic cells results in shoot-forming callus – the ability to regenerate shoots is dominant when one of the partners is a tumour cell. It would seem probable that the fusion of protoplasts, subprotoplasts or microplasts (Bilkey & Cocking

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1980) from undifferentiated tumour tissue carrying foreign genes, and of protoplasts prepared from wild-type plants, will result in regenerated normal plants in which foreign genes are expressed (Wullems *et al.* 1980) (figure 4).

An *Escherichia coli* plasmid vehicle would not be capable of interacting with walled cells, and plant protoplasts will be essential for any attempts at transformation with, for instance, dominant drug resistance genes from bacteria, if no *Agrobacterium* vector is used. With cereal protoplasts it may be essential to use a plasmid vehicle other than that of *Agrobacterium* (such as that of *E. coli*), if natural transformation of cereal protoplasts by *Agrobacterium* or its plasmid should prove impossible.

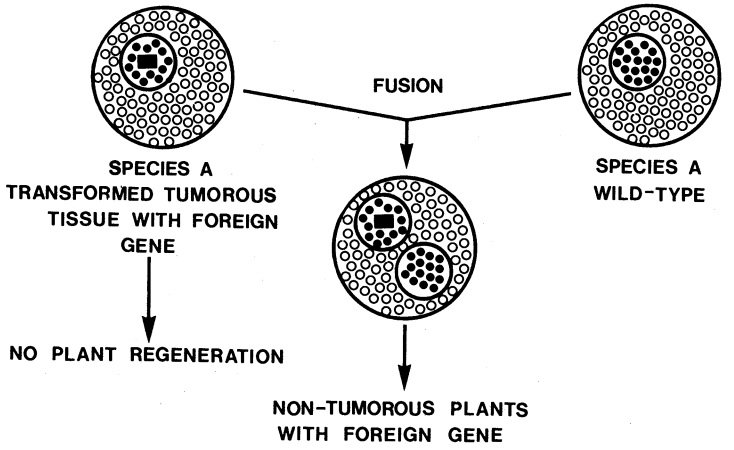

FIGURE 4. Regeneration of transformed non-tumorous plants from transformed tumorous tissue with the use of protoplast fusion.

5. CONCLUSIONS

From what has already been described about the opportunities from the use of protoplasts for plant breeding, it will be evident that much more work will be required before the use of protoplasts adds significantly to the armoury of the plant breeder. What is clearly required is a continuing dialogue with plant breeders to ensure that the objectives of manipulations with protoplasts are closely aligned to those of the breeder. The contribution from the use of protoplasts will only be significant if the existing breeding problems and objectives with the use of conventional methods are uppermost in the thinking and manipulations of protoplast workers.

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Discussion

E. Thomas (*Rothamsted Experimental Station, Harpenden, Herts., U.K.*). Professor Cocking stated that a special feature of plant protoplast culture was the variation in agronomic characters observable in regenerated plants, which is undetectable by other tissue culture systems; potato was given as the example. Recent, but as yet unpublished, data from Holland, very clearly demonstrates in potato that a large amount of variation also occurs from calluses of complex explant origin. Thousands of variant plants have been obtained from such calluses, probably indicating cytological instability during callus culture rather than a unique feature of plant protoplasts. Because of the comparatively difficult nature of protoplast technique in crop plants, perhaps it would be easier, cheaper and far more rapid to obtain variant plants by more conventional tissue culture methods. Further, the use of such variants in plant breeding may be questionable, if we cannot direct a selection pressure for the particular agronomic character that we are seeking without adversely affecting other important agronomic characters. This may be particularly true of vegetatively propagated crops such as potato.

E. C. Cocking. This large amount of variation occurring from calluses of complex explant origin is not unexpected, and re-emphasizes that plant tissue culture *per se* appears to be an unexpectedly new and novel source of genetic variation. Only further detailed studies on a range of potato varieties will reveal whether *extra* variation is revealed as a result of protoplast cloning. Generally, in plant breeding it is difficult to direct a selection pressure for a particular agronomic character without adversely affecting other important agronomic characters; those studying variation in plants regenerated from potato protoplasts have themselves highlighted this type of difficulty. These questions will only be resolved after suitable field trials in which such plants are exposed to environmental pressures to eliminate any that are simultaneously deficient in some critical feature.

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