[Plant protoplast technology has made considerable progress in the last decade and has opened up new avenues for genetic improvement of higher plants. The possibilities are many, ranging from isolation of valuable mutants to direct introduction of alien genes, some of which, though, might seem a bit futuristic at the moment. The advances made in this field along with their possible applications have been brought out clearly by the authors in this article.—Ed.]

IN VITRO GENETIC MANIPULATION OF HIGHER PLANTS

S. BHASKARAN, S. K. MUKHERJEE, REHANA MAJID, N. GUPTA AND B. VATSYA

Nuclear Research Laboratory, Indian Agricultural Research Institute, New Delhi 110 012, *India*

INTRODUCTION

THERE has of late been a growing awareness among plant scientists that in order to evolve efficient crop plants that can meet the production and quality requirement of the future, it will be imperative to incorporate characters from organisms that are beyond the barriers of sexual compatibility which is unattainable through conventional breeding procedures. Para-sexual genetic manipulation through the protoplast technology, that has developed especially over the last decade, holds considerable promise in this direction. The fact that totipotency (ability to regenerate whole plants from isolated single cells) has been demonstrated in isolated protoplasts (cells stripped of their wall) from an ever-growing list of higher plant species makes genetic manipulation in higher plants at the cellular and sub-cellular level a feasible propsosition. Protoplasts of higher plants can be mutagenized to obtain useful mutants that cannot otherwise be isolated, fused to yield interspecific and intergeneric hybrids and transformed by exogenous DNA and possibly by isolated cytoplasmic genophores. The progress made in this field and the possibilities of the production of novel crop plant varieties through this technique are discussed below.

ISOLATION OF MUTANTS

The use of cell culture techniques in selecting mutants has many attractions. It offers the possibility of handling large populations in short space and time, a definite advantage over screening of large plant populations in the field. Due to the absence of constraints of differentiation and haplontic and diplontic competition the recovery of the locked variation is tremendously improved. Shepard[1] has observed a wide spectrum of variation among the potato plants raised through protoplast culture. The possibility of raising haploids and differentiating mutant clones into whole plants further adds to the utility of the technique.

Most of the published work on induced mutagenesis at cellular level concerns selection of resistant mutants, as resistant mutations are generally dominant and are easily detected even in the diploid state by selecting cell colonies growing on the medium containing the

drug. Mutants resistant to over 30 drugs in more than 50 species have been reported (Maliga[2]). A number of them are resistant to the amino acids or their analogues, others show resistance to antibiotics, growth inhibitors, base analogues, phytotoxins, herbicides, chilling and salt. More recently a number of *mutants* resistant to 6-azauracil[3] FUdR[4], abscissic acid[5] and *Phytophthora infestans* toxin[6,7] have been reported.

In the majority of the amino acid analogue resistant *mutants* resistance is due to over-synthesis of the amino acid followed by its accumulation in the cells[8–10]. If the over-synthesized amino acid is found to get accumulated in the storage organs of the differentiated plants, such mutants will be useful from the point of view of improved protein quality. Similarly mutants resistant to phytotoxins, herbicide, chilling, aluminium and salt would be of great agricultural interest.

Apart from their applied value, resistant mutants are also useful in basic studies. For example, BUdR resistance in plant cells was found to exhibit a phenomenon different from the animal cells[11]. In animal cells resistance to BUdR is due to lack of thymidine kinase which phosphorylates the analogue, as the first step in its cellular utilization. Most of the plant cell lines, resistant to BUdR, incorporated BUdR into their DNA and somehow survived[10] while others did not incorporate the analogue due to the over-synthesis of thymidine monophosphate[11]. Similarly Bright and Northcote[12] observed about half the enzyme, hypoxanthine guanosine phosphoribosyl transferase (HGPRT) as opposed to the animal cells where this enzyme is completely lacking in the mutants.

Resistant mutants have also been used as genetic markers for selecting hybrid cells in studies with somatic fusion (see section on somatic hybridization). However, for selecting hybrids *in vitro* auxotrophs are more important as they permit detection of the hybrid product through complementation. Yet the progress with isolation of auxotrophs has been slow. The reasons for this slow progress are : Non-availability of true haploids, presence of multiple copies of genes, presence of isoenzymes for a number of biosynthetic pathways and lack of proper understanding of the plant biochemical systems[10]. Thus attempts to isolate auxotrophs result in leakiness of the mutants and prevent their easy detection.

The earlier workers used the BUdR suicidal technique of Puck and Kao[13] for isolating auxotrophs[14,15]. Later attempts to isolate auxotrophs through this technique were unsuccessful[16]. Using selective media for screening auxotrophs, five auxotrophic variants were isolated in haploid cell cultures of *Nicotiana tabacum*[8]. Nitrate reductase auxotrophs were isolated in *N. tabacum* and *Pisum sativum* by screening for chlorate resistance[17,18]. Calcium pantothenate and adenine auxotrophic cell lines were isolated in *Datura innoxia* by non-selective screening procedure[19,20]. A few auxotrophic mutants have also been used in identifying the hybrid product in somatic fusion studies (see section on somatic hybridization). Other classes of variants are the autotrophs and the visual mutations. These mutants have not been of much consequence as the characters proved to be unstable.

Somatic Hybridization

Fusion of protoplasts derived from two distinct plant species presents three different possibilities for parasexual manipulations : (i) production of stable amphidiploid plants after the fusion of protoplasts from two diploid species, (ii) transfer of partial nuclear genetic information from one species to another and (iii) transfer of cytoplasmic genetic information.

The fusion of mechanically isolated protoplasts has been known since 1909[21] but these fusions were uncontrolled, scarce and generally non-reproducible[22,23] The first induced inter- and intra-specific fusion was achieved by using

sodium nitrate[24]. Later workers have used high Ca^{++}, high pH (pH 10·5) and high temperature (37° C)[25], and, polyethylene glycol (PEG)[26] to obtain high fusion frequencies. It should now be possible with the above procedures to fuse protoplasts of any two higher plant species, irrespective of their taxonomic relationships[27]. However the hybrid cells formed after somatic fusion are usually lost, because the unfused protoplasts of both the parental species and the homokaryons, derived from the fusion of protoplasts of the same species, outgrow the hybrid cells. Therefore, the recovery of the hybrid calli from cultures growing on normal nutrient media is highly unlikely. Successful fusion experiments, therefore, depend on the efficient screening systems which facilitate identification and selection of fusion products.

Different selection schemes for picking up the hybrid cells are based on gene complementation involving non-allelic chlorophyll deficiency mutations[28–45], auxin autotrophy[46,48], resistance to antibiotics and analogues of amino acids[49], amino acid auxotrophs[50] and nitrate reductase deficiency[51]. Selection of fusion products by individually picking up heterokaryons[52,53] or by enriching fusion products on density gradient[54] have also been tried. In addition, heterosis-like growth of hybrid clones has sometimes aided their selection.

These selective methods, to recover hybrid cells after plant protoplast fusion, are known only in a few instances and, therefore, limit the progress of somatic hybridization in plants. The first successful attempt to produce somatic hybrids in higher plants was between leaf protoplasts of *Nicotiana glauca* and those of *N. langsdorfii* by Carlson *et al.*[46]. They used 0·25 M solution of sodium nitrate for 30 min and reported a fusion frequency of about 25%. The selection was based on auxin autotrophy of hybrid cells. The hybrid plants regenerated from the hybrid calli were shown to be the same as the sexual hybrids. A few other examples, in which selection methods have been used for the recovery of hybrid cells and production of whole plant, are listed in Table I.

Different sensitivities of the parents to phytotoxins as shown by Majid and Cocking (unpublished) in *Solanum tuberosum* and *S. nigrum* vis-a-vis *Phytophthora infestans* toxin may be exploited for selecting disease resistance cell lines. Another elegant system would be to use different toxins, each of which destroy one parental protoplast type, leaving only resistant hybrid fusion products as survivors[55]. There is a strong need to develop a selection system, which could generally be applicable to all plant genera. Among all the selection systems used for recovering somatic hybrids, an albino complementation system combined with nutritional mutants holds great promise.

For the formation of stable hybrid cells, protoplast fusion must be followed by nuclear fusion, which occurs during the synchronous mitosis of the parental nuclei, involving a common mitotic apparatus[58]. This is a rare event because of the natural asynchrony between the parent protoplasts[27]. Thus synchronization of the parent cells, which is possible through an *in vitro* starvation–replenishment technique[59], may help in inducing nuclear fusion in heterokaryons at high frequencies. However, in some animal cell culture systems, nuclear fusion has been shown to take place through simultaneous entry into mitosis of the parental nuclei that were in different phases of the cell cycle (*e.g.*, one at G_1 and the other at late G_2) by the influence of one nucleus over the other[60]. Such a phenomenon is yet to be discovered in higher plant systems.

Somatic hybrids obtained after protoplast fusion show three kinds of chromosomal behaviour : (i) complete retention in the regenerated hybrid plants of the chromosomes of both parents[30,31,39]; (ii) loss of chromosomes of either parent, as in the case

TABLE I

Somatic Hybrids	Selection System	Reference
A. *Sexually Compatible* :		
1. *Nicotiana glauca + N. langsdorfii*	Auxin Autotrophy of Hybrid Cells	46, 47, 48
2. *N. tabacum + N. tabacum*	(*a*) Complementation of chlorophyll deficiency	28,29
	(*b*) Genetic complementation of nutritional auxotrophs	51
3. *Petunia parodii + P. hybrida*	Complemertation of chlorophyll deficiency	30, 31
4. *Daucus carota + D. capillifolius*	do.	32
5. *Datura innoxia + D. innoxia*	do.	33, 34
6. *N. tabacum + N. sylvestris*	do.	35
7. *N. tabacum + N. knightiana**	do.	36
8. *P. parodii + P. inflata**	Albino complementation with medium specificity	37
9. *N. tabacbm + N. glauca*	Semidominant albino system	38
10. *N. tabaaum + N. rustica*	Complementation of chlorophyll deficiency	39
B. *Sexually Incompatible* :		
1. *N. sylvestris + N. knightiana*	Kanamycin resistance	49
2. *D. innoxia + D. stramonium*	Complementation of chlorophyll deficiency	40
3. *D. innoxia + D. discolor*	Complementation of chlorophyll deficiency	40
4. *D. innoxia + D. sanguinea*	do.	41
5. *Solanum tuberosum + Lycopersicon esculentum*	do.	42
6. *Daucus carota + Aegopodium podagraria*	Albino system	97
7. *Datura innoxia + D. candida*	Complementation of chlorophyll deficiency	43
8. *D. innoxia + Atropa belladona*	do.	44
9. *Arabidopsis thaliana + Brassica campestris*	Mechanical isolation	61
10. *P. parodii + P. parviflora*	Albino complementation with medium specificity	45
C. *Cytopalsmic Hybrids*		
1. *N. sylvestris + N. tabacum*	X-ray irradiation with medium specificity	65, 71
2. *N. tabacum* (*N. debneyi* cytoplasm) + *N. tabacum*	Morphological markers	69
3. *P. hybrida + P. axillaris*	Medium specificity	70, 72

*Unilaterally compatible.

of *Vicia + Petunia* fusion[56] where most of the chromosomes of either one or the other parent were lost and the number of retained chromosome seemed to get stabilized with passage of time; (iii) preferential elimination of the chromosomes of one of the parents as in the case of *Nicotiana glauca + Glycine max* hybrid cell lines where all or most of the *Nicotiana* chromosomes were eliminated[52]. In this system, analysis of isozyme patterns revealed

coincidence in the loss of protein bands with *progressive chromosome elimination*[57]

Chromosome elimination has also been observed in hybrid plants produced by intergeneric fusions between *Solanum tuberosum* + *Lycopersicon esculentum*[42], *Arabidopsis thaliana* + *Brassica napus*[61] and *Datura innoxia* + *Atropa belladona*[44]. According to Schieder[62], partial chromosome elimination in these hybrids seems to favour morphogenesis. From the available examples it appears that in fusion products involving sexually incompatible and widely separated species, the phenomenon of chromosome elimination is fairly common. Stabilization of only one or a few chromosomes, from one of the parents in somatic hybrids following chromosome elimination, is actually desirable, because the purpose of somatic hybridization involving distantly related or unrelated plant species is not to combine two alien genomes but to incorporate only a partial genome from one species into the genome of the other. Such a system might also be useful (just as the man + mouse cell hybrids which are routinely used for human gene mapping) for location of genes on specific chromosomes of a plant species, provided its individual chromosomes can be identified through improved banding techniques.

Cybrids

The semi-autonomous cytoplasmic organelles have genomes of their own, which are responsible wholly or partly for some traits (*e.g.*, cytoplasmic male sterility[63], photosynthetic apparatus and carbon assimilation enzymes[64]), that a plant breeder might like to incorporate into the nuclear genome background of another species. Thus at times it may be desirable to obtain cytoplasmic hybrids (cybrids) containing the cytoplasm of both but the nucleus of only one of the parents. Such a combination is attainable by any of the following means: (1) X-irradiation of the protoplasts of one of the parents leading to the degeneration of its nuclei in the hybrid cells[65], (2) directional and total elimination of chromosomes of one of the parents in proliferating hybrid cells[52] and (3) fusion of nucleated protoplasts of one parent with enucleated protoplasts of the other. Enucleated protoplasts can be produced in large number by increasing the osmolarity of the incubating enzyme mixture during protoplast isolation[66]

The mixing of two cytoplasms would conceivably offer an opportunity for recombination and/or complementation of genes carried in the cytoplasmic genophores. However, the question of the stability of the two cytoplasms has not yet been adequately resolved. But from the few published reports, on the polypeptide patterns of Fraction I protein (Ribulose biphosphate carboxylase/oxygenase, the large and small sub-units of which are coded by the plastid and nuclear genomes respectively), it seems that in somatic hybrids there is a strong tendency to retain only one of the two plastomes[67–73]. The failure to detect the polypeptide pattern of the large sub-unit of Fraction I protein characteristic of one kind of chloroplast may not be a conclusive evidence of the latter's absence, because the plastid might be present without its DNA being expressed in the novel nucleo-cytoplasmic combination. A more reliable approach of comparing the electrophoretic distribution of restriction endonuclease generated fragments of chloroplast DNA of the somatic hybrid with those of both the parents has been adopted by Belliard *et al.*[69] although arriving at the same conclusion as in the above-mentioned studies involving Fraction I protein. On the other hand, through similar finger printing of the mitochondrial DNA restriction fragments in the case of *Nicotiana tabacum* + *N. debneyi* cytoplasmic hybrids, which derived the stable cytoplasmic male sterility and flower malformation trait from the first parent, it was deduced that not only are the mitochondria of both the parents present, there was also recombination between their DNA's[74]. This finding is also of great significance from the point of view of hybrid vigour, which is considered to be the result of complementation of

mitochandrial functions[75], although whether or not the presence of both the parental and recombinant mitochandria in the cybrid will result in heterosis cannot be said with much confidence at the moment. At present, the inter-specific transfer of cytoplasmic male sterility in tobacco[65,72] and petunia[71] is the available example of practical applicability of cybrids. With further understanding of the functions of the chloroplast and mitochondrial DNA, *in vitro* cybrid production might prove to be a valuable and versatile tool for the plant breeder, while inter-specific and inter-generic cybrids themselves would be excellent systems in the study of the biology and evolution of cell organelles.

Uptake of Organelles and DNA

The idea of introducing alien genetic information directly into plant protoplasts through the uptake of isolated organelles or pure DNA is indeed very attractive. Success has been reported in the transplantation into higher plant protoplasts of isolated nuclei[76,77], higher plant chloroplasts[78], algal chloroplasts[79] and even cells of yeast[80] and algae[81,82]. But such transfers were ineffective as the transferred organelles/organisms degenerated in the new cytoplasmic environment and/or the resultant protoplasts failed to divide, though formation of new cell wall was noted in some cases. An endo-symbiotic relationship necessitates a delicate agreement between a certain number of genes in both the partners. Thus a forced intra-cellular relationship will not be effective unless the partners naturally meet or are 'engineered' to meet this requirement. Thus effective transfer of organelles may be possible only within a species[83,84] or at best between closely related species while transfer of whole microorganisms into higher plant cells to achieve a stable endosymbiotic relationship is too futuristic an idea at the moment.

It has been amply demonstrated that isolated and purified DNA, when suitably protected against extra-cellular nucleases either by poly-L-ornithine or by entrapment in liposomes, can enter plant protoplasts and remain largely intact at least for some time, if not indefinitely[85–88] but these reports highlighted the uptake phenomenon without attempting to demonstrate genetic transformation of the recipient protoplasts by the exogenous DNA. Certain bacterial genes were transferred and made to express in plant tissue cultures through specialized transducing phages[89] though the newly acquired traits were lost after several passages of sub-culture.

In contrast to bacteriophages and bacterial 'R' plasmids, the DNA of cauliflower mosaic virus (CaMV, one of the few DNA plant viruses known) and that of the Ti plasmid of *Agrobacterium tumefaciens* (the pathogen responsible for the crown gall disease in several dicot species) are two potential and more promising vectors for transferring foreign DNA and ensuring its stability, expression, and replication in higher plant cells. The CaMV DNA has been ligated with bacterial plasmids and cloned in *E. coli* cells[90,91]. It has also been adequately shown that such cloned CaMV DNA can infect host plants as effectively as the native virus[91], though the capability of engineered CaMV DNA to cause transformation remains to be seen.

Still more promising is the Ti plasmid of *Agrobacterium tumefaciens* which has been shown to integrate into multiple sites in the higher plant genome[92] all restricted to the nuclear DNA[93] and to express genes carried by it in plant cells[94] Recently Davey *et al.*[65] have succeeded in transforming *Petunia* cells to auxin autotrophy (a tumour trait) by the uptake of the Ti plasmid into the host protoplast. The next step obviously is to attempt transformation of plant cells using an engineered non-tumour inducing derivative of the Ti plasmid that can mediate stable integration and ensure expression of alien genes in higher plant cells.

An idea of transferring the '*nif*' genes to the Ti plasmid through recombinant DNA techniques and using such engineered plasmids to integrate the '*nif*' genes into the genomes of non-leguminous dicot plants has been put forth[96]. Considering the fact that the nitrogenase enzymes are irreversibly inactivated by oxygen, it has been thought more practicable to introduce the '*nif*' genes into an engineered PS II lacking chloroplast and make it coexist in a common cytoplasm by complementation with a photosynthetically complete chloroplast defective for some other function[64]. *Transfer of genes for seed proteins, mRNA* of which are abundantly available in developing seeds, from legumes to cereals, with a view to improve the seed protein quality of the latter is an exciting proposition[99]. Results of experiments in these directions are awaited.

Conclusion

From the above discussion it is evident that *in vitro* genetic modification of higher plants is a definite possibility and it is not unreasonable to expect that in the near future this technology can be extended to the major crop plant species. But at the same time we do not wish to convey the impression that the access to variability, both in qualitative and quantitative terms, in the context of *in vitro* plant improvement is limitless or that all or most *in vitro* manipulations would lead to an improvement over the parent. A better understanding of the genetic and molecular basis of the agronomic traits alongwith further advances and refinement of techniques will be needed before *in vitro* modification of crop plant genomes can become a routine tool for the future plant breeder.

1. Shepard, J. F., Bidney, D. and Shahnin, E., *Science*, 1980, **208**, 17.
2. Maliga, P., *Proc. 4th Int. Congr. Plant Tissue and Cell Culture*, University of Calgary, Alberta, Canada, Aug. 20–25, 1978, p. 381.
3. Jones, G. E. and Hann, J., *Theor. Appl. Genet.*, 1979, **54**, 81.
4. Sung, Z. R. and Jacques, S., *Planta*, 1980, **148**, 389.
5. Wang, J. R. and Sussex, I. M., *Ibid.*, 1980, **148**, *103*.
6. Behnke, M., *Theor. Appl. Genet.*, 1979, **55**, 69.
7. —, *Ibid.*, 1980, **56**, 151.
8. Gupta, N., *Ph.D. Thesis* submitted to the Post-Graduate School of IARI, 1978.
9. Widholm, J. M., In : *Plant Tissue Culture and Its Biotechnological Applications, Eds.* Barz, W., Reinhard, E. and Zenk, M. H., Springer-Verlag, Berlin—Heidelberg, New York, 1977, pp. 112.
10. Widholm, J. M., *I.A.P.T.C. Newsletter* No. 23, 1978, p. 2.
11. Ohyama, K., *Environ. Expt. Botany*, 1976, **16**, *209*.
12. Bright, S. W. J. and Northcote, D. H., *Planta (Berl.)*, 1975, **123**, 79.
13. Puck, T. and Kao, F., *Proc. Nat. Acad. Sci. (U.S.A.)*, 1967, **58**, 1227.
14. Carlson, P. S., Science, 1970, **168**, 487.
15. Zyrd, J. P., *Pl. Sci. Lett.*, 1976, **6**, 157.
16. Aviv, D. and Galun, E., *Pl. Sci. Lett.*, 1977, **8**, 299.
17. Muller, A. J. and Grafe, R., *Mol. Gen. Genet.*, 1978, p. 67.
18. Feenstra, W. J. and Jacobson, E., *Theor. Appl. Genet.*, 1980, **58**, 39.
19. Savage, A. D., King, J. and Gamborg, O. L., *Pl. Sci. Lett.*, 1979, **16**, 367.
20. King, J., Horsch, R. B. and Savage, A. D., *Planta*, 1980, **149**, 480.
21. Kuster, E., *Ber. Dtsch. Bot. Ges.*, 1909, **27**, 589.
22. Michel, W., *Arch. Exp. Zellforsch Besonders Gewebezuecht.*, 1937, **20**, 230.
23. Hoffmeister, L., Protoplasma, 1954, **43**, 278.
24. Power, J. B., Cummins, S. E. and Cocking, E. C., *Nature*, 1970, **255**, 1016.
25. Keller, W. A. and Melchers, G., *Z. Naturforsch. Teil. B*, 1973, **28**, 737.
26. Kao, K. N. and Michayluk, M. R., *Planta*, 1974, **115**, 355.
27. Vasil, I. K., Ahuja, M. R. and Vasil, V., *Advances in Genetics*, 1979, **20**, 127.
28. Melchers, G. and Labib, G., *Mol. Gen. Genet.*, 1974, **135**, 277.
29. Gleba, Y. Y., Butenko, R. G. and Sytnik, K. M., *Dokl. Acad. Nauk. SSSR*, 1975, **221**, 1196.
30. Power, J. B., Frearson, E. M., George, D., Evans, P. K., Berry, S. F., Hayward, C. and Cocking, E. C., *Plant Sci. Lett.*, 1976, **7**, 51.
31. —, —, —, —, —, — and —, *Nature*, 1976, **263**, 500.
32. Dudits, D., Hadlaczky, G., Levi, E., Feyer, O., Haydu, Z. and Lazar, G., *Theor. Appl. Genet.*, 1977, **51**, 127.

33. Schieder, O., *Planta*, 1977, **137**, 253.
34. —, *Mol. Gen. Genet.*, 1978, **162**, 113.
35. Melchers, G., In *International Cell Biology*, 1976–77, eds. Brinkley, B. R. and Porter, K. R., Rockefeller Univ. Press, New York, 1977, p. 207.
36. Maliga, P., Kiss, Z. R., Nagy, A. H. and Lazar, G., *Mol. Gen. Genet.*, 1978, 163, 145.
37. Power, J. B., Berry, S. F., Chapman, J. V. and Cocking, E. C., *Theor. Appl. Genet.*, 1979, **55**, 97.
38. Evans, D. A., Wetter, L. R. and Gamborg, O. L., *Physiol. Plant.*, 1980, **48**, 225.
39. Douglas, G. C., Keller, W. A. and Setterfield, G., *Can. J. Bot.*, 1981, **59**, 208.
40. Schieder, O., *Mol. Gen. Genet.*, 1978, **162**, 113.
41. —, *Cytobiol.*, 1978, **18**, 175.
42. Melchers, G., Sacristan, M. D. and Holder, A. A., *Carlsberg. Res. Commun.*, 1978, **43**, 203.
43. Schieder, O., *I.A.P.T.C. Newsletter*, 1979, No. 28.
44. Krumbiegel, G. and Schieder, O., *Planta*, 1979, **145**, 371.
45. Power, J. B., Berry, S. F., Chapman, J. V. and Cocking, E. C., *Theor. Appl. Genet.*, 1980, **57**, 1.
46. Carlson, P. S., Smith, H. H. and Dearing, R. D., *Proc. Natl. Acad. Sci. U.S.A.*, 1972, **69**, 2292.
47. Smith, H. H., Kao, K. N. and Combatti, N. C., *J. Hered.*, 1976, **67**, 123.
48. Chupeau, Y., Missionier, C., Hommel, M. C. and Goujand, J., *Mol. Gen. Genet.*, 1978, **165**, 239.
49. Maliga, P., Lazar, G., Joo, F., Nagy, A. H. and Menczel, L., *Ibid.*, 1977, **157**, 291.
50. Grimsley, N. H., Ashton, N. W. and Cove, D. J., *Ibid.*, 1977, **143**, 97.
51. Glimelius, K., Eriksson, T., Graffe, R. and Muller, A. J., *Physiol. Plant.*, 1978, **44**, 273.
52. Kao, K. N., *Mol. Gen. Genet.*, 1977, **150**, 225.
53. Gleba, Y. and Hoffmann, F., *Mol. Gen. Genet.*, 1978, **165**, 257.
54. Harms, Ch. T. and Potrykus, I., *Theor. Appl. Genet.*, 1978, **53**, 49.
55. Earle, E. D., *I.A.P.T.C. Newsletter*, No. 31, 1980.
56. Binding, H. and Nehls, R., *Mol. Gen. Genet.*, 1978, **164**, 137.
57. Wetter, L. R., *Ibid.*, 1977, **150**, 231.
58. Kao, K. N., Constabel, F., Michayluk, M. R. and Gamborg, O. L., *Planta*, 1974, **120**, 215.
59. Mukherjee, S. K. and Bhaskaran, S., In *Proceedings of National Symposium on Plant Tissue Culture, Genetic Manipulation and Somatic Hybridization of Plant Cells*, eds. Rao, P. S. Heble, M. R. and Chadha, N. S., B.A.R.C., Bombay, 1980, p. 154.
60. Bryant, J., In *Molecular Aspects of Gene Expression in Plants*, ed. Bryant, J., Acad. Press, London, 1976, p. 1.
61. Gleba, Y. Y. and Hoffmann, F., *Naturwissenschaften*, 1979, **66**, 547.
62. Schieder, O., *I.A.P.T.C. Newsletter*, No. 33, 1981.
63. Levings, C. S. III, Kim, B. D., Pring, D. R., Conde, M. F., Mans, R. J., Laughman, J. R. and Gabey Laughman, S. J., *Science*, 1980, **209**, 1021.
64. Bogorad, L., *Genetic Engineering*, Vol. I, eds. Setlow, J. K. and Hollaender, A., Plenum Press, N.Y., 1979, p. 181.
65. Zelcer, A., Aviv, D. and Galun, E., *Z. Pflanzenphysiol.*, 1978, **90**, 397.
66. Vatsya, B. and Bhaskaran, S., *Plant Sci. Lett.*, 1981 (In press).
67. Kung, S. D., Gray, J. C., Wildman, S. G. and Carlson, P. S., *Science*, 1975, **187**, 353.
68. Chen, K., Wildman, S. G. and Smith, H. H., *Proc. Natl. Acad. Sci., U.S.A.*, 1977, **74**, 5109.
69. Belliard, G., Pelletier, G., Vedel, F. and Quetier, F., *Molec. Gen. Genet.*, 1978, **165**, 231.
70. Izhar, S. and Power, J. B., *Plant Sci. Lett.*, 1979, **14**, 49.
71. Aviv, D., Fluhr, R., Edelman, M. and Galun, E., *Theor. Appl. Genet.*, 1980, **56**, 145.
72. Izhar, S. and Tabib, Y., *Theor. Appl. Genet.*, 1980, **57**, 241.
73. Gleba, Y. Y., *Plant Cell and Tissue Culture—Principles and Applications*, Ohio State University Press, Columbus (In press).
74. Belliard, G., Vedel, F. and Pelletier, G., *Nature*, 1979, **281**, 401.
75. Sarkissian, I. V., *Z. Pflanzenzuchtung*, 1972, **67**, 53.
76. Potrykus, I. and Hoffmann, F., *Z. Pflanzenphysiol.*, 1973, **69**, 287.
77. Lorz, H. and Potrykus, I., *Theor. Appl. Genet.*, 1978, **53**, 251.
78. Davey, M. R., Freeman, E. M. and Power, J. B., *Plant Sci. Lett.*, 1976, **7**, 7.
79. Bonnett, H. T. and Eriksson, T., *Planta*, 1974, **120**, 71.
80. Davey, M. R. and Power, J. B., *Plant. Sci. Lett.*, 1975, **5**, 267.
81. Burgoon, A. C. and Bottino, P. J., *J. Hered.*, 1976, **67**, 223.
82. Meeks, J. C., Malmberg, R. L. and Wolk, C. P., *Planta*, 1978, **139**, 55.
83. Carlson, P. S., *Proc. Natl. Acad. Sci. U.S.A.*, 1973, **70**, 598.
84. Landgren, C. R. and Bonnett, H. T., *Plant Sci. Lett.*, 1979, **16**, 15.
85. Suzuki, M. and Takebe, I., *Z. Pflanzenphysiol.*, 1976, **78**, 421.

86. Lurquin, P. F. and Kado, C. I., *Mol. Gen. Genet.*, 1977, **154**, 113.
87. Hughes, B. G., White, F. G. and Smith, M. A., *Plant Sci. Lett.*, 1978, **14**, 303.
88. Lurquin, P. F., *Nucleic Acids Res.*, 1979, **6**, 3773.
89. Doy, C. H., Gresshoff, P. M. and Rolfe, B. G., *Proc. Natl. Acad. Sci. U.S.A.*, 1973, **70**, 723.
90. Langridge, J., In *Proceedings of I.A.E.A. Symposium on Seed Protein Improvement in Cereals and Grain Legumes*, Vol. II, I.A.E.A., Vienna, 1979, p. 403.
91. Howell, S. H., Walker, L. L. and Dudley, R. K., *Science*, 1980, **208**, 1265.
92. Van Montagu, M. and Schell, J., In *Plasmids of Medical Environmental and Commercial Importance*, ed. Timmis, K., Elsevier, Amsterdam, 1979, p. 71.
93. Willimitzer, L., De Beuckeleer, M., Van Montagu, M. and Schell, J., *Nature*, 1980, **287**, 359.
94. Hernalsteens, J. P., Van Villet, F., De Beuckeleer, M., Depicker, A., Engler, G., Lemmers, M., Holster, M., Van Montagu, M. and Schell, J., *Ibid.*, 1980, **287**, 654.
95. Davey, M. R., Cocking, E. C., Freeman, J., Pearce, M. and Tudor, I., *Plant Sci. Lett.*, 1980, **18**, 307.
96. Schell, J. and Van Montagu, M., In *Genetic Engg. for Nitrogen Fixation*, ed. Hollaender, A., Plenum Press, New York, 1977, p. 159.
97. Dudits, D., Hadlaczky, G., Bajskar, G. Y., Koncz, C., Lazar, G. and Horvath, G., *Plant Sci. Lett.*, 1979, **15**, 101.